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# What Flow Cytometry Can Tell Us About Marine Micro-Organisms – Current Status and Future Applications

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## 1. Introduction

Born in the field of medicine for the analysis of mammalian cell DNA, flow cytometry (FCM) was first used in microbiology studies in the late 1970s thanks to optical improvements and the development of new fluorochromes (Steen & Lindmo, 1979; Steen, 1986). Its initial applications in clinical microbiology are dated to the 1980s (Steen & Boyne, 1981; Ingram et al., 1982; Martinez et al., 1982; Steen 1982; Mansour et al., 1985), and, by the end of that decade, FCM had also become popular in environmental microbiology (Burkill, 1987; Burkill et al., 1990; Yentsch et al., 1983; Yentsch & Pomponi, 1986; Yentsch & Horan, 1989; Phinney & Cucci, 1989). Today, it is a poweful and commonly used tool for the study of aquatic micro-organisms. FCM has thus become a precise alternative to microscopic counts, increasing the number of both the micro-organisms detected and the samples that can be analyzed. The advantages of FCM include single-cell detection, rapid analysis (5000 cells per second or more), the generation of multiple parameters, a high degree of accuracy and statistically relevant data sets.

The significance of flow cytometry can be summarized as the measure (-metry) of the optical properties of cells (cyto-) transported by a liquid sheath (flow) to a light source excitation (most often a laser) (Shapiro, 2003).

FCM facilitates single cell analyses of both cell suspension, such as eukariotic and prokariotic cells, and "non cellular" suspension, such as microbeads, nuclei, mitochondria and chromosomes.

A typical flow cytometer is formed by different units: the light source, the flow cell, the hydraulic fluidic system, several optical filters, a group of photodiodes or photomultiplier tubes and, finally, a data processing unit (Veal et al., 2000; Longobardi, 2001; Shapiro, 2003; Robinson, 2004; Diaz et al., 2010).

In a flow cytometer, individual cells pass in a single file within a hydrodynamically focused fluid stream. Single cells are centered in the stream so that they intercept an excitation source, meaning that scatter and/or fluorescence signals can be collected and optically separated by dichroic filters and detectors. The data collected are then converted into digital information. Finally, software displays data as events along with their relative statistics.

The light scattering properties are detected as FALS (forward angle light scatter) and RALS (right angle light scatter). FALS, collected in the same direction as the incident light (0-13° conic angle with respect to the incident point of the laser), is measured in the plane of the laser beam and provides information on cell size, while RALS is usually measured at 90º (70-110° conic angle) to the beam and provides data on cell granularity or the internal structure of the cell (Hewitt & Nebe-Von-Caron, 2004) (Fig. 1).

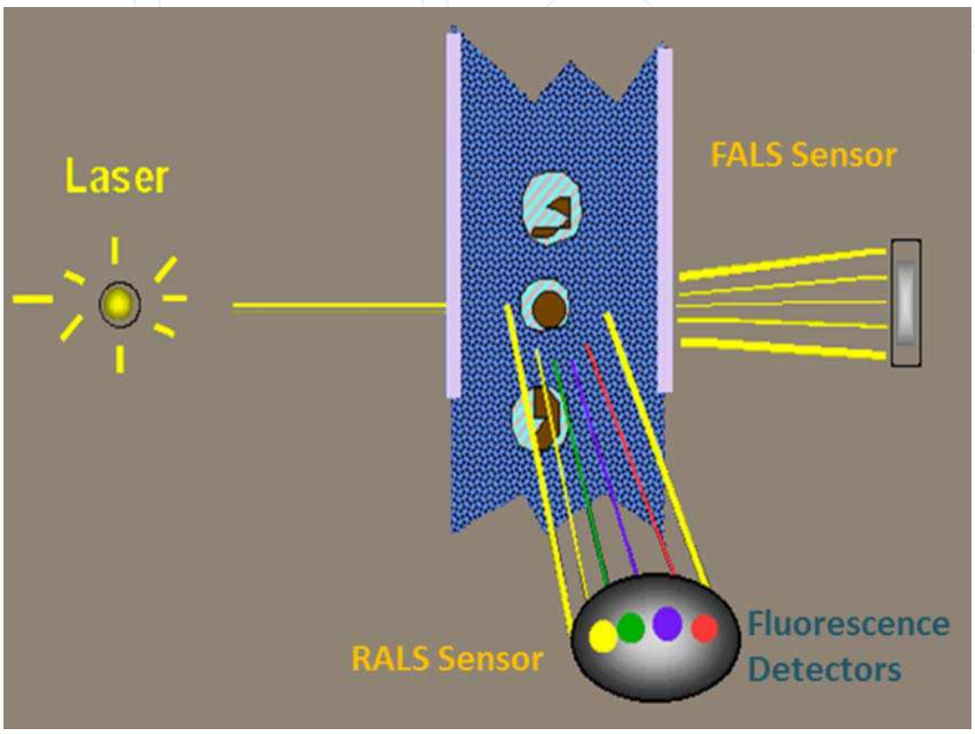



Fig. 1. Light fractions scattered and fluorescence by an excited single cell

Together with the FALS and RALS data, fluorescent information can also be collected, which includes signals from autofluorescence or induced fluorescence.

Each single value can be amplified, and stored events are commonly represented in a monoparametric histogram or biparametric dot plot. One-parameter histograms represent the number of cells or particles per channel (y-axis) versus the scattering or fluorescence intensity (x-axis). Dot plots are the most common graphic representations of the relative distribution of different cell populations.

Regions and gates can be made to better separate and analyze populations of interest. Furthermore, on the basis that the dyes used to stain cells have overlapping emission spectra, the compensation is normally made to reduce interference.

While basic instruments may only permit the simultaneous collection of two or three fluorescence signals, the more complex and expensive research instruments mean that it is possible to obtain more than 14 parameters (Winson & Davey, 2000; Chattopadhyay et al., 2008) depending on the laser equipment utilized. Selection of the lasers will depend on the range of wavelengths needed for the excitation of the selected fluorochromes.

Some flow cytometers have the ability to physically separate different sub-populations of interest (cell sorting) depending on their cytometric characteristics (stream-in-air), thus permitting the recovery and purification of cell subsets from a mixed population for further applications (Bergquist et al., 2009; Davey, 2010).

In natural samples in particular, a very important advantage of FCM is the opportunity to analyze micro-organisms with minimal pre-treatment and without the need for cultivation steps, also taking into account that the most of natural bacteria are resistant to cultivation (Fig. 2). Furthermore, FCM is particularly well-suited for the investigation of natural picoplankton. This is because of their small size (<2 μm; Sierbuth, 1978), which renders the analysis thereof difficult by more traditional methods. Particularly due to the rapidity with which data can be obtained, flow cytometry has been routinely used over the last few decades for the analysis of different types of micro-organisms in marine samples (Porter et al., 1997; Yentsch & Yentsch, 2008; Vives-Rego et al., 2000; Wang et al., 2010). It is now commonly accepted as a reference technique in oceanography.

Knowledge of seawater microbial diversity is important for understanding community structure and patterns of distribution. In the ocean water column, organisms <200 μm include a variety of taxa, such as free viruses, autotrophic bacteria (cyanobacteria, which include the group known formerly as prochlorophytes), heterotrophic bacteria, protozoa (flagellates and ciliates) and small metazoans (Legendre et al., 2001), all of which have different morphological, ecological and physiological characteristics.

Heterotrophic and autotrophic bacteria, viruses and authotrophic picoeukaryotes represent marine picoplankton (2- 0,2 μm), while the larger fraction of micro-organisms is divided into nano-plankton (20-2 μm) and micro-plankton (200-20 μm).

Among these taxa, bacteria are very important because they play a crucial role in most biogeochemical cycles in marine ecosystems (Fenchel, 1988), taking part in the decomposition of organic matter and the cycling of nutrients. Bacteria are also an important source of food for a variety of marine organisms (Das et al., 2006), and their activity has a major impact on ecosystem metabolism and function. Both autotroph and heterotroph micro-organisms constitute two fundamental functional units in ecosystems, where the former generally dominate eutrophic systems and the latter generally dominate oligotrophic systems (Dortch & Postel, 1989; Gasol et al., 1997). An extensive body of literature has documented the great importance of the activity of algae in terms of the size of picoplankton in the global primary production of aquatic ecosystems (Craig, 1985; Stockner & Antia, 1986; Stockner, 1988; Callieri & Stockner, 2002). Picocyanobacteria are a diverse and widespread group of photosynthetic prokaryotes and belong to the main group of primary producers (Castenholz & Waterbury. 1989; Rippka, 1988). Picoeukaryotes, meanwhile, are a diverse group that is widely distributed in the marine environment, and they have a fundamental role in aquatic ecosystems because of their high productivity. Like bacteria, marine viruses are thought to play important roles in global and small-scale biogeochemical cycling. They are also believed to influence community structure and affect bloom termination, gene transfer, and the evolution of aquatic organisms. Viruses are the most numerous 'lifeforms' in aquatic systems, being about 15 times more abundant than total of bacteria and archaea. Data from literaure seem to indicate that the abundance of marine viruses is linked to the abundance of their hosts, so that changes in the prokaryotic host populations will affect viral abundance (Danovaro et al., 2011).

Given that the vast majority of the biomass [organic carbon (OC)] in oceans consists of micro-organisms, it is expected that viruses and other prokaryotic and eukaryotic micro-organisms will play important roles as agents and recipients of global climate change (Danovaro et al., 2011).

Accordingly, the accurate determination of micro-organism abundance, biomass and activity is essential for understanding the aquatic ecosystem. Consequently, the aim of this review is to provide a general overview of the applications of flow cytometric tecniques to studies in marine microbiology.

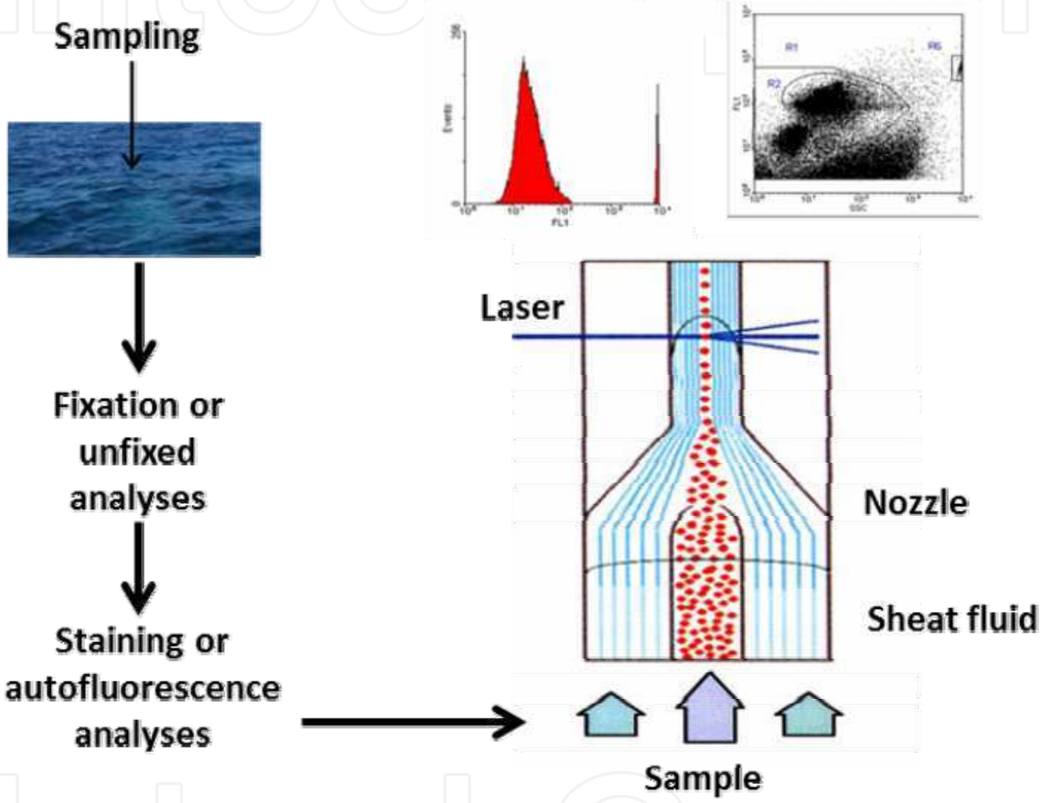



Fig. 2. Scheme of the main step: from sampling to the flow cytometric data

## 2. Autofluorescence analyses

The opportunity to measure fluorescence by flow cytometry is a key aspect in microbial ecology, since light-scattering characteristics alone are not usually enough to uncover much detail about either the taxonomic affinities or the physiological status of micro-organisms (Davey & Kell, 1996). Phytoplanktonic micro-organisms are an ideal subject for flow cytometric analysis because they are naturally autofluorescent by virtue of their complement of photosynthetic pigments. Most of these pigments can absorb the blue light of the 488 nm line of an Argon laser, meaning that they can be distinguished because of their unique fluorescence emission spectra. Standard filter arrangements in a dual laser system (488 and 633 nm lasers) can distinguish and quantify chlorophyll fluorescence (red ex, em > 630 nm), phycoerythrin (PE) fluorescence (blue ex, em 570 nm) and phycocyanine (PC)

fluorescence (red ex, em >630 nm) (Callieri, 1996; Callieri & Stockner, 2002). Accordingly, flow cytometric data collected from natural phytoplankton assemblages can be used to identify and classify phytoplankton based on scattering characteristics (size) and fluorescence (pigmentation) (for an example, see Figure 3).

