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# Assessment of a new technique combining a viability test, whole-cell hybridization and laser-scanning cytometry for the direct counting of viable *Enterobacteriaceae* cells in drinking water

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## Abstract

A new direct approach, called direct viable count (DVC)-FISH-ScanRDI, combining viability measurement, specific detection and sensitive enumeration of highly diluted *Enterobacteriaceae* cells, was assessed during the summer in water samples from a North American drinking water treatment plant and its distribution system. Major results of this field investigation show a higher sensitivity of the DVC-FISH-ScanRDI approach in enumerating viable *Enterobacteriaceae* cells in distributed drinking water, relative to a culture-based method, and the increased concentration of viable but non-culturable (VBNC) *Enterobacteriaceae* cells in distributed water for temperatures above 18 °C.

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**Keywords:** *Enterobacteriaceae*; Drinking water; DVC-FISH-ScanRDI

## 1. Introduction

The sensitive enumeration of highly diluted viable fecal indicator bacteria is a priority for several activity sectors linked to the use of drinking water. The development, during the last decade, of fluorescent probes allowing a direct characterization of cells at sin-

gle-cell level [1] has resulted in a much better understanding of the physiological behavior of bacterial cells exposed to environmental pressures [2]. It is now acknowledged that, in water environments under some conditions, injured bacterial cells can evolve into a viable but non-culturable (VBNC) state, and are undetectable with the culture-based methods currently used for water quality assessment [2,3]. The direct viable count (DVC) procedure was proposed by Kogure et al. [4] to assess viability at single-cell level [5,6]. Indeed, the ability of sensitive cells to elongate in the presence of nutritive resources and a cellular division inhibitor reflects a potential active cellular metabolism and the synthesis of various intracellular constituents (proteins, RNA)

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[7]. The DVC procedure had previously been applied to measure the proportion of viable cells in water samples without taxonomic specificity [7,8] or for a taxonomic group strongly represented in drinking water biofilm bacterial communities ( $\beta$  proteobacteria) [9,10]. In these works, taxonomical information was investigated by the whole-cell hybridization technique performed on 16S rRNA [DVC-fluorescent in situ hybridization, FISH].

In earlier work [11], a highly specific and quantitative sensitivity method was proposed, combining the DVC assay, the whole-cell hybridization technique and laser-scanning cytometry (LSC, ScanRDI, Chemunex, Ivry-sur-Seine, France) for the detection and enumeration of rare viable *Enterobacteriaceae* cells in water samples. The aim of the present study was to assess the sensitivity of this new approach (DVC–FISH–ScanRDI) for the direct counting of viable *Enterobacteriaceae* in a North American treatment plant and its associated drinking water distribution system (DWDS).

## 2. Materials and methods

### 2.1. Sampling stations

Over a 10-week period in the summer of 2001 (from 01/06/18 to 01/08/21), water samples were collected at four sampling stations on the Sainte-Rose treatment plant and distribution system (Fig. 1): raw water (RW, inlet freshwater), biological activated carbon filtered water (BACFW), treated chlorinated water (CW, finished water) and distributed water (DW). The RW was surface water from the Milles Iles River in Quebec, Canada. General characteristics of the RW were reported by

Andersson et al. [12]. The treatment line comprises dynamic settling (Alum + SiO<sub>2</sub>), rapid sand/anthracite filtration, ozonation, biological activated carbon filtration, pH adjustment and disinfection with Cl<sub>2</sub>, and has a nominal capacity of 110,000 m<sup>3</sup> day<sup>-1</sup>. The DW sampling point is commonly used by the utility for regulatory monitoring. It is in a remote location and has a known history of low chlorine residuals. Water samples were collected in sterilized 1 L bottles. Turbidity and temperature were measured on each sample and free chlorine was measured for CW and DW [13].

### 2.2. Bacterial analysis

Chlorine present in microbiological samples was neutralized by the addition of sodium thiosulfate (0.01% final concentration). Heterotrophic plate counts (HPC) and enterobacteria CFU counts were enumerated using the membrane filtration method on R2A (7 days at 20 °C) and on MacConkey media (24 h at 35 ± 2 °C), respectively [11,13]. Total cells were enumerated after nucleic acid-DAPI staining [11].

