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AQUATIC FLOW CYTOMETRY: ACHIEVEMENTS AND PROSPECTS. *M. RECKERMANN and F. COLIJN (eds.)*

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

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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 epifluorescence 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-

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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 Jernaes 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), Trousellier *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 Trousellier *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 ($> 2\ \mu m^3\ cell^{-1}$) are many times larger than planktonic or soil bacteria (typically $<0.2\ \mu m^3\ cell^{-1}$, and often $< 0.06\ \mu m^3\ cell^{-1}$), 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-ß-hydroxybutirate (e.g. Srienc *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. Bernarder *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

Argus-100, BioRad Bryte and Bruker ACR 1400-Sp), and were for a while a cheap and practical alternative to the laser-based flow cytometers. These instruments had the added advantage of combining UV excitation in a small machine, since UV lasers required a high power source and thus, expensive and sophisticated refrigeration systems. At a time when blue-excitable stains for DNA quantification were almost non existent, this was a powerful reason to purchase an arc-lamp cytometer to study bacteria. Another advantage of these instruments was the possibility of easily changing excitation wavelengths (Peters, 1979).

Some of the properties of the molecules used to stain the cells, which often were antibiotic molecules that affected the structure of the DNA, were used to characterize bacterial species. Van Dilla *et al*. (1983), for example, used a combination of a GC-specific stain (chromomycin A) and an AT specific stain (Hoechst 33258), in combination with light scatter, to differentiate mixed bacterial cultures and determine their base-pair content. Other approaches to detecting specific microorganisms in water, food or in body fluids were developed combining several of the variables provided by the cytometers: light scatter (as surrogates of size and internal structure), protein and DNA content (Ingram *et al*., 1982; Miller and Quarles, 1990; Allman *et al*., 1992) or by the use of 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 μm^3) 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, Bernander *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 fluoresce 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 (dye characteristics adapted from Haugland 1996 and Davey and Kell 1996).

Stain	Binds to:	Exc. / Em. (nm)	Type of sample	References
Ethidium bromide (EthBr)	DNA and RNA	518 / 605	cultures	Paau *et al*. 1977, Pinder *et al*. 1990
Propidium iodide (PI)	DNA and RNA	535 / 617	cultures	Bailey *et al*. 1977, Hutter and Eipel 1979, Miller and Quarles 1990
			cooling towers	Tyndall *et al*. 1985
Fluorescein isothiocyanate (FITC)	protein	495 / 520	cultures	Bailey *et al*. 1977, Miller and Quarles 1990, Allman *et al*. 1990
Chromomycin A3	DNA (GC)	340 / 470	cultures	Boye *et al*. 1983, van Dilla *et al*. 1983
Acridine orange (AO)	RNA*	460 / 650*	cultures	Nishimura *et al*. 1995
			seawater	Nishimura *et al*. 1995
Mithramycin and EthBr	DNA and RNA	425 / 550	cultures	Boye *et al*. 1983, Thorsen *et al*. 1992, Allman *et al*. 1992, Steen *et al*. 1994
DAPI	DNA	358 / 461	cultures	van Dilla *et al*. 1983
			marine and freshwater	Robertson and Button 1989
HOECHST 33342	DNA (AT)	350 / 461	cultures	van Dilla *et al*. 1983
			marine	Monger and Landry 1993
Benzoxazinone-kanamycin (BVC kanamycin)	cell surfaces	495 / 616	cultures	Depierreux *et al*. 1990
TO-PRO-1	DNA and RNA**	515 / 531	marine	Li *et al*. 1995
TOTO-1	DNA and RNA**	514 / 533	marine	Li *et al*. 1995, Zubkov *et al*. 1998
SYTO-13	DNA and RNA**	488 / 514	freshwater	del Giorgio *et al*. 1996
			marine	Guindulain *et al*. 1997, Lebaron *et al*. 1998
YOYO-1	DNA and RNA	491 / 509	cultures	Marie *et al*. 1996
YO-PRO-1	DNA and RNA	491 / 509	cultures	Marie *et al*. 1996
PicoGreen	dsDNA	480 / 520	cultures	Marie *et al*. 1996, Veldhuis *et al*. 1997
			marine	Sieracki *et al*. 1999
SYBRGreen I	DNA and RNA	494 / 521	marine	Marie *et al*. 1997
			freshwater	Lebaron *et al*. 1998
SYTOX	dsDNA	504 / 523	cultures	Veldhuis *et al*. 1997
SYTO-9, 11, BC	DNA (and RNA)	480-510 / 500-520	freshwater and marine	Lebaron *et al*. 1998
SYBRGreen II	RNA (and DNA)	492 / 521	freshwater and marine	Lebaron *et al*. 1998
SYTO-17	DNA and RNA	633 / 675	cultures	Comas and Vives-Rego 1997
SYTO-16	DNA and RNA	488 / 518	cultures	Ibrahim *et al*. 1997
SYPRO	Protein	550 / 630	cultures	Zubkov *et al*. 1999

* 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)

easily cross bacterial membranes, or are essentially imperment 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 in some degree. Most of the stains have selective binding to DNA and to RNA, with different fluorescence yields, at least *in vitro*. However, even those that are marketed as preferentially staining RNA tend to also stain DNA (see the comparison of SybrGreen 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 *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 *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 *et al.* (1993) and Heldal *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 *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 *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 *et al.* (1995) and del Giorgio *et al.* (1996) introduced blue-excitable stains for enumerating plankton bacteria. Li *et al.*

TABLE 2. – Some technical details offered in most of the papers to date where natural planktonic bacteria have been enumerated by FC.

