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Flow Cytometry as a Powerful Tool for Monitoring Microbial Population Dynamics in Sludge

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1. Introduction

Flow cytometry is a technology that simultaneously measures and analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size (represented by forward angle light scatter), relative granularity or internal complexity (represented by right-angle scatter), and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system which records how the cell or particle scatters incident laser light and emits fluorescence. A wide range of dyes, which may bind or intercalate with different cellular components, can be used as labels for applications in a number of fields, including molecular biology, immunology, plant biology, marine biology and environmental microbiology. Interest in rapid methods and automation for prokaryotic cell studies in environmental microbiology has been growing over the past few years.

There are several available methods for the detection and enumeration of microorganisms in raw and processed environmental samples. Culture techniques are the most common, but a major disadvantage of these is their failure to isolate viable but nonculturable organisms (Davey & Kell, 1996). Actually, in both natural samples and axenic cultures in the laboratory, there is clear evidence of the presence of intermediate cell states which remain undetectable by classical methods (Kell *et al.*, 1998). In recent years, this fact has generated a great confusion in the scientific community as to the concept of cell viability. The reality is that the absence of colonies on solid media does not necessarily mean that cells are dead at the time of sampling (Nebe-von-Caron *et al.*, 2000).

Various other methods have been developed in order to investigate the problems associated with culture-based detection systems. Among these methods, flow cytometry has become a valuable tool for rapidly enumerating microorganisms and allowing the detection and discrimination of viable culturable, viable nonculturable and nonviable organisms. There is also the possibility that numerous (or even rare) microbial cells could be detected against a background of other bacteria or nonbacterial particles by combining flow cytometry and specific fluorescent probes. In this case, the objective is to label cells with different structural properties or else differing in their activity or functionality.

Since the practical and accurate microbial assessment of environmental systems is predicated on the detection and quantification of various microbial parameters in complex matrices, flow cytometry represents an accurate tool in environmental microbiology and in particular for bioprocesses monitoring.

Considerable research has been devoted over recent decades to the optimisation and control of biological wastewater treatment processes. Many treatment processes have been studied so as to increase the methane potential of sludge with a rate-limiting hydrolysis stage of organic matter associated with microbial cells. Although a great deal of information about sludge minimisation processes is currently available in WWTP (i.e., sonication, ozonation or thermal treatment), little data is available as to its fundamental mechanisms, especially microbial changes.

The most common parameter used for quantifying activated sludge is the content of suspended solids, expressed as Total Suspended Solids (TSSs) or Volatile Suspended Solids (VSSs). However, VSSs do not coincide with the effective bacterial biomass in activated sludge because they also include endogenous biomass (the residue produced by bacterial death and lysis) and organic non-biotic particulate matter fed into the plant with the influent wastewater (Foladori *et al.*, 2010a). On the one hand, the bacterial biomass in activated sludge is generally estimated by theoretical calculations based on substrate mass balances using kinetic and stoichiometric parameters (Henze, 2000). On the other hand, knowledge of the amount of bacterial biomass and the physiological state of microorganisms in an activated sludge system represents key parameters for understanding the processes, kinetics and dynamics of substrate removal (Foladori *et al.*, 2010b).

Early investigations which aimed to recover bacteria from activated sludge for quantification were based on cultivation methods. For a long time, no routine methods have been proposed to rapidly quantify the bacterial biomass in activated sludge and wastewater. To obtain a more accurate view of bacterial populations, the application of *in situ* techniques or direct molecular approaches are needed (Foladori *et al.*, 2010b). With regard to the recovery of bacteria from complex matrices such as sludge in order to count them, sonication has been proposed in several studies so as to disaggregate activated sludge flocs while maintaining cell viability (Falcioni *et al.*, 2006a;Foladori *et al.*, 2007;Foladori *et al.*, 2007 and Foladori *et al.*, 2010a) or to induce cell lysis and bacteria inactivation (Zhang *et al.*, 2007). In the same manner, the characterisation of the impact of enhanced hydrolysis by the pre-treatments mentioned above (sonication, ozonation and thermal treatment) in terms of microbial activity (active cells able to convert organic matter) and viability (cell lysis with the resulting release of intracellular material) remain fundamental for sludge reduction optimisation (Prorot *et al.*, 2011).

The procedure recently developed by Ziglio *et al.* (2002) to disaggregate sludge flocs before staining (with dyes), and flow cytometry analysis has demonstrated that fluorescent dyes combined with this technique can be a valuable tool for the assessment of the viability and activity of an activated sludge mixed-bacterial population. These studies indicated that flow cytometry allows a rapid and accurate quantification of the total bacterial population, including the viable nonculturable fraction, and consequently that flow cytometry represents an appropriate tool for activated sludge investigations.

This review seeks to highlight the interest of the technique of flow cytometry for quantitative and qualitative bacterial biomass monitoring in activated or anaerobic sludge.

In the first part, we review the basic principles of flow cytometry and its use in different areas in environmental microbiology. As cells differ in their metabolic or physiological states, we presented the flow cytometry potentialities in order to allow for the detection of different subpopulations according to their structural or physiological parameters. We describe the strategies used for cell detection (scattering and fluorescence signals) and the cellular targets associated with fluorescent probes which are currently used in assays related to microbial assessment in environmental systems, and especially those related to sludge investigations. The second part is devoted to a discussion of the concept of cell viability and functionality, with a detailed review of the different intermediate physiological states between cellular life and death. Next, a concise revision concerning the most recent applications of flow cytometry related to cell analyses and quantification in sludge processes is presented. Finally, a general conclusion provides an overview of the main perspectives related to this powerful technique for the sludge treatment process.

2. Flow cytometry technique

Flow cytometry is a quantitative technology for the rapid individual analysis of large numbers of cells in a mixture, using light scattering and fluorescence measurements. The power of this method lies both in the wide range of cellular parameters that can be determined and in its ability to obtain information on how these parameters are distributed in the cell population.

2.1 Basic principles

Flow cytometry uses the principles of light scattering, light excitation and the emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells within the size range of 0.5 um to 40 um diameter. A common flow cytometer is formed by several basic units (Díaz *et al.*, 2010): a light source (lasers are most often used), a flow cell and hydraulic fluidic system, several optical filters to select specific wavelengths, a group of photodiodes or photomultiplier tubes to detect the signals of interest and, finally, a data processing unit (Figure 1).

Light scattering occurs when a particle or a cell deflects incident laser light. The extent to which this occurs depends on the physical properties of a cell, namely its size and internal complexity. Factors that affect light scattering are the cell membrane, the nucleus and any granular material inside the cell. Cell shape and surface topography also contribute to the total light scatter. The forward scatter light (FSC - light scatter at low angles) provides information on cell size, although there is no direct correlation between size and FSC (Julià *et al.*, 2000). Light scattered in an orthogonal direction can also be collected by a different detector (a side scatter or SSC detector), which provides information about granularity and cell morphology.