#### 2.2.1. Direct viable count procedure

For direct viable cell enumeration, a modified DVC procedure [11], was applied to cells filtered on a 25-mm, 0.45- $\mu$ m black polyester membrane (type CB04, Chemunex, Ivry-sur-Seine, France). The assay included a bacterial cell metabolism revivification step in the presence of a nutritive source and a DNA gyrase inhibitor (nalidixic acid), which stops cell division and increases the intracellular rRNA content and the cellular length of sensitive cells. Samples were filtered through a 25-mm, 0.45- $\mu$ m pore-size black polyester membrane filter (type CB04,

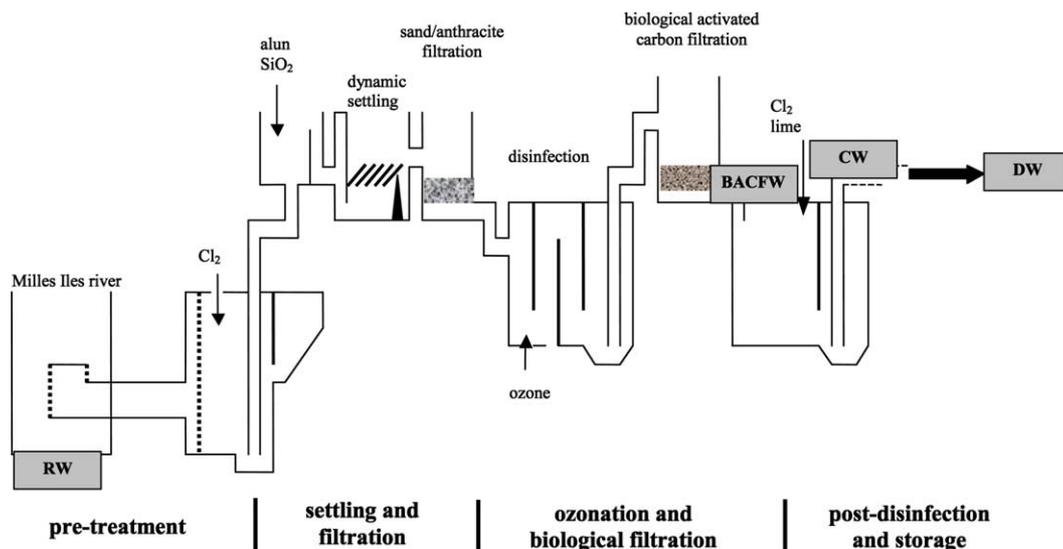


Fig. 1. Location of sampling stations through the Sainte-Rose treatment plant and DWDS. RW, raw water; BACFW, biological activated carbon filtered water; CW, chlorinated water; DW, distributed water.

Chemunex, Ivry-sur-Seine, France). The individual membranes were placed on a cellulose pad (25 mm, Millipore, Bedford, MA, USA) soaked in 650  $\mu\text{l}$  of non-selective nutritive broth (70% phosphate-buffered saline [130 mM NaCl, 2.5 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2; PBS], 0.036% yeast extract, 0.36% casamino acid) containing nalidixic acid (final concentration 10  $\mu\text{g ml}^{-1}$ , Sigma) in Petri dishes. The water samples were incubated at  $35 \pm 2$  °C for 4 h. An untreated DVC control was made up for each sample analyzed.

### 2.2.2. Fluorescent *in situ* hybridization procedure

Prior to hybridization, the cells were fixed with a paraformaldehyde solution [11]. The oligonucleotide probe use, has been described by Loge et al. [14] (ENT1, 5'-CCGCTTGCTCTCGCGAG-3'). The probe (ThermoElectron Corporation, Ulm, Germany), was conjugated at its 5' end with horseradish peroxidase (HRP). A fluorescein-tyramide kit, the Tyramide-System-Amplification (TSA)-direct system (Perkin-Elmer, Life Science, Woodbridge, Ont., Canada) was used to reveal the hybridized cells. As well, the cells plotted on the membrane were permeabilized by treating them with ethanol and lysozyme [11]. Hybridization was then performed at 45 °C for 2.5 h in a hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.001% sodium dodecyl sulfate (SDS), 20% dionized formamide, 0.2% diethyl pyrocarbonate) containing a probe with a final concentration of 5 ng  $\mu\text{l}^{-1}$ . Cells were washed in buffer (180 mM NaCl, 20 mM Tris-HCl, pH 7.2, 5 mM EDTA, 0.001% SDS) at 48 °C for 30 min and, finally, stained using the TSA-direct system (fluorescein kit) as suggested by the manufacturer.