Reference	Instrument	Laser (power)	Flow rate μl min^{-1}	Sheath fluid	Fixative	Stain	Pretreatment
Robertson and Button 1989	Ortho Cytofluorograph IIS	UV 5W	5	dH_2O	Ethanol 75%	DAPI (2.5 μg ml^{-1})	-
Button and Robertson 1989	Ortho Cytofluorograph IIS	UV 5W	5	dH_2O	Ethanol 75%	DAPI (2.5 μg ml^{-1})	-
Monfort and Baleux 1992	ACR-1400-SP Bruker*	UV 100W	-	-	Formalin 3.7%	DAPI (2.5 μg ml^{-1})	-
Button and Robertson 1993	Ortho Cytofluorograph IIS	UV 5W	5	dH_2O	Formalin 0.5%	DAPI (0.5 μg ml^{-1})	Triton X-100 (0.1%)
Troussellier *et al.* 1993	ACR-1400-SP Bruker*	UV	-	-	PFA 0.5-4%	DAPI (-)	-
Monger and Landry 1993	Coulter EPICS 753	UV 200 mW	25 – 40	-	PFA 1%	HOECHST 33342 (0.5 μg ml^{-1})	-
Heldal *et al.* 1994	Argus 100-4	UV	20	-	Glut 2%	DAPI (20 μg ml^{-1})	-
Campbell *et al.* 1994	Coulter EPICS 753	UV 200 mW	-	-	PFA 0.2 %	HOECHST 33342 (0.5 μg ml^{-1})	-
Troussellier *et al.* 1995	ACR 1400-SP Bruker*	UV	5	-	several	DAPI (2.5 μg ml^{-1})	-
Li *et al.* 1995	FACSort	Blue	12	-	Glut 1%, PFA 1%	TOTO (0.3-0.5 μM) TO-PRO (3-5 μM)	Triton X-100 (0.1%)
del Giorgio *et al.* 1996	FACScan	Blue 15 mW	12	Hematall	Glut 1%, FA 3%	SYTO 13 (2.5 μM)	-
Binder *et al.* 1996	Coulter EPICS 753	UV 200 mW	-	-	Glut 0.12 %	HOECHST 33342 (0.5 μg ml^{-1})	-
Landry *et al.* 1996	Coulter EPICS 753	UV 200 mW	-	-	PFA 0.9%	HOECHST 33342 (0.8 μg ml^{-1})	-
Button *et al.* 1996	Ortho Cytofluorograph IIS	UV 5W	5	dH_2O	Formalin 0.5%	DAPI (0.5 μg ml^{-1})	Triton X-100 (0.1%)
Jellett *et al.* 1996	FACSort	Blue 15 mW	12	Filt Seawater	PFA 1%	TO-PRO-1 (3 μM)	Triton X-100 (0.1%)
Buck *et al.* 1996	Coulter EPICS 753	UV 200 mW	-	-	PFA 0.2 %	HOECHST 33342 (0.5 μg ml^{-1})	-
Pile *et al.* 1996	Coulter EPICS 753	UV 225 mW	-	-	-	HOECHST 33342 (0.5 μg ml^{-1})	-
Campbell *et al.* 1997	Coulter EPICS 753	UV 200 mW	-	-	PFA 0.2 %	HOECHST 33342 (1 μg ml^{-1})	-
del Giorgio *et al.* 1997b	FACScan	Blue 15 mW	12	Hematall	-	SYTO 13 (2.5 μM)	-
Marie *et al.* 1997	FACSort	Blue 15 mW	-	-	Glut 0.1%	SybrGreen I (10^{-4})	RNAse, 30 mM potassium citrate
	Coulter EPICS 541	UV 500 mW	-	-	Glut 0.1%	HOECHST 33342 (0.45 μg ml^{-1})	
Wallner *et al.* 1997	FACStar Plus	UV 200 mW	-	-	PFA 3%	DAPI (1 μM)	-
Guindulain *et al.* 1997	Coulter EPICS Elite	Blue 15mW	-	-	PFA 2%	SYTO 13 (2.5 μM)	Triton X-100 (0.1%)
Pile 1997	Coulter EPICS 753	UV 225 mW	-	-	-	HOECHST 33342 (0.5 μg ml^{-1})	-
Zubkov *et al.* 1998	FACSort	Blue 15mW	45	-	Glut 0.2%	TOTO (0.4 μM)	Triton X-100 (0.1%)
Peters *et al.* 1998	FACSCalibur	Blue 15mW	19	dH_2O	PFA 1% + Glut 0.05%	SYTO 13 (2.5-5 μM)	-
Campbell *et al.* 1998	Coulter EPICS 753	UV 200 mW	-	-	PFA 0.2 %	HOECHST 33342 (1 μg ml^{-1})	-
López-Amorós *et al.* 1998	Coulter XL	Blue 15mW	-	-	PFA 0.2%	SYTO 13 (0.125 μM)	Triton X-100 (0.1%)
Jacquet *et al.* 1998b	FACSort	Blue 15mW	-	Filt Seawater	PFA 1% + Glut 0.05%	SybrGreen I (10^{-4})	30 mM potassium citrate
Lebaron *et al.* 1998	FACSCalibur	Blue 15mW	-	-	Formalin 2%	(several)	sodium or potassium citrate
Ribes *et al.* 1998a, 1998b	Coulter EPICS 753	UV 225 mW	-	-	-	HOECHST 33342 (0.5 μg ml^{-1})	-
Marie *et al.* 1999	FACSort	Blue 15mW	50	-	PFA + G / Glut 0.1-0.5%	SybrGreen I (10^{-4})	(several)
Gasol and Morán 1999	FACSCalibur	Blue 15mW	19	dH_2O	PFA 1% + Glut 0.05%	SYTO 13 (2.5-5 μM)	-
Gasol *et al.* 1999	FACSCalibur	Blue 15mW	19	dH_2O	PFA 1% + Glut 0.05%	SYTO 13 (2.5-5 μM)	-
Sherr *et al.* 1999	FACScan	Blue 15mW	12	Hematall	-	SYTO 13 (2.5 μM)	-
Sieracki *et al.* 1999	FACScan	Blue 15mW	20 - 50	-	PFA 1%	PicoGreen (10^{-2})	-
Vives-Rego *et al.* 1999	Coulter XL	Blue 15mW	-	-	none	SYTO 13 (2.5 μM)	-
Mostajir *et al.* 1999	ACR 1400-Sp Bruker*	UV 100W	-	-	Formalin 3.7%	DAPI (2.5 μg ml^{-1})	-
Chatila *et al.* 1999	ACR 1400-Sp Bruker*	UV 100W	-	-	Formalin 3.7%	DAPI (2.5 μg ml^{-1})	-
Karl *et al.* 1999	Coulter EPICS 753	UV 200 mW	-	-	PFA 1 %	HOECHST 33342 (1 μg ml^{-1})	-
Gin *et al.* 1999	Coulter EPICS 753	UV 200 mW	-	-	Glut 0.1 %	HOECHST 33342 (0.5 μg ml^{-1})	-
Troussellier *et al.* 1999	FACSCalibur	Blue 15mW	-	-	-	SYTO 13 (5 μg ml^{-1})	-
Servais *et al.* 1999	FACSCalibur	Blue 15mW	-	-	-	SYTO 13 (5 μg ml^{-1})	-
Lebaron *et al.* 1999	FACSCalibur	Blue 15mW	-	-	-	SYTO 13 (5 μg ml^{-1})	-

