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ORIGINAL ARTICLE

Rapid detection of *Escherichia coli* in waters using fluorescent *in situ* hybridization, direct viable counting and solid phase cytometry

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Keywords*E. coli*, fluorescent *in situ* hybridization, solid phase cytometer, viable cell, water quality.**Correspondence**Julia Baudart, Laboratoire ARAGO, Avenue Fontaulé, F-66650 Banyuls-sur-Mer, France.
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Abstract**Aims:** We developed an improved Fluorescent *In Situ* Hybridization FISH-based method to detect viable *Escherichia coli* cells by solid phase cytometry (SPC), and results were compared to those obtained by the standard culture method.**Methods and Results:** The method includes a direct viable count (DVC) assay, multi-probes labelled and unlabelled (helpers) to detect specifically viable *E. coli* cells and to enhance SPC cell counts. We demonstrate that helpers increase the fluorescence intensity of hybridized *E. coli* cells as detected by SPC and assess the high specificity of the DVC-FISH procedure on a large panel of cultured strains. Application to seawater, freshwater and wastewater samples showed a good correlation between SPC cells counts and standard plate counts.**Conclusion:** The high specificity of the procedure was demonstrated as well as its accuracy for detecting and counting viable *E. coli* cells in environmental samples.**Significance and Impact of the Study:** The developed approach may be used to monitor faecal contamination sources and to investigate the occurrence of viable *E. coli* in natural environments.**Introduction**

Bacteriological control of water quality requires the detection and quantification of a faecal contamination indicator to signal the presence of a potentially harmful pathogen of faecal origin. Among the indicators of faecal contamination, *Escherichia coli* is now recognized in Europe as the best one by the European Bathing Water Quality Directive (EEC, 2006); however, most of the standard methods used for the quantification of *E. coli* require at least 18 h. Therefore, rapid methods for detecting and counting *E. coli* in recreational waters (seawater and freshwater) are needed to better predict any contamination and for environmental management. This need is reinforced by some well-known limitations of culture-based methods: (i) the low specificity of solid media used for the detection of *E. coli* that requires biochemical

confirmation steps and delays the results by 2 or 3 days (Maheux *et al.* 2008) and (ii) the presence of starved and stressed slow-growing cells or viable but nonculturable (VBNC) micro-organisms in aquatic environment (Garcia-Armisen *et al.* 2005). The significance of VBNC cells in causing disease is today unclear. Nevertheless, VBNC cells should be detected along in addition to culturable cells, because VBNC cells can potentially become pathogenic when favourable conditions are again present (Besnard *et al.* 2000). Alternative methods should allow the detection of all viable cells and provide results within a few hours.

A few rapid methods have been developed to enumerate viable and VBNC *E. coli* cells without any culture step. Some of these methods are based on the detection of the β -D-glucuronidase activities (Van Poucke and Nelis 2000; Garcia-Armisen *et al.* 2005; Lebaron *et al.* 2005).

Nevertheless, limitations have been reported about their specificity (Fiksdal and Tryland 2008; Baudart *et al.* 2009).

Other methods based on Fluorescent *In Situ* Hybridization (FISH) to analyse rRNA sequences can detect the presence of cells of either *E. coli* or members of the *Enterobacteriaceae* in water samples (Prescott and Fricker 1999; Jansen *et al.* 2000; Regnault *et al.* 2000; Baudart *et al.* 2002; Garcia-Armisen and Servais 2004).

FISH-based methods are particularly interesting because of the high specificity of 16S rDNA sequences (Amann *et al.* 1995). However, the majority of studies that use these methods for cell detection in environmental samples have limited applications because of the very low fluorescence signal after hybridization. These low fluorescence signals are mostly owing to the low ribosomal content of starved cells (Amann *et al.* 1995) and/or to the low accessibility of some rRNA sequences because of the conformational state of the rRNA molecule (Fuchs *et al.* 1998). To overcome the ribosomal content limitations, some treatments as the catalysed reporter deposition (CARD)-FISH technique and/or the direct viable count (DVC) assay before the FISH procedure were proposed. The CARD-FISH technique increases the fluorescence signal of hybridized cells using horseradish peroxidase-labelled probes (Lebaron *et al.* 1997; Baudart *et al.* 2002). Nevertheless, this method is still time-consuming because it requires many procedural steps. In addition, it does not resolve the problem of the inaccessibility of some rRNA sequences (Fuchs *et al.* 1998). The DVC assay applied before the FISH procedure offers the advantage of inducing the ribosomal RNA synthesis of responsive cells (considered as viable) and therefore allows the specific detection of viable cells at a family, genus or species level (Nishimura *et al.* 1993; Kalmbach *et al.* 1997; Villarino *et al.* 2000; Kenzaka *et al.* 2001; Baudart *et al.* 2002; Garcia-Armisen and Servais 2004). To increase the accessibility of some rRNA sequences when ineffective for FISH, Fuchs *et al.* (2000) reported the use of unlabelled DNA oligonucleotides (helpers) adjacent to the target

sequences with labelled DNA oligonucleotides, which can open inaccessible rRNA regions for FISH. In this study, we designed and tested the effect of helpers for two probes published as specific for the detection of *E. coli* in water by FISH and proposed a combined DVC-FISH method for the detection and quantification of viable *E. coli* cells by solid phase cytometry (SPC) in different types of water (seawater, freshwater and wastewater).

