Methods for microbiological quality assessment in drinking water: a comparative study

K. Helmi, F. Barthod, G. Méheut, A. Henry, F. Poty, F. Laurent and N. Charni-Ben-Tabassi

ABSTRACT

The present study aimed to compare several methods for quantifying and discriminating between the different physiological states of a bacterial population present in drinking water. Flow cytometry (FCM), solid-phase cytometry (SPC), epifluorescence microscopy (MSP) and culture method performances were assessed by comparing the results obtained for different water samples. These samples, including chlorinated and non-chlorinated water, were collected in a drinking water treatment plant. Total bacteria were quantified by using SYBR Green II (for FCM) and 4′,6′-diamino-2-phenylindole (DAPI) (for MSP), viable and non-viable bacteria were distinguished by using SYBR Green II and propidium iodide dual staining (for FCM), and active cells were distinguished by using CTC (for MSP) and Chemchrome V6 (for FCM and SPC). In our conditions, counts using microscopy and FCM were significantly correlated regarding total bacteria and active cells. Conversely, counts were not significantly similar using solid-phase and FCM for active bacteria. Moreover, the R2A medium showed that bacterial culturability could be recovered after chlorination. This study highlights that FCM appears to be a useful and powerful technique for drinking water production monitoring.

Key words | bacteria, culture, flow cytometry, microscopy, solid-phase cytometry, viability

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INTRODUCTION

Microbiological monitoring of drinking water quality has to be suitably accurate to improve decision-making regarding treatment and distribution management. The total bacteria level is routinely controlled using standard detection methods based on a culture requiring several days to obtain a result. This approach is not compliant with regards to health risk active management.

Moreover, as bacteria can be oligotrophic or autotrophic (Wang *et al.* 2010), less than 1% of bacteria found in the environment are culturable. Consequently culture methods may minimize the bacterial concentration present in a sample (Phe *et al.* 2005). These bacterial fractions are called viable but non-culturable (VBNC) and may still present an active metabolism. These bacteria may have the potential to recover proliferation capability when conditions become favourable (Kell *et al.* 1998; Vives-Rego *et al.* 2000). In the environment, bacteria can be damaged or inactivated by several different ways including a lack of nutrients, cell aging, predation by protozoa or even viral lysis. In addition, drinking water processing leads to bacteria removal, stress or injury via physical and chemical treatment. Indeed, these treatments exhibit different impacts on bacterial integrity, such as cell wall, metabolism activity or nucleic acids.

The integrity of the cell wall is widely known as a means of discriminating between viable (fraction including culturable and VBNC) and non-viable bacteria, assuming that cells with injured membranes are not able to tolerate an electrochemical gradient and consequently are not able to multiply (Vives-Rego *et al.* 2000). The membrane integrity can be assessed by using various dyes, which can be incorporated exclusively in cells presenting a damaged membrane (Joux & Lebaron 2000; Vives-Rego *et al.* 2000;

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Gregori *et al*. 2001; Manini & Danovaro 2006). Regarding the research of bacteria still presenting an active metabolism, membrane potential or enzymatic activity can be highlighted.

DAPI (4′,6′-diamino-2-phenylindole) is a DNA-binding molecule (with preferential recognition of the A-T base pair), staining bacteria without distinction of viable or non-viable cells, and it emits a blue fluorescence under UV excitation. The principle of marking the bacteria using SYBR Green II is similar to that using DAPI. Indeed, SYBR Green is a nucleic acid dye capable of entering all cells, regardless of their physiological sate. The main difference lies in the fact that the fluorescence of the molecule is green when excited. Propidium iodide (PI) is a fluorescent nucleic acid dye, intercalating bases without particular affinity for specific sequences and with a stoichiometry of one molecule for 4–5 base pairs. PI is used to indicate that cell wall integrity is intact penetrating only cells presenting injured membranes. For active metabolism highlighting, 5-(and 6)-carboxyfluorescein diacetate (5(6)-CFDA) allows the detection of esterase activity in the bacterium. This non-fluorescent molecule has the ability to penetrate passively and to accumulate in most cells. Once inside, the complex is enzymatically hydrolyzed by non-specific intracellular esterases, giving a fluorescent polar product called carboxyfluorescein. This fluorescent product is maintained only in the cells that have intact plasma membranes. CTC (5-cyano-2,3-ditolyl tetrazolium chloride) is a fluorescent marker for the detection of bacterial respiration through dehydrogenase activity (an enzyme of the respiratory chain). CTC emits a red fluorescence once reduced by the enzymes of the respiratory chain. However, it has been shown that under certain conditions, bacteria do not reduce CTC despite metabolic activity (Servais *et al*. 2001).