*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 FACSCalibur, 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

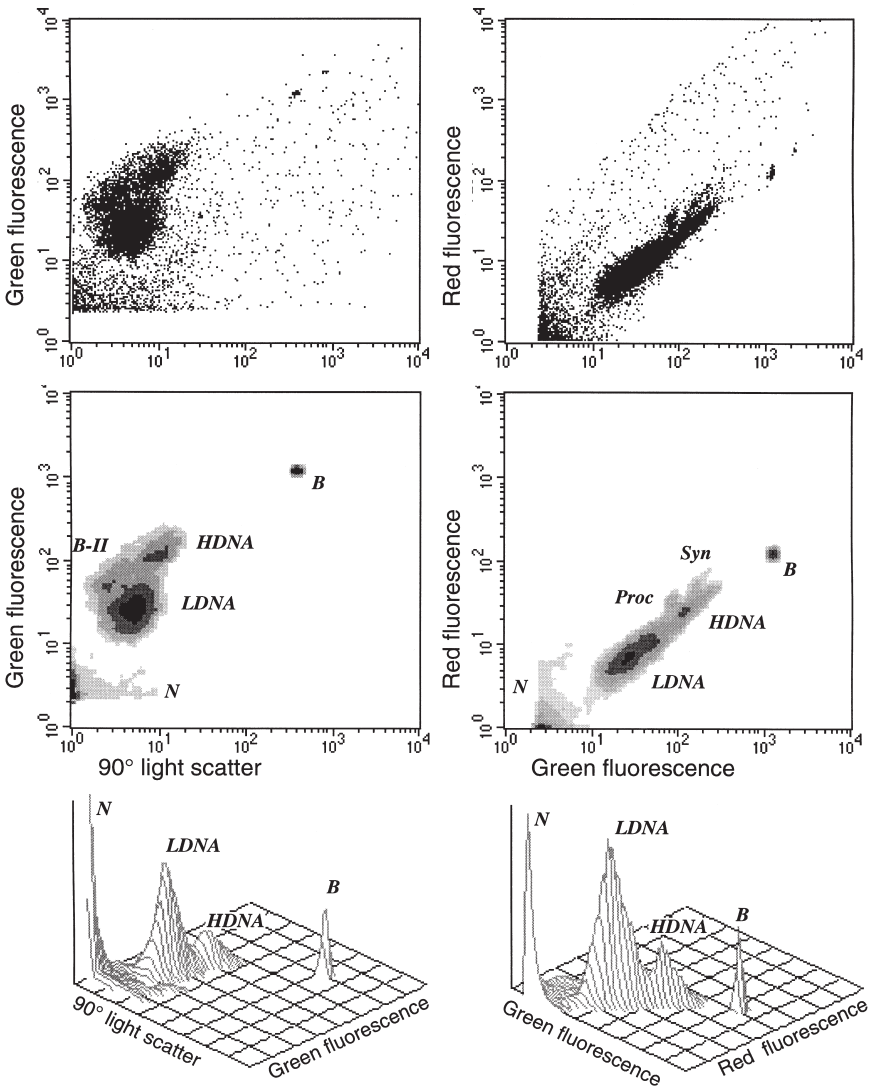

FIG. 1. – Flow cytometric analysis of a surface open Atlantic ocean sample after staining with SYTO 13. In the upper panels, 10000 events are displayed after acquisition in log mode. In the left side of the graph, a representation of 90° light scatter (SSC or WALS) vs. green fluorescence (FL1 in our 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 (Crissman *et al*., 1978; Lebaron *et al*., 1998; Troussellier *et al*., 1999).

Some degree of post-fixation cell disappearance

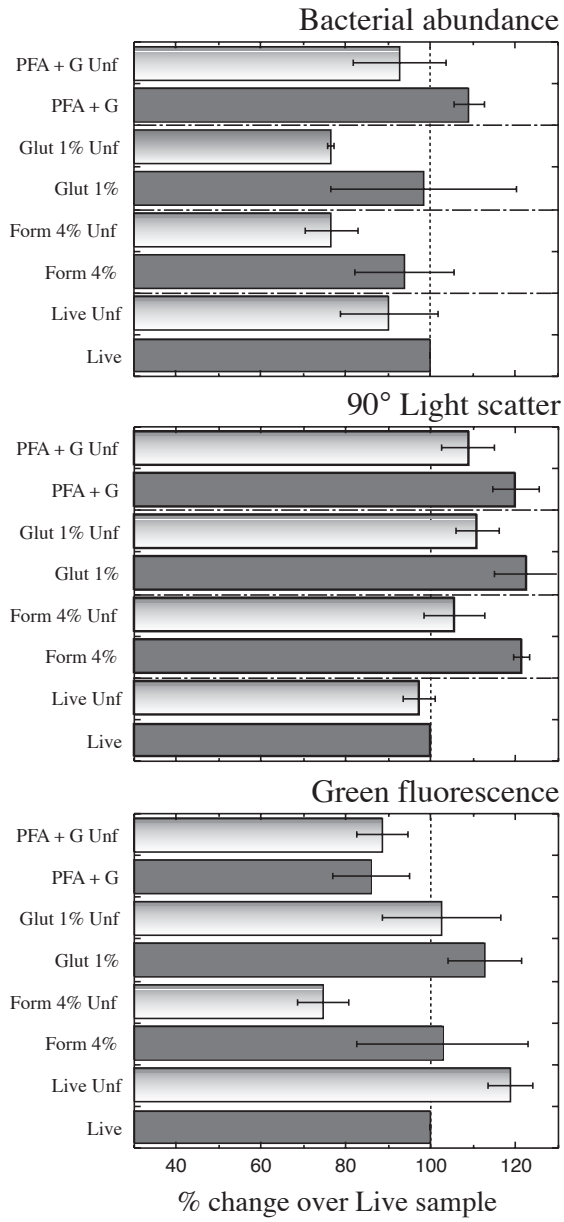

FIG. 2. – Effect of some fixation protocols on bacterial abundance, bacterial side scatter and bacterial green fluorescence after staining with SYTO 13. The values are expressed as percentage of the "live" treatment (unfixed and unfrozen). Average plus standard error (or range) of two samples: a coastal Mediterranean one, and an aged-water open Mediterranean sample. Treatments are: No fixation ("live"), 