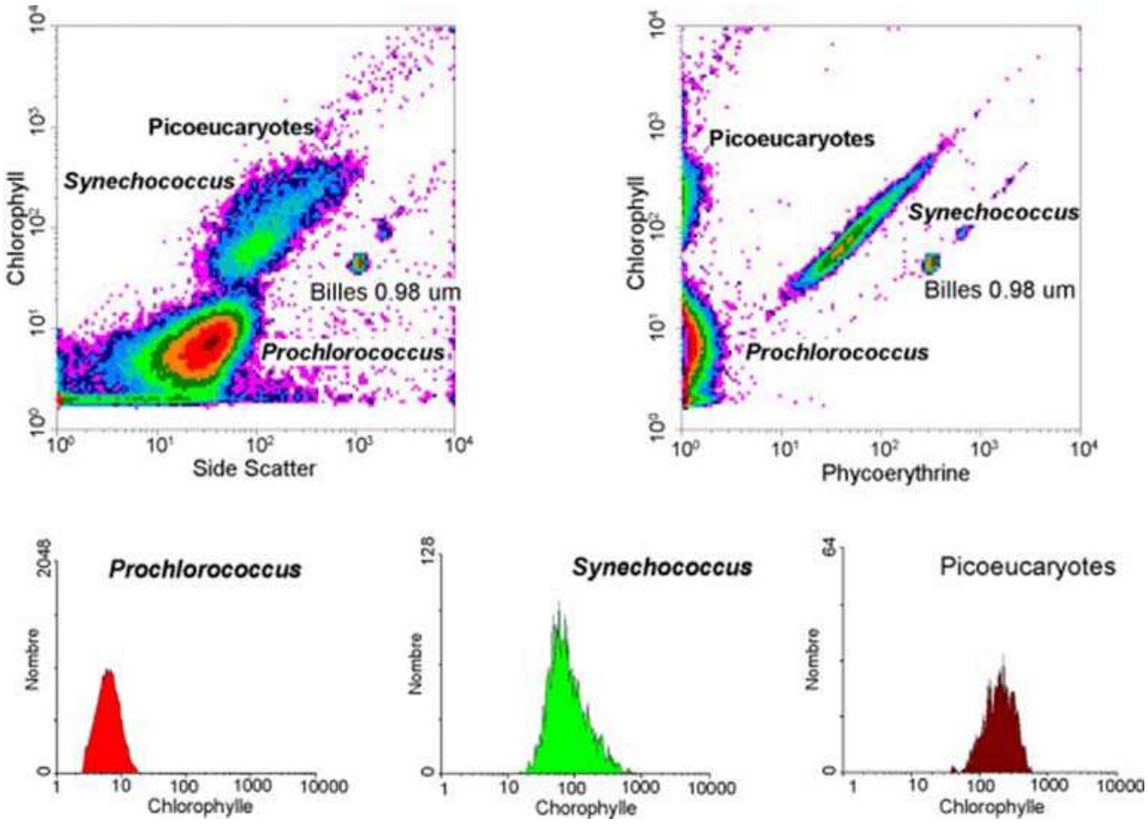



Fig. 3. Autotrophic picoplankton by flow cytometry. Image provided by Daniel Vaulot, CNRS, Station Biologique de Roscoff, France

The use of flow cytometry in aquatic microbial ecology increased our knowledge of the structure of phytoplankton assemblages (Olson et al., 1993). Based on flow cytometric analyses, phytoplankton are typically divided into Cyanobacteria (*Synechococcus*, *Prochlorococcus*) and small (pico-) and large eukaryotes. They are also able to define the distributions and dynamics of each group (e.g. Olson et al., 1990; Campbell et al., 1994; Li, 1995; Lindell & Post, 1995; Partensky et al., 1996; Campbell et al., 1997). The phycoerythrin (PE)-containing *Synechococcus* can be distinguished from *Prochlorococcus,* which are similar in size, but do not produce the 'orange' fluorescence that is typical of phycoerythrin. Eukaryotic phytoplankton, meanwhile, are distinguished based on their larger scatter and chlorophyll fluorescence signals.

The application of flow cytometry to marine samples led to the discovery of a primitive, prokaryotic picocyanobacteria of the Prochlorophyta group (Chisholm et al., 1988), with divinyl chlorophyll-*a* (chl-*a*) as the principal light-harvesting pigment and divinyl chlorophyll b (chl-*b*), zeaxanthin, alfa-carotene and a chl-*c*-like pigment as the main accessory pigments (Goericke & Repeta, 1993).

In some cases, the larger cells may be further distinguished based on their scattering characteristics (coccolithophorids) or the presence of both PE and chlorophyll (cryptophytes) (Olson et al., 1989; Collier & Campell, 1999).

Many authors have reported the distributions and dynamics of each photosynthetic group in the water column in different marine environments (Li, 1995; Campbell & Vaulot, 1993; Vaulot & Marie, 1999). As both cyanobacteria and picoeukaryotes are widely distributed in the marine environment, they play a fundamental role in aquatic ecosystems because of their high productivity.

Cyanobacteria are a diverse group of unicellular and multicellular photosynthetic prokaryotes (Castenholz & Waterbury, 1989; Rippka, 1988); they are often referred to as blue-green algae, even though it is now known that they are not related to any of the other algal groups.

Seasonal patterns of picoplankton abundance have been observed in many studies, revealing a strong relation with water temperature. A study on picophytoplankton populations conducted by Alonso and colleagues (2007) in north-west Mediterranean coastal waters showed a peak during the winter for picoeukaryotes, and peaks in spring and summer for *Synechococcus*. Meanwhile, *Prochlorococcus* was more abundant from September to January.

Zubkov et al. (2000) found that *Prochlorococcus spp*. were the dominant cyanobacteria in the northern and southern Atlantic gyres and the equatorial region, giving way to *Synechococcus* spp. in cooler waters. *Synechococcus* cells also become more numerous and even reach blooming densities near the tropical region affected by the Mauritanian upwelling. Finally, the concentrations of Picoeukaryotes tend to be at their height in temperate waters.

The small coccoid prochlorophyte species, *Prochlorococcus marinus*, were found to be abundant in the North Atlantic (Veldhuis & Kraay, 1990), the tropical and subtropical Pacific Ocean (Campbell et al., 1994), the Mediterranean (Vaulot et al., 1990) and in the Red Sea (Veldhuis & Kraay, 1993).

A monitoring study conducted in the Central Adriatic Sea (authors' unpublished results) revealed the presence of cyanobacteria, pico-eukariotes and nano-plankton (Fig. 4), while prochlorococcus were absent throughout the entire year.

Other authors (Marie et al., 2006) have underlined the similarity of the distribution of picoeukariotes to that of total chlorophyll-*a* in the Mediterranean Sea, with maximum concentrations reaching around $2x10^2$ cell/ml.

Shi and co-authors (2009) have characterized photosynthetic picoeukaryote populations by flow cytometry in samples collected in the south-east Pacific Ocean, registering abundances from $6x10^2$ to $3{,}7x10^4$ cell/ml. Meanwhile, 18S rRNA gene clone libraries were constructed after flow sorting.

## 3. Total cell counting

Total cell counting is one of the most important functions of flow cytometry. The rapidity and accuracy of the data obtained overcome the limitations (e.g. time-consuming, subjectiveness linked to the operator) of other techniques such as epifluorescence microscopy.

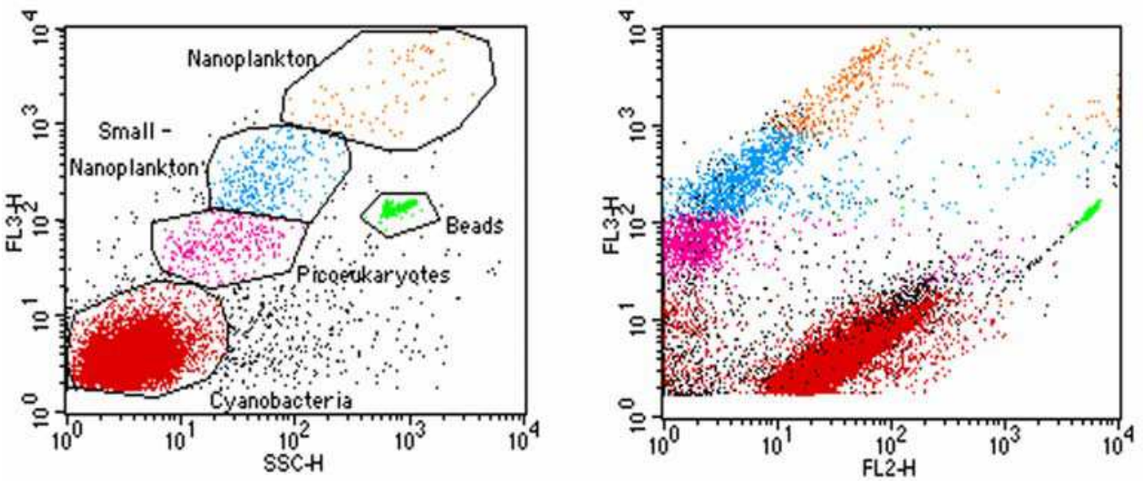



Fig. 4. Example of dot plot showing autothrophic micro-organisms in the Central Adriatic Sea

Flow cytometric countings can be determined with high statistical confidence. Some flow cytometers are equipped with volumetric counting hardware that enables the absolute cell count to be made through a predefined volume. Yet most cytometers do not have this equipment, and, in such circumstances, cell counting is perfomed by: 1) the addition of synthethic counting beads; 2) the calibration of the flow rate; and 3) weighing the sample before and after conducting any analyses. The addition of precounted beads is now also possible with commercially available beads for "absolute counting" (e.g. Coulter Flowcount beads, Cytocount counting beads, DakoCytomation, and Trucount tubes by Becton Dickinson). Accompanying datasheets provide the exact number per µl of beads to use (Cantineaux et al., 1993; Brando et al., 2000, Manti et al., 2008). The number of cells per microlitre is obtained by the following formula:

Number of cells = (cell events/beads events)*
(bead number/µl)* Dilution Factor

Other methods have proposed the use of standard beads (Polysciences latex beads), as well described by Gasol and del Giorgio in 2000. Briefly, the beads have to be counted every day and must be sonicated to avoid aggregation.

