Total and Viable *Legionella pneumophila* Cells in Hot and Natural Waters as Measured by Immunofluorescence-Based Assays and Solid-Phase Cytometry^{▽†}

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A new method was developed for the rapid and sensitive detection of viable *Legionella pneumophila*. The method combines specific immunofluorescence (IF) staining using monoclonal antibodies with a bacterial viability marker (ChemChrome V6 cellular esterase activity marker) by means of solid-phase cytometry (SPC). IF methods were applied to the detection and enumeration of both the total and viable *L. pneumophila* cells in water samples. The sensitivity of the IF methods coupled to SPC was 34 cells liter⁻¹, and the reproducibility was good, with the coefficient of variation generally falling below 30%. IF methods were applied to the enumeration of total and viable *L. pneumophila* cells in 46 domestic hot water samples as well as in cooling tower water and natural water samples, such as thermal spring water and freshwater samples. Comparison with standard plate counts showed that (i) the total direct counts were always higher than the plate counts and (ii) the viable counts were higher than or close to the plate counts. With domestic hot waters, when the IF assay was combined with the viability test, SPC detected up to 3.4×10^3 viable but nonculturable *L. pneumophila* cells per liter. These direct IF methods could be a powerful tool for high-frequency monitoring of domestic hot waters or for investigating the occurrence of viable *L. pneumophila* in both man-made water systems and environmental water samples.

Legionnaires' disease was first recognized in July 1976. The pathogenic agent isolated was identified as a bacterium and was called *Legionella pneumophila*. Since then, more than 50 species of *Legionella* and 64 serogroups have been identified, and at least 20 species have been associated with the disease in humans (15). Nevertheless, *L. pneumophila* is still the most pathogenic species, accounting for more than 90% of legionellosis cases, and serogroup 1 is the etiological agent responsible for more than 80% of the legionellosis cases diagnosed worldwide (30, 31). *Legionella* bacteria, described as opportunistic human pathogens, are widespread in natural aquatic environments and in artificial water systems and can also survive for a long time in a low-nutrient environment (7, 9, 29). The majority of outbreaks have been traced to aerosols contaminated with these organisms from either cooling towers or water distribution systems (i.e., drinking water distribution systems and hot sanitary waters) (16). The analysis of water samples collected from a source suspected of amplifying *Legionella* is a valuable means of preventing the health risk posed by potential sources of legionellosis. The rapid monitoring of *Legionella* in water systems has therefore become a priority in preventing and controlling the disease. The health risk can be measured by a microbiological laboratory experienced in *Le-*

gionella detection by determining the number of organisms present in water samples. Culture methods are the most commonly used diagnostic methods for *Legionella* infections. Nevertheless, these methods have some recognized limitations: they are time-consuming (taking up to 10 days) (12), they are not very sensitive, microbial contamination may inhibit *Legionella* growth, and viable but nonculturable (VBNC) bacteria can be present but not detected (26). Consequently, although culture methods are still considered the "gold standard" for the detection of *Legionella* in water, they are not well suited for real-time monitoring or for risk assessment and management of *Legionella* in water systems. The development of quicker and more sensitive methods without the cultivation step is the main priority for water quality assessment. These methods should allow the detection of all viable cells, including VBNC *Legionella*, in water systems in a few hours, so that *Legionella* proliferation can be prevented and rapidly controlled.

Immunofluorescence (IF) assays have already been proposed as a faster method of monitoring *Legionella* at hot water facilities and in cooling towers (3, 11, 28). These methods have some advantages with respect to detecting specific microbes in environmental water samples: they are easy to implement in a laboratory, and results can be obtained within a few hours. The targeted cells can be concentrated efficiently on a membrane filter and directly counted at the cellular level by use of an epifluorescence microscope or solid-phase cytometry (SPC) (3, 19). However, some caution is required when IF analysis is applied to environmental water samples: the antibodies must be highly specific for *Legionella*, and the targeted cells must be quantified accurately within a wide range of concentrations (3, 14). A further advantage of IF assays is that they can be

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combined with both specific detection and viability criteria (10, 22).