### 2.2.3. Direct enumeration of *Enterobacteriaceae* cells by the Scan RDI

After hybridization, the membranes were scanned by the Scan RDI solid phase cytometer as described previously [11]. Typical targeted fluorescent signals were discriminated from raw data by a set of internal

discriminants (Chemunex). Results were plotted on a schematic membrane on which fluorescent events were positioned ( $X, Y$  coordinates). At the end of the analysis procedure, all events positively selected after the discrimination process were validated by epifluorescence microscopy (BX60 with a WIBA filter, Olympus) fitted on a motorized stage driven by the cytometer's computer. With this validation step, it is possible to confirm fluorescent events as targeted fluorescent bacterial signals or as false positive signals (autofluorescent particles). In this study, two discriminant parameters, determined by the Scan RDI software, were used to characterize hybridized *Enterobacteriaceae* cells: the peak intensity of the dominant peak expressed in arbitrary fluorescence units (FU) which is linked to the fluorescence intensity and the product of two internal parameters: scan line number (nL)  $\times$  scan sample number (nS), which are linked to the cellular length. These parameters were, respectively, considered to be an expression of the intracellular content of the 16S rRNA ribosomal targets and of the rate of cell elongation. Elongated cells showing higher fluorescence intensity than control *Enterobacteriaceae* cells were considered to be viable cells presenting potential synthesis activity [DVC-FISH(+)].

The volumes analyzed using this direct method, were similar to those analyzed using the culture-based method. They ranged between 1 and 5 ml for the RW and were 100 ml each for the BACFW, CW and DW. All analyses were performed in duplicate.

### 2.3. Statistical analysis

The Spearman correlation coefficient was applied to establish the relationship between direct counts of viable *Enterobacteriaceae* cells (DVC-FISH-ScanRDI counts) and temperature measured in drinking water sample. Statistical analyses were performed using the PRISM 1.2 software, and statistical significance was assessed at  $p < 0.01$ .

Table 1

Average value and range for each water quality parameter analyzed during the period of investigation ( $n = 10$ )

	Water samples <sup>a</sup>			
	RW	BACFW	CW	DW
Temperature (°C)	24.3 (20.7–26.9)	24.3 (20.7–26.9)	24.3 (20.7–26.9)	17.5 (16–20)
Turbidity (NTU)	6.50 (3.40–10.90)	0.04 (0.04–0.05)	0.23 (0.18–0.34)	0.23 (0.20–0.30)
Chlorine residual ( $\text{mg l}^{-1}$ )	–	–	0.7 (0.5–0.9)	<0.1
Heterotrophic plate counts (100 $\text{ml}^{-1}$ )	$3.2 \times 10^5$ ( $1.1 \times 10^6$ – $2.5 \times 10^3$ )	$2.6 \times 10^4$ ( $3.0 \times 10^3$ – $6.1 \times 10^4$ )	75.4 (13–380)	$3.7 \times 10^5$ ( $2.1 \times 10^3$ – $1.0 \times 10^7$ )
Total cells (100 $\text{ml}^{-1}$ )	$4.6 \times 10^8$ ( $3.3 \times 10^8$ – $6.8 \times 10^8$ )	$2.7 \times 10^7$ ( $1.6 \times 10^7$ – $4.1 \times 10^7$ )	$3.5 \times 10^7$ ( $1.4 \times 10^7$ – $6.4 \times 10^7$ )	$4.2 \times 10^7$ ( $3.0 \times 10^7$ – $7.4 \times 10^7$ )

The minimum and maximum values are noted in parentheses. NTU, nephelometric turbidity unit.