Materials and methods

Escherichia coli-specific oligonucleotide probes and helpers

Two 16S rRNA probes (Ecoli and Colinsitu) targeting specific sequences for *E. coli* were selected from literature (Table 1) (McGregor *et al.* 1996; Jansen *et al.* 2000; Regnault *et al.* 2000). The specificity of both oligoprobes was verified, in first, *in silico* by using the probe match function of the Ribosomal Database Project software package (Maidak *et al.* 2001) and the BLAST function of the NCBI website (Altschul *et al.* 1997). Unlabelled helpers for Ecoli and Colinsitu probes (HEc_L, HColin_L and HColin_R) were designed using the ARB software (Ludwig *et al.* 2004) with regard to their (i) location (adjacent to the target sequences with *E. coli* DNA oligonucleotides), (ii) *in silico* specificity (included *E. coli* species), (iii) length and (iv) melting temperature (T_m) very similar to those of the Ecoli and Colinsitu probes (Table 1) as recommended by Fuchs *et al.* (2000). All oligonucleotides were synthesized by ThermoFisher Scientific (Ulm, Germany), and probes were conjugated at their 5' end with fluorescein (fluo).

The effect of helpers on the fluorescent intensity of hybridized cells was investigated on *E. coli* ATCC 11775. Fluorescent intensity was quantified by solid phase cytometer (SPC, ScanRDI™; AES-Chemunex, Ivry sur Seine, France). Different probe combinations were tested for FISH assays: single labelled probe without helpers, a couple of labelled probes without helpers, single labelled probe with helpers and a couple of labelled probes with

Table 1 The 16S rRNA-targeted oligonucleotide probes used in this study

Probe/helper*	Sequence (5'-3')	rRNA target and position	Brightness class†	References
Ecoli	GCAAAGGTATTAACCTTACTCCC	16S, 453-475	VI	McGregor <i>et al.</i> (1996); Jansen <i>et al.</i> (2000)
HEc_L	TTCTCCCCGCTGAAAGT	16S, 435-452	III	This study
Colinsitu	GAGACTCAAGATTGCCAGTATCAG	16S, 637-660	VI	Regnault <i>et al.</i> (2000)
HColin_L	ATGCAGTCCAGGTTGAG	16S, 617-636	IV-VI	This study
HColin_R	ACCTGGAATTCTACCCCTCTAC	16S, 661-684	IV	This study

*Helpers are noted with the prefix H.

†As reported in Fuchs *et al.* (1998).

their helpers (as reported in Table 3). Fluorescence was quantified in parallel from cells in stationary growth phase and from DVC-treated cells to evaluate the effect of the DVC assay on the fluorescent intensity of cells detected by SPC.

Direct Viable Count (DVC) test

The viability of cells was tested by a modified DVC assay developed by Kogure *et al.* (1979) and described in Baudart *et al.* (2002). Sample was filtered on 0.40- μm black polyester membrane (CB04, Cycloblack, 25 mm; AES-Chemunex), then placed on a 25-mm cellulose pad (labelling pad; AES-Chemunex) soaked with 550 μl of nonselective low nutritive broth [70% phosphate buffer saline (PBS) (pH 7.2, 130 mmol l⁻¹ NaCl, 2.5 mmol l⁻¹ KCl, 10 mmol l⁻¹ Na₂HPO₄, 1.8 mmol l⁻¹ KH₂PO₄), 0.036% yeast extract, 0.36% casamino acid] containing nalidixic acid (final concentration 10 $\mu\text{g ml}^{-1}$; Sigma Aldrich, Saint Quentin Fallavier, France) and incubated at 37°C for 4 h. Cells were then hybridized by the FISH procedure. Cells that responded positively to the DVC assay become elongated, show high fluorescent signal according their high ribosomal content and can be considered as viable (Baudart *et al.* 2002). An untreated DVC control was made up for each sample analysed by the SPC.

FISH protocol

After filtration of DVC-treated or untreated sample, the CB04 membrane was transferred to a labelling pad soaked in 600 μl of 80% ethanol for 3 min at room temperature. The membrane and pad were left in Petri dish at room temperature for 4 \pm 1 min and then dried at room temperature for 3 \pm 1 min. Membrane was placed on 50 μl of hybridization buffer [900 mmol l⁻¹ NaCl, 20 mmol l⁻¹ Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate, 20% deionized formamide, 0.5% bovine serum albumin, 0.1 mg ml⁻¹ Poly(A), 0.01% Evans blue] containing respective probes (final concentration of each probe and helpers was 2.5 ng μl^{-1}) in hybridization chambers and incubated at 46°C for 90 min in a covered water-bath. After hybridization was complete, each membrane was placed on a labelling pad soaked in 550 μl of washing buffer [40 mmol l⁻¹ NaCl, 20 mmol l⁻¹ Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate, 5 mmol l⁻¹ EDTA, 0.01% Evans blue] and then stored until analysis at room temperature.

Specificity control of probe sets

The set of probe given the higher fluorescent intensity measured by SPC was then applied on a large panel of

bacterial strains included 27 *E. coli* and 44 non-*E. coli* strains culture collections as well as isolates from the environment (Table 2) to test their specificity. Tests were performed from cells in the late stationary growth phase (low rRNA content). Bacterial strains were grown overnight on tryptic casein soya broth (TSB; AES-Chemunex) at 37°C for the *Enterobacteriaceae* strains and at 30°C for non-*Enterobacteriaceae* strains. One percentage of the overnight preculture was inoculated into a fresh TSB and then cultured for 18–24 h at the same temperature that preculture. Following the cultivation procedure, 1% of cell suspension was added to a PBS solution, and then 100 μl of this cell suspension was filtered on CB04 membranes. Each membrane was then treated by the DVC assay and FISH protocol (see below). Finally, membranes were air-dried and mounted with an antibleaching media (Citifluor AF1; Citifluor Ltd., Houdon, UK) for observation by epifluorescence microscopy using a WIBA filter block for the fluorescein (FITC) detection (Olympus model AX70, Hamburg, Germany). A negative control (without oligonucleotides) was performed to test the auto-fluorescence of the protocol.

