



HAL
open science

New Rapid Methods to Track Diluted Enterobacteriaceae Cells in Drinking Waters

Julia Baudart, Amandine Olaizola, Patrick Laurent, Josée Coallier, Michèle Prévost

► **To cite this version:**

Julia Baudart, Amandine Olaizola, Patrick Laurent, Josée Coallier, Michèle Prévost. New Rapid Methods to Track Diluted Enterobacteriaceae Cells in Drinking Waters. WQTCConference, WQTC; AWWA, Nov 2001, Tennessee, United States. <hal-03952326>

HAL Id: hal-03952326

<https://hal.science/hal-03952326v1>

Submitted on 23 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

New Rapid Methods to Track Diluted *Enterobacteriaceae* Cells in Drinking Waters

Julia Baudart¹, Amandine Olaizola¹, Patrick Laurent¹, Josée Coallier¹ and Michèle Prévost²

¹ Ecole Polytechnique de Montréal, NSERC Industrial Chair on Drinking Water, CP6079, Succ. Centre-Ville, Montréal, Québec, Canada, H3A 3A7

² Vivendi Water - USFilter - John Meunier, 4105 rue Sartelon, St-Laurent, Québec, Canada, H4S 2B3

Abstract

Drinking water quality assessment involves the specific, sensitive and rapid detection of bacterial indicators in water samples. This work assesses the sensitivity of a new FISH method, combined with a physiological assay for direct enumeration at single-cell level of highly diluted viable *Enterobacteriaceae* cells in drinking waters. The approach (DVC-FISH) was completed by the use of a new direct detection device, the solid-phase laser cytometer (ScanRDI). The DVC-FISH method combined with ScanRDI detection make it possible to detect as little as one targeted cell in approximately 10^8 non-targeted cells plotted on a membrane. This new approach as applied to a drinking water distribution system was investigated.

Key words: *Enterobacteriaceae*, DVC-FISH, solid-phase laser cytometer, drinking water.

1. Background and problem statement

Drinking water quality assessment involves the specific and rapid detection of bacterial indicators (total coliforms, *E coli*) in water samples. Since their presence is often the main cause of non-compliance and subsequent boiling events, quick identification of bacterial contamination sources in distribution systems is a major goal of utilities (Besner *et al.*, 2001). However, depending on environmental pressures, variable proportions of cells can be disseminated in waters in viable but non-culturable (VBNC) states (Colwell and Grimes, 2000). Consequently, bacterial indicators and pathogenic bacteria entering the distribution system in non-culturable states cannot be detected through classical routine culturing procedures. The occurrence of VBNC indicators and pathogenic cells in drinking waters is currently unknown. Their existence may change our approach to health-related risks linked to the dissemination of non-culturable pathogenic cells in drinking waters.

2. Project description

Molecular and cellular tools applied to the specific detection of bacterial cells in water samples can be useful for increasing the rapidity, sensitivity and specificity of analysis. With these methods, a high degree of sensitivity and specificity can be achieved without the need for a complex cultivation step. Because culturing is not required, they permit a direct identification of bacteria within hours, instead of the days required with the traditional methods. Among them, fluorescently labeled DNA probes combined with *in situ* hybridization (fluorescent *in situ* hybridization, FISH) applied to 16S rRNA targets permits rapid identification of bacteria at a cellular level in natural habitats.

On the one hand, the quantitative limitations of classical enumeration devices used for direct detection of fluorescently labeled cells (such as the epifluorescence microscope or the flow cytometer) are a major problem in the direct enumeration of diluted targeted cells in water samples ($< 10^5$ targeted cells distributed on a membrane) (Joux and Lebaron, 2000).

On the other, FISH application in oligotrophic environments (such as drinking water) might be limited by the low ribosome content of starved/stressed bacteria (Nishimura *et al.*, 1993; Kalmbach *et al.*, 1997).

The development of rapid method at the single-cell level combines simultaneously both physiological test, which increase the intracellular rRNA content in starved/stressed cells, and taxonomic probes could be useful approach for the identification and enumeration of viable or active specific pathogen or indicator cells in drinking water.

In this work, the sensitivity of a new FISH method which includes a physiological assay [the direct viable count (DVC), as described by Kogure *et al.* (1979)] for enumerating highly diluted viable *Enterobacteriaceae* cells in drinking waters is assessed. The final step in this approach (DVC-FISH) involves the use of a new direct detection device, the solid-phase laser cytometer (ScanRDI). Actually, the ScanRDI (Chemunex, Ivry sur Seine, France) allows a rapid enumeration of fluorescent labeled cells (in less than 3 min) with a very low quantitative detection limit (1 to 300 fluorescent cells).