<sup>a</sup> RW, raw water; BACFW, biological activated carbon filtered water; CW, treated chlorinated water; DW, distributed water.

### 3. Results and discussion

#### 3.1. Relationship between cultural and direct *Enterobacteriaceae* counts

The temperature, turbidity, residual chlorine, heterotrophic plate count and total cell count values measured at the various sampling points displayed classical water quality variations of a treatment line during the study period (Table 1). The CFU (MacConkey media) and direct (DVC–FISH–ScanRDI) counts of *Enterobacteriaceae* were compared for each type of water (Fig. 2). All RW and BACFW sample counts were positive with both methods. However, higher direct counts were measured on 80% of the RW samples (Fig. 2(a)) and 100% of the BACFW samples (Fig. 2(b)). No *Enterobacteriaceae* cells were detected in the CW with either method (Fig. 2(c)). These results agree with those of Lisle et al. [15] and Tryland et al. [16], who found a significant reduction in culturability and DVC counts following chlorine disinfection. Seventy percent of the DW sample counts were positive by the DVC–FISH–ScanRDI method, while no *Enterobacteriaceae* were detected by the culture-based method (Fig. 2(d)). The direct counts measured on positive DW samples ranged from  $1.5 \pm 0.7$  to  $135 \pm 39.7$  cells  $\times 100$  ml $^{-1}$ . It is worth noting that at this point, the chlorine concentration was negligible (Table 1), and that the CW and DW samples always tested negative with the standard m-Endo culture-based method for detecting total coliforms in drinking water [13] (data not shown).

#### 3.2. Occurrence of viable *Enterobacteriaceae* cells in drinking water samples

During this investigation, 81.5% of the water samples showed higher counts (between 0.1 and 2 log) with the direct method (DVC–FISH–ScanRDI) than with colony counts (CFU on MacConkey). *Enterobacteriaceae* cells detected by the DVC–FISH–ScanRDI approach were easily identified during the microscopic confirmation step. Hybridized cells were characterized by fluorescence intensities decreasing from  $267.2 \pm 112.9$  FU in raw water samples,  $179.9 \pm 27.6$  FU in biological activated carbon filtered water samples, and  $188.5 \pm 50.6$  FU in distributed water samples. Cellular length index (nL  $\times$  nS) shows same evolution according the water sample and decrease from  $35.0 \pm 15.0$  U in RW samples,  $20.9 \pm 3.2$  U in BACFW samples, and  $21.9 \pm 3.6$  U in DW samples. The decrease of fluorescence intensity and cellular length from the raw water to treated waters and the absence of viable *Enterobacteriaceae* cells in treated chlorinated samples seems indicate a negative effect of environmental pressures linked to treatment system on the DVC sensitivity of cells.