Consequently, regarding the wide range of existing physiological states and fluorescent dyes, the design of a single suitable method for bacterial viability assessment is a challenge. In this context, several alternative techniques are available to quantify and estimate bacterial physiological states in a short period of time, as opposed to culture methods. Optical systems coupled with fluorescent dyes specific of a physiological state are commonly used to overcome the problems associated with cultural techniques. Among them, microscopy and cytometry techniques present several advantages compared with standard methods. Both approaches allow the counting and discrimination of bacterial cells according to their physiological state using different principles or combinations (Joux & Lebaron 2000).

These techniques allow the application of a wide range of staining protocols (Joux & Lebaron 2000; Vives-Rego *et al*. 2000; Gruden *et al*. 2004; Czechowska *et al*. 2008; Hammes & Egli 2010; Hammes *et al*. 2012) and multiparametric data acquisition, increasing the chance of providing more reliable results regarding the characterization of different physiological states.

However, it is unclear to what extent the data obtained through these different cultivation-free techniques are similar regarding the same information. This study was dedicated to a comparison of methods highlighting one or several bacterial physiological states in natural water samples, including flow cytometry (FCM), solid-phase cytometry (SPC), epifluorescence microscopy and culture methods. The main objective was to select and propose the most adapted technique for drinking water active management, according to technical performance including time to result and level of information related to bacterial quantification. The novelty of this work lies in the fact that statistical tests were performed in order to have an objective approach regarding data interpretation.

MATERIALS AND METHODS

Water samples

Environmental samples were collected from a drinking water treatment plant once a week for 5 weeks. At the end of the process, the water underwent the following treatment: after pH adjustment, water was transferred to a disinfection basin for shock chlorination. CT values (concentration × time) were calculated taking into account the contact time in the disinfection basin and the residual chlorine measured at the basin outlet. In addition, the CT value involves a corrective coefficient (hydraulic efficiency) to determine the effective residence time in the basin, which reflects the degree of short-circuit in the basin. Its use ensures that 90% of the treated water was in contact with the disinfectant for the required time. After the shock chlorination, residual

chlorine underwent an intermediate dechlorination using sodium bisulfite and the water was transferred to tanks for storage. Water was then dechlorinated a second time and chlorination was performed in order to reach an appropriate concentration residual concentration for distributed water. The sampling points, including non-chlorinated, highly chlorinated and finished water, were selected in order to assess to what extent the tested techniques enabled quantification of bacteria and to highlight their viability according to different treatments. Water characteristics and treatment information are presented in Table 1. Each sampling day included samples (in duplicate) collected before (non-chlorinated water) and after (highly chlorinated water) the disinfection basin. Finished water samples were also collected in order to highlight possible bacterial regrowth. All samples were collected in plastic bottles containing sodium thiosulfate in order to neutralize residual chlorine, and were processed within 24 h of sampling.

Flow cytometry

All experiments were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with an argon air-cooled laser, providing 15 mW at 488 nm. The applied protocol enables discrimination between non-viable, viable and active bacteria and was adapted from a method initially developed for freshwater and seawater application, described in a previous study (Gregori *et al.* 2001). The development was performed using pure strains of *Escherichia coli* and *Enterococcus faecalis* (data not shown). To distinguish non-viable and viable bacteria, the principle is based on a nucleic acid double staining, involving the use of two dyes simultaneously: SYBR Green II (SGII, Molecular Probes, Oregon, USA) as a permeant coupled with propidium iodide (PI, Sigma Chemical Co.) as an impermeant. This approach takes advantage of the fluorescence resonance energy transfer which occurs when both probes are linked to nucleic acids. The principle consists of a full quenching of the permeant probe green fluorescence (SGII) by the impermeant probe (PI), generating only a red fluorescence that allows the identification of cells presenting a compromised membrane (non-viable bacteria). Conversely, a lack of quenching means that bacteria present an intact membrane and so are viable (green fluorescence). Briefly, for 980 μL of cell suspension, 10 μL of stock solution of PI (final concentration of 10 mg mL^{-1}) and 10 μL of a 1-log diluted SGII commercial solution in dimethyl sulfoxide (final concentration of 1:1000 v/v) were added simultaneously. Samples were then incubated for 20 min at room temperature in the dark. To differentiate active bacteria, the esterase activity was highlighted using Chemchrome V6 (CV6, AES-Chemunex, France) which allows the reduction of dye extrusion conversely to analogous staining molecules. Fluorescent molecules, which are produced after cleavage and internalized within bacteria, emit a green fluorescence while inactive bacteria are not able to hydrolyze the substrate and are therefore not visualized. Briefly, 100 μL of each sample was added to 890 μL of Chemsol B16 buffer (AES-Chemunex, France) containing 10 μL of CV6. Samples were incubated for 30 min at 30 °C in the dark and were analyzed immediately after incubation or kept at 4 °C prior to analysis in order to avoid excessive background noise. Green fluorescence of cells stained with SGII or CV6 dyes was detected in the FL1 channel (530 ± 15 nm) and red fluorescence of cells stained with PI was detected in the FL3