4% formalin, 1% glutaraldehyde and 1% Paraformaldehyde plus 0.01% glutaraldehyde. UNF: indicates that after fixation the sample was frozen in liquid nitrogen, left for a few minutes and later unfrozen to run the analyses.

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 Troussellier *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 decided 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 the prokaryotes and wants to infer growth rates from those data (e.g. Vaulot *et al*., 1995), but it is not necessary for regular enumeration 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 subpopulations 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-

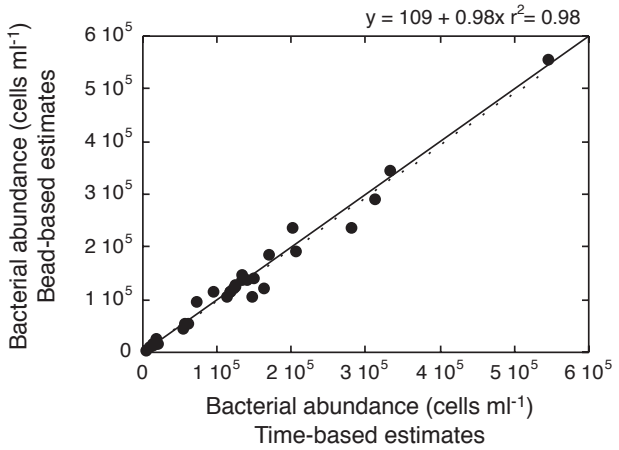

FIG. 3. – Comparison of bacterial concentrations obtained from the relationship of cells detected to the number of beads detected (the beads coming from a solution of known concentration added as internal standards: "bead-based"); and bacterial concentrations calculated from the sample delivery rate of the machine (calibrated gravimetrically at the beginning of the session: "time-based").