Flow rate calibration can be performed by weighing a tube containing water, processing various volumes, estimating the time needed for each volume to go through and then reweighing the tube. This makes it possible to calculate the mean of the flow rate per minute (Paul, 2001).

The third method is comprised of estimating volume differences: the volume of the sample is measured by a micro-pipette before and after the run through the flow cytometer. However, these measurements are not as precise as those obtained using weight differences.

The flow cytometric counting of non-fluorescent cells is possible through the staining of nucleic acids (or other cellular components) with fluorescent dyes. There are commercially available probes that allow the direct counting of marine bacteria, such as, for example, the nucleic acid dyes Syto-9, Syto-13 (Lebaron et al., 1998; Vives-Rego et al., 1999), SYBR Green I

and II (Lebaron et al., 1998; Marie et al., 1997), Pico Green (Sieracki et al., 1999; Marie et al., 1996), TO-PRO 1, and TOTO-1 (Li et al., 1995). Their use permits the separation of cells from abiotic particles and background signals in a water sample. An initial selection step is represented by the threshold, usually in the typical channel fluorescence (e.g. green fluorescence when SYBR Green I is used). In order to better visualize cells, a dot plot containing the scatter signal (FCS or SSC) against fluorescence signals (green or red fluorescence) is recommended.

Figure 5 shows a marine sample stained with SYBR Green I and analyzed by a FACScalibur flow cytometer (Becton Dickinson).

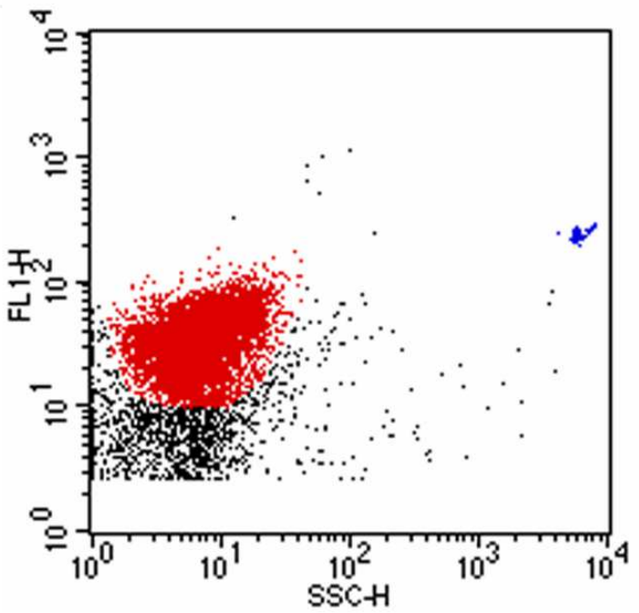



Fig. 5. Dot plot SSC *vs.* FL1 showing bacteria population stained with SYBR Green I

The affinity of the cyanine dyes, TOTO- 1 and YOYO- 1, and their monomeric equivalents, YO-PRO- 1 and TO-PRO-l, decreases significantly with increasing ionic strength, meaning that their use is inappropriate for the analysis of seawater samples (Marie et al., 1996). Other dyes, such as the SYBR Greens I and II, SYTOX Green and the SYTO family, are less dependent on medium composition and can therefore be used to count marine bacteria (Marie et al., 1999b; Lebaron et al., 1998). As SYBR Green I (SYBR-I) has a very high fluorescence yield, its use is recommended for enumerating bacteria from marine samples (Table 1).

Zubkov and collegues (2000) determined the total number of picoplankton in marine samples using the fluorochromes TOTO-1 iodide and SYBR Green I. These dyes bind strongly to nucleic acids, but SYBR Green 1 penetrates cell membranes, whereas it is necessary to use detergent to aid the penetration of TOTO-1 into cells (Li et al., 1995; Marie, et al., 1996; Marie, et al., 1997). The number of bacteria found in subsamples stained by SYBR Green I were the same as the TOTO-1 counts for the same samples. The results obtained were evidence that the intensity of fluorescence with SYBR Green 1 was greater than with TOTO-1; at the same time, SYBR Green I improved the recognition of cells with low

staining, helping the separation of their signal from the background noise level. This confirms that SYBR Green is more adaptable for the analysis of marine bacteria.

In a study reported by Gregori and colleagues (2001), SYBR Green II expresses a higher selectivity for RNA, with a quantum yield of 0.54, while also maintaining a strong affinity for double-stranded DNA, with a quantum yield of 0.36, about half that of SYBR Green I.

In 1999, Gasol and co-workers published a study on a comparison of different nucleic acid dyes and techniques, such as flow cytometric and epifluorescence microscopy. They found that Syto13 counts correlate well with DAPI and SYBR Green I counts, generating slightly lower fluorescence yields than those of the other fluorochromes. This was particularly true in seawater, meaning that, without dismissing the potential of other stains, this fluorochrome is a viable alternative to the total counting of marine planktonic bacteria.

Alonso and co-authors published (Alonso et al., 2007) a monthly study in Blanes Bay, which revealed that the abundance of heterotrophic prokaryotes (ranging from 0,5 $x10^6$ to 1,5$x10^6$ cell/ml) roughly followed the pattern of Chl-a.

In general, heterotrophic bacterial abundances followed the distribution of total picophytoplankton, revealing seasonal changes in their distribution, as reported for the subtropical northern Pacific Ocean (Campbell & Vaulot, 1993; Zubkov et al., 2000).

Lasternas and colleagues (2010) produced results from a cruise on the Mediterranean Sea during the summer of 2006. The composition and viability of pelagic communities were studied in relation to nutrient regimes and hydrological conditions. It was found that the picoplankton fraction dominated the pelagic community across the study region, with bacterioplankton being the most abundant (mean ± SE 7,73 ± 0,39$x10^5$ cells/ml) component.

## 4. Detection of viruses

Viruses control microbial and phytoplankton community succession dynamics (Fuhrman & Suttle, 1993; Suttle, 2000; Castberg et al., 2001; Weinbauer, 2004; Weinbauer & Rassoulzadegan, 2004; Sawstrom et al., 2007; Rohwer & Thurber, 2009). They also play an important role in nutrient (Wilhelm & Suttle, 1999) and biogeochemical cycling (Fuhrman, 1999; Mathias et al., 2003; Wang, et al., 2010).

Initial studies of viruses in aquatic environments were performed using either transmission electron microscopy (TEM) (Bergh et al., 1989; Borsheim et al., 1990; Sime-Ngando et al., 1996; Field, 1982) or epifluorescence microscopy (EFM) (Hennes & Suttle, 1995; Chen et al., 2001; Danovaro et al., 2008). The use of EFM combined with the development of a variety of highly fluorescent nucleic acid specific dyes soon became the accepted study method, because it involved faster and less expensive technology. Nowadays, viruses (especially bacteriophages) are still typically counted by EFM using fluorochromes such as SYBR Green I, SYBR Green II, SYBR Gold or Yo- Pro I (Xenopoulos & Bird, 1997; Marie et al., 1999a,b; Shopov et al., 2000; Hewson et al., 2001a,b,c; Chen et al., 2001; Middelboe et al., 2003; Wen et al., 2004; Duhamel & Jacquet, 2006). These techniques are selective for viruses that are infectious to a specific host, but they are very time-consuming.

In 1999, however, Marie and colleagues (Marie et al., 1999a,b) successfully proposed the use of flow cytometry for the analysis of viruses in the water column. Other authors then

applied FCM to virus studies (Marie et al., 1999a,b; Brussaard et al., 2000; Chen et al., 2001; Jacquet et al., 2002a,b).

The protocol proposed by Marie and collagues in 1999 included the use of SYBR Green I to stain virus nucleic acids. This protocol was revised and optimized by Broussard in 2004.

Viruses are too small in particle size (less than 0.5 micron) to be discriminated solely on the basis of their light scatter properties using the standard, commercially available, benchtop flow cytometers. As most flow cytometers are not designed for the analysis of these small and abundant particles, attention to detail must be paid to obtain high quality data. It is, therefore, crucial to determine the level of background noise with the use of an adequate negative control such as a 0. 2µm pore-size filtered liquid of a comparable composition.

Brussaard (2004) has shown that a variety of viruses of different morphologies and genome sizes could be detected by flow cytometry. Indeed, flow cytometry (FCM) data suggested that two virus groups (V-I and V-II) were present in natural water samples (Marie et al., 1999; Wang et al., 2010).

In their research, Wang et al. (2010) revealed a viral abundance ranging from $7{,}06 \times 10^6$ VLP $ml^{-1}$ to $5{,}16 \times 10^7$ VLP $ml^{-1}$, with the average being $2{,}47 \times 10^7$ VLP $ml^{-1}$. The V-II group was the dominant virioplankton, and had lower DNA compositions than the V-I group.

## 5. DNA content

The use of nucleic acid dyes for the detection of bacterioplankton cells revealed a tendency to cluster into distinct fractions based on differences in individual cell fluorescence (related to the nucleic acid content) and side and forward light scatter signals. There were at least two major fractions: cells with a high nucleic acid content (HNA cells) and cells with a low nucleic acid content (LNA cells) (Robertson & Button, 1989; Li et al., 1995; Marie et al., 1997; Gasol et al., 1999; Troussellier et al., 1999; Zubkov et al.,2001; Lebaron et al., 2001; Sherr et al., 2006) (Fig. 6). In a recent study, Bouvier and co-authors (2007) underlined that despite the large presence of these clusters in aquatic ecosystems (fresh to salt water, eutrophic to oligotrophic environments), there is still no consensus among scientists about their ecological significance.

The results obtained by Bouvier and others (Bouvier et al., 2007) support the notion that it is more likely that the existence of these two fractions in almost all of the bacterioplankton assemblages is the result of complex processes involving both the passage of cells from one fraction to another as well as bacterial groups that are characteristic of either HNA or LNA fractions.

The findings by Zubkov et al., (2007), which were based on the results of fluorescence *in situ* hybridization, revealed that 60% of heterotrophic sorted bacteria, with low nucleic acid content, were comprised of SAR11 clade cells.

The SAR11 clade has the smallest genome size among free-living bacteria (Giovannoni et al., 2005), and they are also the most abundant class of the bacterial ribosomal RNA genes detected in seawater DNA by gene cloning.

Many authors have presented data about the presence of HNA and LNA, not only in marine environments, but also in freshwater (Boi et al., in prep.) and in lakes (Stenuite et al., 2009).

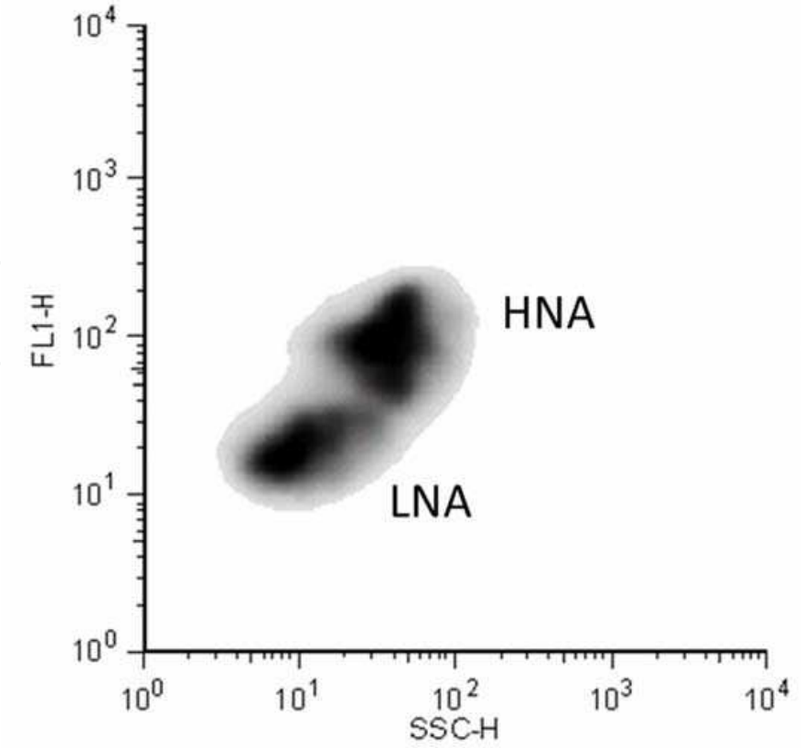



Fig. 6. Dot plot SSC *vs.* FL1 showing HNA and LNA cells stained with SYBR Green I

## 6. Physiological states

There is a wide and extensive variety of stains used in combination with FCM, with different degrees of specificity (Collier & Campell, 1999). Numerous classifications are available according to several criteria (Davey & Kell, 1996; Vives –Rego et al., 2000; Shapiro, 2000).