The most common type of quantitative analysis using flow cytometry data involves creating a histogram of fluorescence events to count the number of cells with the attached probe. This effectively creates a set of data which gives a ratio of the cells in a population with a particular structural parameter or else with a specific functional property. Except for fluorescence naturally produced by some intracellular compounds, fluorescence signals are generally produced consecutive to the staining of sample-containing cells with dyes related

to structural or functional parameters as physiological probes. These dyes are specifically bound and - after excitation with the laser beams - fluoresce, giving quantitative information on the respective cell parameters.

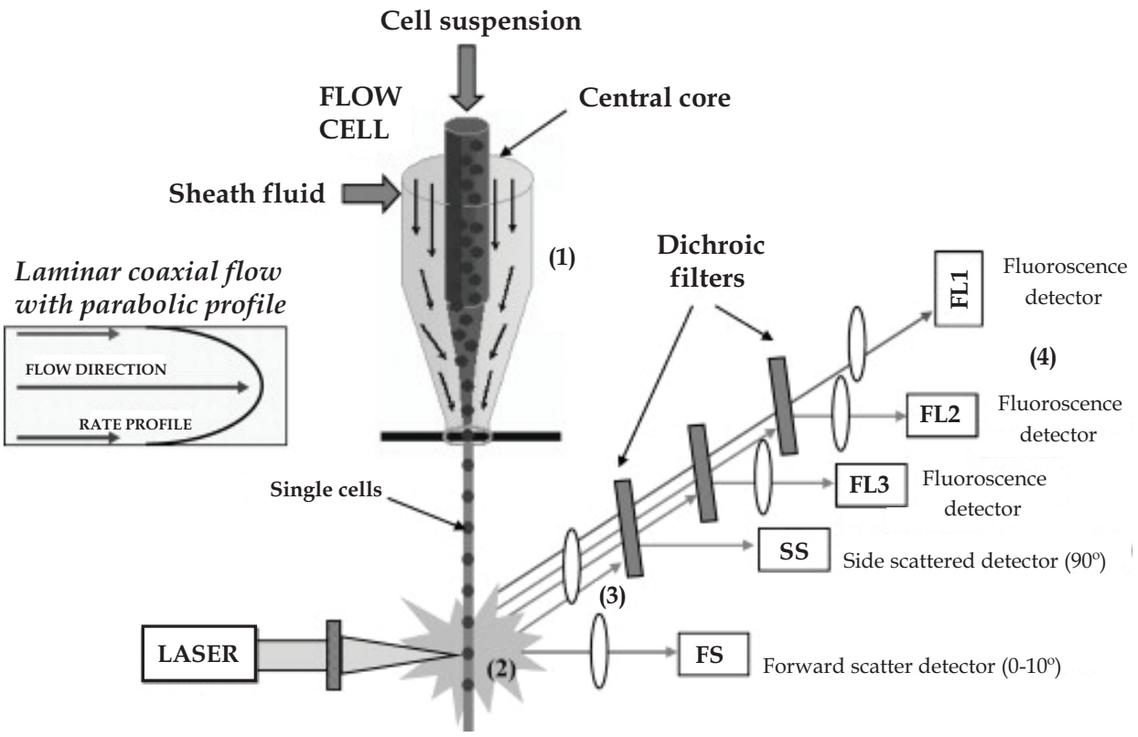


Fig. 1. Scheme of and principles behind a typical flow cytometer (from (Díaz *et al.*, 2010): the cell suspension or mixture containing cells is hydro-dynamically focused in a sheath fluid before passing excitation sources, such as laser beams. The combined flow is reduced in diameter, forcing the cells into the centre of the stream (1). These cells are aligned to pass, single file, through a laser beam and impact with the laser in a confined site, emitting different signals related to diverse cell parameters (2). For each cell or particle, a multi-parametric analysis is made using a combination of dyes which have different properties and subcellular specificities. The emitted and scattered light resulting from the cell-laser intersection are divided into appropriate colours using a group of wavelength-selective mirrors and filters (3). The signals are collected by the detection system, which is formed by a collection of photodiodes, two scattering and different fluorescence (FL1, FL2, FL3) detectors (4). Finally, signals are sent to a computer, thereby obtaining a representation of the distribution of the population with respect to the different parameters

The intensity of the optical signals generated (scattering and/or fluorescence signals) is therefore correlated to structural and/or functional cell parameters (Davey & Kell, 1996). A combination of light-scattering and fluorescence measurements on stained or unstained cells allows for the detection of multiple cellular parameters. Depending on the dye used, many of these measurements can be done simultaneously on the same cells. The scatter and fluorescence signals detected can be combined in various ways to allow for the detection of subpopulations (Comas-Riu & Rius, 2009). This contrasts with spectrophotometry, in which

the percentage of the absorption and transmission of specific wavelengths of light is measured for a bulk volume of the sample.

2.2 Cell parameter measurements

Flow cytometric assays have been developed to determine both cellular characteristics (such as size, membrane potential and intracellular pH) and the levels of cellular components (such as DNA, protein or surface receptors). Flow cytometry is generally used in microbial cell analysis for rapid counting, the study of heterogeneous bacterial populations, strain improvement in industrial microbiology, and in order to sort bacteria for further molecular analysis (Díaz *et al.*, 2010);(Müller & Nebe-von-Caron, 2010)(Müller *et al.*, 2010)(Comas-Riu & Rius, 2009). In microbiological applications using one or several dyes, the main objective is the labelling of cells with different structural properties or differing in their activity or functionality (Díaz *et al.*, 2010)(Achilles *et al.*, 2006).

Measurements that reveal the heterogeneous distribution in bacterial cell populations are important for bioprocesses because they describe the population better than the average values obtained from traditional techniques (Rieseberg *et al.*, 2001), and consequently they provide a valuable tool for bioprocess design and control (Díaz *et al.*, 2010). Although many different measurements are possible, only those most related to the study of microbial population dynamics in environmental and water systems will now be discussed. An earlier review has already summarised the flow cytometry potentialities for single-cell analysis in environmental microbiology (Czechowska & Johnson, 2008).