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 5x10^5 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 μm) 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 μl 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

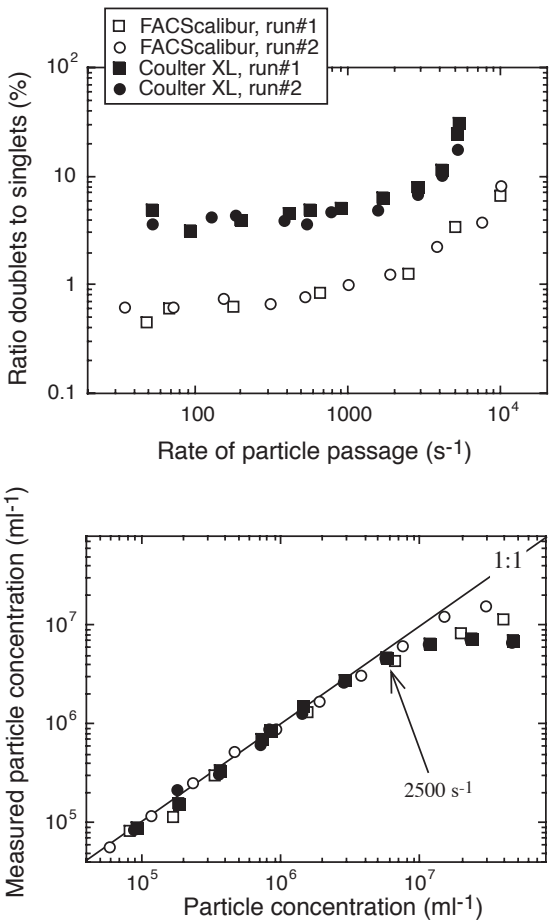

FIG. 4. – Effects of the speed of particle passage through two different flow cytometers (Coulter XL and Becton and Dickinson's FACScalibur). The speed was changed by serially diluting a fluorescent bead solution: Upper panel, the amount of beads detected as doublets. Lower panels, relationship between the actual particle concentration and the flow cytometrically measured particle concentration.

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 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 *et al*., 1999) or in dH_2O if the samples are fixed (authors' obs.). We have been succesful at enumerating bacteria from solar salt ponds where they are at concentrations above 10^7 ml^{-1} in salinities around 250‰ (Gasol and Pedrós-Alió, unpublished). Dilution of the PFA-fixed sample in dH_2O served here two purposes: to reduce coincidence and simultaneously to reduce salinity so that salt did not interfere with the nucleic acid stain. Some researchers, however, are routinely counting at rates at or above 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 doublets (two particles seen as one) increases exponentially (Fig 4a). By relating then the observed particle concentration to the expected concentration, we were able to find out the limits of a FACScalibur and of a Coulter XL, which were very similar and broke out at particle passage rates of around 2000 s^{-1}, equivalent to total particle concentrations of several million particles per ml. Even though this procedure can be used to find out the limits of any machine, it will always be safe to keep the rates of particle passage 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, for example, 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} (Shalapyonok *et al*., 1998).

As discussed above, double laser excitation is required to completely resolve *Prochlorococcus* when staining with DAPI or HOECHST (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*.