Table 1 | Chlorination procedure and water characteristics for each sampling day

	Chlorination basin			Storage tank			Finished water		
Sampling date	Contact time	Chlorine (mg/L)	CT (mg/L.min)	Contact time	Chlorine (mg/L)	CT (mg/L.min)	Temperature (°C)	pH	Chlorine (mg/L)
7 Jul	2 h 48 min	1.54	186.30	2 h 45 min	1.08	68.58	24.76	7.50	< 0.5
21 Jul	1 h 48 min	1.57	155.58	3 h 13 min	2.13	157.80	24.52	7.51	< 0.5
28 Jul	3 h 29 min	1.80	270.21	2 h 59 min	1.13	77.60	23.40	7.55	< 0.5
5 Aug	3 h 52 min	1.54	257.18	3 h 22 min	1.13	87.54	22.98	7.54	< 0.5
10 Aug	3 h 59 min	1.45	250.15	3 h 16 min	1.14	85.88	22.65	7.53	< 0.5

CT = (concentration × time).

channel (>620 nm). Also, 1-μm standardized fluorescent microspheres (Fluoresbrite YG, Polysciences, USA) were systematically added to each sample as an internal reference. Analyses were performed at a flow rate of 20 μL per minute on average and with a 1-min duration for collection strain suspensions and 2 min for environmental samples. All parameters were collected in logarithmic signal and then analyzed using Cell Quest (Becton Dickinson, California, USA).

Solid-phase cytometry

The solid-phase cytometer device (ChemScan RDI, Chemunex, France) consisted of a laser-scanning unit (488-nm argon laser) able to scan a 25-mm diameter membrane filter within 3 min. Two photomultiplier tubes with wavelength windows were set for the green (500–530 nm) and for the amber (540–585 nm) regions of the emission spectrum of fluorescein, detecting the fluorescence light emitted by the labelled bacteria. Active cells were analysed with CV6 staining protocol following the instructions provided by the manufacturer (AES-Chemunex, France). Briefly, after filtration on a 0.2-μm membrane, a pre-labelling revitalization step (ChemSol A4) and a counterstain (CSE/2) involving a 60-minute incubation at 37 °C was first performed. Then, for viable microorganism labelling, bacteria were stained using CV6 dye and the membrane was incubated for 30 min at 30 °C in the dark prior to analysis by ChemScan. The fluorescence signals were collected by two photomultipliers (wavelengths of 500–530 and 540–585 nm) and processed by software enabling the instrument to discriminate between stained bacteria and background electronic noise or autofluorescent particles. Scan results were displayed as coloured spots which were then visually confirmed using epifluorescence microscopy.

Epifluorescence microscopy

Microscopical analyses were performed on an epifluorescence microscope (DMRB, Leica, Germany) equipped with a 100 W light source (HBO, OSRAM, Germany) with a ×1000 magnification. Total bacteria count was obtained by using 4,6-diamino-2-phenylindole (DAPI) (Sigma, USA) for nucleic acid staining. Briefly, a 200-mL aliquot was stained by adding 1% (v/v) of a DAPI solution (0.25 mg·mL^{-1}) for 20 min at room temperature in the dark. The sample was then filtered through a 25-mm black polycarbonate membrane with a pore size of 0.2 mm (GTBP, Millipore, USA). Before the filtration step, 2% (v/v) formaldehyde (Merck, Germany) was added to the solution for fixation and preservation of the sample. Reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, USA) was used to identify viable respiring cells. Briefly, 200 mL of sample was filtered through a 25 mm black polycarbonate membrane with a pore size of 0.2 mm (Nucleopore, France) in a sample collector (Millipore, USA). Then, 5 mL of a CTC solution (0.64 mg·mL^{-1}) was added in the well and incubated at room temperature for 2 h. For CTC, DAPI and nucleic acid dual staining, the membrane was then placed on a slide and a bacteria count was performed by counting at least 10 microscopic fields containing a minimum of 20 cells.