Actually, the data mainly sought in environmental microbiology has been focused on the analysis of the physiological state of bacteria in different microbial ecosystems, including sludge. Plate-culturing techniques only reveal a small proportion (viable and cultivable bacteria) of the total microbial population. This can be explained mainly by the inability of microorganisms that are either stressed or which have entered into a non-cultivable state to growth on conventional plating techniques (Giraffa, 2004)(Lahtinen *et al.*, 2005). One promising tool of flow cytometry consists of characterising and distinguishing different the physiological states of microorganisms at the single-cell level (Joux & Lebaron, 2000) (Nebe-von-Caron *et al.*, 2000). The ability of flow cytometry to distinguish between different physiological states is important for assessing the growth of microorganisms in oligotrophic environments (Berney *et al.*, 2007), the survival of pathogenic microorganisms (Vital *et al.*, 2007) and the effects of bactericidal treatments or different environmental stresses on microbial activity (Prorot *et al.*, 2008)(Ziglio *et al.*, 2002)(Foladori *et al.*, 2010a)(Booth, Ian R, 2002). When employed in conjunction with fluorescent dyes, flow cytometry is able to measure various biological parameters (i.e., nucleic acid content, metabolic activity, enzyme activity and membrane integrity), allowing the detection of microorganisms at viable, viable but non-cultivable (or intermediate) and non-viable states (Joux & Lebaron, 2000)(Walberg *et al.*, 1999).

One widely-used strategy for analysing viable and dead bacterial cells in environmental samples was done on the basis of membrane integrity, coupling a cell-impermeant dye - such as propidium iodide - and a cell-permeant dye, like most of the SYTO family or SYBR (Berney *et al.*, 2007)(Ziglio *et al.*, 2002). Dyes from the SYTO family and propidium iodide are nucleic acid-binding probes and are, with others, well described.

In this approach, all cells are supposed to incorporate SYTO and fluoresce green. Only dead or damaged cells (considered as associated with the loss of cell membrane integrity) are permeable for propidium iodide, and the cells thus fluoresce red (Czechowska & Johnson, 2008). Thanks to this approach, the transition phases between viable and non-viable states have been observed for different microbial ecosystems (e.g., when bacterial cells in drinking water are irradiated by UVA) (Berney *et al.*, 2007). In this case, cells in intermediate states displayed high levels of both green and red fluorescence. For this purpose, Molecular Probes Inc. (Leiden, The Netherlands) has developed a fluorescent stain - the LIVE/DEAD BacLight™ bacterial viability kit - which is composed of SYTO9™ and propidium iodide. This last dye can also be used alone to assess membrane integrity (Shi *et al.*, 2008).

Another method is proposed to deduce cellular activity from the amount of nucleic acid within the cell (Kleinsteuber *et al.*, 2006)(Servais *et al.*, 2003). However, this method is based on the use of a nucleic acid binding fluorescent dye that has been recently critically discussed by (Bouvier *et al.*, 2007). From a wide range of environmental communities, they have demonstrated that nucleic acid contents do not necessary correlate with differences in metabolic activity. The use of the redox dye 5-cyano-2,3ditolyl tetrazolium chloride (CTC) is also available for discriminating between active (respiring cells) and non-active (non-respiring cells) populations of microorganisms, although this technique does not always show consistent results (Longnecker *et al.*, 2005;Czechowska & Johnson, 2008).

The uptake of a growth substrate by a cell represents the first step of metabolism. Therefore, fluorescently marked substrates can be used to obtain information on either substrate transport mechanisms, enzyme activity or the viability of cells (Sträuber & Müller, 2010). For example, the non-fluorescent esterase substrate fluorescein di-acetate (FDA) is cleaved by esterases in viable cells, releasing fluorescein which stains the cells green. FDA could ideally be used in combination with propidium, which stains non-viable cells red (Veal *et al.*, 2000).

3. Cell viability and functionality

3.1 Cell viability and physiological target sites

The impact of micro-organisms on the environment has been widely investigated via studies based on the growth characteristics of viable cells, and on the related consequences of microbial proliferation. Bacteria were the most extensively studied, using quantitative methods set on the determination of the number of colony-forming units. If the plate count methods could not be used, because of a wide variety of reasons (unknown growth requirements, auxotrophic micro-organisms, etc.), cultures in liquid media followed by a statistical treatment of values could lead to interesting interpretations, although with much less accuracy than those obtained with Petri plates.

However, cells which are unable to cultivate may possess basic metabolic capacities which could influence the environment accordingly (Sträuber & Müller, 2010). Furthermore, and in a theoretical sense, attention should be paid to dead cells resulting from severe - and thus irreversible - structural damage. Dead cells may release numerous molecules, ranging from small-sized ones to macromolecules. They may induce the cryptic growth of other living cells or act as chelating agents, and the listing of such possible forms of interference is far from exhaustive.

FCM was first applied to eukaryotic cells, but in the late 1970s this technology began to be used for the study of prokaryotic cells (mostly bacteria) (Steen *et al.*, 1982; Allman *et al.*, 1992) and yeasts (Scheper *et al.*, 1987). FCM gained interest with its application to industrial microbial processes (Díaz *et al.*, 2010). Other fields of application have appeared to be of interest, such as the optimisation of SRP (sludge reduction processes) (Prorot *et al.*, 2008; Prorot *et al.*, 2011).

The development of fields of application for FCM was accompanied with research seeking a better understanding of cellular bacterial viability. Apart from irreversibly dead cells, the main cellular states were commonly sorted into two classes: viable and able to cultivate cells and viable but unable to cultivate cells.

This classification into three groups (irreversibly dead, cultivable, viable but non-cultivable cells) could be improved by taking into account various intermediate states, and especially those concerning viable but non–cultivable cells. This requires the fixing beforehand of an adequate definition of cellular viability. The viability of a cell is its capacity to live. We chose, as point of departure, the definition back by a high degree of scientific authority, namely the NASA definition (definition 1) (Joyce *et al.*, 1994):

(Definition 1) "Life is a self-sustained chemical system capable of undergoing Darwinian evolution"

The publication of this definition aroused numerous amendments aiming to approach it in a more precise way at the level of the cell. Luisi (1998) proposed several interesting amendments based on various points of view. The first definition emanates from the point of view of the biochemist (definition 2):

(Definition 2) "a system which is spatially defined by a semipermeable compartment of its own making and which is self-sustaining by transforming external energy/nutrients by its own process of components production"

Considering the geneticist's view, the same author in the same paper proposed definition 3:

(Definition 3) "a system which is self-sustaining by utilising external energy/nutrients owing to its internal process of components production and coupled to the medium via adaptive changes which persist during the time history of the system"

At present, no definitive definition has met with general approval. Some attempts have been made to investigate the different states of bacterial viability. The existence of three bacterial viability states was admitted by Barer (1997):

1. dead cells
2. viable but non-cultivable cells (VBNC)
3. colony-forming cells

Bogosian (2001) investigated the intermediate cellular state(s) of VBNC cells. This author discussed works relating the (weakly) possible resuscitation of injured bacteria exhibiting the characteristics of VBNCs for a long time before their resuscitation by using appropriate techniques. The need for a better understanding of such intermediate cellular states appeared after the work of several authors who developed cytological methods for investigating them and who provided interesting data concerning these states (Czechowska & Johnson, 2008; Sträuber & Müller, 2010).