The DVC-FISH approach was optimized to *E. coli* cultured cells in the stationary growth phase. Quantitative sensitivity limitations of the DVC-FISH approach combined with ScanRDI enumeration were evaluated from drinking water samples according to the density of the bacterial

microflora associated with the sample and according to the concentrations of spiked *E. coli* cells. This approach was applied to the detection of VBNC *Enterobacteriaceae* cells in samples from a North American water distribution system.

3. Materials and methods

3.1. DNA Probe

The *Enterobacteriaceae* targeted 16S rRNA probe used here and previously described by Loge *et al.* (1999) has the following sequence: 5'-CCGCTTGCTCTCGCGAG-3' (ENT1-probe). Hybridizations were performed using a DNA oligonucleotide probe conjugated with horseradish peroxidase (HRP). This enzyme was then revealed by a FITC-tyramide substrate (TSA system, Perkin-Elmer, Life Science). The direct TSA system application allows a 10- to 20-fold increase in signal intensity over fluorescein-monolabeled probes (Schönhuber *et al.*, 1997).

3.2. Solid phase DVC-FISH procedure

3.2.1. DVC procedure. Cellular viability, represented by metabolic synthesis activity, was measured by a direct viable count, a modified Kogure *et al.* (1979) procedure. The assay included a bacterial cell metabolism revivification step in the presence of a DNA gyrase inhibitor (nalidixic acid), which inhibits cell division and increases the intracellular rRNA content of sensitive cells. An optimized DVC protocol applied to *Enterobacteriaceae* cells plotted on a membrane after filtration was developed. Samples were filtered through a 25mm, 0.45µm-pore-size black polyester membrane filter (type CB04, Chemunex, Ivry-sur-Seine, France). The DVC procedure was developed using a broth consisting of a non-selective medium (70% phosphate-buffered saline [PBS], 0.036% yeast extract, 0.36% casamino acid) (Joux and Lebaron, 1997) containing nalidixic acid (final concentration 10 µg.ml⁻¹). The cells were incubated at 35 ± 2°C for 2 hr for samples containing cultured cells and for spiked drinking water samples. A control, untreated by the DVC procedure was realized for each water sample analyzed.

3.2.2. Fluorescent *in situ* hybridization. Following cell fixation with paraformaldehyde diluted at 4% in PBS at 4°C for 4-16 hr, fixed cells were subjected to an *in situ* hybridization procedure. Individual membranes were placed on cellulose pads and soaked in baths containing solutions of 50, 80 and 94% ethanol, and incubated at room temperature for 4 min in each bath. The membranes were then treated with a lysozyme-TRIS-EDTA solution and incubated at room temperature for 10 min. The enzyme reaction was stopped by incubation in a Tris-EDTA (TE) buffer for 5 min. Fifteen microliters of hybridization buffer warmed to 45°C and containing an ENT1-HRP probe (final concentration 5ng.µl⁻¹) were poured onto the membranes. Coverslides were added to limit evaporation during the hybridization period (45°C for 2.5 hr). The membranes were then washed with buffer and incubated at 48°C for 30 min. They were then treated with TNT buffer (0.1M Tris-HCl [pH7.5], 0.15 M NaCl, 0.05% Tween 20) and incubated at room temperature for 15 min. To reveal HRP, the membranes were placed on 100 µl of FITC-

tyramide (1/50) and incubated in the dark and at room temperature for 10 min and then washed with TNT buffer at room temperature. They were then analyzed using the ScanRDI.

3.3. Cells enumeration by the ScanRDI

The direct detection and enumeration of DVC-FISH *Enterobacteriaceae* cells was realized on a solid-phase laser cytometer (ScanRDI). The membranes were scanned by an argon laser (488nm emission wavelength), and fluorescent events emitting at 515nm were detected. These optical characteristics are compatible with those of the fluorescein dye conjugated with tyramide substrate. Typical targeted fluorescent signals were discriminated from the raw data by a set of internal discriminants (Mignon-Godefroy *et al.*, 1997). Results were plotted on a schematic membrane and fluorescent events positioned thereon (X,Y coordinates). At the end of the analysis procedure, all events positively selected following the discrimination process were validated by epifluorescence microscope (Olympus, BX60, with a WIBA filter) fitted on a motorized stage driven by the cytometer's computer (Mignon-Godefroy *et al.*, 1997; Catala *et al.*, 1999). This validation step makes it possible to confirm fluorescent events as targeted fluorescent bacterial signals or as false-positive signals (autofluorescent particles). Two discriminant parameters determined by the ScanRDI software were used here to characterize hybridized *Enterobacteriaceae* cells: peak intensity of the dominant peak expressed in arbitrary fluorescent units (FU) and a number of scan lines (SL), which were linked to the fluorescence intensity and to the cellular length of the detected events respectively. These parameters were considered to be an expression of the intracellular content of the 16S rRNA ribosomal targets and of the rate of cell elongation. Cells elongated and showing higher fluorescence intensity than control *Enterobacteriaceae* cells were considered to be viable cells presenting potential synthesis activity [DVC-FISH(+)].