SPC detection and enumeration

Escherichia coli SPC counts were measured as following: membranes were placed after FISH procedure into the SPC's sample holder and on to a 25-mm black cellulose membrane (support pad, 0.45 μm pore size; AES-Chemunex), which was previously saturated with 100 μl of washing buffer. The SPC system (ScanRDI™; AES-Chemunex) scans the sample on the support pad with an argon laser beam (488 nm emission wavelength) recording all fluorescent events at 500–530 and 540–570 nm with two photomultiplier tubes. Fluorescent events are discriminated as targeted fluorescent bacterial signal or as false-positive (autofluorescent particles) using a set of discriminant parameters (described in Mignon-Godefroy *et al.* 1997). The discriminant parameters were as follow: peak intensity, 50–2000; secondary/primary ratio, 0.1–0.8; number of lines, 1–50; and number of samples, 1–100.

Analytical results are plotted in two dimensions where all discriminated fluorescent events are shown in terms of *x* and *y* coordinates on a schematic figure of the membrane. One membrane can be scanned in three minutes, including the discrimination step.

The validation step using epifluorescent microscopy was then performed, using a BH2 epifluorescent microscope (Olympus) with a FITC filter block installed and a motorized stage driven by the ScanRDI™ system.

Fluorescent yellow-green 2- μm reference beads (standard G; AES-Chemunex) were used as external standard of fluorescence, and measures of cellular fluorescence was

Table 2 Bacterial strains used for specificity control of the direct viable count–Fluorescent *In Situ* Hybridization (DVC–FISH) assay

Bacterial strains	Origin*	Taxonomic family	DVC–FISH assay	
			With probes*	Without probes
Non-<i>Escherichia coli</i> strains				
<i>Bacillus subtilis</i>	ATCC 6653	<i>Bacillaceae</i>	–	–
<i>Brevundimonas diminuta</i>	ATCC 11569	<i>Caulobacteraceae</i>	–	–
<i>Citrobacter freundii</i>	ATCC 8090	<i>Enterobacteriaceae</i>	–	–
<i>Enterobacter aerogenes</i>	ATCC 13048	<i>Enterobacteriaceae</i>	–	–
<i>Enterobacter cloacae</i> type Co1303	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Ent. cloacae</i> type Co2301	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Enterobacter sakazakii</i> type Ba1803	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Enterobacter gergoviae</i>	CIP 761T	<i>Enterobacteriaceae</i>	–	–
<i>Enterococcus faecalis</i>	CIP 106877	<i>Enterococcaceae</i>	–	–
<i>Enterococcus faecium</i>	CIP 5855	<i>Enterococcaceae</i>	–	–
<i>Klebsiella oxytoca</i>	ATCC 13182	<i>Enterobacteriaceae</i>	–	–
<i>Kl. oxytoca</i>	CIP 7932	<i>Enterobacteriaceae</i>	–	–
<i>Klebsiella pneumoniae pneumoniae</i>	ATCC 13883	<i>Enterobacteriaceae</i>	–	–
<i>Kl. pneumoniae pneumoniae</i> type Ba0504	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Kl. pneumoniae pneumoniae</i> type Ba0508	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Kl. pneumoniae pneumoniae</i> type Co1805	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Kl. pneumoniae rhinoscleromatis</i>	ATCC 13884	<i>Enterobacteriaceae</i>	–	–
<i>Klebsiella sp.</i> type Ba0506	Seawater	<i>Enterobacteriaceae</i>	–	–
<i>Lactobacillus lactis lactis</i>	CIP 7057	<i>Lactobacillaceae</i>	–	–
<i>Micrococcus luteus</i>	ATCC 9341	<i>Micrococcaceae</i>	–	–
<i>Pseudomonas aeruginosa</i>	ATCC 19429	<i>Pseudomonadaceae</i>	–	–
<i>Ps. aeruginosa</i>	ATCC 27853	<i>Pseudomonadaceae</i>	–	–
<i>Ps. aeruginosa</i>	ATCC 15442	<i>Pseudomonadaceae</i>	–	–
<i>Pseudomonas putida</i> type B494	Pharmaceutic	<i>Pseudomonadaceae</i>	–	–
<i>Pseudomonas syringae</i> type cc94	Pharmaceutic	<i>Pseudomonadaceae</i>	–	–
<i>Proteus mirabilis</i>	ATCC 10005	<i>Enterobacteriaceae</i>	–	–
<i>Raoultella ornithinolytica</i>	CIP 103434T	<i>Enterobacteriaceae</i>	–	–
<i>Raoultella planticola</i>	ATCC 33531	<i>Enterobacteriaceae</i>	–	–
<i>R. planticola</i>	ATCC 33558	<i>Enterobacteriaceae</i>	–	–
<i>Raoultella terrigena</i>	ATCC 33257	<i>Enterobacteriaceae</i>	–	–
<i>Salmonella enterica</i> Typhimurium LT2	ATCC 43971	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Typhimurium	CIP 103446	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Typhimurium type S54	Freshwater	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Indiana type S47	Freshwater	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Indiana type S55	Freshwater	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Enteritidis type S191	Freshwater	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Parayphi B type S44	Freshwater	<i>Enterobacteriaceae</i>	–	–
<i>Salm. enterica</i> Virchow type S109	Freshwater	<i>Enterobacteriaceae</i>	–	–
<i>Staphylococcus aureus</i>	ATCC 6538	<i>Staphylococcaceae</i>	–	–
<i>Serratia marcescens</i>	CIP5864	<i>Enterobacteriaceae</i>	–	–
<i>Shigella boydii</i>	CIP 82-50	<i>Enterobacteriaceae</i>	+	–
<i>Shigella dysenteriae</i>	CIP 57-28	<i>Enterobacteriaceae</i>	+	–
<i>Shigella flexneri</i>	CIP 82-48T	<i>Enterobacteriaceae</i>	–	–
<i>Shigella sonnei</i>	CIP 82-49	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> strains				
<i>E. coli</i>	ATCC 11775	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i>	ATCC 8739	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i>	ATCC 104130	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> O157:H7	ATCC 35150	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> O126:B16	OOB	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> K12	OOB	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Co0601	Seawater	<i>Enterobacteriaceae</i>	+	–