Thus we proposed a definition based on and adapted from the former definitions in order to improve the classification of the various states of cellular life, by taking into account the "intermediate viability states" (concerning the VBNCs). This approach is referred to by definition 4.

(Definition 4) "Cellular viability is the property of any system bounded by a semipermeable membrane of its own manufacturing and potentially capable of auto-speaking, of reproducing almost as before by making its own constituents from energy and\or from outer (foreign) elements and to evolve according to its environment"

Accordingly, we admitted that a bacterium could be classified as viable if the following criteria were satisfied:

1. maintenance of membrane integrity (structure and functions)
2. normal gene expression, protein synthesis and division (scissiparity) control
3. maintenance of metabolic activity (for both catabolism and anabolism)

We hypothesised the existence of five physiological states according to environmental conditions (varying from the worst to the most favourable ones for the cell):

State 1: corresponds to the worst one (irreversible damage). Different states evolve up to the most favourable one (state 5, or standard cell growth, allowing colony formation). The originality of the classification lies in the appearance of new intermediate states (3 and 4):(

State 3: this state corresponds to wounded cells but differs from state 2, because they can resuscitate under well-defined conditions

State 4: this state corresponds to endospores. Endospores can form colonies when inoculated in favorable growth media. Nevertheless endospores cannot be classified within state 5, because typical bacteria of state 5 form colonies originating from viable vegetative cells (the physiology of which greatly differing from the endospores one)

Additional comments should be made in order to complete the data provided by Table 1.

State 1: This state should correspond to cells which were in contact with harsh physico-chemical conditions, for instance after a Ultra High Temperature (UHT) thermal treatment as generally proceeds in the food industry (135°-150° for 15 seconds). The cellular corpses may be observed via microscopy, but no detectable sign of life can be determined (e.g., enzymatic activities). Extreme pH values, violent osmotic shock or starvation conditions can also lead to death or the appearance of such a physiological state.

The influence of dead microbial cells should not be underestimated, because they can provide nutrients for the growth of other bacteria inoculated in the medium after the physico-chemical conditions return to acceptable levels. Some dead cell components can also interfere with biofilms' evolution. Working in the field of bacterial adhesion, Mai-Prochnow *et al. (*2004) studied the development of the multicellular biofilm of *Pseudomonas tunicata* They discovered a novel 190-kDa autotoxic protein produced by this Pseudomonas, designated AlpP. They found that this protein was involved in the killing of the biofilm and its detachment. An Δ*AlpP* mutant derivative of *P. tunicata* was generated, and this mutant did not show cell death during biofilm development. Thus, (MaiProchnow *et al.*, 2004)

proposed that AlpP-mediated cell death plays an important role in the development of the multicellular biofilm of *P. tunicata* and the subsequent dispersal of surviving cells within the marine environment.

State	State characteristics	Examples of causes of such damages	Some effects on the environment
1	Irreversible death, cells still observable using microscopy. No sign of any biological activity is detectable (e.g., residual enzymatic activity). Loss of membrane integrity.	Excessive physico-chemical parameters values (temperature, pH, ionic strength, etc.), the action of drugs, chemical effectors, radiations, biological inhibitors, prolonged starvation, etc.	No biological activity is detected. Nevertheless, chemicals of a biological origin may interfere. The presence of dead cell components (EPS) can favour biofilm formation. They can also provide nutrients for further cryptic growth.
2	Wounded cell, possibility of repair(s) allowing survival, membrane integrity remains intact, but the cell is non-cultivable.	The same as above but to a much lower extent.	Resting cells with their effects remaining to be determined. In addition to the potential role of chemicals as nutrients and/or biofilms' starters, some residual enzymatic activities to be determined could influence the environment (for instance oxidation or reduction processes).
3	Wounded cell, possibility of repair(s) allowing survival. Physiological state close to state 2. Regrowth might be possible, but under well-defined conditions and after a long "lag phase".	The same type as above (state 2).	Some pathogenic bacteria lose their capacity for growth after a prolonged starvation period in a media poor in nutrients. After re-inoculation in living organisms, pathogenicity reappears after a period of time, which can be of a great magnitude.
4	Wounded cells, possibility of repair(s), growth possible when conditions are favourable. After adaptation, the characteristics of survivors appear to be identical to the ones of the initial cells. This is the case of so-called "spores forming cells"	Intermediate between the conditions of states 1 and 2.	Regrowth of pathogens in products which are badly sterilised.
5	Viable cells, cultivable (colony-forming).	These are the standard colony forming units on Petri plates, or else cultivable in adequate liquid media	The usual effects of living bacteria (positive or negative for the environment).

Table 1. The different physiological bacterial cellular states

State 2: A recent paper by Ben Said *et al. (*2010) provides an excellent example of this category. They irradiated a strain of E. coli with UV. They noted a 99.99% decrease of viable cells (colony forming units) from 45 mJ/cm^2. In studying the potential evolution of the cells' viability, they employed a useful tool : the Qb phage (RNA). They checked the lytic effect of this phage on the population before and after irradiation (doses higher than 45 mJ/cm^2 were investigated up to 120 mJ/cm^2). They studied the P'/P_0 ratio as a function of the irradiation dose (0 for the blank and from 45 to 120 mJ/cm^2 irradiation doses). P' was the Qb phage units number after 18 hours of incubation at 37°C, and P_0 was the initiated free-phage concentration at time 0.

At time 0 (UV dose = 0), this ratio was close to 10^4. At 45 mJ/cm^2 the ration fell to 10^3, whereas a 99.99% decrease of viable cells was determined. The presence of 0.01% residual cultivable cells could not justify by itself the P'/Po ration value (10^3) if only viable and cultivable cells would allow the phage's growth. This showed that, even if the major part of the bacterial population was killed by UV, the dead cells could nevertheless induce Qb phage replication. This also showed that UV did not integrally destroy any "vital" function of *Escherichia coli* cells..The ones implied in phage multiplication would have been affected to a low extent or just preserved. For higher UV doses, the P'/P_0 ration proportionally decreased as a function of the UV dose, and at 120 mJ/cm^2 the ratio was still around 10^1. This showed that much higher UV doses appeared to be required in order to really affect the major vital functions of the cells.

Qb phage replication depends, at first, on its fixation on the cell membrane. For a second time, the RNA has to cross over the membrane to reach the cytoplasm. Once in the cytoplasm replication of Qb phage only occurs if intact or repaired components of the host cells are available. This experiment proved that the membrane's integrity was persistent and a major part of the cellular components remained active, whereas cellular division could not occur.