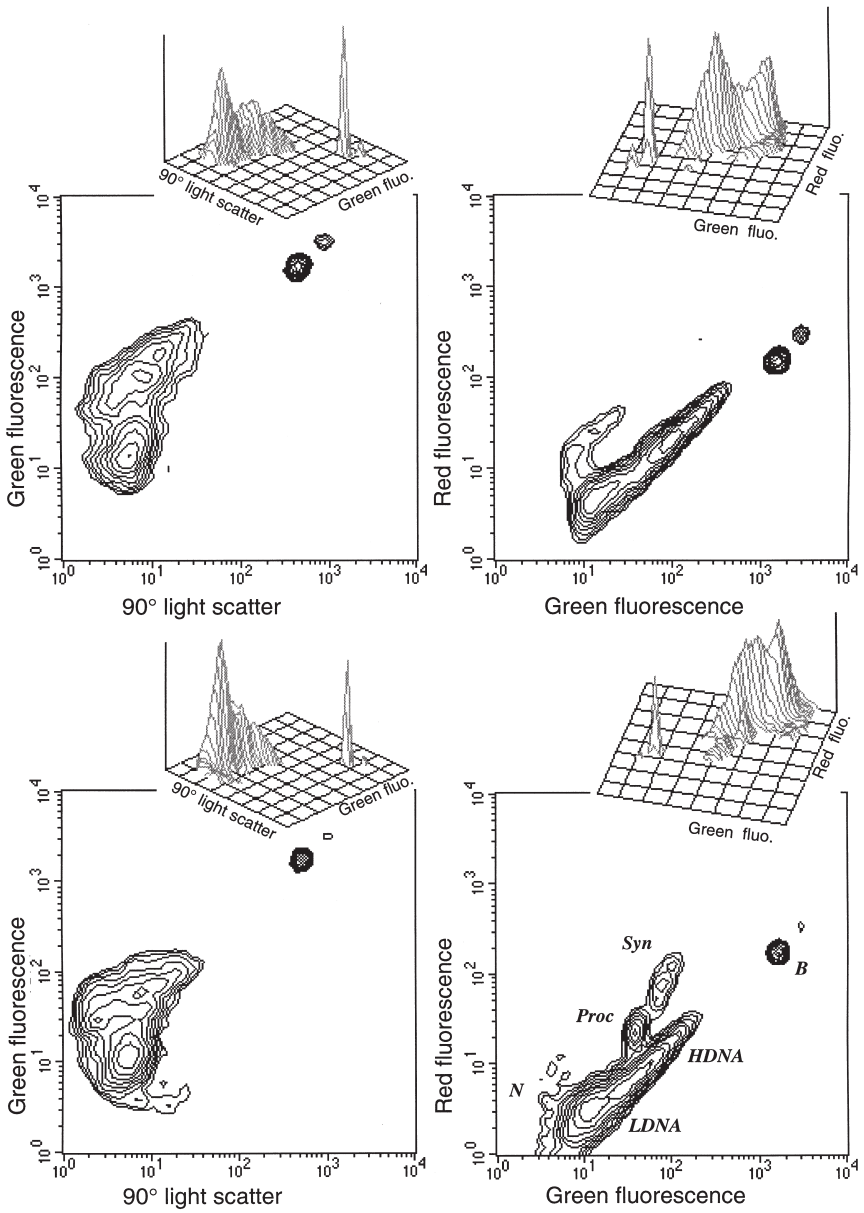

FIG. 5. – Flow cytometric analysis of a coastal (upper) and an open ocean (lower) Atlantic sample after staining with 5 μM SYTO 13. The panels present contour density plots of 10000 acquired events. In the left side of the graphs, a representation of 90° light scatter (SSC or WALS) vs. green fluorescence (FL1 in our instrument). In the right side, a representation of green fluorescence vs. red fluorescence (FL3 in our instrument). As in Fig.1, no electronic compensation was applied to the fluorescences, and slightly more voltage was applied to the FL3 photomultipliers than to the FL1 photomultipliers. One step of data smoothing was applied, and a threshold of 1% eliminated isolated data for these contour density plots. Interpretation of the different subpopulations that appear is as described in Fig.1: B: Yellow-green 1 μm Polysciences latex beads. N: electronic noise. HDNA: Bacteria with High DNA content . LDNA: Bacteria with Low DNA content. Syn: *Synechococcus*. Proc: *Prochlorococcus*.. Insert in the graphs, a three-dimensional representation of the same data. Note that the 3-D representation of the FL1 vs. FL3 plots has been turned around to show the peaks of the photosynthetic prokaryotes.

(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. 5, upper) or very difficult (Fig. 5, 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

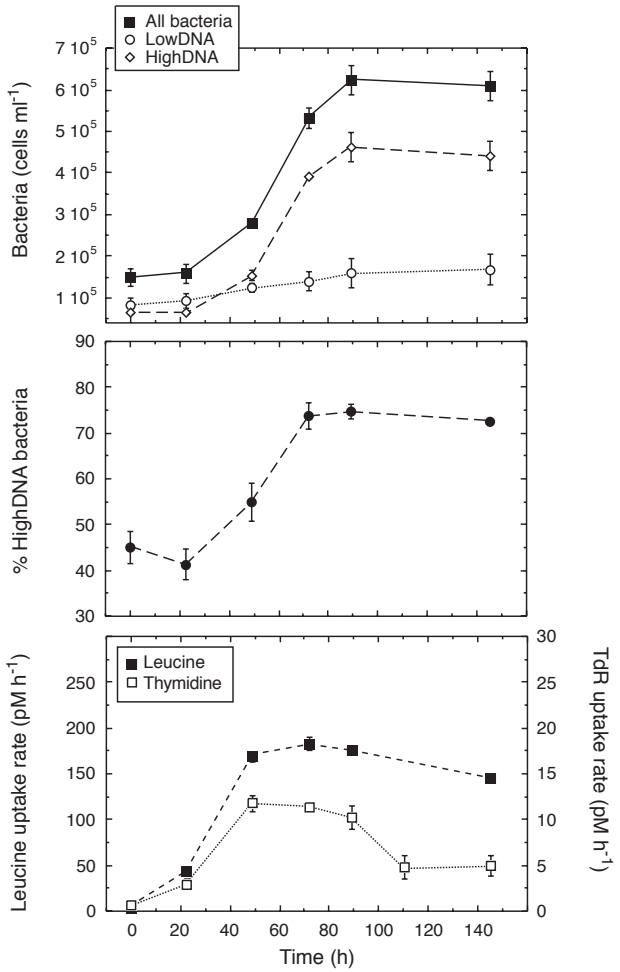

FIG. 6. – A dilution-growth experiment with open North Atlantic water. Water was filtered through 0.8 μm and diluted 2:10 with 0.2 μm-filtered water. Upper panel: evolution of total bacteria, HighD NA bacteria and LowDNA bacteria. Central panel: evolution of the % contribution of HighDNA bacteria to total bacteria. Lower panel: Evolution of the uptake rates of Leucine and thymidine (standard methods as described in Kemp *et al*. 1993). Average plus standard error of two replicated bottles. Data courtesy of Carlos Pedrós-Alió.