Table 2 (Continued)

Bacterial strains	Origin*	Taxonomic family	DVC–FISH assay	
			With probes*	Without probes
<i>E. coli</i> type Ba0601	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Co2304	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Co2703	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Ba3010	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Co3001	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Co0402	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Ba0503	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Ba0513	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Co0602	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Ba1104	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Ba1105	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type Ba1107	Seawater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S31	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S877	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S1024	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S1025	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S1027	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S1029	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S1030	Freshwater	<i>Enterobacteriaceae</i>	+	–
<i>E. coli</i> type S1035	Freshwater	<i>Enterobacteriaceae</i>	+	–

*ATCC, American Type Culture Collection; CIP, Institut Pasteur Collection, France; OOB, Culture collection maintained by the Oceanographic Observatory of Banyuls sur mer–France.

expressed as the ratio between fluorescence measured and fluorescence of standard.

Counting of *Escherichia coli* cells by SPC (*Escherichia coli* SPC counts)

The specificity and the sensitivity of the total procedure included the cells counting by SPC were tested from cultured strains (11 *E. coli* and 21 non-*E. coli*) and naturally contaminated water samples. Cultured strains were prepared as described previously, and suspensions cells were diluted in PBS before filtration of 100 μ l of approximately from 10^2 to 10^3 cells ml⁻¹. The *E. coli* SPC counts were compared to the plate counts measured on nonselective media (Nutrient agar; BD Biosciences, Le Pont de Claix, France). Plate counts were counted from the same volume analysed in SPC spread over on nonselective media, Nutrient agar, for 24 h at 37°C for *Enterobacteriaceae* strains and 30°C for non-*Enterobacteriaceae* strains.

Occurrence of *E. coli* was investigated with the both DVC–FISH–SPC and standard culture methods from freshwater and sea-bathing water samples contaminated by input of faecal bacteria from a wastewater treatment plant (WWTP) outfalls and located in the French Mediterranean coast. The wastewater sample was collected from the same site. *Escherichia coli* SPC counts were compared to standardized Most Probable Number (MPN)

method (ISO 9308-3) using microplates (Biokar Diagnostics, Beauvais, France) for *E. coli* enumeration. In this method, based on the defined substrate approach, 200 μ l aliquots of different dilutions (1/2, 1/20, ...) of each sample were added to microplate wells containing the substrate, 4-methylumbelliferyl- β -D-glucuronide, in a dehydrated form. This substrate is hydrolysed by β -D-glucuronidase releasing the fluorescent compound methylumbelliferone (MUF) that is detectable under ultraviolet light. The microplates were incubated for 36–48 h at 44°C, and the MUF fluorescence was measured for each well under UV light. *Escherichia coli* abundance was based on the number of positive wells and a statistical analysis using Poisson's law. The detection limit of this method is 15 culturable *E. coli* (100 ml)⁻¹. The expected coefficient of variation between replicates using this method is about 30% (Lebaron *et al.* 2005; Prats *et al.* 2008).

SPC analyses were performed on 25 ml samples of freshwater and seawater and on 1 ml of wastewater diluted 10⁴-fold in PBS.

Total cells count

Total cells counts were enumerated from water samples to estimate the relative proportion of *E. coli* in total bacteria. Sub-samples were fixed with formaldehyde (2% final concentration), and total cell concentrations were

determined by flow cytometry (FascCalibur; Beckton Dickinson, Le Pont de Claix, France) using the SybrGreen I procedure (Lebaron *et al.* 1998).

Statistical analysis

Normal distribution of data was tested using the Shapiro–Wilk test, and statistical comparisons between different cell concentrations were made using the *t* test for normally distributed data and the Mann–Whitney test when normality was not confirmed. The relation between SPC counts and standard plate counts was determined by linear regression models (Spearman test for $\alpha = 0.05$). All statistics were conducted using XL stat software (Addinsoft, Paris, France).

Results

Helpers design

On 16S rRNA map, the Ecoli and Colinsitu probes were respectively located in the helices 18 and 22. These targeting sites are located within the brightness class VI, qualified as ‘totally blocked sites’ by Fuchs *et al.* (2000) and showing a very low *in situ* accessibility of oligonucleotide probes (Table 1). Helper sequences to the Ecoli and Colinsitu probes were designed using the ARB software and selected to bind to the rRNA of *E. coli* in regions immediately adjacent to the probes and according to their *in situ* accessibility. Helper sequences were designed *in silico* as a function of their specificity (including at least *E. coli* strains but not exclusively), length and *T_m* (closed to the *T_m* of labelled probes). The Colinsitu helpers,

HColin_L and HColin_R, bind to regions 617–636 and 661–684, respectively. The Ecoli helper Hec_L binds to the 435–452 region. No helper covering the region upper 476 was designed because this region is not conserved for all *E. coli* species. The predictive brightness classes of the designed helpers were class IV for the two Colinsitu helpers and class III for Hec_L and showed a better *in situ* accessibility than their corresponding probes.