The authors hypothesised that the transformation of vegetative cells into VBNC could be a strategy developed by the cell in order to survive the action of UV.

State 3: This state is related to bacteria which were in contact with unfavourable growth conditions that greatly affected cellular viability, but which were able to give rise to colonies on Petri plates when treated adequately. The word "resuscitation" was often cited to describe this phenomenon. (Steinert *et al*., 1997; Whitesides & Oliver, 1997). Steinert *et al*..) (1997) studied *Legionella pneumophila*, an aquatic bacterium responsible for Legionnaire's disease in humans. The legionellae usually parasitise free-living amoebae which provide the accurate environment for the proliferation of these bacteria. When starved (inoculation in low nutrient media), *L. pneumophila* can enter into a VBNC state. These authors inoculated sterilised tap water by a suspension of *L. pneumophila* at 10^4 cells/ml. After 125 days of incubation in tap water, no colony-forming unit appeared on the routine plating media. Counts were made in parallel using the Acridine Orange Direct Count (AODC) method and hybridisation with 16S rRNA-targeted oligonucleotide probes: cells were still detectable.

After this incubation period, cells of *Acanthamoeba castellanii* were added. This led to the "resuscitation" of *L. pneumophila* cells that became cultivable. This tended to show that during the starvation period, the damage that affected the cell was reversible, at least for these latter cells and that the amoeba provided enough elements for reversing the VBNC state. The notion of a survival strategy could be implied in this phenomenon.

A similar phenomenon was evidenced for *Vibrio vulnificus*, a human pathogen responsible for wound infections often leading to septicaemia. For *V. vulnificus*, the VBNC state can be induced by incubation at temperatures below 10°C. Whitesides & Oliver (1997) studied the reversibility of this phenomenon for the latter bacterium. Cells were incubated at 5°C for several days. The total counts were determined via AODC and made-viable CFU by the routine plate count method. Starting from a population of 10^7 viable cells/ml, no CFU occurred after 4 days of incubation, whereas the AODC did not show any significant decrease of the total count. At day 4, the medium was placed for 24h at c.a. 22°C. On day 5 (24h temperature upshift) the CFU value was close to approximately 2.10^6 CFU/ml, apparently showing a "resuscitation" phenomenon. Once more, the damage affecting cells placed at a low temperature could be partially repaired by the temperature upshift described here.

State 4: Bacterial endospores give rise to vegetative cells able to form colonies, but the procedure implied in the "daughter" cells' formation greatly differs from that implied by standard bacterial division. In addition, the structure of the mother cell completely differs from that of the daughter vegetative cells. Furthermore, both sporulation and germination appear as real physiological crises, lasting a relatively long time (10-12 hours) and generally accompanied with the production of highly pathogenic toxins.

The aptitude of bacterial endospores to give rise to viable, cultivable but structurally different cells does not make it possible to classify according to the three previous groups. This validates the existence of a separate state, referred to as State 4 and different from State 5 described below.

State 5: The bacteria are able to form colonies on Petri plates. Their growth in liquid media is accompanied with an increase of optical density (shape, morphology and constitution are identical for "daughter" and "mother" cells. Mutation phenomena are not discussed here). The optimal viability criteria of bacteria of State 5 are those previously noted:

1. maintenance of membrane integrity (structure and functions)
2. normal genes expression, proteins synthesis and division (scissiparity) control
3. maintenance of metabolic activity (for both catabolism and anabolism)

3.2 Fluorescent dyes

There are dozens of Fluorochromes which can aid flow cytometry studies and their number is constantly increasing. The aim of this chapter is not to study them all but rather to show the diversity of dye/cell interactions and the variety of information available according to the type of fluorochrome employed.

Table 2 summarises the properties and the mode of interaction of some of the most commonly used fluorochromes. We classified them into three groups according to the nature of their interactions with cells:

Interactions with nucleic acids. This can provide information about the nuclear content and state of the cell. A given dye can provide indirect information about the physiological state of a given bacterium. For instance, propidium iodide - which binds to DNA or RNA - is normally excluded from healthy cells, being a membrane impermeant. However, if the membrane has been damaged or altered it can more easily cross the latter and stain intracellular components.

Table 2. Continues on next page

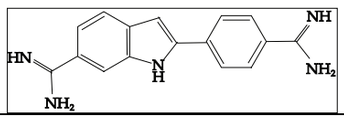
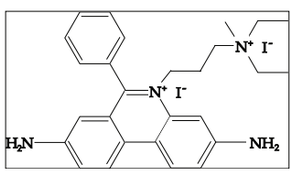
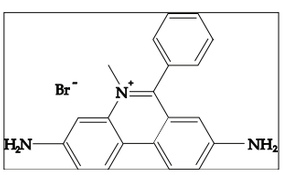
Dye	Structure	λ_{em} (nm) and λ_{ex}(nm)	Reference
Interactions with nucleic acids			
DAPI 4',6'-diamidino-2-phénylindole		451 357	Kapusci (1995 Zink *et al.*
Propidium iodide 3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenantridinium diiodide		631 370/560	Lecoeur (Moore *e* (1998 Jones & (1987
Ethidium bromide 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide		622 370/530	Ohta *et al.*

Table 2. Continues on next page

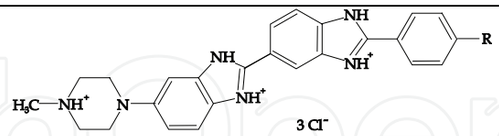
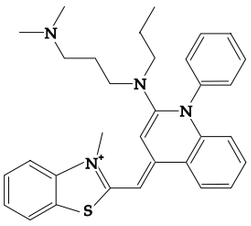
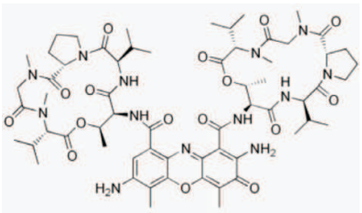
Dye	Structure	λ_{em} (nm) and λ_{ex}(nm)	Referen
Interactions with nucleic acids			
Hoechst 33342, 33258, 34580 Bisbenzimides derivatives	 33342, R = - CH_2CH_3 / 33258, R = -OH / 34580, R = -$N(CH_3)_2$	402 For 33342 365 For 33342	Latt & (1 Alle (2 Port Warin
SYBR green [2-[N-(3-dimethylaminopropyl)- N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium		520 497	Ohta *et* Bacho (2 Kiltie (1
7-AAD 7 amino-actinomycin D		650 488	Schm (1