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 begining 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

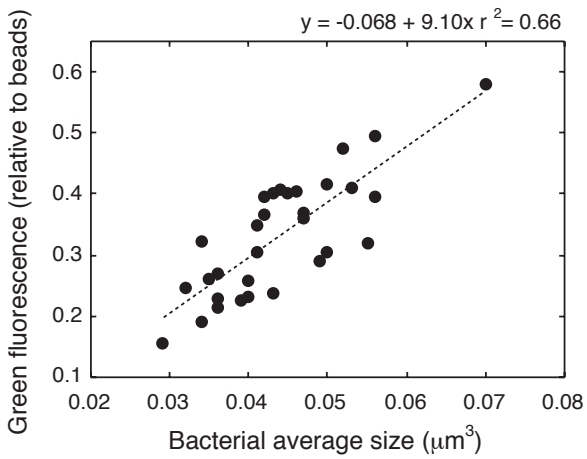

FIG. 7. – Relationship between average bacterial size (obtained by image analysis of DAPI preparations following the procedure of Massana *et al.* 1997), and average fluorescence (relative to beads) of the SYTO 13-stained sample run in a FACSort flow cytometer. Samples from the plankton of Lake Cromwell (Quebec). Data from Prairie Y. *et al.* (in prep.).

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. DNA content also seemed to covary with bacterial protein in most of the assayed bacterial species.

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

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

Stain	Mode of action	Exc/Em	applied in	References
AO (acridine orange)	Different color when linked to DNA or other things	460 / 650	cultures	Nishimura *et al.* 1995 Darzynkiewicz and Kapuscinski 1990, McFeters *et al.* 1991
PI (propidium iodide)	excluded by living cells	536 / 623	cultures cultured bact in seawater	Jepras *et al.* 1995 López-Amorós *et al.* 1995b
EthBr (ethidium bromide)	excluded by living cells	510 / 595	cultures	Paau *et al.* 1977, Pinder *et al.* 1990
Oxonols (JC-1, oxonol VI, $DiBaC_4(3)$)	accumulate when energy-defficient	488 / 525	cultures	Mason *et al.* 1995, Deere *et al.* 1995, Jepras *et al.* 1995, López-Amorós *et al.* 1995a Comas and Vives-rego 1997, Beck and Huber 1997
FDG (fluorescein-galactopyranose)	Activity of the enzime ß-galactosidase	494 / 518	cultures	Nir *et al.* 1990, Miao *et al.* 1993
Fluorescein diacetate (FDA)	cleaved by intracellular enzims	492 / 517	cultures	Diaper *et al.* 1992
CFDA, Chemchrome B, calcein blue AM, SFDA...	cleaved by intracellular enzims	(488-520/560)	cultures compost freshwater	Diaper and Edwards 1994a, Jepras *et al.* 1995, Jacobsen *et al.* 1997, Beck and Huber 1997 Diaper and Edwards 1994b Porter *et al.* 1995a
CTC	indicator of respiratory-chain activity	480 / >585	cultures freshwater marine	Kaprelyants and Kell 1993a, López-Amorós *et al.* 1997 del Giorgio *et al.* 1997b, Yamaguchi and Nasu 1997 López-Amorós *et al.* 1998, Sieracki *et al.* 1999
Rh123 (rhodamine 123)	accumulated in live cells	510 / 580	cultures cult bact in SW	Diaper *et al.* 1992, Davey *et al.* 1993, Kaprelyants and Kell 1992 López-Amorós *et al.* 1995b
Cyanine dyes ($DiOC_6(3)$, $DiOC_2(3)$...)	accumulated in live cells	488/ 520-560 488 / > 600	cultures cultures	Mason *et al.* 1995, Monfort and Baleaux 1996 Novo *et al.* 1999
c-SNARF-1 AM	intracellular pH	~488 / ~610	cultures	Leyval *et al.* 1997
Calcafluor white, Tinopal CBS-X...	excluded by living cells	347 / 436 325 / 430	cultures cultures	Mason *et al.* 1995 Davey and kell 1997
SYTOX Green	excluded by living cells	504 / 523	cultures	Roth *et al.* 1997, Veldhuis *et al.* 1997
TOPRO-1	excluded by living cells	515 / 531	freshwater	del Giorgio *et al.*, in press
TOPRO-3	excluded by living cells	642 / 661	cultures	Davey *et al.* 1999
16S rRNA probes	attach to ribosomes	-	cultures	Amann *et al.* 1990; Wallner *et al.* 1993
DVC*	live cells elongate when in presence of ABs	-	cultures seawater	Thorsen *et al.* 1992, Joux *et al.* 1997 Nishimura *et al.* 1995
BacLight Live / Dead	(Syto9 / PI)	-	cultures	Joux *et al.* 1997, Jacobsen *et al.* 1997

* 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.