Culture

For heterotrophic plate count (HPC) of environmental water samples, 1 mL of the appropriate dilution was transferred into a sterile Petri dish and mixed with about 20 mL of R2A or PCA (NF EN ISO 6222) media. For R2A, colonies were counted after 11 days of incubation at 20 °C and for PCA, colonies were enumerated after 48 h of incubation at 36 and 22 °C. All culturable counts are expressed as CFU mL^{-1} (colony forming units per milliliter).

Statistical analysis

Differences between the data series were assessed by Wilcoxon test. The raw data used for statistical analysis were the bacteria concentration per milliliter in the initial sample. All statistical tests were performed using the XLSTAT software (Addinsoft, New York, USA).

RESULTS

Results overview

A comparison of four techniques highlighting bacterial physiological state were performed on water samples including

non-chlorinated, highly chlorinated and finished water. Samples were analyzed in parallel by FCM, SPC, microscopy (MSP) and culture. The results are presented in Figure 1 (logarithmic scale). Active bacteria were detected in 100% of non-chlorinated water samples by microscopy (CTC), solid-phase cytometry (CV6) and flow cytometry (CV6), which was correlated to HPC counts. None of the bacteria presenting enzymatic activity were detected using FCM in chlorinated samples (highly chlorinated and finished water). Conversely, using microscopy, active bacteria were detected in five highly chlorinated water samples out of eight, and eight finished water samples out of 10. Similarly, SPC highlighted their presence in two finished water samples out of eight. Regarding culture (R2A), CFU were observed in three highly chlorinated water samples out of eight and four finished water samples out of 10. However, for chlorinated samples, detection results obtained by R2A culture are coincident three times regarding microscopy and only once regarding SPC. Non-viable bacteria were detected by FCM using PI (coupled with SGII) after chlorination while no viable bacteria, using SGII (coupled with PI), and no active bacteria (CV6) were detected. It is important to note that storage time could have an impact on the results, even if residual chlorine was neutralized using sodium thiosulfate. This is particularly the case regarding culture-based methods as bacteria may lose their ability to grow during a 24-hour period, unlike techniques using vital dyes.

Statistical correlation

In order to rigorously compare the methods that are expected to provide the same information on bacterial physiological states, a statistical test (Wilcoxon test) was applied using counts obtained by FCM, SPC and microscopy (Table 2) assessing total and active bacteria. The results showed a significant correlation between microscopy (DAPI) and flow cytometry (SGII) concerning total bacteria quantification. Similarly, active bacteria enumerated by microscopy (CTC) were correlated with results obtained using flow or solid phase cytometry (CV6). Interestingly, the results were not correlated between FCM and SPC (both using CV6). Indeed, the counts recorded by FCM were systematically ten times higher than SPC.