The most valuable source lists on fluorescent probes for flow cytometry are the *Handbook of Fluorescent Probes and Research Chemicals* (Haugland, 1996) and the catalogue of Molecular Probes, Inc. (Eugene, OR, USA; www.invitrogen.com). The current edition, which is the 11th, lists a range of dyes with different spectral characteristics and high specificities for nucleic acids.

Some fluorochromes bind specifically to cell molecules (nucleic acids, proteins and lipids) while increasing their fluorescence. Others accumulate selectively in cell compartments, or modify their properties through specific biochemical reactions in response to changes in the environment, such as pH, membrane polarization (cyanines, oxonols) or enzymatic activity (fluorogenic substrates) (Fig. 7).

A number of commercial kits are available which allow microbiologists to enumerate and determine physiological states and Gram status (Davey et al., 1999; Haugland et al., 1996; Winson & Davey, 2000).

Knowledge of the living/non-living and active/inactive states of cell populations is fundamental to understanding the role and importance of micro-organisms in natural ecosystems. Several probes, or a combination thereof, have been used to assess bacteria

physiological states (Lebaron et al., 1998; Joux & Lebaron, 2000; Gregori et al., 2001). Among others, an interesting application of FCM in microbiology is the determination of viability, even if this is one of the most fundamental properties of a cell that is difficult to define and measure.

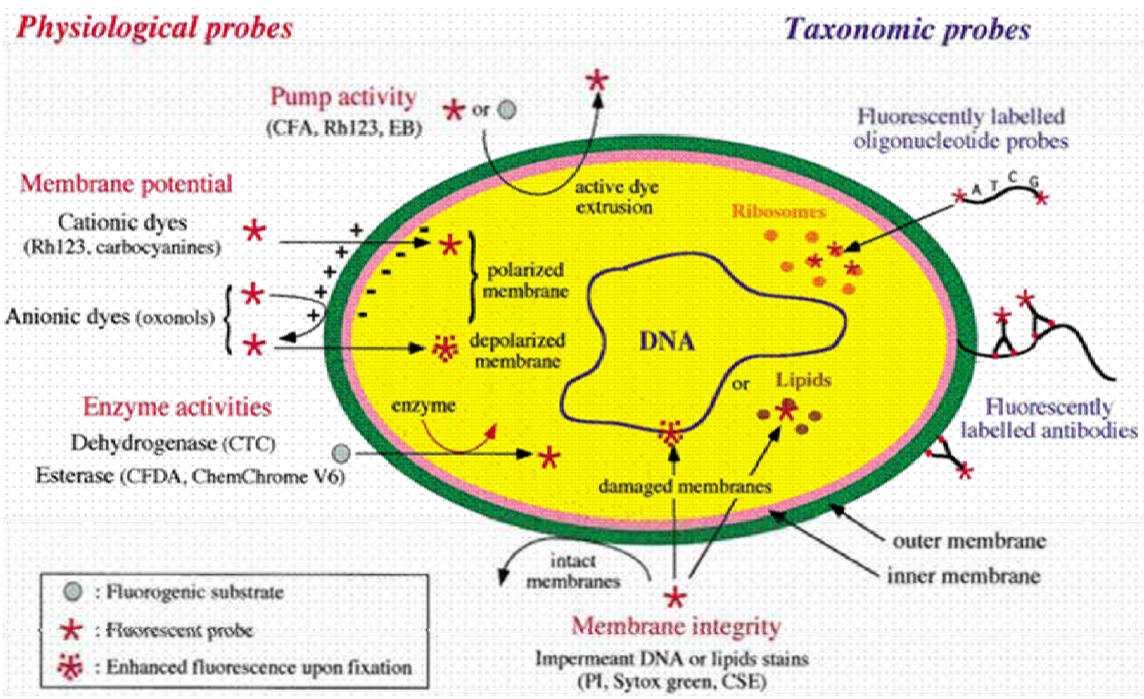



Fig. 7. Different cellular target sites for physiological and taxonomic fluorescent dyes from Joux & Lebaron, 2000

Many approaches are based on membrane integrity, such as the Life/Dead kits (e.g. the LIVE/DEAD BacLight bacterial viability kit from Molecular Probes) that are based on the rely of the propidium iodide based assessment of dead cells. Usually, a combination of SYBR Green dyes or Sytox 9 and PI is used to analyze dead cell numbers.

Barbesti and co-authors (2000) proposed a protocol for the assessment of viable cells based on nucleic acid double staining (NADS). The NADS protocol uses, simultaneously, a permeant dye, such as SYBR Green (Lebaron et al., 1998), and an impermeant one, as propidium iodide (Jones & Senft. 1985; Lopez-Amoros, 1997; Sgorbati et al., 1996; Williams et al., 1998). The efficiency of the combined staining is magnified by the energy transfer from SYBR Green to PI when both are bound to the nucleic acids, as described by Barbesti and colleagues (2000). Both dyes can be readily excited with the blue light from the laser or arc lamp of relatively simple and portable flow cytometers; the green nucleic acid probes lead to energy transfer from SYBR Green to the red PI fluorescence in the case of double staining (Barbesti et al., 2000; Falcioni et al., 2008; Manti et al., 2008). In order to better distinguish dead from viable cells, a dot plot containing fluorescence signals (green *vs* red fluorescence) is recommended (Fig. 8). Membrane intact cells that are considered to be viable emit a green fluorescence that is only due to the incorporation of SYBR Green. Cells with a damaged membrane will enable PI to enter and to bind some nucleic acids, with a corresponding increase in red and a decrease in green fluorescence.

In 2001, Gregori and co-authors optimized the double staining protocol, comparing two dyes belonging to the SYBR Green family. SYBR Green II expresses greater selectivity for RNA, while keeping a strong affinity for double-stranded DNA of about half that of SYBR Green I. The authors thus concluded that using SYBR Green II on marine samples was better.

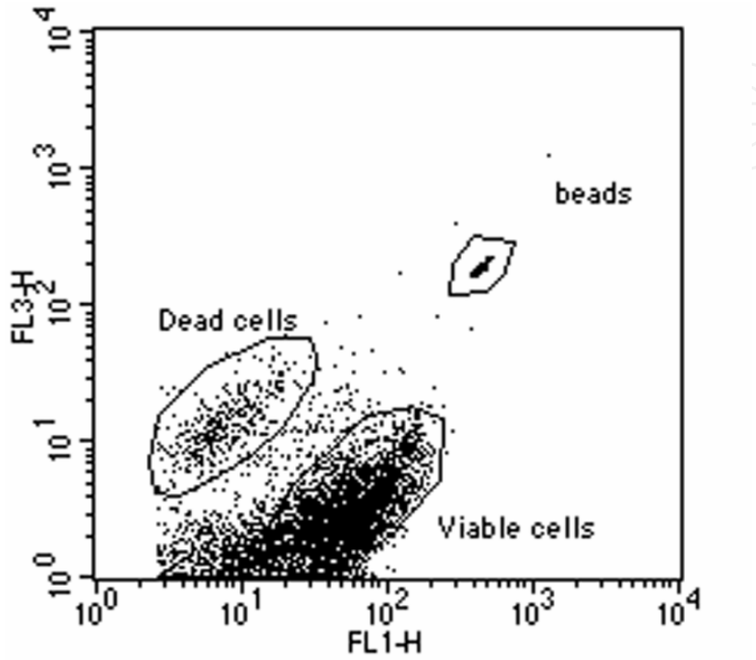



Fig. 8. Dot plot FL1 *vs.* FL3 of a marine sample stained with SYBR Green I and PI

Cell viability can be tested by assessing esterase activity or bacterial respiration. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) in flow cytometry has been used to assess ''active bacteria'' in seawater (del Giorgio et al., 1997), and is referred to cells that have an active electron transport system and are capable of reducing the tetrazolium salt (CTC) (Table 1). Because CTC is reduced to a brightly fluorescent formazan, it is possible to enumerate respiring cells with great sensitivity, precision and speed.

While the use of this method has increased over the last few years (e.g. Sherr et al. 1999; Jugnia et al., 2000; Haglund et al., 2002), there have also been a number of studies that are highly critical of CTC as a means of distinguishing metabolically active cells (e.g. Ullrich et al., 1996, 1999; Karner & Fuhrman, 1997; Servais et al., 2001). Some authors have stated that CTC could be toxic for some bacteria, while in some cases the results obtained would underestimate the real activity of bacteria, especially in natural seawater (Gasol & del Giorgio, 2000). Although abundances of CTC+ cells in natural samples tend to be well correlated to measures of either bacterial production (e.g. del Giorgio et al., 1997; Sherr et al., 1999) or respiration (Smith, 1998), the proportion of total cells scored as CTC+ tends to be too low, generally less than 20%, and sometimes less than just a few percent (Smith & del Giorgio, 2003).

5 (and 6)-carboxyfluorescein diacetate (CFDA) was employed to detect esterase activity in living cells in seawater samples. CFDA is a non-fluorescent molecule, but upon intracellular

enzymatic cleavage produces a green fluorescent compound that can be detected by FCM (Gasol & del Giorgio, 2000) (Table 1). Some authors (Yamaguchi et al., 1994; Schupp & Erlandsen, 1987; Yamaguchi & Nasu, 1997) coupled 6CFDA with proidium iodide to distinguish active from inactive cell membranes. Accordingly, after 6CFDA-PI double staining, bacterial cells with esterase activity display only green CFDA fluorescence, while damaged cells show only red PI fluorescence.

| | DYE | EX/EM | REFERENCE |
|---|---|---|---|
| NUCLEIC ACID PROBES | SYTO- 13 | 485-508/ 498-527 | Andrade et al., 2003<br>Gasol et al., 1999<br>Gasol & del Giorgio, 2000<br>Alonso et al., 2007 |
| | SYBR Green I/II | 497-520 | Lebaron et al., 1998, Gregori et al., 2001,<br>Marie et al., 1997 |
| | Propidium Iodide (PI) | 536-623 | Barbesti et al., 2000<br>Gregori et al., 2001 |
| | TOTO-1 | 509-533 | Guindulain et al., 1997; Zubkov et al., 2000 |
| DEHYDROGENASE ACTIVITY | CTC | 480/580-660 | Gasol et al., 1995, Sherr et al. 1999, Servais et al., 2001; Pearce et al., 2007 |
| ENZIMATIC ACTIVITY | CFDA | 492/517 | Gregori et al., 2001; Pearce et al., 2007 |

Table 1. Shows some available dyes used for the analysis of marine micro-organisms, their excitation and emission maximal wavelengths, along with some selected references

Another interesting application of FCM to microbiology requires the use of fluorochromes conjugated to antibodies or oligonucleotides for the detection of microbial antigens or DNA and RNA sequences to directly (Vives-Rego et al., 2000; Amann et al., 1990a; Marx et al., 2003; Temmerman et al., 2004) identify micro-organisms in natural ecosystems (Amann et al., 1990b; Amann et al., 2001; Wallner et al., 1997; Biegala et al., 2003).