3.4. Colony counts

Culturable cells were enumerated by means of the plate count procedure after sample filtration through a 47mm, 0.45µm pore-size membrane (type HVLP, Millipore). Colony forming unit (CFU) enumeration was performed on m-Endo agar for cultured cell samples and for spiked *E. coli* drinking water samples. The m-endo medium is recommended for standard detection and enumeration of the coliform group in drinking water samples (APHA, 1998). For spiked drinking water samples, analyses were performed after filtration of 100ml of sample.

3.5. Total cell counts

Samples were fixed with formaldehyde (final concentration 3.7%). Total cells were enumerated after nucleic acid staining with DAPI (4', 6'-diamidino-2-phenylindole, final concentration 10µg.ml⁻¹) for 15 min in the dark. Stained samples were then filtered through a 25mm, 0.2µm pore-size black polycarbonate membrane filter under low vacuum. Membranes were mounted in oil for microscopic observation. Total cells were counted by epifluorescence microscopy (BX60, Olympus) with a WU bloc filter under a 100X immersion objective.

4. Results

4.1 DVC-FISH procedure optimized on *E.coli* cultured cells

The optimized solid phase DVC-FISH protocol was developed on *E. coli* ATCC 25922 cultured cells in the stationary growth phase. Hybridized cells with and without the DVC procedure (Fig. 1) were analyzed by the ScanRDI.

Peak intensities and numbers of scan lines measured by the ScanRDI for both untreated and treated cells were measured. Untreated DVC cells showed weak fluorescence intensity signals, detectable by microscopy but not by the ScanRDI (fluorescence intensity signals below the detection threshold). By comparison, treated DVC cells presenting a positive response showed a high fluorescence intensity signal and an increase in cell length. Peak intensity and scan line numbers were 399.8 ± 10.7 FU (mean value, $n = 152$) and 4.1 ± 0.5 SL (mean value, $n = 152$) respectively.

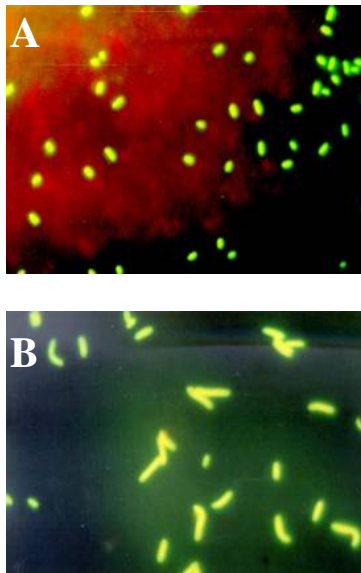


FIG. 1. In *situ* hybridizations performed on *E. coli* ATCC 25922 cells in the stationary growth phase with: (A) the ENT1-HRP probe; and (B) the ENT1-HRP probe following the DVC-FISH assay (nalidixic acid $10 \mu\text{g}\cdot\text{ml}^{-1}$, incubation for 2 hrs in a nutritive broth at $35 \pm 2^\circ\text{C}$). Hybridized cells were revealed with FITC-tyramide (TSA system).

The specificity of the DVC-FISH-ScanRDI approach was evaluated from other strains, including *Enterobacteriaceae* and no-*Enterobacteriaceae* strains. Sensitivity to DVC-FISH treatment was confirmed from all *Enterobacteriaceae* strains tested. Positive DVC-FISH cells showed the same order of fluorescence intensity signal and cell length as the *E. coli* cells when the DVC-FISH treatment was applied. No fluorescence signals were detected for no-*Enterobacteriaceae* strains (data not shown).