Table 2. Continues on next page

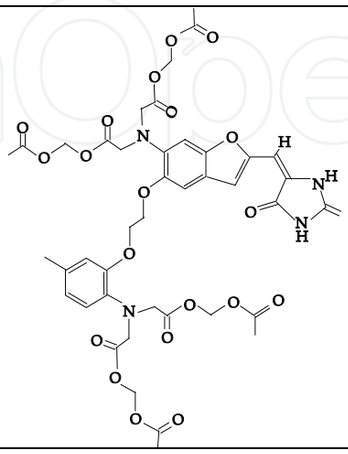
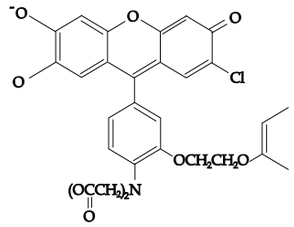
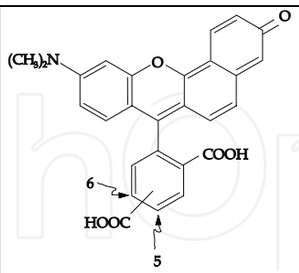
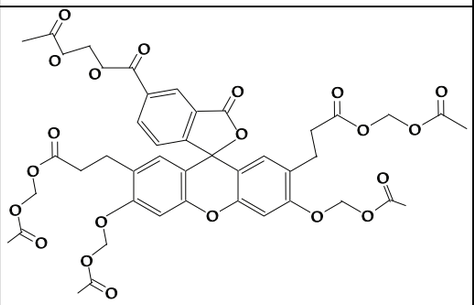
Dye	Structure	λ_{em} (nm) and λ_{ex}(nm)	Referenc
Intracellular calcium indicators			
Fura Red Glycine,N-[2-[(acetyloxy)methoxy]-2-oxoethyl]-N-[5-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-2-[(5-oxo-2-thioxo-4-imidazolidinylidene)methyl]-6-benzofuranyl]-,(acetyloxy)methyl ester		660 450/500	Kurebay (19 Nov Rabinovi
Fluo-3 1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2 -amino-5 -methylphenoxy)ethane-,N,N,N -tetraacetic Acid Pentaacetoxymethyl Ester		526 506	Merritt *e* Caputo & (19

Table 2.

Dye	Structure	λ_{em} (nm) and λ_{ex}(nm)	Reference
Miscellaneous indicators			
SNARF 1 ® (seminaphtorhodafluor-1-acetoxymethylester		580/630 488	Wieder *et* Ribou *et a*
BCECF-AM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester		530 440	Ozkan & Muthara Dascalu *e*
Cascade blue	Cascade Blue acetyl azide is the amine-reactive derivative of the trademarked and patented sulphonated pyrene that Molecular Probes uses to prepare its Cascade Blue dye–labelled proteins	423 399	Whitaker 1991

Calcium flow indicators. For instance, Ca^{2+} has important roles in bacterial cell differentiation, such as the sporulation of *Bacillus* (Herbaud *et al.*, 1998).

Miscellaneous dyes. This group gathers together dyes able to provide interesting information covering a wide range of physiological properties, for instance intracellular pH, the presence or absence of specific enzymatic activities, etc., which are related to the metabolic activity of a given cell.

Each dye has to be chosen according to the type of answer expected, and several of them may be used to improve our understanding of the cellular state of a given bacterium.

New techniques allowed the attachment of fluorochromes to antibodies. This research is under development for finer cytological approaches (both on and inside the cell) as well as specific applications, such as the research of pathogens in the food industry (Comas-Riu & Rius, 2009).

4. Applications of FCM to sludge samples analyses

4.1 General considerations: Sludge matrix composition

In biological wastewater treatment systems, most of the microorganisms are present in the form of microbial aggregates, such as sludge flocs. Basic floc formation is due to a growth-form of many species of natural bacteria. Floc-forming species share the characteristics of the formation of an extracellular polysaccharide layer, also termed glycocalyx. This material - which consists of polysaccharides, proteins and sometimes cellulose fibrils - "cements" the bacteria together to form a floc. Floc formation occurs at lower growth rates and at lower nutrient levels, essentially starvation or stationary growth conditions. The size of activated sludge flocs ranges from very small aggregates of only a few cells (few µm) to large flocs of more than 1 mm. In most activated sludges, the flocs are typically 10 to 100 µm in diameter, relatively strong and not easy to break apart (Figure 2).

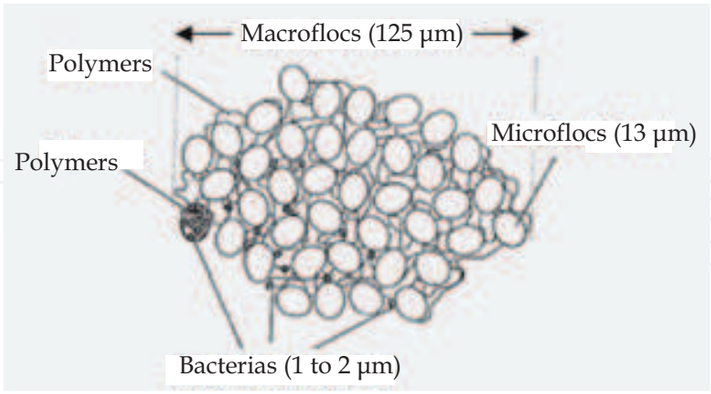


Fig. 2. Schematic representation of activated sludge flocs and their typical size (Jorand *et al.*, 1995)

An activated sludge floc consists of many different components: bacterial cells, various types of extracellular polymeric substances (EPS), adsorbed organic matter, organic fibres, and inorganic compounds (Figure 3). This basic composition is common to all flocs, but the relative proportion of the components and the exact types of chemical compounds or types

of microorganisms vary from plant to plant. The organic matter is usually the largest fraction of the dry matter weight of sludge (60 to 80%) whereas the inorganic fraction (ions adsorbed in the EPS matrix, attached minerals, etc.,) is much less abundant. The EPS matrix consists of various macromolecules, such as proteins, polysaccharides, nucleic acids, humic substances, various heteropolymers and lipids. The macromolecules are partly exopolymers produced by bacterial activity and lysis and hydrolysis products, but they are also adsorbed from the wastewater (Wilén *et al.*, 2003).