## 7. Bacteria identification with antibodies and nucleic acid probes (FISH)

Immunodetection techniques utilize the specificity of the antibody/antigen association as a probe for recognizing and distinguishing between micro-organisms. Parallel, immunological detection methods can provide quantitative data, including in relation to the sensitivity of the method used. The application of immunology in phytoplankton research started when Bernhard and co-authors (1969) developed antibodies against two species of diatoms, but it was in the 1980s that immunological techniques for species identification were actually applied in marine research. The first species investigated were prokaryotes (Dahl & Laake, 1982; Campbell et al., 1983); later Hiroish et al. (1988) and Shapiro et al. (1989) conducted studies on eukaryotic organisms.

The use of antibodies in combination with FCM is a powerful tool for the specific detection and enumeration of micro-organisms in medical, veterinary and environmental microbiology

(Cucci & Robbins, 1988; Porter et al., 1993; Vrieling *et al*., 1993a; McClelland & Pinder, 1994; Vrieling & Anderson, 1996; Kusunoki et al., 1998; Chitarra et al., 2002). Antibodies also have a role to play in determinations of the physiological characteristics of cells; Steen and colleagues used fluorescently labelled antibodies as part of a flow cytometric method of antigenicity determination (Steen et al., 1982) that may vary according to growth conditions (Davey & Winson, 2003).

The availability of antibodies against bacteria is limited mostly to the research and identification of pathogens (e.g. Kusunoki et al., 1996; Kusunoki et al., 1998; McClelland & Pinder, 1994; Tanaka et al., 2000).

Barbesti and colleagues (Barbesti et al., 2000) performed bacterial viability measurement and identification tests using a Cy5-labelled monoclonal antibody combined with SYBR Green I and propidium iodide.

A recent study (Manti et al., 2010) conducted in natural seawater samples reports the immunodetection of *Vibrio parahaemolyticus* and an examination of the specificity and sensitivity of the polyclonal antibody used.

As described above for antibodies, oligonucleotides allow the detection and recognition of micro-organisms in a mixed population. The phylogenetic heterogeneity of micro-organisms can be studied with analyses of ribosomal RNA sequences. Fluorescence *in situ* hybridization (FISH) is based on the omology of an oligonucletide probe with a target region in an individual microbial cell.

In natural samples, however, the signal derived from the use of labelled oligonucleotide probes is often undetectable because of the low rRNA content. Among other methods, FISH with horseradish peroxidase (HRP)-labelled oligonucleotide probes and tyramide signal amplification, also known as catalyzed reporter deposition (CARD), is especially suitable for aquatic habitats with small, slow growing, or starving bacteria (Diaz et al., 2007).

Oligonucleotide probes labelled (directly or indirectly) with fluorescent markers can be detected by epifluorescence and confocal microscopy, or by flow cytometry (Giovannoni et al, 1988; De Long et al, 1989; Amann et al., 1990a; 1990b; 2001; Pernthaler et al., 2001). Several publications have reported the combination of rapidity and the multi-parametric accuracy of flow cytometry, with the phylogenetic specificity of oligonucleotide FISH probes as a powerful emerging tool in aquatic microbiology (Yentsch & Yentsch, 2008; Hammes & Egli, 2010; Muller & Vebe-Von-Caron, 2010; Wang et al., 2010).

The combination of FCM and FISH has been successfully applied to describe microbial populations dispersed in a liquid suspension derived from different media (Lim et al., 1993; Joachimsthal et al., 2004; Rigottier-Gois et al., 2003; Barc et al., 2004; Lange et al., 1997; Wallner et al., 1993 and 1995; Miyauchi et al., 2007).

Only a few studies (Lebaron et al., 1997; Gerdts & Luedk, 2006; Kalyuzhnaya et al., 2006; Yilmaz et al., 2010) have combined FISH and FCM for the analysis of acquatic microbial communities. The main limitation of combining CARD-FISH and FCM is that the former is commonly performed and optimized on a solid support (i.e. polycarbonate membrane filters; Pernthaler et al., 2002), while the latter requires liquid samples with a well dispersed suspension of single cells (Shapiro, 2000). Schonhuber and co-authors (1997) have bridged the two methodologies while working with liquid suspensions, although the proposed

permeabilization procedure was not ideal for the detection of large bacterial groups with different cell walls. Meanwhile, Biegala and colleagues (2003) successfully performed a CARD-FISH-FCM protocol for the detection of marine picoeukaryotes, while Sekar and co-authors (2004) proposed the enumeration of bacteria by flow cytometry identified by *in situ* hybridization.

A recent study (Manti et al., 2011) proposed an improved protocol for the flow cytometric detection of CARD-FISH stained bacterial cells, remarking on the importance of improving the identification and quantification of phylogenetic populations within heterogeneous, natural microbial communities.

## 8. Conclusions

Flow cytometry is a powerful technique with a wide variety of potential applications in marine microbiology. Due to its characteristics, FCM has contributed to the knowledge of free living planktonic microbial community structures and their distribution.

The employment of new techniques and probes normally used in other ecosystems or in clinical microbiology could enhance the field of application of flow cytometry and so the studies of marine assemblages.

Furthermore, modern flow cytometers also provide quantitative data and image analyses for the detection of microbial subgroups, thereby extending the field of flow cytometry applications (Andreatta et al., 2004; Olson & Sosik, 2007).

Last but not least, the development of a portable and cheap flow cytometer, and/or imaging system with a reliable interpretation may render the monitoring of microbial communities in marine ecosystems faster and efficient.

## 9. References

Alonso-Saez, L., Balaguè, V., Sa, E.L., Sanchez, O., Gonzalez, J.M., Pinhassi, J., Massana, R., Pernthaler, J., Pedros-Aliò, C. & Gasol, J.M. (2007). Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiology and Ecology*, 60, pp. 98–112

Amann R., Krumholz L. & Stahl, D.A. (1990b). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, 172, pp. 762-770

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. & Stahl, D.A. (1990a). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied Environmental Microbiology,* 56, pp. 1919–1925

Amann, R.I., Fuchs, B.M. & Behrens, S. (2001). The identification of micro-organisms by fluorescence in situ hybridization. *Current Opinion in Microbiology,* 12, pp. 231–236

Andrade, L., Gonzalez, A.M., Araujo, F.V. & Paranhos, R. (2003). Flow cytometry assessment of bacterioplankton in tropical marine environments. *Journal of Microbiological Methods*, 55, pp. 841-850

Andreatta, S., Wallinger, M.M., Piera, J., Catalan, J., Psenner, R., Hofer, J.S. & Sommaruga, R. (2004). Tools for discrimination and analysis of lake bacterioplankton subgroups

measured by flow cytometry in a high-resolution depth profile. *Aquatic Microbial Ecology*, 36, pp. 107-115

Barbesti S., Citterio S., Labra M., Baroni M.D., Neri M.G. & Sgorbati, S. (2000). Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. *Cytometry,* 40, pp. 214-218

Barc, M.C., Bourlioux, F., Rigottier-Gois, L., Charrin-sarnel, C., Janoir, C., Boureau, H., Doré, J. & Collignon, A. (2004). Effect of amoxicillin-Clavulanic acid on human fecal flora in a gnotobiotic mouse assessed with fluorescence hybridization using group-specific 16S rRNA probes in combination with flow cytometry. *Antimicrobial Agents Chemotherapy*, 48, pp. 1365–1368

Bergh, O., Borsheim, K.Y., Bratbak, G. & Heldal, M. (1989). High abundances of viruses found in aquatic environments. *Nature,* 340, pp. 467–468

Bergquist, P.L., Hardiman, E.H., Ferrari, B.C. & Winsley, T. (2009). Applications of flow cytometry in environmental microbiology and biotechnology. *Extremophiles*, 13, pp. 389-401

Bernhard, M.B., Lomi, G., Riparbelli, G., Saletti, M. & Zattera, A. (1969). Un metodo immunologico per la caratterizzazione del fitoplancton. *Estratto dalle Pubblicazioni Stazione Zoologica Napoli*, 37, pp. 64-72

Biegala, I.C., Not, F., Vaulot, D. & Simon, N. (2003). Quantitative assessment of picoeucaryotes in natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification-fluorescence in situ hybridization and flow cytometry. *Applied Environmental Microbiology,* 69, pp. 5519–5529

Borsheim, K.Y., Bratbak, G. & Heldal, M. (1990). Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. *Applied Environmental Microbiology*, 56, pp. 352– 356

Bouvier, T., del Giorgio P. A. & Gasol, J.M. (2007). A comparative study of the cytometric characteristics of High and Low nucleic-acid bacterioplankton cells from different aquatic ecosystems. *Environmental Microbiology*, 9(8), pp. 2050–2066

Brando B., Barnett D., Janossy G., Mandy F., Autran B., Rothe G., Scarpati B., D'Avanzo G., D'Hautcourt J.L., Lenkei R., Schmitz G., Kunkl A., Chianese R., Papa S. & Gratama, J.W. (2000). Cytofluorimetric methods for assessing absolute numbers of cell subsets in blood. *Cytometry.* 42, pp. 327-346

Brussaard, C.P.D. (2004). Optimization of Procedures for Counting Viruses by Flow Cytometry. *Applied Environmental Microbiology*, 70, pp. 1506– 1513

Brussaard, C.P.D., Marie, D. & Bratbak, G. (2000). Flow cytometric detection of viruses. *Journal of Virology Methods,* 85, pp. 175–182

Burkill P.H., Mantoura, R.F.C. & Cresser, M. (1990). The rapid analysis of single marine cells by flow cytometry. *Philosophical Transactions of the Royal Society,* 333, pp. 99-112

Burkill, P.H. (1987). Analytical flow cytometry and its application to marine microbial ecology, In: *Microbes in the sea*, Sleigh, M.A., pp. 139-166, Wiley, New York, Chichester

Callieri, C. (1996). Extinction coefficient of red, green and blue light and its influence on picocyanobacterial types in lakes at different trophic levels. *The journal Memorie dell'Istituto Italiano di Idrobiologia*, 54, pp. 135-142

Callieri, C. & Stockner, J.G. (2002). Freshwater autotrophic picoplankton: a review. *Journal of Limnology* 61, 1-14

Campbell, L. & Vaulot, D. (1993). Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station ALOHA). *Deep Sea Research Part I: Oceanographic Research Papers*, 40, pp. 2043-2060

Campbell, L., Carpenter, E.J. & Iacono, V.I. (1983). Identification and enumeration of marine chroococcoid cyanobacteria by immunofluorescence. *Applied Environmental Microbiology*, 46, pp. 553-559

Campbell, L., Nolla, H. A. & Vaulot, D. (1994). The importance of Prochlorococcus to community structure in the central North Pacific Ocean. *Limnololy and Oceanography,* 39, pp. 954–961

Campbell, L., Liu, H., Nolla, H. A. & Vaulot, D. (1997). Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean and Station ALOHA during the 1991–1994 ENSO event. *Deep Sea Research*. 44, pp.167–192

Cantineaux, B., Courtoy, P. & Fondu, P. (1993). Accurate flow cytometric measurement of bacteria concentrations. *Pathobiology*, 61, pp. 95-97

Castberg, T., Larsen, A., Sandaa, R.A., Brussaard, C.P.D., Egge, J.K., Heldal, M., Thyrhaug, R., van Hannen, E.J. & Bratbak, G. (2001). Microbial population dynamics and diversity during a bloom of the marine coccolithophorid *Emiliania huxleyi* (Haptophyta). *Marine Ecology Progress Series,* 221, pp. 39–46

Castenholz, R. W. & Waterbury, J. B. (1989). Group I. Cyanobacteria, In: *Bergey's Manual of Systematic Bacteriology*, Staley, J.T., Bryant, M.P., Pfennig, N. & Holt, J.G., pp. 1710-1728, Williams &Wilkins, Baltimore

Chattopadhyay, P.K., Hogerkorp C.M. & Roederer, M. (2008). A chromatic explosion: the development and future of multiparameter flow cytometry. *Immunology*, 125, pp. 441–449