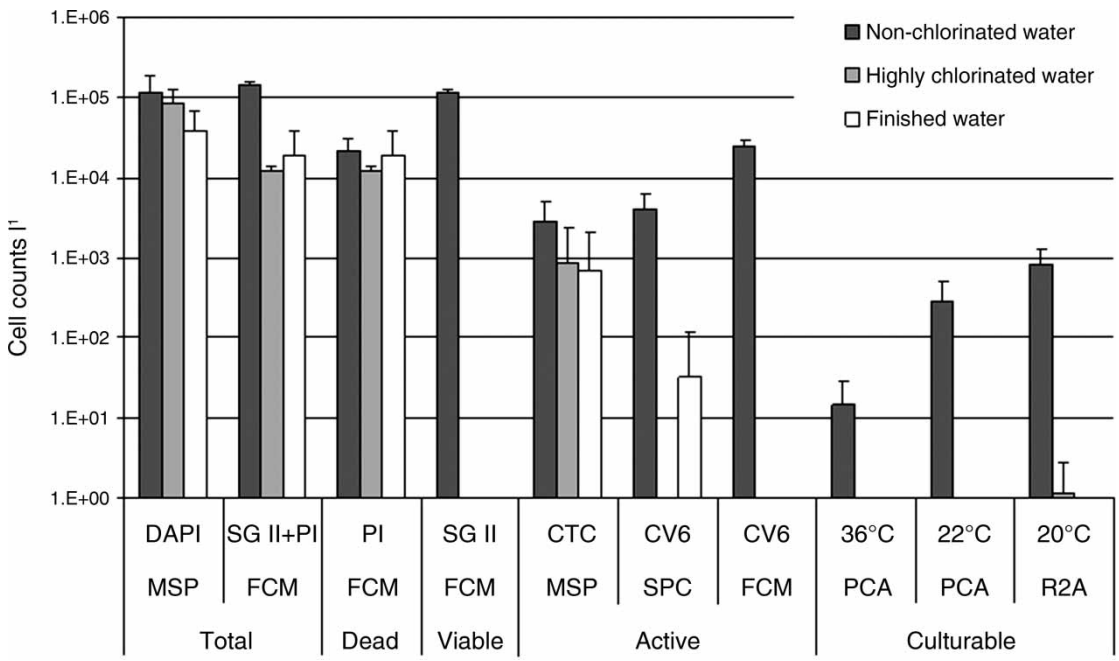

Figure 1 | Comparison of four different methods for bacterial quantification and physiological state assessment. DAPI = 4,6-diamino-2-phenylindole, FCM = flow cytometry, SPC = solid-phase cytometry, MSP = epifluorescence microscopy, SGII = SYBR Green II, PI = propidium iodide and CV6 = chemchrome V6. For non-chlorinated water $n = 10$ except for FCM $n = 8$ (SGII and PI), for chlorinated water $n = 8$ except for FCM $n = 6$ (SGII and PI) and for produced water $n = 10$ except for FCM $n = 7$ (SGII and PI) and $n = 9$ (CV6).

Table 2 | Statistical analysis (Wilcoxon test) for analytical methods comparison, p (*n*)

	SGII (FCM)	CTC (MSP)	CV6 (FCM)
DAPI (MSP)	0.137 (22)	N/a	N/a
CTC (MSP)	N/a	N/a	0.181 (25)
CV6 (SPC)	N/a	0.246 (26)	0.005 (25)

N/a = not applicable, *n* = number of analyses taken into account for statistical test, DAPI = 4,6-diamino-2-phenylindole, FCM = flow cytometry, SPC = solid-phase cytometry, MSP = epifluorescence microscopy, SGII = SYBR Green II, and CV6 = chemchrome V6.

Time to result

In addition to the detection performances, another important aspect is the processing time related to each method to provide data on bacteria physiological state (Table 3). FCM is able to acquire four types of information within 1 h regarding physiological states while SPC obtains a piece of information within 2 h 30 min and epifluorescence microscopy provides two kinds of information within 4 h. Regarding culture methods, time required to obtain colony forming unit counts ranges from 48 h to 11 days, depending on the media and incubation temperature.

DISCUSSION

The aim of the study focused on the comparison of four methods which allows the assessment of the physiological state of bacteria in drinking water samples. Concerning non-chlorinated natural samples, counts (total and active bacteria) obtained using microscopy and FCM were not significantly different. Conversely, despite using the same dye to detect active bacteria (Chemchrome V6), counts were notably different when using SPC and FCM. Indeed, a 1-log difference was systematically observed between the two methods. This could be explained by the fact that, for SPC, the fluorescence intensity threshold is set to detect only highly active bacteria. As a result, bacteria exhibiting weak fluorescence intensity, suggesting a lower esterase activity (including injured cells presenting a remaining enzymatic activity), were not taken into account during the enumeration. Conversely, this category of bacteria was detected by FCM, suggesting that about 10% of the population in the non-chlorinated analyzed samples may present a low or residual enzymatic activity. However, the observed discrepancies between both methods were not observed in a previous study, showing similar results regarding flow and SPC when using Chemchrome V6 staining (Parthuisot *et al.* 2000). This may be explained by the fact that this comparison was performed using collection strains whereas in the present study, the evaluation was achieved with environmental samples, suggesting a possible matrix impact on indigenous bacterial behaviour.