DVC assay and helpers effects

Assays for *E. coli* ATCC 11775 were performed using Ecoli and/or Colinsitu probes with and without helpers. The fluorescent signals and cells numbers detected and quantified by SPC were compared between different combinations of probes applied to DVC-treated and untreated cells (Table 3).

Untreated DVC control cells exhibited a weak fluorescence detectable by epifluorescence microscopy, but no cells were detected by the SPC. The fluorescence of untreated cells was below the threshold of the SPC (which corresponded to 50 units of fluorescence or 0.06 when fluorescence was standardized by fluorescent beads, standard G). This is in agreement with Baudart *et al.* (2002), in which no detectable fluorescent signal for untreated cells hybridized with a CARD–FISH procedure was observed.

DVC-treated cells were detected by SPC showing fluorescence intensity above the threshold, except for the single fluo-Colinsitu probe, with which no signal was detected. The combination of different probes offered a range of relative fluorescence intensities (mean and standard deviation) from 0.15 (0.02) for Ecoli probe to 0.82

Table 3 Mean relative fluorescence intensity and counts of *Escherichia coli* ATCC 11775 cells detected by solid phase cytometry (SPC)

Probes*	Untreated cells		DVC-treated cells	
	Fluorescence intensity	SPC counts ml ⁻¹	Fluorescence intensity	SPC counts ml ⁻¹
Without helpers				
Fluo-Ecoli	nd	0	0.15 (0.02)	2.0 (0.0)
Fluo-Colinsitu	nd	0	nd	0
Fluo-Colinsitu/Fluo-Ecoli	nd	0	0.18 (0.02)	2.5 (0.7)
With helpers				
Fluo-Ecoli/Hec_L	nd	0	0.30 (0.01)	40.5 (13.4)
Fluo-Colinsitu/HColin_L/HColin_R	nd	0	0.25 (0.00)	32.0 (12.7)
Fluo-Colinsitu/Fluo-Ecoli/HColin_L/HColin_R/Hec_L	nd	0	0.82 (0.25)	48.0 (9.9)

The fluorescence values of cells are expressed in relative fluorescent units after standardization with a fluorescent standard G.

Standard deviations in parentheses were calculated for the means of duplicate membrane counts.

nd, no cell detected by the SPC; DVC, direct viable count.

*Helpers are noted with the prefix H.

(0.25) for the complete combination including two probes (Colinsitu and Ecoli) and three helpers (HEc_L, HColin_L and HColin_R) (Table 3). Both probes (Ecoli and Colinsitu) supplemented with their helpers improved the SPC counts and amplified by 5.6- and 4.5-fold the fluorescent intensity of *E. coli* cells respectively, compared to single labelled probes or two labelled probes without helpers, as illustrated on Fig. 1. Therefore, the probe set that consists in Colinsitu and Ecoli probes complemented by three helpers was chosen for specificity control assays and *E. coli* enumeration in water samples.

Specificity control assay

The specificity of the probe set (fluo-Colinsitu and fluo-Ecoli, HEC_L, HColin_R and HColin_L) was evaluated *in situ* for a wide spectrum of bacterial strains provided from reference collections or isolated from different sources (seawater, freshwater, pharmaceutical products) as well as from different geographical origins. Twenty-seven *E. coli* strains and 44 non-*E. coli* strains were used to assess the specificity of the DVC-FISH assays to detect the *E. coli* (Table 2).

All *E. coli* strains (100%) yielded a positive fluorescent signal with the probe set, and no fluorescent signal was generated with the non-*Enterobacteriaceae* strains. For the non-*E. coli* members of *Enterobacteriaceae*, a cross-hybridization was observed with *Shigella boydii*, *Shigella dysenteriae* and *Shigella sonnei*. The cross-hybridization with the Colinsitu probe for *Shigella* sp. was already mentioned in Regnault *et al.* (2000), who reported 100% of *rrs* sequence homology from the Colinsitu target sequence (position 637–660) for *E. coli*, *Escherichia fergusonii*, *Shigella flexneri*, *Sh. dysenteriae*, *Sh. sonnei* and *Sh. boydii*. Similarly, the specificity for the Ecoli sequence and helpers (HEC_L, HColin_R and HColin_L) was tested *in silico* and showed matches for *E. coli*, *Escherichia albertii*, *Sh. flexneri*, *Sh. dysenteriae*, *Sh. sonnei* and *Sh. boydii*. No auto-fluorescent signal (without oligonucleotides) was detected for all tested bacterial strains.

Escherichia coli detection by SPC

The DVC-FISH procedure was assessed on *E. coli* ($n = 11$) and non-*E. coli* strains ($n = 21$), including *Shigella* sp., stemmed from collection or environmental isolates (Table 4). The fluorescent intensities of cells were quantified by SPC, and cells numbers were compared to plate counts measured on non selective media.

The untreated DVC control cells exhibited weak fluorescence detectable by epifluorescence microscopy, but no cells were detected by SPC because the fluorescence was below the threshold. In contrast, the cells of DVC-treated