4.2. Sensitivity of the DVC-FISH-ScanRDI approach with respect to the density of bacteria-associated microflora and the concentrations of spiked *E. coli* cells

The quantitative limitations of the DVC-FISH-ScanRDI approach were analyzed on drinking water samples spiked with *E. coli* ATCC 25922 cultured cells and presenting different densities of bacteria-associated microflora. *E. coli* cells in the stationary growth phase were added at various low concentrations (ranging between approximately 1 and 1000 culturable cells per 100ml) to three types of water:

- i. water without endogenous microflora made up of pre-filtered sterile milli-Q water (water A),
- ii. water with endogenous microflora made up of 10% tap water mixed with 90% pre-filtered sterile milli-Q water ($1.8 \pm 0.2 \cdot 10^7$ total cells.100ml⁻¹, water B)
- iii. water with endogenous microflora made up of 100% tap water ($1.8 \pm 0.2 \cdot 10^8$ total cells.100ml⁻¹, water C). The free chlorine level of this water was considered to be zero (results below the detection limit of the method).

DVC-FISH counts enumerated with the ScanRDI were compared to colony counts measured on an m-endo medium after 24 hrs of incubation. All analyses were performed after filtration of a 100ml sample.

Relationships between DVC-FISH-ScanRDI *E.coli* counts and colony counts are presented in Figure 2.

When DVC-FISH-positive cells were present in the sample, cells were easily detected by the ScanRDI and confirmed by epifluorescence microscopy. DVC-FISH-positive cells were characterized by: (i) peak fluorescence intensity ranging between 326.2 ± 30.5 FU (water A) and 422.8 ± 110.6 FU (water C); and (ii) cell length ranging between 3.6 ± 0.26 SL (water A) and 3.8 ± 0.7 SL (water B).

A good correlation between DVC-FISH-ScanRDI and CFU counts was obtained for the three types of water: values ranged between 1.07 (water B) and 0.71 (water C). Furthermore, the coefficients of determination ($R^2=0.999$ (waters A and C) and $R^2=1.00$ (water B) are highly significant.

The DVC-FISH method combined with ScanRDI detection offers a very low quantitative detection limit, making it possible to detect as little as one targeted cell (one viable *E. coli* cell in this study) mixed within a high total cell density (approximately 10^8 total cells.100ml⁻¹). Nevertheless, high concentrations of autofluorescent particles may be a limitation to an exact enumeration of diluted targeted cells.

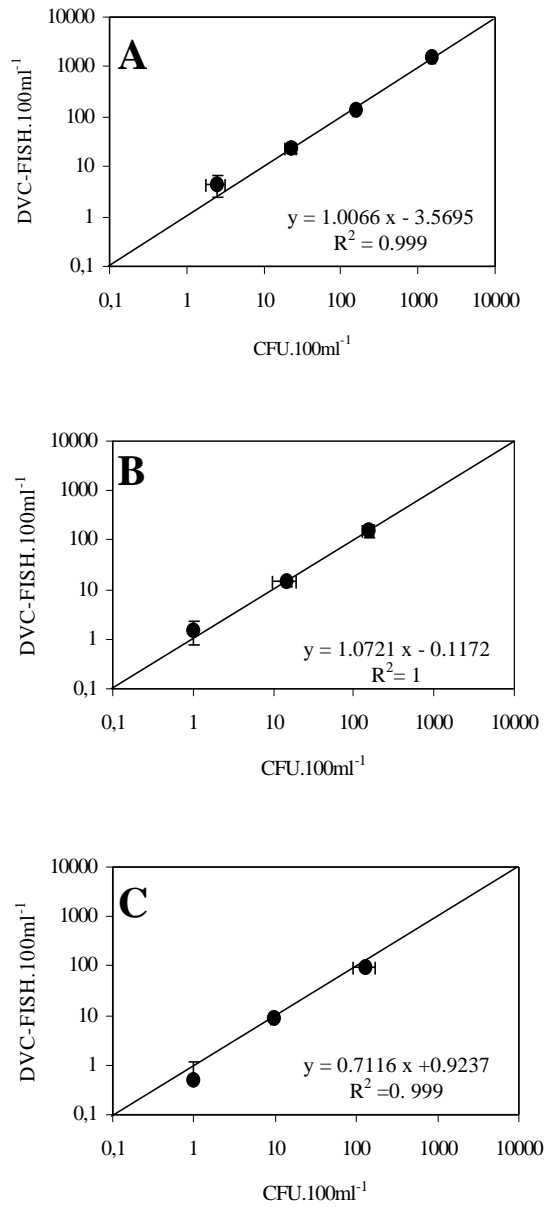


FIG. 2. Relationships between numbers of *E.coli* cells enumerated by the DVC-FISH-ScanRDI method and CFU counts in 100ml of water A (A), water B (B) and water C (C). Horizontal and vertical bars are standard deviations calculated on the mean value of duplicate CFU counts and DVC-FISH-ScanRDI positive events respectively.