Shock chlorination led to important CT values, ranging from 156 to 250. Surprisingly, despite a high chlorine disinfection level, active bacteria were detected in 72% of chlorinated samples using CTC by microscopy (taking into account highly chlorinated and finished water samples). This may be explained by extracellular deposits of formazan (red fluorescent salt converted by active bacteria) which could be erroneously included during enumeration (Cools *et al.* 2005). In the same way, active bacteria were detected in 11% of finished water samples using solid-phase cytometry (CV6) while FCM showed 0% of positive samples. This difference may be due to the fact that the pre-labelling step applied on the membrane (using ChemSol A4) for SPC analysis involves a revitalization procedure, whereas this step is not included in the FCM protocol. Culturable bacteria (R2A) were highlighted in 39% of the chlorinated samples but some discrepancies were observed. Indeed, only three positive samples by microscopy out of 13 and one positive sample by SPC out of two were positive by culture as well. These discrepancies could be explained by R2A agar characteristics. Indeed, in contrast to PCA media, R2A is very low in nutrients and is incubated for a long period (11

Table 3 | Processing time for the four compared detection methods

Method	Dye or media	Processing time	Physiological state information
FCM	SGII/PI	1 h	Total, non-viable and viable
	CV6		Active
SPC	CV6	2 h 30 min	Active
Epifluorescence microscopy	DAPI	1 h	Total
	CTC	3 h	Active
Culture	PCA	48 h	Culturable
	R2A	11 days	Culturable

days at 22 °C), stimulating the growth of stressed and/or chlorine-tolerant bacteria. These observations underscore the difficulty of linking active and culturable bacteria counts (Kell *et al.* 1998).

Regarding bacterial population dynamics, and taking into account the average of FCM quantification ($n = 8$) of non-chlorinated samples, the viable and non-viable fractions represent 80.6 and 14.8% of the total bacteria, respectively. In addition, the active and the culturable bacteria parts represent 21.4 and 0.7% of the viable bacterial population, respectively, which is consistent with observations reported from previous studies (Vives-Rego *et al.* 2000; Berney *et al.* 2008). These observations suggest that 79.9% of the total bacteria may be VBNC bacteria. Previous studies reported that VBNC cells should be taken into account regarding health risk assessment (Alleron *et al.* 2008; Falcioni *et al.* 2008), as a fraction may recover their culturability potential under favourable conditions (Kell *et al.* 1998; Vives-Rego *et al.* 2000). In addition, non-culturable pathogen bacteria may be capable of expressing virulence factors, such as toxins (Rahman *et al.* 1994). It is important to note that this information regarding VBNC populations cannot be obtained either by microcopy or by SPC, underlining the gain provided by FCM. On the other hand, for chlorinated water samples analyzed by FCM, 100% were identified as non-viable bacteria and 0% as viable (including active bacteria), demonstrating that the disinfection conditions using chlorine were efficient regarding bactericidal effect. Few FCM studies were carried out using SYBR Green II and propidium iodide dual staining for drinking water monitoring application. A previous work (Phe *et al.* 2005) corroborates the fact that using SGII and PI with FCM is reliable for assessing bacteria injuries due to chlorine disinfection. Moreover, this method appears to be reliable compared to a common method based on bacterial high and low nucleic acid content (HNA/LNA) assessment by measurement of emitted fluorescence intensity. Previous studies have shown that HNA/LNA could not be directly be linked to bacterial viability. Indeed, bacterial populations present different DNA content or cell sizes depending on the aquatic ecosystem (Bouvier *et al.* 2007; Ramseier *et al.* 2011) and the main part of LNA bacteria could still be viable (Gregori *et al.* 2001).

Concerning processing time and data collection, FCM presents a non-negligible advantage as less than 1 h is required to obtain information on bacterial population including total, non-viable, viable and active fractions. Otherwise, to obtain some of the data, it is necessary to perform several time-consuming methods in parallel, each providing a single piece of information. In view of these observations and although no method is ideal, FCM appears to be a reliable alternative technique to labour-intensive methods as it allows rapid quantification and differentiation of cell clusters according to their physiological state: viable bacteria, active bacteria and non-viable bacteria. As a result, this work, in agreement with previous studies (Berney *et al.* 2008; Hammes *et al.* 2008, 2012), suggests that this technique is useful for drinking water production monitoring.

CONCLUSIONS

- FCM is a powerful method for drinking water quality assessment regarding bacterial enumeration and physiological states distinction. Indeed, this method enabled viable quantification of active and non-viable bacteria with a time to result of less than 1 h.
- This study allowed the comparison of several methods allowing the assessment of biocide impact on a bacterial population. The chlorination effect on bacterial viability was obviously highlighted in real water samples using FCM.
- The results obtained under our conditions are encouraging. Thus, further work has to be undertaken using the FCM principle to improve and extend this application for the specific detection of other microorganism types such as parasitic protozoa, viruses, or endospores by combining nucleic acid probes, fluorescent antibodies and/or vital dyes.

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