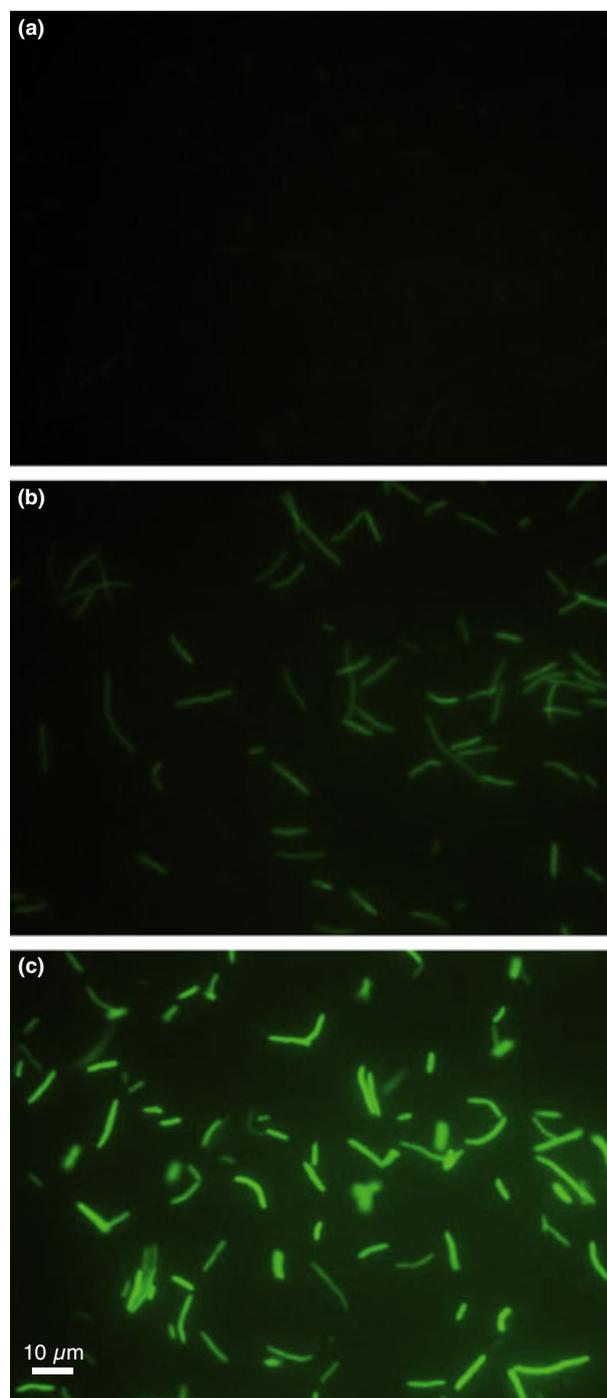


Figure 1 Epifluorescence micrographs of *Escherichia coli* (ATCC 11775) treated by the Direct Viable Count assay and hybridized with (a) one probe, Fluo-Colinsitu, (b) Fluo-Colinsitu and Fluo-Ecoli duo probes, (c) Fluo-Colinsitu and Fluo-Ecoli duo probes and three helpers.

E. coli strains were easily detected by SPC, and the counts obtained by both SPC and the plate count method were not significantly different (*t* test, $P = 0.414$, $\alpha = 0.05$).

Table 4 Plate and solid phase cytometry (SPC) counts for various *Escherichia coli* and non-*E. coli* strains

Strains (references)	Plate counts ml ⁻¹	SPC counts ml ⁻¹	Fluorescence intensity*
<i>Bacillus subtilis</i> ATCC 6653	43.0 (1.4)	0	nd
<i>Brevundimonas diminuta</i> ATCC 11568	59.0 (12.7)	0	nd
<i>Citrobacter freundii</i> ATCC 8090	94.5 (3.5)	0	nd
<i>Enterobacter aerogenes</i> ATCC 13048	121.5 (19.1)	0	nd
<i>Enterobacter sakazakii</i> type Ba1803	59.0 (7.1)	0	nd
<i>Klebsiella oxytoca</i> CIP 7932	75.5 (0.7)	0	nd
<i>Lactobacillus lactis lactis</i> CIP 7057	36.5 (0.7)	0	nd
<i>Micrococcus luteus</i> ATCC 934	69.0 (7.1)	0	nd
<i>Proteus mirabilis</i> ATCC10005	136.5 (3.53)	0	nd
<i>Pseudomonas aeruginosa</i> ATCC 27853	141.5 (2.1)	0	nd
<i>Pseudomonas syringae</i> type cc94	48.0 (0.0)	0	nd
<i>Raoultella planticola</i> ATCC 33531	60.0 (5.6)	0	nd
<i>Salmonella enterica</i> Enteritidis type S191	54.5 (12.0)	0	nd
<i>Salm. enterica</i> Indiana type S47	20.0 (2.8)	0	nd
<i>Salm. enterica</i> Paratyphi B type S44	39.0 (7.1)	0	nd
<i>Salm. enterica</i> Typhimurium type S54	36.0 (12.7)	0	nd
<i>Shigella boydii</i> CIP 82-50	57.5 (6.4)	63.0 (7.1)	0.23 (0.03)
<i>Shigella dysenteriae</i> CIP 57-28	173.0 (7.1)	49.0 (12.7)	0.15 (0.00)
<i>Shigella flexneri</i> CIP 82-48T	49.0 (1.4)	0	nd
<i>Shigella sonnei</i> CIP 82-49	27.0 (1.4)	12.0 (0.0)	0.41 (0.05)
<i>Staphylococcus aureus</i> ATCC 6538	17.0 (5.6)	0	nd
<i>E. coli</i> ATCC 11775	190.5 (4.9)	152.5 (75.7)	0.82 (0.26)
<i>E. coli</i> ATCC 8739	155.5 (3.5)	173.5 (20.5)	0.89 (0.01)
<i>E. coli</i> O157 : H7 ATCC 35150	46.0 (5.6)	54.5 (2.1)	0.71 (0.16)
<i>E. coli</i> type S877	84.5 (0.7)	107.0 (16.9)	0.58 (0.03)
<i>E. coli</i> type S31	19.0 (7.1)	13.5 (2.1)	0.45 (0.04)
<i>E. coli</i> type S1025	74.5 (2.1)	88.0 (1.41)	0.70 (0.08)
<i>E. coli</i> type S1027	103.5 (21.9)	92.5 (3.5)	0.48 (0.17)
<i>E. coli</i> type S1024	60.5 (10.6)	76.5 (17.7)	1.07 (0.07)
<i>E. coli</i> type S1035	95.0 (11.3)	109.0 (42.2)	1.06 (0.02)
<i>E. coli</i> type Co0601	57.0 (1.4)	69.0 (4.2)	0.40 (0.01)
<i>E. coli</i> type Ba0601	34.0 (2.8)	41.5 (4.9)	0.54 (0.02)

Standard deviations in parentheses were determined from two replicated membranes.

nd, no fluorescent signal detected by SPC.