In our conditions, viable *E.coli* cell numbers were underestimated by an order of 30% compared with CFU counts, when 100ml of tap water were analyzed (water C). In these cases, and due to these large numbers of autofluorescent particles plotted on the membrane, microscopic confirmation becomes tedious. Consequently, only a small part of the membrane can be analyzed for the purposes of this confirmation step. Total enumeration is thus obtained on the extrapolation of cell numbers detected, weighted by the proportion of confirmed positive events. This extrapolation could account for the underestimation. A solution would be to perform the analysis on a smaller volume of sample to decrease the number of false-positive events detected on the membrane.

4.3. Drinking water distribution system application: a study case

The DVC-FISH-ScanRDI approach was conducted on a North American water treatment plant and distribution system. The raw water here is subject to seasonal fluctuations in concentrations of ammonia, DOC and temperature (1 to 25°C). The treatment plant has a nominal capacity of 110,000 m³/day (with an average annual flow of 50,000 m³/day). The treatment line includes dynamic settling (Alum+SiO₂), rapid sand/antracite filtration, ozonation, BAC filtration, pH adjustment and disinfection with ClO₂.

A weekly survey of the bacteriological quality of raw water (RW, inlet water), biological activated carbon filtered water (BACFW), chlorinated water (CW, finished water) and distribution system water (DSW) was performed over 6 weeks, in June and July 2001, to assess the sensitivity of the DVC-FISH-ScanRDI approach in tracking viable *Enterobacteriaceae* cells compared with traditional cultured-based methods.

In this study, culturable cells were enumerated by the plate count procedure following sample filtration on a volume sample according to the sample origin. For RW, analyses were performed on volumes ranging from 1 to 5 ml, and, for BACFW, CW and DSW, analyses were performed on 100ml water samples. Two media were analyzed: the m-endo medium and the MacConkey medium, the latter a specific medium for the isolation of *Enterobacteriaceae* (APHA, 1998).

Owing to the stress level of endogenous cells present in RW, BACFW, CW and DWS, the incubation period of DVC treatment was increased to 4 hrs (35±2°C).

During the investigated period, total cells ranged between $4.6 \pm 1.4 \cdot 10^8$ total cells.100ml⁻¹ (raw water, mean value, n=6), $2.2 \pm 0.6 \cdot 10^7$ total cells.100ml⁻¹ (BAC filtered water, mean value, n=6), $4.5 \pm 2.1 \cdot 10^7$ total cells.100ml⁻¹ (chlorinated water, mean value, n=6) and $4.6 \pm 1.8 \cdot 10^7$.100ml⁻¹ (distribution system water, mean value, n=6). During this period, free chlorine levels were equal to 0.6 ± 0.1 for chlorinated water (mean value, n=6) and <0.05 mg Cl₂.l⁻¹ for distribution system water (n=6).

Weekly results of DVC-FISH-ScanRDI and CFU counts following incubation for 24 hrs on both m-endo and MacConkey media (35 ± 2°C) are reported in Figure 3.

This weekly survey of bacteriological quality and the physiological status of *Enterobacteriaceae* cells showed variable relations between DVC-FISH-ScanRDI counts and CFU counts according to the type of water and the week of analysis.