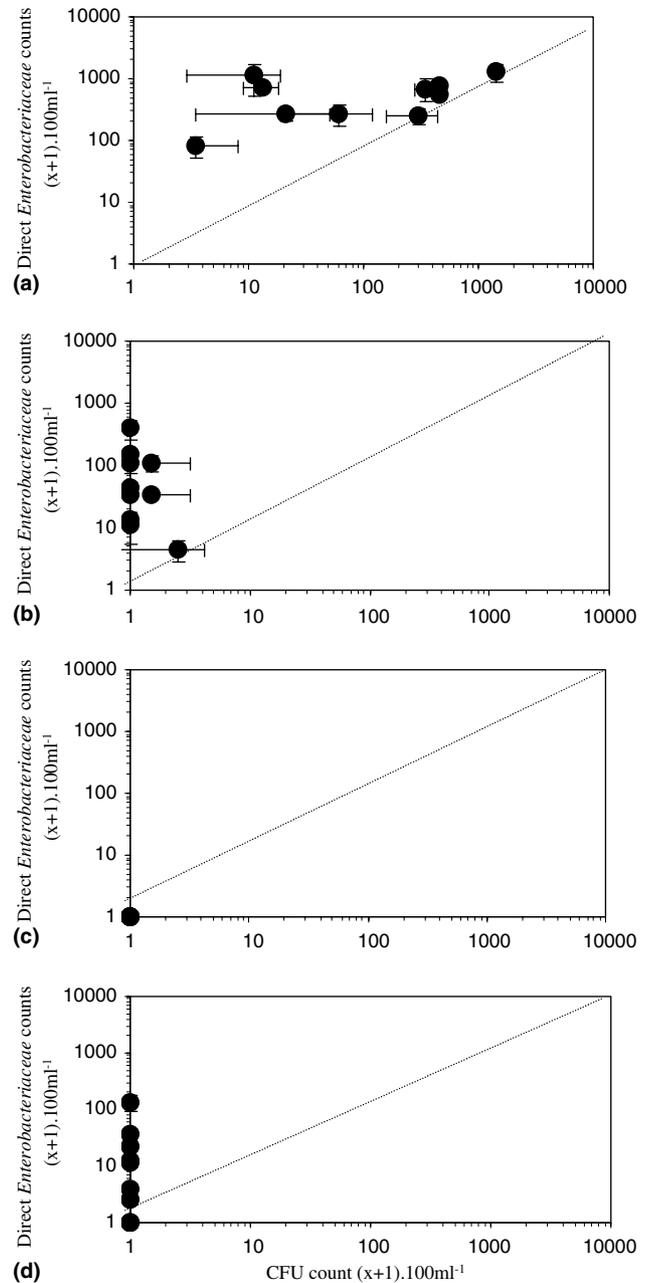


Fig. 2. Log–log relation between direct *Enterobacteriaceae* counts (DVC–FISH–ScanRDI) and CFU counts on MacConkey medium according to type of water. (a) RW, (b) BACFW, (c) CW, and (d) DW. The broken line represents a 1:1 relation. The horizontal and vertical bars are standard deviations calculated from duplicate plate and DVC–FISH–ScanRDI counts.

The lowest number of *Enterobacteriaceae* cells measured by the direct method in proportion to total cells (measured after DAPI staining) was  $5.6 \times 10^{-8}$  in DW, which agrees with the sensitivity of the detection level defined from lab experiments [11]. By contrast, higher direct counts of viable *Enterobacteriaceae* were measured on the DW samples at temperatures above 18 °C and the log-transformed direct counts were positively

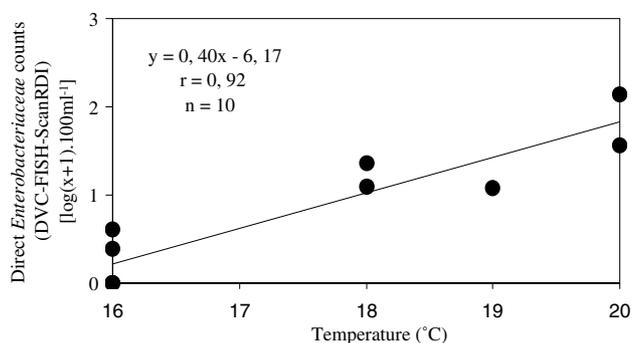


Fig. 3. Relationship between mean log-transformed direct *Enterobacteriaceae* counts measured by the DVC–FISH–ScanRDI method and temperature on DW samples during the summer period studied.

linearly correlated with temperature values ( $p < 0.01$ , Spearman test) (Fig. 3), while no significant relationship was found between direct counts and turbidity values. These results agree with those of other works, which has shown an increase in total coliform occurrence in DWDS at temperatures above 15 °C [17–19].

#### 4. Conclusions

This work assessed the greater sensitivity of the DVC-FISH method combined with ScanRDI for the direct counting of viable *Enterobacteriaceae* in various types of water, including drinking water samples, compared to the classical culture-based method. Moreover, the FISH technique performed on a highly conserved sequence of 16S rRNA has the potential to allow very specific detection of viable *Enterobacteriaceae* cells without the time-consuming cultivation and biochemical confirmation steps. The combination of the whole-cell hybridization technique with a direct viable test (such as the DVC) and ScanRDI detection make possible the enumeration of highly diluted viable *Enterobacteriaceae* cells in a single day. Results of this investigation indicate that some viable but non-culturable *Enterobacteriaceae* cells may be increasingly present in DWDS as a function of temperature, which could increase the potential risk of North America DWDS contamination during the summer period.

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