*Values are expressed in relative fluorescent units after standardization with a fluorescent standard G.

The mean fluorescence intensities of *E. coli*-hybridized cells ranged between 0.40 and 1.07. Detection of *Sh. boydii*, *Sh. dysenteriae* and *Sh. sonnei* strains was confirmed by SPC towing to the matching of Colinsitu and Ecoli probes with 16S rRNA sequences of *Shigella* sp. as previously reported.

The fluorescent intensities measured for *Shigella* species were generally below those measured from *E. coli* strains and below 0.24 relative fluorescent units excepted for *Sh. sonnei* (0.41). Consequently, the SPC settings were changed to allow the discrimination of *E. coli* cells in waters by fixing a minimum value for peak intensity to 250 FU (corresponding to 0.3 relative units when standardized with the standard G). This threshold allows the detection of all tested *E. coli* strains and few *Sh. sonnei* cells as reported in Table 4.

Enumeration of viable *Escherichia coli* cells in naturally contaminated waters

The robustness of the DVC-FISH procedure combined with SPC cell detection for counting viable *E. coli* was tested on naturally contaminated freshwater, seawater and wastewater samples. Results of bacterial concentrations are reported in Table 5. SPC counts were compared to MPN counts enumerated with the ISO 9308-3 standard method recommended for the enumeration of *E. coli* in waters.

The reproducibility of each method was investigated by determining the coefficient of variation from three replicates of each sample. The coefficients of variation (CVs) of the DVC-FISH-SPC counts ranged between 13.1 and 33.5% and between 0 and 37.1% for the MPN method

Table 5 Mean bacterial concentrations, coefficients of variation (%) and fluorescence intensities of direct viable count-treated *Escherichia coli* cells as determined by solid phase cytometry (SPC) from different contaminated water samples

Water samples	Total cells (100 ml) ⁻¹	<i>E. coli</i> MPN counts (100 ml) ⁻¹	<i>E. coli</i> SPC counts (100 ml) ⁻¹	Fluorescence intensity*
Seawater	1.36 × 10 ⁸ (2.9%)	15.0 (0.0%)	58.7 (21.6%)	0.47 (14.9%)
Freshwater	1.16 × 10 ⁸ (0.0%)	244.7 (37.1%)	383.3 (33.5%)	0.51 (7.8%)
Wastewater	3.95 × 10 ¹⁰ (0.0%)	1.5 × 10 ⁷ (35.3%)	1.8 × 10 ⁷ (13.1%)	0.52 (13.5%)

Coefficient of variation (%) in parentheses was calculated between three replicates.

MPN, Most Probable Number.

*Values are expressed in relative fluorescent units after standardization with a fluorescent standard G.

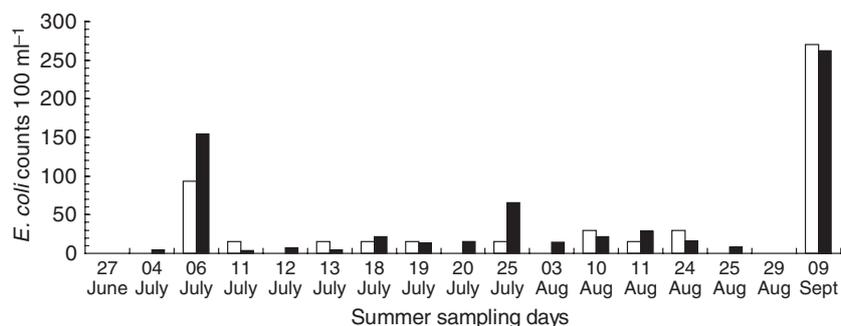
(Table 5). SPC counts were not significantly different from standard MPN counts for all samples (Mann–Whitney test, $P = 0.473$, $\alpha = 0.05$). As expected, the relative proportion of viable *E. coli* cells determined by SPC, when compared to total cell counts, increased from seawater to wastewater. These proportions ranged from 3.09×10^{-7} in seawater to 3.30×10^{-6} in freshwater and 4.47×10^{-4} in wastewater. The occurrence of *E. coli* cells was also investigated by monitoring seawater quality during one summer period (Fig. 2). Seventeen samples were analysed with both the DVC–FISH–SPC approach and the MPN standard method. DVC–FISH–SPC counts were not significantly different from MPN concentrations (Mann–Whitney, $P = 0.422$, $\alpha = 0.05$). For two samples (2/17), *E. coli* was not detected by both methods. Ten samples (10/17) were positives with both methods with SPC counts that were close to MPN counts, whereas five samples (5/17) were only positives by the SPC approach. For these five samples, SPC counts were very low [between 3.5 and 14.5 viable *E. coli* (100 ml)⁻¹] and below the detection limit of the MPN standard method [15 *E. coli* (100 ml)⁻¹].