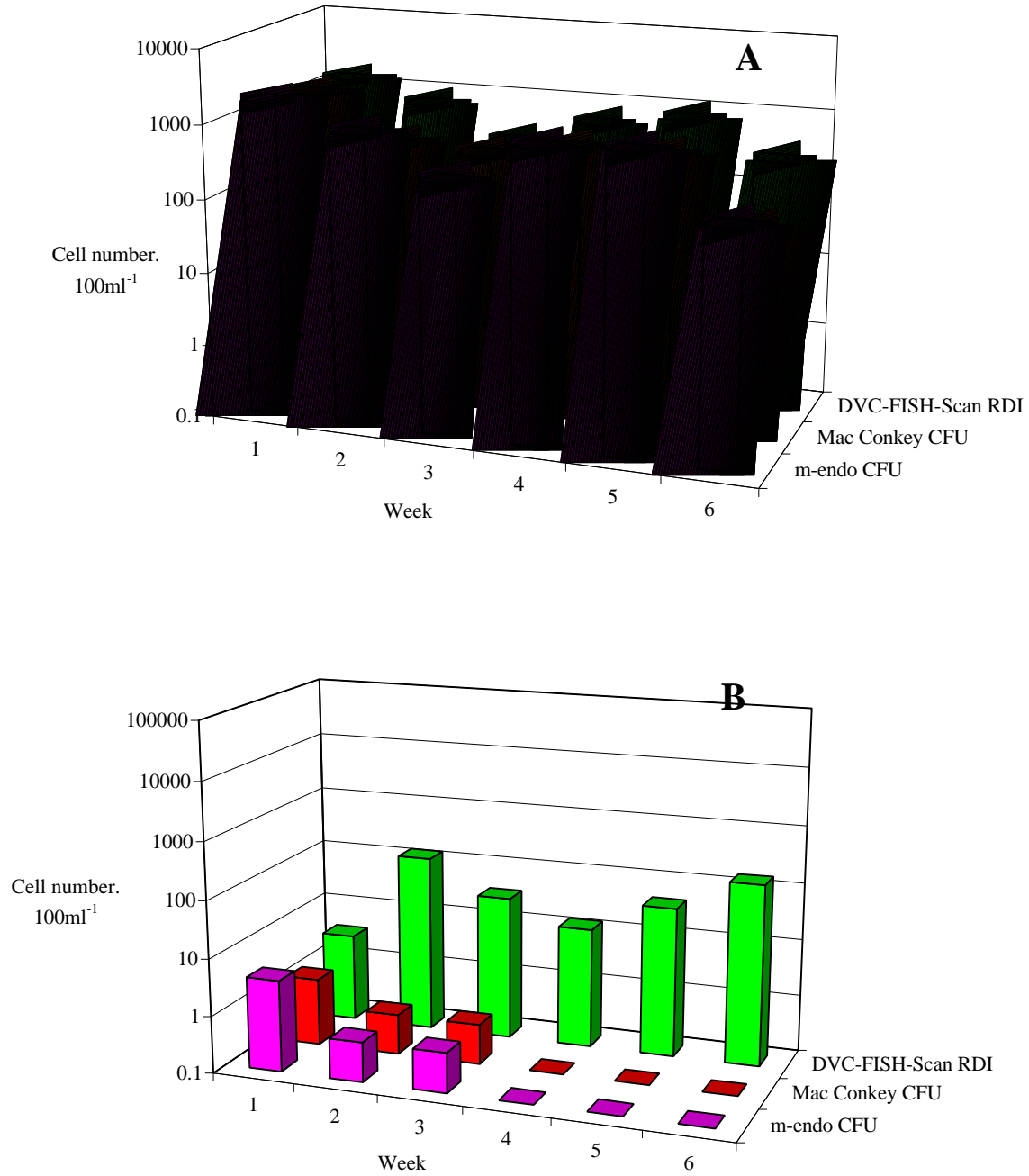


FIG. 3. Weekly DVC-FISH counts enumerated by the ScanRDI and CFU counts following 24 hrs of incubation on MacConkey and m-endo media on RW (A), BACFW (B), CW (C) and DSW (D).