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 tetrazolium salts such as CTC, which is reduced to a formazan by the enzymes of the electron transport system, and FDA, which produce a fluorescent product upon intracellular cleavage by active estereases.

The overwhelming majority of physiological probes currently in use are fluorescent and can be used in conjunction 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 ($DiBAC_4(3)$, 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 nonfluorescent 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 Via-

bility kit, or the combination of ChemChrome V6 and CSE, Catala *et al*., 1999) are continously 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; Ullrich *et al*., 1996) at the concentrations used, and might not work in some circumstances (Thom *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; Sieracki *et al*., 1999). 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).

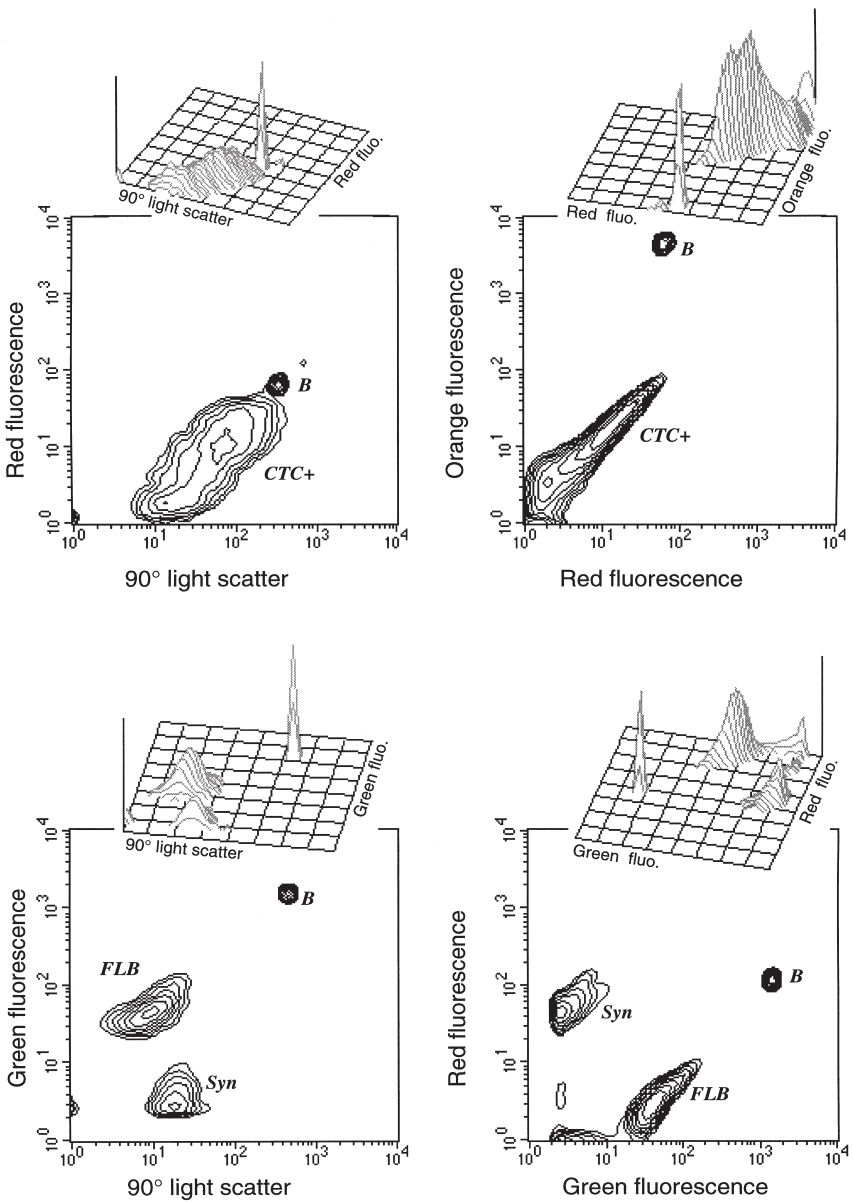

FIG.8. – Upper panels. Flow cytometric analysis of a Mediterranean plankton sample after incubation with 5 mM CTC for a few hours showing the presence of a bacterial population that took up the salt and reduced it to the fluorescent CTF (labeled as CTC+). Yellow beads (B) were included in the run. The panels present contour density plots of 10000 acquired events. In the left side of the graphs, a representation of 90° light scatter (SSC) vs. red fluorescence (FL3). In the right side, a representation of orange fluorescence (FL2) vs. red fluorescence. Note that the 3-D representation of the FL2 vs. FL3 plot has been turned around to better show that the main peak is separated from the background. Lower panels. Flow cytometric signature of FITC-stained *Pseudomonas diminuta* (FLB) added as a tracer for grazer experiments to a Mediterranean plankton sample particularly rich in *Synechococcus* (labeled as Syn). Yellow beads (B) were included in the run. The panels present contour density plots of 10000 acquired events. In the left side of the graphs, a representation of 90° light scatter (SSC) vs. green fluorescence (FL1). In the right side, a representation of red fluorescence (FL3) vs. green fluorescence. Note that the 3-D representation of the FL1 vs. FL3 plot has been turned around to better show the separation of both populations.

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 ß-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 ^{14}C-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-Dominguez *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 of bacteria and picoalgae 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 antarctic 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.

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