Chen, F., Lu, J.-R., Binder, B.J., Liu, Y.C. & Hodson, R.E., (2001). Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR Gold. *Applied Environmental Microbiology,* 67, pp. 539–545

Chisholm, S.W., Olson, R.J. Zettler, R., Goericke, R., Waterbury, J.B. & Welschmeyer, N.A. (1988). A novel free-living prochlorophyte abundant in the oceanic euphotic zone. *Nature*, 334, pp. 340-343

Chitarra, L.G., Langerak, C.J., Bergervoet, J.H.W. & Bulk, R.W. (2002). Detection of the plant pathogenic bacterium Xanthomonas campestris pv. campestris in seed extracts of Brassica sp. applying fluorescent antibodies and flow cytometry. *Cytometry,* 47, pp. 118–126

Collier, J.L. & Campbell, L. (1999). Flow cytometry in molecular aquatic ecology. *Hydrobiologia,* 401, pp. 33-53

Craig, S.R. (1985). Distribution of algal picoplankton in some European freshwaters, *Proceedings of 2nd International Phycology Congress*, Copenhagen, August 1985

Cucci, T.L. & Robins, D. (1988). Flow cytometry and immunofluorescence in aquatic sciences, In: *Immunochemical approaches to coastal, estuarine and oceanographic questions*, Yentsch, C.M., Mague F.C., & Horan P.K., pp. 184-193, Springer- Verlag, Berlin

Dahl, A.B. & Laake, M. (1982). Diversity dynamics of marine bacteria studied by immunofluorescent staining on membrane filters. *Applied Environmental Microbiology*, 43, pp. 169-176

Danovaro, R., Dell'Anno, A., Corinaldesi, C., Magagnini, M., Noble, R., Tamburini, C. & Weinbauer, M. (2008). Major viral impact on the functioning of benthic deep-sea ecosystems. *Nature*, 454, pp. 1084-1087

Danovaro, R., Corinaldesi, C., Dell'Anno, A., Fuhrman J.A., Middelburg, J.J., Noble, R.T. & Suttle, C.A. (2011). Marine viruses and global climate change. *FEMS Microbiology Reviews,* 35, pp. 993–1034

Das, S., Lyla P.S. & Khan, S.A. (2006). Marine microbial diversity and ecology: importance and future perspectives. *Current Science*, 90, pp. 1325-1335

Davey, H.M. (2010). Prospects for the automation of analysis and interpretation of flow cytometric data. *Cytometry Part A*, 77, pp. 3-5

Davey, H.M. & Kell, D.B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiology and Molecular Biology Reviews,* 60, pp. 641-696

Davey, H.M. & Winson, M.K. (2003). Using Flow Cytometry to Quantify Microbial Heterogeneity. *Current Issues in Molecular Biology*, 5, pp. 9-15

Davey, H.M., Kaprelyants, A.S., Weichart, D.H. & Kell, D.B. (1999). Microbial Cytometry, In: *Current protocols in cytometry*, J. P. E. A. Robinson, John Wiley & Sons, Inc., New York. N.Y

del Giorgio, P.A., Prairie, Y.T. & Bird, D.F. (1997). Coupling Between Rates of Bacterial Production and the Abundance of Metabolically Active Bacteria in Lakes, Enumerated Using CTC Reduction and Flow Cytometry. *Microbial Ecology*, 34, pp. 144–154

DeLong, E.F., Wickham, G.S. & Pace, N.R. (1989). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science,* 243, pp. 1360-1363

Diaz, E. González, T., Joulian, C. & Amils, R. (2007). The Use of CARD-FISH to Evaluate the Quantitative Microbial Ecology Involved in the Continuous Bioleaching of a Cobaltiferrous Pyrite. *Advanced Materials Research,* 21, pp. 565-568

Diaz, M., Herrero, M., Garcia, L.A. & Quiros, C. (2010). Application of flow cytometry to industrial microbial bioprocesses. *Biochemical Engineering Journal*, 48, pp. 385–407

Dortch, Q. & Postel, J.R. (1989). Biochemical indicators of N utilization by phytoplankton during upwellings off the Washington coast. *Limnology and Oceanography*, 34, pp. 758-773

Duhamel, S. & Jacquet, S. (2006). Flow cytometric analysis of bacteria- and virus-like particles in lake sediments. *Journal of microbiological methods,* 64, pp. 316-332

Falcioni, T., Papa, S. & Gasol, J.M. (2008). Evaluating the Flow-Cytometric Nucleic Acid Double-Staining Protocol in Realistic Situations of Planktonic Bacterial Death. *Applied Environmental Microbiology*, 74, pp. 1767-1779

Fenchel, T. (1988). Marine plankton food chains. *Annual Review of Ecology, Evolution, and Systematics*, 19, pp. 19-38

Field, A.M. (1982). Diagnostic virology using electron microscopy. *Advances in virus research,* 27, pp. 1 –69

Fuhrman, J.A. (1999). Marine viruses and their biogeochemical and ecological effects. *Nature,* 399, pp. 541–548

Fuhrman, J.A. & Suttle, C.A. (1993). Viruses in marine planktonic systems. *Oceanography,* 6, pp. 51–63

Gasol J.M., del Giorgio P.A. & Duarte, C.M. (1997). Biomass distribution in marine planktonic communities. *Limnology and Oceanography*, 45, pp. 789-800

Gasol J.M., del Giorgio P.A., Massana R. & Duarte, C.M. (1995). Active versus inactive bacteria: size-dependence in a coastal marine plankton community. *Marine Ecology Progress Series,* 128, pp. 91-97

Gasol, J.M., Zweifel U.L., Peters, F., Fuhrman, J.A. & Hagstro, A. (1999). Significance of Size and Nucleic Acid Content Heterogeneity as Measured by Flow Cytometry in Natural Planktonic Bacteria. *Applied Environmental Microbiology*, 65, pp. 4475–4483

Gasol, J.P. & Del Giorgio, P.A. (2000). Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina*, 64, pp. 197-224

Gerdts, G. & Luedk, G. (2006). FISH and chips: Marine bacterial communities analyzed by flow cytometry based on microfluidics. *Journal of Microbiolial Methods*, 64, pp. 232–240

Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D., Bibbs, L., Eads, J., Richardson, T.H., Moordewier, M., Rappè, M.S., Short, J.M., Carrington, J.C. & Mathur, E.J. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science,* 309, pp. 1242–1245

Giovannoni, S.J.,Delong, E.F., Olsen, G.J. & Pace, N.R. (1988). Phylogenetic Group-Specific Oligodeoxynucleotide Probes for Identification of Single Microbial Cells. *Journal of Bacteriology*, 170, pp. 720-726

Goericke, R. & Repeta, D.J. (1993). Chlorophylls a and b and divinyl-chlorophylls a and b in the open subtropical North Atlantic Ocean. *Marine Ecology Progress Series*, 101, pp. 307-313

Gregori, G., Citterio, S., Ghiani, A., Labra, M., Sgorbati, S., Brown, S. & Denis, M. (2001). Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. *Applied and Environmental Microbiology,* 67, pp. 4662-4670

Guindulain T., Comas, J. & Vives-Rego, J. (1997). Use of Nucleic Acid Dyes SYTO-13, TOTO-1, and YOYO-1 in the Study of Escherichia coli and Marine Prokaryotic Populations by Flow Cytometry. *Applied and Environmental Microbiology,* 63, pp. 4608-4611

Haglund, A.L., Tornblom, E., Bostrom, B. & Tranvik, L. (2002). Large differences in the fraction of active bacteria in plankton, sediments, and biofilm. *Microbial Ecology*, 43, pp. 232–241

Hammes, F. & Egli, T. (2010). Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. *Analytical and bioanalytical chemistry,* 397, pp. 1083–1095

Haugland, R. P. (1996). In: *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Spence, M.T.Z., Molecular Probes, Eugene, OR

Hennes, K.P. & Suttle, C.A. (1995). Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. *Limnololgy and Oceanography*, 40, pp.1050–1055.

Hewitt, C. J. & Nebe-Von-Caron, G. (2004). The application of multi-parameter flow cytometry to monitor individual microbial cell physiological states. *Advances in Biochemical Engineering/Biotechnol*ogy, 89, pp. 197-223

Hewson, I., O'Neil, J.M. & Dennison, W.C. (2001a). Virus-like particles associated with Lyngbya majuscula (Cyanophyta; Oscillatoria) bloom decline in Moreton Bay, Australia. *Aquatic Microbial Ecology*, 25, pp. 207– 213

Hewson, I., O'Neil, J.M., Furhman, J.A. & Dennison, W.C. (2001c). Virus-like particle distribution and abundance in sediments and overlying waters along eutrophication gradients in two subtropical estuaries. *Limnology and Oceanography,* 47, pp. 1734– 1746

Hewson, I., O'Neil, J.M., Heil, C.A., Bratbak, G. & Demison, W.C. (2001b). Effects of concentrated viral communities on photosynthesis and community composition of co-occurring benthic microalgae and phytoplankton. *Aquatic Microbial Ecology*, 25, pp. 1-10

Hiroishi, S., Uchida, A., Nagasaki, K. & Ishida, Y. (1988). A new method for identification of inter- and intra-species of the red tide algae Chattonella antiqua and Chatonella marina (Raphidophyceae) by means of monoclonal antibodies. *Journal of Phycology*, 24, pp. 442-444

Ingram, M., Cleary, T. J, Price, B. J. & Castro, A. (1982). Rapid detection of Legionella pneumophila by flow cytometry. *Cytometry*, 3, pp. 134–147

Jacquet, S., Havskum, H., Thingstad, F.T. & Vaulot, D. (2002a). Effect of inorganic and organic nutrient addition on a coastal microbial community (Isefjord, Denmark). *Marine Ecology Progress Series,* 228, pp. 3–14

Jacquet, S., Heldal, M., Iglesias-Rodriguez, D., Larsen, A., Wilson, W. & Bratbak, G., (2002b). Flow cytometric analysis of an Emiliana huxleyi bloom terminated by viral infection. *Aquatic Microbial Ecology,* 27, pp. 11-124

Joachimsthal, E.L., Ivanov, V., Tay, S.T.-L. & Tay, J.-H. (2004). Bacteriological examination of ballast water in Singapore harbor by flow cytometry with FISH. *Marine Pollution Bulletin*, 423, pp. 334–343

Jones, K.H. & Senft, J.A. (1985). An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *Journal of Histochemistry and Cytochemistry*, 33, pp. 77–79

Joux, F. & Lebaron, P. (2000). Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection*, 2, pp. 1523−1535

Jugnia, L.B., Richardot, M., Debroas, D., Sime-Ngando, T.S. & Devaux, J. (2000). Variations in the number of active bacteria in the euphotic zone of a recently flooded reservoir. *Aquatic Microbial Ecology*, 22, pp. 251–259

Kalyuzhnaya, M.G., Zabinsky, R., Bowerman, S., Baker, D.R., Lidstrom, M.E. & Chistoserdova, L. (2006). Fluorescence in situ hybridization-flowcytometry-cell sorting-based method for separation and enrichment of type I and 335 type II methanotroph populations. *Applied and Environmental Microbiology,* 72, pp. 4293–4301

Karner, M. & Fuhrman, J.A. (1997). Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. *Applied and Environmental Microbiology,* 63, pp. 1208–1213

Kusunoki, H., Kobayashi, K., Kita, T., Tajima, T., Sugii, S. & Uemura, T. (1998). Analysis of enterohemorrhagic Escherichia coli serotype 0157: H7 by flow cytometry using monoclonal antibodies. *Journal of Veterinary Medical Science*, 60, pp. 1315–1319

Kusunoki, H., Tzukamoto, T., Gibas, C.F.C., Dalmacio, I.F. & Uemura, T. (1996). Application of flow cytometry for the detection of Escherichia coli O157. *Journal of Food Hygiene Japanese Society*, 37, pp. 390-394