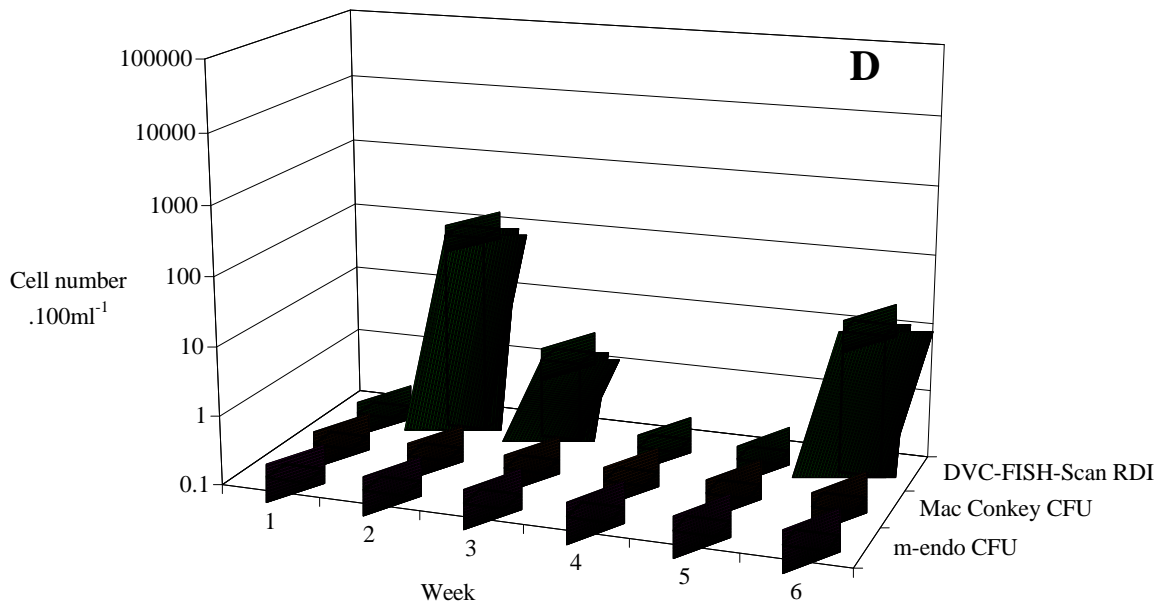
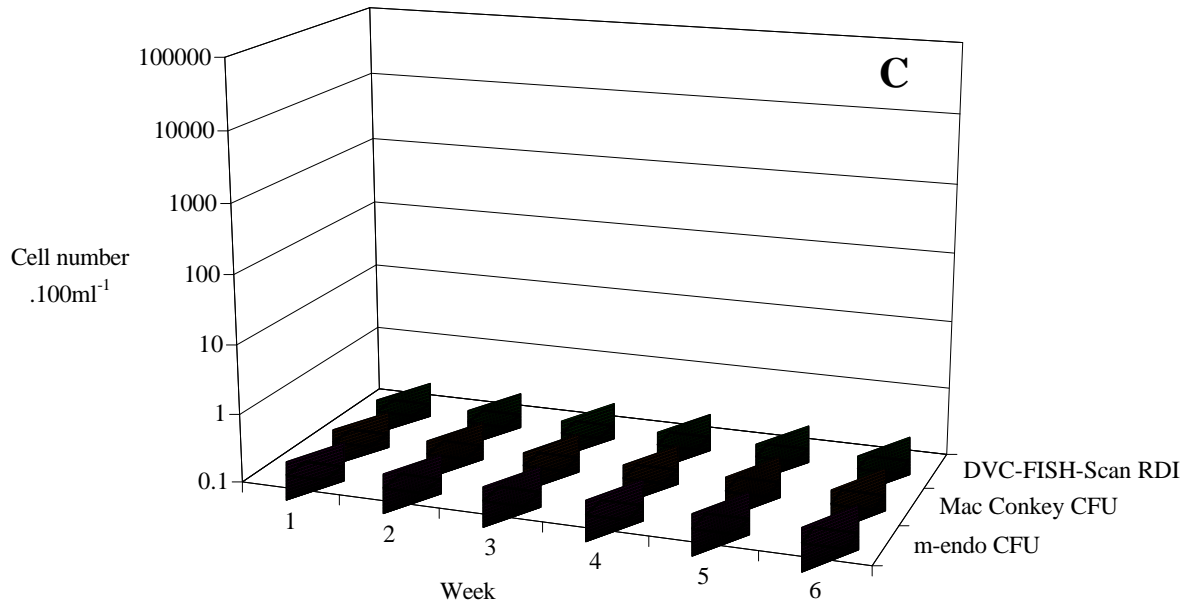


FIG. 3- (continued)

For RW samples (Fig. 3A), the DVC-FISH-ScanRDI counts are similar to the CFU counts (m-endo), except in one of six cases where an additional 48% of VBNC cells were detected.

Contrary to the RW samples, for BACFW samples (Fig. 3B), 5 samples out of 6 presented higher DVC-FISH-ScanRDI counts than CFU counts, and 50% of the samples presented DVC-FISH-ScanRDI positive counts but no CFU counts. For all BACFW samples, numbers of *Enterobacteriaceae* VNBC cells ranged between 98.5 and 100%.

For CW samples (Fig. 3C), no DVC-FISH-ScanRDI and no CFU colony counts were detected in any sample.

For DSW samples (Fig. 3D), 3 samples out of 6 showed DVC-FISH-ScanRDI positive counts and no CFU counts. As a consequence, the proportion of *Enterobacteriaceae* VBNC cells was 100% when DVC-FISH counts were performed.

When DVC-FISH-ScanRDI events were detected, viable *Enterobacteriaceae* cells showed high fluorescence intensity and scan line numbers (cell length index), as reported in Table 1. Nevertheless, fluorescence intensity and cell length decreased from raw water to distribution system waters, and could reflect, depending on the type of water, the different levels of stress of targeted cells.