Discussion

The development of rapid methods for the detection and quantification of *E. coli* is a major goal for a large number of industries linked with water use and for environmental agencies. In the past decade, various rapid methods were

proposed for the fast and specific quantification of *E. coli* in water samples. Most of these methods are based on a defined medium containing specific enzymatic substrate as β -D-glucuronidase (GLUase activity) for *E. coli* detection. Recently, a quantitative one-step enzyme-based detection method performed in <2 h was proposed by Garcia-Armisen and Servais (2004) to detect and quantify *E. coli* cells in freshwater or wastewater without any cultivation step and, more recently, by Lebaron *et al.* (2005) in seawater for monitoring water quality of bathing coastal areas. The one-step enzymatic test is interesting for its rapidity; however, cross-reactions have been reported when applied with seawater (Pisciotta *et al.* 2002; Fiksdal and Tryland 2008; Baudart *et al.* 2009). The DVC–FISH procedure is an alternative to circumvent the cross-reaction problem. Garcia-Armisen *et al.* (2005) proposed a DVC–FISH assay using a single monolabelled probe, named Colinsitu, to detect and enumerate *E. coli* cells in freshwater and wastewater samples contaminated by faecal coliforms by means of epifluorescent microscopy. They reported a linear correlation between log unit transformed GLUase activity and viable *E. coli* counts determined by DVC–FISH and epifluorescence microscopy. These authors suggested the use of both methods to detect viable *E. coli* in waters. Nevertheless, epifluorescence microscopy has quantitative limitations for enumeration of low *E. coli* concentrations, as those reported by the European sea-bathing guidelines [i.e., 250 *E. coli* (100 ml)⁻¹, EEC, 2006] or for drinking waters (Lemarchand *et al.* 2001; Lisle *et al.*

Figure 2 *Escherichia coli* concentrations measured by the solid phase cytometry (direct viable count–Fluorescent *In Situ* Hybridization method) (black bars) and the Most Probable Number method (ISO 9803-3) (white bars) from sea-bathing water samples collected on the French Mediterranean coast.



2004). The enumeration of stained fluorescent cells with SPC such as the Scan RDI instrument offers an ultra-sensitive quantification of targeted cells for the detection of rare cells in the water volume analysed (generally 100 ml), such as *E. coli* or more generally, as pathogens (Pyle *et al.* 1999; Pougard *et al.* 2002; Cools *et al.* 2005; West *et al.* 2006; Aurell *et al.* 2004). Our results showed that DVC–FISH performed with either single or two monolabelled probes is not efficient to allow cell detection by SPC. The ScanRDI has a lower detection sensitivity of the fluorescence than the epifluorescence microscope and requires a fluorescence amplification system for cell detection. The multi-probes approach using several labelled oligonucleotides could increase the fluorescent signal of hybridized cells (Lebaron *et al.* 1997). Nevertheless, specific 16S rRNA sequences for some bacterial species are not always available, and not all rRNA sequences are equal for their accessibility (Fuchs *et al.* 1998, 2000). Several DNA oligonucleotide probes for FISH application have been proposed to detect and to quantify *E. coli* cells and are available from the literature such as Ecoli (Jansen *et al.* 2000), Colinsitu (Regnault *et al.* 2000), Eco1167 (Neef *et al.* 1995) and EC1531 (Poulsen *et al.* 1994). However, a preliminary investigation to assess the specificity of these probes has shown cross-hybridization with other 23S rRNA–targeted probes (Eco1167 and EC1531) of *Enterobacter aerogenes* (data not shown). Consequently and because of the higher specificity of 16S rRNA–targeted probes, we retained Ecoli and Colinsitu probes for this study.

The analysis of the targeted 16S rRNA sites for Ecoli and Colinsitu probes revealed that they are located within helices 18 and 22, which correspond to the VI brightness class with very low accessibility (Fuchs *et al.* 1998). In this work, helper sequences for Ecoli and Colinsitu probes were designed according to Fuchs *et al.* (2000). Our results confirmed those of Fuchs *et al.* (2000) suggesting that in case of regions with a low *in situ* accessibility (helices 18 and 22), hybridization signals can be improved by combining fluorescent probes with helpers to open inaccessible sites. In this study, combining two labelled probes with three helpers resulted in a 4.5–5.5 increase of fluorescence signals when compared to those obtained with single or two labelled probes and, therefore, in an increase of SPC counts. This increase in fluorescence signals is essential when detection of hybridized cells is performed by SPC. Indeed, SPC has lower detection sensitivity than epifluorescence microscope, but in contrast, it allows the detection of rare events (Lemarchand *et al.* 2001). The method developed in this study has made possible the quantification of low *E. coli* concentrations in waters as those reported by European Bathing Water Quality Directive (EEC, 2006). Nevertheless, caution is required because helpers can modify the specificity

of the probe. All helpers designed in this study included *E. coli* in their specificity assays but were not exclusively specific to *E. coli*. This point was discussed by Fuchs *et al.* (2000), who suggested that helpers should ideally have the exact specificity of the probe, but this is a condition hard to fulfil because helpers have to be placed either adjacent to the specific probe target site or opposing it. Our results have shown that widening of target group sometimes does not affect the specificity of the FISH assay.

The application of the DVC–FISH procedure developed in this work offers an ultra-sensitive enumeration of viable *E. coli* cells by SPC in different water samples contaminated by faecal material. Interestingly, this method provides results that are not significantly different from those found with the standardized MPN counts.

The cellular identification by the FISH method is a powerful tool in terms of specificity when based on highly conserved sequences. This study shows that the unlabelled helpers approach is easy to perform, quite inexpensive and is recommended for increasing the fluorescent signal when the traditional FISH technique is combined with SPC and applied to *E. coli* detection in waters. The method developed in this work is rapid, specific, sensitive and allows an accurate detection and quantification of viable *E. coli* cells in different types of waters and may constitute an interesting and relevant tool to further investigate the ecology and significance of *E. coli* in the natural environment. One limitation to the routine use of this method is the high cost of SPC machines. Although the technique remains of great interest, there is an urgent need to develop a new generation of cheaper methodologies.

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