Lange, J.L., Thorne, P.S. & Lynch, N. (1997). Application of flow cytometry and fluorescent in situ hybridization for assessment of exposures to airborne bacteria. *Applied and Environmental Microbiology,* 63, pp. 1557–1563

Lasternas, S., Agustí, S. & Duarte, C.M. (2010). Phyto- and bacterioplankton abundance and viability and their relationship with phosphorus across the Mediterranean Sea. *Aquatic Microbial Ecology,* 60, pp. 175-191

Lebaron, P., Català, P., Fajon, C., Joux, F., Baudart, J. & Bernard, L. (1997). A new sensitive, whole-cell hybridization technique for the detection of bacteria involving a biotinylated oligonucleotide probe targeting rRNA and tyramide signal amplification. *Applied and Environmental Microbiology,* 63, pp. 3274-3278

Lebaron, P., Parthuisot, N. & Catala, P. (1998). Comparison of Blue Nucleic Acid Dyes for Flow Cytometric Enumeration of Bacteria in Aquatic Systems. *Applied and Environmental Microbiology,* 64, pp. 1725–1730

Lebaron, P., Servais, P., Agogue, H., Courties, C. & Joux, F. (2001a). Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Applied Environmental Microbiology,* 67, pp. 1775–1782

Legendre, L., Courties, C. & Troussellier, M. (2001). Flow cytometry in oceanography 1989-1999: environmental challenges and research trends. *Cytometry,* 44, pp. 164-172

Li, W. K. W., Jellett, J. F. & Dickie, P. M. (1995). DNA distribution in planktonic bacteria stained with TOTO or TO-PRO. *Limnology and Oceanography,* 40, pp. 1485–1495

Li, W.K.W. (1995). Composition of ultraphytoplankton in the central North Atlantic. *Marine Ecology Progress Series*, 122, pp. 1–8

Lim, E.L., Amaral, L.A., Caron, A. & Delong, F. (1993). Application of rRNA-Based Probes for Observing Marine Nanoplanktonic Protists. *Applied and Environmental Microbiology,* 59, pp. 1647-1655

Lindell, D. & Post, A.F. (1995). Ultraphytoplankton succession is triggered by deep winter mixing in the Gulf of Aqaba (Eilat), Red Sea. *Limnology and Oceanography,* 40, pp. 1130–1141

Longobardi Givan, A. (2001). Flow Cytometry: First Principles. In: *Current protocols in cytometry*, J. P. E. A. Robinson, John Wiley & Sons, Inc., New York. N.Y

Lopez-Amoros, R., Castel, S., Comas-Riu, J. & Vives-Rego, J. (1997). Assessment of Escherichia coli and Salmonella viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123:DiBAC4(3), propidium iodide, and CTC. *Cytometry,* 29, pp. 298–305

Mansour, J.D., Robson, J.A., Arndt, C.W. & Schulte, T.H. (1985). Detection of Escherichia coli in blood using flow cytometry. *Cytometry*, 6, pp. 186–190

Manti, A., Boi, P., Amalfitano, S., Puddu, A. & Papa, S. (2011). Experimental improvements in combining CARD-FISH and flow cytometry for bacterial cell quantification. *Journal of Microbiological Methods*, 87, pp. 309-315

Manti, A., Boi, P., Falcioni, T., Canonico, B., Ventura, A., Sisti, D., Pianetti, A., Balsamo, M. & Papa, S. (2008). Bacterial Cell Monitoring in Wastewater Treatment Plants by Flow Cytometry. *Water Environmental Research*, 80, pp. 346–354

Manti, A., Falcioni, T., Campana, R., Sisti, D., Rocchi, M. Medina, V., Dominici, S., Papa S. & Baffone, W. (2010). Detection of environmental Vibrio parahaemolyticus using a polyclonal antibody by flow cytometry. *Applied Environmental Reports*, 2, pp. 158-165

Marie, D., Bruussard, C., Bratbak, G. & Vaulot, D., (1999a). Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied Environmental Microbiology,* 65, pp. 45– 52

Marie, D., Partensky, F., Jacquet, S. & Vaulot, V. (1997). Enumeration and Cell Cycle Analysis of Natural Populations of Marine Picoplankton by Flow Cytometry Using the Nucleic Acid Stain SYBR Green I. *Applied and Environmental Microbiology,* 63, pp. 186–193

Marie, D., Partensky, F., Vaulot, D. & Brussaard, C.P. (1999b). Enumeration of phytoplankton, bacteria, and viruses in marine samples, In: *Current protocols in cytometry*, J. P. E. A. Robinson, John Wiley & Sons, Inc., New York. N.Y

Marie, D., Vaulot, D. & Partensky, F. (1996). Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. *Applied and Environmental Microbiology*, 62, pp. 1649–1655

Martinez, O.V., Gratzner, H.G, Malinin, T.I. & Ingram, M. (1982). The effect of some beta-lactam antibiotics on *Escherichia coli* studied by flow cytometry. *Cytometry*, 3, pp. 129–133

Marx, A., Hewitt, C.J., Grewal, R., Scheer, S., Vandre, K., Pfefferle, W., Kossmann, B., Ottersbach, P., Beimfohr, C., J. Snaidr, Auge, C. & Reuss, M. (2003). Anwendungen der Zytometrie in der Biotechnologie, *Chemie Ingenieur Technik,* 75, pp. 608–614

Mathias, M., Lasse, R., Grieg, F.S., Vinni, H. & Ole, N. (2003). Virus-induced transfer of organic carbon between marine bacteria in a model community. *Aquatic Microbial Ecology,* 33, pp. 1–10

Mattanovich, D. & Borth, N. (2006). Applications of cell sorting in biotechnology, *Microbial Cell Factories,* 5, pp. 1–11

McClelland, R.G. & Pinder, A.C. (1994). Detection of low levels of specific Salmonella species by fluorescent antibodies and flow cytometry. *Journal of Applied Bacteriology*, 77, pp. 440–447

Middelboe, M., Glud, R.N. & Finster, K. (2003). Distribution of viruses and bacteria in relation to diagenic activity in an estuarine sediment. *Limnology and Oceanography*, 48, pp. 1447–1456

Miyauchi, R., Oki, K., Aoi, Y. & Tsuneda, S. (2007). Diversity of Nitrite Reductase Genes in "Candidatus Accumulibacter phosphatis"-Dominated Cultures Enriched by Flow Cytometric Sorting. *Applied Environmental Microbiology,* 73, pp. 5331–5337

Muller, S. & Nebe-von-Caron, G. (2010). Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. *FEMS Microbial Ecology*, 34, pp. 554–587

Olson, R. J., Zettler, E.R. & Anderson, O.K. (1989). Discrimination of eukaryotic phytoplankton cell types from light scatter and autofluorescence properties measured by flow cytometry. *Cytometry,* 10, pp. 636–643

Olson, R. J., Zettler, E. R. & DuRand M.D. (1993). Phytoplankton analysis using flow cytometry, In: *Handbook of methods in aquatic microbial ecology*, Kemp, P. F., Sherr, B. F., Sherr E. B., & Cole, J. J., Lewis pp. 175-186, Boca Raton, FL

Olson, R.J. & and Sosik, H.M. (2007). A submersible imaging-in-flow instrument to analyze nano and microplankton: Imaging FlowCytobot. Limnology and Oceanograohy: Methods, 5, pp. 195–203

Olson, R.J., Chisholm, S.W., Zettler, E.R. Altabet, M.A. & Dusenberry, J.A. (1990). Spatial and temporal distributions of prochlorophyte picoplankton in the North Atlantic Ocean. *Deep-Sea Research*, 37, pp. 1033- 1051

Packard, T.T. (1985) Measurements of electron transport activity in microplankton. *Advances in Aquatic* Microbiology, 3, pp. 207–261

Partensky, F., Blanchot, J., Lantoine, F., Neveux, J. & Marie, D. (1996). Vertical structure of picophytoplankton at different trophic sites of the tropical northeastern Atlantic Ocean. *Deep Sea Research*, 43, pp. 1191–1213

Paul, J.H. (2001). Marine microbiology, In: *Methods of microbiology*. Whitton, B., & Potts, M., pp. 563–589, The Netherlands: Kluwer Academic Publishers, Elsevier Academic press

Pearce, I., Davidson, A. T., Bell, E. M. & Wright, S. (2007). Seasonal changes in the concentration and metabolic activity of bacteria and viruses at an Antarctic coastal site. *Aquatic Microbial Ecology,* 47, pp. 11–23

Pernthaler, A., Pernthaler, J. & Amann, R. (2002). Fluorescence 359 in situ hybridization and catalysed reporter deposition for the identification of marine bacteria. *Applied Environmental Microbiolology*, 68, pp. 3094–3101

Pernthaler, J., Glöckner, F.O., Schönhuber, W. & R. Amann. (2001). Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Methods in Microbiology,* 30, pp. 208-210

Phinney, D. A. & Cucci, T.L. (1989). Flow cytometry and phytoplankton. *Cytometry*, 10, pp. 511-521

Porter, J., Deere, D., Hardman, M., Clive, E. & Pickup, R. (1997). Go with the flow - use of flow cytometry in environmental microbiology. *Microbiology Ecology*, 24, pp. 93-101

Porter, J., Edwards, C., Morgan, A.W. & Pickup, R.W. (1993). Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. *Applied Environmental Microbiology,* 59, pp. 3327–3333

Rigottier-Gois, L., Le Bourhis, A.G., Gramet, G., Rochet, V. & Doré, J. (2003). Fluorescenthybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. *FEMS Microbiology and Ecology*, 43, pp. 237–245.

Rippka, R. (1988). Recognition and identification of cyanobacteria. *Methods in enzymology,* 167, pp. 28-67

Robertson, B.R. & Button, D.K. (1989). Characterizing aquatic bacteria according to population, cell-size, and apparent DNA content by flow-cytometry. *Cytometry,* 10, pp. 70–76

Robinson, J.P. (2004). Flow cytometry, In: *Encyclopaedia of Biomaterials and Biomedical Engineering,* Bowlin, G.L., & Wnek, G., pp. 630–640, Marcel Dekker, Inc., New York

Rohwer, F. & Thurber, R.V. (2009). Viruses manipulate the marine environment. *Nature,* 459, pp. 207–212

Sawstrom, C., Graneli, W., Laybourn-Parry, J. & Anesio, A.M. (2007). High viral infection rates in Antarctic and Arctic bacterioplankton. *Environmental Microbiology*, 9, pp. 250–255

Schonhuber, W., Fuchs, B., Juretschko, S. & Amann, R. (1997). Improved Sensitivity of whole-Cell Hybridization by the combination of Horseradish Peroxidase-Labeled Oligonucleotides and Tyramide Signal Amplification. *Applied Environmental Microbiology,* 63, pp. 3268–3273

Schupp, D.G. & Erlandsen, S.L. (1987). A new method to determine Giardia cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. *Applied Antibacterial and Antifungal Agents,* 22, pp. 65–68

Sekar, R., Fuchs, B.M., Amann, R. & Pernthaler, J. (2004). Flow sorting of marine bacterioplankton after fluorescence in situ hybridization. *Applied Environmental Microbiology*, 70, pp. 6210-6219

Servais, P., Agoguè, H., Courties, C., Joux, F. & Lebaron, P. (2001). Are the actively respiring cells (CTC+) those responsible for bacterial production in aquatic environments? *FEMS Microbiology Ecology*, 35, pp. 171-179

Sgorbati, S., Barbesti, S., Citterio, S., Bestetti, G. & De Vecchi, R. (1996). Characterization of number, DNA content, viability and cell size of bacteria from natural environments using DAPI PI dual staining and flow cytometry. *Minerva Biotechnology,* 8, pp. 9–15

Shapiro, H.M. (2000). Microbial analysis at the single-cell level: tasks and techniques. *Journal of Microbiology Methods*, 42, pp. 3–16