Table 1: Cellular characteristics of *Enterobacteriaceae* treated by the DVC-FISH procedure and detected by the ScanRDI from natural water samples.

Water sample	Fluorescence intensity (FU)	Cell length (SL)
RW	298.5 (92.2)	3.6 (0.7)
BACFW	174.8 (26.1)	2.4 (0.2)
CW	no detected	no detected
DSW	171.6 (32.8)	2.5 (0.2)

Values in parentheses are standard deviations calculated on the mean value of positive counts from all analyzed membranes for each type of water.

5. Conclusion and significance to industry

Rapid detection and enumeration of highly diluted bacterial cells is a challenge, particularly in the drinking water field. The combination of 16S rRNA-targeted oligonucleotide probes, physiological assay by means of DVC and the ScanRDI cytometer, greatly increases the sensitivity of diluted viable labeled cell detection and identification, and may represent a useful tool for rapid water quality assessment. This enhanced detection capacity could have a great impact on reducing health-related events linked to drinking water contamination. The first field application of the DVC-FISH-ScanRDI showed higher sensitivity of this new tool for stressed *Enterobacteriaceae* detection in drinking water samples, compared with culture-based methods. These results have also made it possible to confirm the existence of VBNC *Enterobacteriaceae* cells in distributed water. However, further work is required on larger numbers of samples and

different drinking water distribution systems and will be performed to confirm these initial results.

The way in which the sensitivity of the DVC-FISH-ScanRDI approach varies with the treatment plant process (including disinfection treatment) will also be investigated.

Finally, large-scale application of the DVC-FISH-ScanRDI approach to DWDS (including more sampling stations and higher-frequency sampling) in combination with a deterministic model would allow us to better understand the origins of bacteriological contamination.

6. References

- APHA, AWWA and AEF.** 1998. Standard methods for the examination of water and wastewater (20th edition). Washington, DC.
- Besner M.C., Gauthier V., Barbeau B., Millette R., Chapleau R. and Prévost M.** 2001. Understanding distribution system water quality. *J. AWWA.* **93**:7, 101-114.
- Catala P., Pathuisot N., Bernard L., Baudart J., Lemarchand K. and Lebaron P.** 1999. Effectiveness of CSE to counterstain particles and dead bacterial cells with permeabilised membranes: application to viability assessment in waters. *FEMS Microbiol. Lett.* **178**: 219-226.
- Colwell R.R. and Grimes D.J.** 2000. Nonculturable microorganisms in the environment. R.R. Colwell and D.J. Grimes editors. ASM Press, Washington, D.C., USA.
- Joux F. and Lebaron P.** 1997. Ecological implications of an improved direct viable count method for aquatic bacteria. *Appl. Environ. Microbiol.* **63**: 3643-3647.
- Joux F. and Lebaron P.** 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbiol. Infec.* **2**: 1523-1535.
- Kalmbach S., Manz W. and Szewzyk U.** 1997. Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of a single cell assessed by formazan reduction and *in situ* hybridization. *FEMS Microbiol. Ecol.* **22**: 265-279.
- Kogure K., Simidu U. and Taga N.** 1979. A tentative direct microscopic method for counting living marine bacteria. *Can. J. Microbiol.* **25**: 415-420.
- Loge F.J., Emerick R.W., Thompson D.E., Nelson D.C. and Darby J.L.** 1999. Development of a fluorescent 16S rRNA oligonucleotide probe specific to the family *Enterobacteriaceae*. *Water Environ. Res.* **71**: 75-83.
- Mignon-Godefroy K., Guillet J-G., and Butor C.** 1997. Solid phase cytometry for detection of rare events. *Cytometry.* **27**: 336-344.
- Nishimura M., Kita-Tsukamoto K. and Kogure K.** 1993. A new method to detect viable bacteria in natural seawater using 16S rRNA oligonucleotide probe. *J. Oceano.* **49**: 51-56.
- Schönhuber W., Fuchs B., Juretschko S. and Amann R.** 1997. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Appl. Environ. Microbiol.* **63**: 3268-3273.

7. Acknowledgments

This research was supported by Vivendi Water and the Industrial Partners of the NSERC (Natural Sciences and Engineering Research Council of Canada) Industrial Chair on Drinking Water; namely, the City of Laval, the City of Montreal, BPR-Triax Consultants and US Filter-John Meunier. The Scan RDI was financed by the Fondation Canadienne pour Innovation .The

authors would like to thank D. Duchesne and Y. Fontaine for their valuable assistance in the sampling procedure.