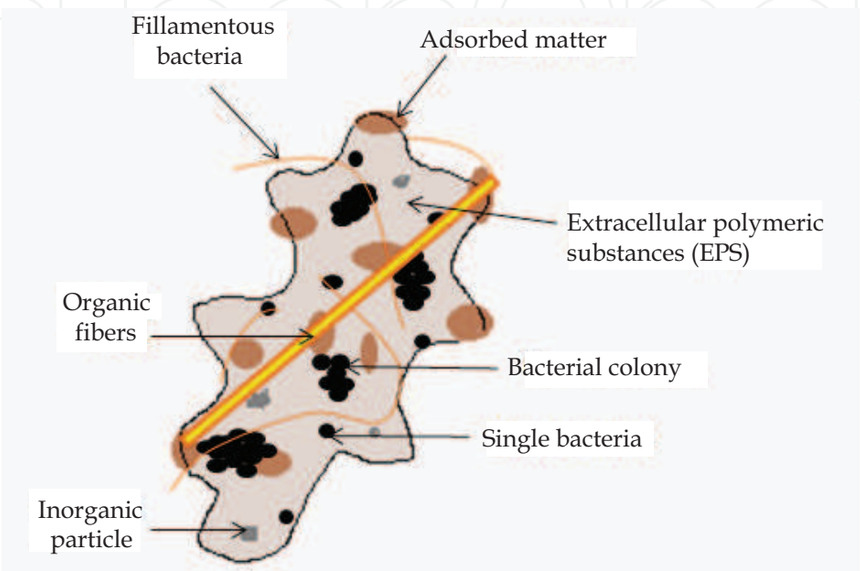


Fig. 3. Schematic illustration of the constituents of activated sludge flocs

It is important to note that polysaccharides - which are assumed to be an important part of bacterial exopolymers - are not present in large amounts in typical sludge flocs (Frolund *et al.*, 1996). Instead, proteins seem to act as the most important "glue" component. The exact function of the large protein pool is not well understood, but exo-enzymatic activity is present (Frolund *et al.*, 1995). Humic substances can form a large fraction in those systems in which they are present in the wastewater and in which the sludge is old. The amount of the extracted EPS and its components and the ratio between each EPS constituent vary, depending on the sample source, the extraction technique and also on the analytical method. While implementing analytical methods for measuring EPS constituents, it is important to know whether they have a high sensitivity to the compound and a low sensitivity to interfering substances (Raunkjaer *et al.*, 1994). The most doubt when choosing the correct analytical method seem to arise with regard to proteins and saccharides.

Thus, only a minor part of organic matter represents the living cells of a biomass. The bacteria can be single, growing in microcolonies or else growing as filaments (Figure 3), but the majority of bacteria grow in aggregates which provide a number of advantages for them when compared to suspended growth. In particular, the presence of EPS components ensures a well-buffered local chemical environment that provides a substrate and important ions, and protection against predators and toxic compounds (Lazarova & Manem, 1995). Furthermore, close proximity to other cells improves interspecies substrates and gene transfer. Considerable effort has been made in recent years in gaining an understanding

about the most important mechanisms controlling the floc structure and biofilm formation. This has been supported by the development of various tools, such as light microscopy, epifluorescence microscopy and confocal laser scanning microscopy for *in situ* studies.

4.2 Disruption procedure

As previously described, in activated sludge samples the major fraction of bacterial cells is attached to aggregates, and this represents a problem for microbiological analysis by flow cytometry. Cytometric analysis requires an homogeneous cell suspension and so the detachment of bacteria from flocs is required (Falcioni *et al.*, 2006b) since in FCM, individual particles are analysed; i.e., for free-living bacteria the properties of single cells are measured (Figure 1).Therefore, cells within activated-sludge flocs cannot reasonably be identified by FCM without an appropriate disruption procedure (Wallner *et al.*, 1995). A fundamental difficulty in efficiently dissociating bacteria from flocculated clumps lies in the balance between using procedures which are hard enough to achieve near-complete detachment and the concomitant risk of cell disruption.

There is no agreement as to which procedure gives the best results with which type of substratum, but different methodologies can be found in the literature, mainly based on chemical (detergent) (Duhamel *et al.*, 2008) or physical treatments (mechanical or ultrasonication) (Ziglio *et al.*, 2002; Foladori *et al.*, 2010b).

Recently, Duhamel *et al.* (2008) developed a method for analysing phosphatase activity in aquatic bacteria at the single cell level using flow cytometry. In this study, the most efficient means for disaggregating/separating bacterial cell clumps was obtained by incubating the sample for 30 min with Tween 80 (10 mg l^{-1}, final concentration). Lake samples were chemically treated after cell staining with the substrate ELF-97 [2-(59-chloro-29-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone] -phosphate (ELF-P) and centrifugation or filtration in order to concentrate cells. Microscopic inspection confirmed that the Tween 80 treatment was efficient at separating the cell clumps. The number of free bacteria and aggregates increased significantly with the addition of Tween 80, up to a final concentration of 10 mg l^{-1}. On the contrary, sonication in a water bath did not generate any increase in the free/attached ratio. Even worse, it led to a significant decrease in total bacteria counts and gave an increase in the filter background.

Buesing & Gessner (2002) tested the effect of 4 detachment instruments (an ultrasonic probe, an ultrasonic bath, an Ultra-Turrax tissue homogeniser and a Stomacher 80 laboratory blender) on the release of bacteria associated with leaf litter, sediment and epiphytic biofilms from a natural aquatic system. They concluded that relatively harsh extraction procedures with an ultrasonic probe turned out to be most appropriate for organic materials, such as decaying leaves and epiphytic biofilms, whereas a more gentle treatment with a Stomacher laboratory blender was preferable for mineral sediment particles.

Ziglio *et al.* (2002) developed a procedure for disaggregating sludge flocs before dye staining and cytometric analysis. The developed procedure, based on mechanical disaggregation (Ultra Turrax), allows a high recovery of bacteria with high accuracy and repeatability, and minimising the damage to the cells' suspension obtained from the disaggregation of the flocs.

In another study, Falcioni *et al.* (2006) compared two different instruments and techniques: sonication and mechanical treatment, in terms of the total detached bacteria number and cell viability. These authors concluded that the treatments used were quite satisfactory, although a complete detachment without bacterial cell death seemed unlikely to be achieved. Although the maximum detachment value was obtained by sonication, mechanical treatment, even if a little bit lower in terms of detachment, showed a good linearity in its results without cell damages, so it could be an alternative method for disaggregating sludge flocs. In addition, they concluded that a combination of the two treatments showed a higher efficiency in terms of bacteria detachment compared with the single treatment with respect to cell viability.

More recently, Foladori *et al.* (2007) also compared mechanical treatment and ultrasonication as pre-treatments for disaggregate activated sludge flocs, with the aim of obtaining a suspension mostly made up of free single cells. According to this study, the pre-treatment based on ultrasonication was more effective than mechanical treatment (after ultrasonication, the maximum range of viable cells was 3.2 times higher than after mechanical treatment). In order to investigate eventual losses in bacterial viability after ultrasonication and mechanical treatment, the ratio of dead/viable free cells was evaluated, and it was found that it did not change significantly for increasing specific energy during sonication or for mechanical treatment times below 20 min.