Shapiro, H.M. (2003). Practical Flow Cytometry. 4th Edition, Wiley-Liss, New York

Shapiro, L.P., Campbell L. & Haugen, E.M. (1989). Immunochemical recognition of phytoplankton species. *Marine Ecology Progress Series*, 57, pp. 219-224

Sherr, B.F., del Giorgio, P.A. & Sherr, E.B. (1999). Estimating the abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. *Aquatic Microbial Ecology*, 18, pp. 117–131

Sherr, E.B., Sherr, B.F. & Longnecker, K. (2006). Distribution of bacterial abundance and cell-specific nucleic acid content in the Northeast Pacific Ocean. *Deep Sea Res Part I Oceanographic Research Papers,* 53, pp. 713–725

Shopov, A., Williams, S.C. & Verity, P.G. (2000). Improvements in image analysis and fluorescence microscopy to discriminate and enumerate bacteria and viruses in aquatic samples. *Aquatic Microbial Ecology*, 22, pp. 103– 110

Sieburth, J. McN., Smetacek, V. & Lenz, J. (1978). Pelagic ecosystem structure: heterotrophic compartments of the plankton and their relationship to plankton size fractions. *Limnology and Oceanography*, 23, pp. 1256-1263

Sieracki, M. E., Cucci, T.L. & Nicinski, J. (1999). Flow Cytometric Analysis of 5-Cyano-2,3-Ditolyl Tetrazolium Chloride Activity of Marine Bacterioplankton in Dilution Cultures. *Applied and Environmental Microbiology,* 65, pp. 2409-2417

Sime-Ngando, T., Mignot, J.-P., Amblard, C., Bourdier, G., Desvilettes, C. & Quiblier-Lloberas, C. (1996). Characterization of planktonic virus-like particles in a French mountain lake: methodological aspects and preliminary results. *Annual Limnology*, 32, pp. 1–5

Smith, E. M. & del Giorgio, P.A. (2003). Low fractions of active bacteria in natural aquatic communities? *Aquatic Microbial Ecology,* 31, pp. 203–208

Smith, E.M. (1998). Coherence of microbial respiration rate and cell-specific bacterial activity in a coastal planktonic community. *Aquatic Microbial Ecology,* 16, pp. 27–35

Steen, H.B. (1986). Simultaneous separate detection of low angle and large angle light scattering in an arc lamp-based flow cytometer. *Cytometry,* 7, pp. 445–449

Steen, H.B. & Lindmo, T. (1979). Flow cytometry: a high-resolution instrument for everyone. *Science,* 204, pp. 403-404

Steen, H.B., Boye, E., Skarstad, K., Bloom, B., Godal, T. & Mustafa, S. (1982). Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects, and quantitation of antibody binding. *Cytometry,* 2, pp. 249–257

Steen, H. & Boye, E. (1981). Growth of Escherichia coli studied by dual-parameter flow cytometry. *Journal of Bacteriology*, 145, pp. 1091–1094

Stenuite, S., Pirlot, S., Tarbe, A.L., Sarmento, H., Lecomte, M., Thill, S., Leporcq, B., Sinyinza, D., Descy, J.P. & Servais, P. (2009) Abundance and production of bacteria, and relationship to phytoplankton production, in a large tropical lake (Lake Tanganyika). *Freshwater biology*, 54, pp. 1300-1311

Stockner, J.G. & Antia, N.J. (1986). Algal picoplankton from marine and freshwater: a multidisciplinary perspective. *Canadian Journal of Fishers and Aquatic Sciences*, 43, pp. 2472-2503

Stockner, J.G. (1988). Phototrophic picoplankton: an overview from marine and freshwater ecosystems. *Limnology and Oceanography*, 33, pp. 765-775

Suttle, C. (2000). Cyanophages and their role in the ecology of cyanobacteria. In The Ecology of Cyanobacteria. Whitton, B., & Potts, M., pp. 563–589. The Netherlands: Kluwer Academic Publishers

Tanaka, Y., Yamaguchi, N. & Nasu, M. (2000). Viability of *Escherichia coli* O157:H7 in natural river water determined by the use of flow cytometry. *Journal of Applied Microbiology*, 88, pp. 228–236

Temmerman, R., Huys, G. & Swings, J. (2004). Identification of lactic acid bacteria: culture-dependent and culture-independent methods. *Trends in Food Science & Technology,* 15, pp. 348–359

Troussellier, M., Courties, C., Lebaron, P. & Servais, P. (1999). Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. *FEMS Microbiology Ecology,* 29, pp. 319–330

Ullrich, S., Karrasch, B. & Hoppe, H.G. (1999). Is the CTC dye technique an adequate approach for estimating active bacterial cells? *Aquatic Microbial Ecology*, 17, pp. 207–209

Ullrich, S., Karrasch, B., Hoppe, H.G., Jeskulke, K. & Mehrens, M. (1996). Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. *Applied Environmental Microbiology*, 62, pp. 4587–4593

Vaulot, D. & Marie, D. (1999). Diel variability of photosynthetic picoplankton in the equatorial Pacific. *Journal of geophysical research*, 104, pp. 3297-3310

Vaulot, D., Parternski, F., Neveux,J., Mantoura, R.F.C. & Llewellyn, C.A. (1990). Winter presence of Prochlorophyte in surface waters in the north-western Mediterranean Sea. *Lymnology and Oceanography,* 35, pp, 1156-1164

Veal, D.A., Deere, D., Ferrari, B., Piper, J. & Attfield, P.V. (2000). Fluorescence staining and flow cytometry for monitoring microbial cells. *Journal of Microbiology Methods,* 243, pp. 191–210

Veldhuis, M.J.W. & Kraay, G.W. (2000). Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *Scientia Marina*, 64, pp. 121-134

Veldhuis, M.J.W. & Kraay, G.W. (1993). Cell abundance and fluorescence of picophytoplankton in relation to growth irradiance and nitrogen availability in the Red Sea. *Netherlands Journal of Sea Research*, 21, pp. 135– 145

Vives-Rego, J., Guindulain, T., Vàzquez-Domínguez, E., Gasol, J.M., Lopez-Amoros, R., Vaquè, D. & Comas, J. (1999). Assessment of the effects of nutrients and pollutants on coastal bacterioplankton by flow cytometry and SYTO-13 staining. *Microbios,* 98, pp. 71–85

Vives-Rego, J., Lebaron P. & Nebe-von-Caron, G. (2000). Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiology Ecology*, 24, pp. 429-448

Vrieling, E.G. & Anderson, D.M. (1996). Immunofluorescence in phytoplankton research: applications and potential. *Journal of Phycology*, 32, pp. 1-16

Vrieling, E.G., Gieskes, W.W.C., Colijn, F., Hofstraat, J.W., Peperzak L. & Veenhuis, M. (1993). Immunochemical identification of toxic marine algae: first results with Prorocentrum micans as a model organism, In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J., & Shimizu, Y., pp. 925-931. Elsevier, Amsterdam.

Wallner, G., Amann, R. & Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of micro-organisms. *Cytometry,* 14, pp. 136–143

Wallner, G., Erhart, R. & Amann, R. (1995). Flow Cytometric Analysis of Activated Sludge with rRNA-Targeted Probes. *Applied Environmental Microbiology*, 61, pp. 1859–1866

Wallner, G., Steinmetz, I., Bitter-Suermann, I. & Amann, R. (1997). Combination of rRNA-targeted hybridization probes and immuno-probes for the identification of bacteria by flow cytometry. *System of Applied Microbiology*, 19, pp. 569–576

Wang, M., Liang, Y., Bai, X., Jiang, X., Wang, F. & Qiao, Q. (2010). Distribution of microbial populations and their relationship with environmental parameters in the coastal waters of Qingdao, China. *Environmental Microbiology,* 12, pp. 1926–1939

Weinbauer, M.G. (2004). Ecology of prokaryotic viruses. *FEMS Microbiology Reviews*, 28, pp. 127–181

Weinbauer, M.G. & Rassoulzadegan, F. (2004). Are viruses driving microbial diversification and diversity? *Environtal Microbiology*, 6, pp. 1–11

Wen, K., Ortmann, A.C. & Suttle, C.A. (2004). Accurate estimation of viral abundance by epifluorescence microscopy. *Applied Environmental Microbiology*, 70, pp. 3862– 3867

Wilhelm, S.W. & Suttle, C.A. (1999). Viruses and nutrient cycles in the sea. *Bioscience*, 49, pp. 781–788

Williams, S.C., Hong, Y., Danavall, D.C.A., Howard-Jones, M.H., Gibson, D., Frisher, M.E. & Verity, P.G. (1998). Distinguishing between living and non-living bacteria: evolution of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. *Journal of Microbiology Methods,* 32, pp. 225–236

Winson, M.K. & Davey H.M. (2000). Flow Cytometric Analysis of Micro-organisms. *Methods,* 21, pp. 231-240

Xenopoulos, M.A. & Bird. D. F. (1997). Virus a` la sauce Yo-Pro: microwave-enhanced staining for counting viruses by epifluorescence microscopy. *Limnology and Oceanography*, 42, pp. 1648–1650

Yamaguchi, N. & Nasu, M. (1997). Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *Journal of Applied Microbiology*, 83, pp. 43–52

Yamaguchi, N., Nasu, M., Choi, S.T. & Kondo, M. (1994). Analysis of the life-cycle of Bacillus megaterium by the fluorescein diacetate/propidium iodide double staining method. *Journal of Antibacterial and Antifungal Agents,* 22, pp. 65–68

Yentsch, C. M. & Pomponi, S.A. (1986). Automated Individual Cell Analysis in Aquatic Research. *International Review of Cytology,* 105, pp. 183-243

Yentsch, C. & Yentsch, C.M. (2008). Single cell analysis in biological oceanography and its evolutionary implications. *Journal of Plankton Research*, 30, pp. 107–117

Yentsch, C.M. & Horan, P.H. (1989). Cytometry in the Aquatic Sciences. *Cytometry*, 10, pp. 497-499

Yentsch, C.M., Horan, P.K., Muirhead, K., Dortch, Q., Haugen, E., Legendre, L., Murphy, L.S., Perry, M.J., Phinney, D.A., Pomponi, S.A., Spinrad, R.W., Wood, M., Yentsch C.S. & Zahuranec, B.J. (1983). Flow Cytometry and Cell Sorting: A Technique for the Analysis and Sorting of Aquatic Particles. *Limnology and Oceanography*, 28, pp. 1275-1280

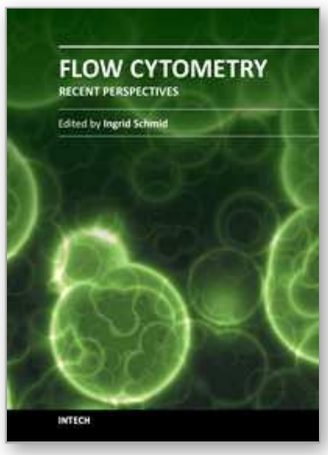
Yilmaz, S., Haroon, M.F., Rabkin, B.A., Tyson, G.W. & Hugenholtz, P. (2010). Fixation-free fluorescence in situ hybridization for targeted enrichment of microbial populations. *ISME Journal*, 4, pp. 1352–1356

Zubkov, M.V, Mary, I. & Woodward E.M.S. (2007). Microbial control of phosphate in the nutrient-depleted North Atlantic subtropical gyre. *Environmental Microbiology,* 9, pp. 2079-2089

Zubkov, M.V., Fuchs, B.M., Burkill, P.H. & Amann, R. (2001). Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. *Applied Environmental Microbiology,* 67, pp. 5210–5218

Zubkov, M.V., Sleigh, M.A., Burkill, P.H. & Leakey, R.J.G. (2000). Picoplankton community structure on the Atlantic Meridional Transect: a comparison between seasons. *Progress In Oceanography*, 45, pp. 369-386

http://www. invitrogen.com

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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