To conclude this part, it appears that it is not possible to apply a standardised method as a sample preparation before flow cytometric analysis. There is no agreement on which procedure gives the best results with which type of substratum. This is particularly true if activated sludge is considered due to its variable composition and variable floc structure, having different shapes, a different porosity, and irregular boundaries and sizes, ranging widely from a few microns (small clumps of microbial cells) to several hundred microns (Figure2).

4.3 Specific applications to the sludge treatment processes

Conventional municipal sewage treatment plants utilise mechanical and biological processes to treat wastewater. The activated sludge process is the most widely used for biological waste water treatment in the world, but it results in the generation of a considerable amount of excess sludge that has to be disposed of (Pérez-Elvira *et al.*, 2006). The cost of the excess sludge treatment and disposal can represent up to 60% of the total operating costs. The main alternative methods for sludge disposal in the EU are landfill, land application and incineration, accounting for nearly 90% of total sludge production in the EU. The land application of sewage sludge is restricted to prevent health risks to people and livestock due to potentially toxic elements in the sewage sludge, i.e., heavy metals, pathogens and persisting organic pollutants (Wei *et al.*, 2003). Therefore, current legal constraints, rising costs and public sensitivity towards sewage sludge disposal necessitate the development of strategies for the reduction and minimisation of excess sludge production. Reducing sludge production in waste water treatment instead of post-treating the sludge that is produced appears to be an ideal solution to this issue, because the problem would be treated at its roots (Pérez-Elvira *et al.*, 2006). The biological sludge production in conventional wastewater treatment plants can be minimised in a number of ways. There is a large number of different processes by which sludge reduction can be achieved, but most of these alternative

technologies involve a disintegration of the organic sludge matrix ("solubilisation") in order to improve its further biodegradation (the concept of "solubilisation" and subsequent cryptic growth)(Camacho *et al.*, 2005).

Sewage sludge disintegration during hydrolysis treatment can be defined as the destruction of sludge by external forces. These forces can be physical, chemical or biological in nature. A result of the disintegration process is numerous changes to a sludge's properties, which can be grouped into three main categories (Müller *et al.*, 2004):

- the destruction of floc structures and the disruption of cells
- the release of soluble substances and fine particles
- biochemical processes

Floc destruction and cell disruption will lead to many changes in a sludge's characteristics and the result is an accelerated and enhanced degradation of the organic fraction of the solid phase. The applied stress during the disintegration causes the destruction of floc structures within the sludge and/or leads to the break-up of microorganisms. If the energy input is increased, the first result is a drastic decrease in particle sizes within the sludge. The destruction of floc structures is the main reason for this phenomenon. The disruption of microorganisms is not so easily determined by the analysis of particle size because disrupted cell walls and the original cells are of a similar size (Müller *et al.*, 2004). For this reason, the use of FCM - which allows a rapid and accurate quantification of the total bacterial population - could provide very specific and useful information about the physiological state of bacteria, including cell disruption (Prorot *et al.*, 2008)) during sludge hydrolysis treatment.

Foladori *et al.* (2007) investigated the effect of the sonication treatment on the viability of bacteria present at different points in a WWTP using FCM after fluorescent nucleic acid staining (SYBR-Green and propidium iodide). In particular, they investigated the effects of sonication on mixed populations of microorganisms in raw wastewater and activated sludge, with particular attention paid to the viability and disruption of bacteria. They concluded that in activated sludge samples, low levels of specific ultrasonic energy (Es) produced a prevalent disaggregation of flocs releasing single cells in the bulk liquid, while the disruption of the bacteria was induced only by very high levels of Es (Es>120 kJ L-1).

Prorot *et al.* (2008) assessed the possibility of using FCM to evaluate the physiological state changes of bacteria occurring during sludge thermal treatment. To this end, they stained bacteria with CTC and SYTOX green was used to evaluate biological cell activity and the viability of cell types contained in the activated sludge. The monitoring of cell activity and viability was performed using FCM analysis both before and after the thermal treatment of the activated sludge. Their results indicated an increase in the number of permeabilised cells and a decrease in the number of active cells, and hence the potential of FCM to successfully evaluate the physiological heterogeneity of an activated sludge bacterial population. The same methodology was used by (Salsabil *et al.*, 2009) to investigate cell lysis after activated sludge treatment using sonication. The use of FCM has shown that this sludge treatment did not lead to cell lysis and, therefore, that the origin of soluble organic matter was essentially extracellular (PEC).

Recently, Foladori *et al.* (2010) analysed how sludge reduction technologies (ultrasonication, high pressure homogenisation, thermal treatment and ozonation) affect the integrity and

permeabilisation of bacterial cells in sludge using FCM after a double fluorescent DNA-staining with SYBR-Green and propidium iodide. Whereas the damage to cells increased for increasing levels of applied energy irrespective of the technology, this methodology allowed them to identify different mechanisms of cell disruption depending on the treatment applied.

Finally, Prorot *et al. (*2011) investigated chemical, physical and biological effects of thermal treatment using a multi-parametric approach. In order to clarify the relationship between sludge reduction efficiency and both chemical and biological modifications, the effects of thermal treatment on activated sludge were investigated by combining the monitoring of cell lysis using flow cytometry (FCM), organic matter solubilisation, floc structure and biodegradability. This complete investigation underlines the necessity to combine all parameters, i.e. chemical, physical and biological effects in order to understand and improve the reduction of sludge production during waste water treatment processes.

5. Conclusion

The improvement of control strategies and process optimisation in biotechnology requires the application of analytical methods which allow for the rapid evaluation of metabolic activities and cell viability in environmental processes. Among the many microbiological methods, FCM stands out for its accuracy, speed and the option of sorting components of interest. Nevertheless, the first point that should be taken care of is that there may remain some bias in specific counts by FCM due to the difficulty of achieve complete disaggregation in flocs without the destruction of a fraction of the microbial cells. The design and commercialisation of new cell probes could clearly improve the understanding of individual cells in environmental processes. For instance, the use of fluorochromes bound to specific antibodies could provide interesting information both on and inside the cell. Finally, the potential of FCM for microbiology is still a long way away from being fully utilised. Because each method (culture-dependent methods, PCR, microscopy and FCM) has various advantages and limitations, a combination of methods might be the most reasonable way to achieve a better understanding of microbial life, especially in the environmental field.

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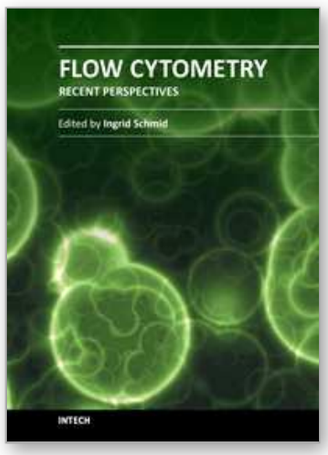

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well 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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