This study aimed at assessing the performances of two IF-based assays for rapid quantification of total and viable *L. pneumophila* cells in waters by using SPC. Viable *L. pneumophila* cells were detected using the IF method proposed by Aurell et al. (3) combined with the use of ChemChrome V6 (CV6), a cellular esterase activity marker, for viability assessment (21). In this paper, the term “viable SPC counts” refers to cells detected by IF and with detectable SPC esterase activity, although there is no direct proof that the detected cells are able to grow and divide under favorable conditions. The use of esterase activity as a viability marker in combination with cell detection by SPC was proposed by several authors for the detection of viable cells in aqueous products or water samples (11, 21, 25). These assays were used to investigate the occurrence of *L. pneumophila* in samples from man-made water systems, such as hot water samples and cooling tower waters, and in natural water samples, such as hot spring waters and freshwaters, and the results were compared to standard plate counts.

MATERIALS AND METHODS

Specificity control of the IF protocol and bacterial strains. Two monoclonal antibodies, specific for *L. pneumophila* serogroup 1 and *L. pneumophila* serogroups 2 to 15, were purchased from Microbiobioscience (Commercy, France). Their specificity for *L. pneumophila* has been controlled by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence methods in previous works (3, 17). In this study, 52 additional strains (*Legionella* and non-*Legionella* species), provided from reference and environmental collections, were tested to complete the specificity control (see Table S1 in the supplemental material). The *Legionella* strains were grown on buffered yeast extract containing α -ketoglutarate (BCYE α) supplemented with L-cysteine and ferric pyrophosphate (Oxoid, Dardilly, France) at 37°C for 48 to 72 h, and non-*Legionella* strains were grown at 30 or 37°C, depending on their optimum growth temperature, for 24 h on nutrient agar (Bio-Rad, Marnes la Coquette, France).

The *L. pneumophila* serogroup 1 strain Philadelphia (ATCC 33152) was also used for performance evaluations (sensitivity and recovery rate) of the IF methods.

Enumeration of total and viable *L. pneumophila* cells by solid-phase cytometry. Total *L. pneumophila* cells were immunostained by fluorescein isothiocyanate (FITC)-conjugated antibodies according to the protocol proposed by Aurell et al. (3). Ten milliliters of each water sample was filtered through a black polyester membrane (0.4- μ m pore size and 25-mm diameter) (CB04; AES-Chemunex, Ivry sur Seine, France). The membrane was then placed on 50 μ l of PBS-T-BSA staining solution (0.1% [vol/vol] Tween 20, 2% [wt/vol] bovine serum albumin [BSA] [Sigma-Aldrich, Saint Quentin Fallavier, France] in phosphate-buffered saline [PBS] adjusted to pH 6.9 [Sigma-Aldrich]). The staining solution contained a primary monoclonal antibody cocktail (the final concentration of anti-*L. pneumophila* serogroup 1 was 4.5 μ g ml⁻¹, and that of anti-*L. pneumophila* serogroups 2 to 15 was 7.3 μ g ml⁻¹), and incubations were performed at 37°C for 60 min. After staining, the membrane was transferred onto a hydrating pad soaked in 500 μ l of PBS-T-BSA and incubated for 2 min to eliminate excess antibodies. Primary antibodies were revealed by a secondary antibody solution containing FITC-conjugated sheep anti-mouse IgG (final concentration of 40 μ g ml⁻¹) (Sigma-Aldrich) diluted in PBS-T-BSA with 0.01% Evans blue (Sigma-Aldrich). The membrane was placed on 50 μ l of secondary antibody solution in petri dishes and incubated at 37°C for 60 min. The membrane was then transferred onto a hydrating pad soaked in 500 μ l of PBS-T-BSA and incubated for a minimum of 2 min at room temperature to eliminate excess antibodies before SPC analysis.

The analysis was performed in triplicate, and the results are expressed as total *L. pneumophila* SPC counts liter⁻¹. The detection limit of the method was 34 total *L. pneumophila* cells liter⁻¹.

Viable *L. pneumophila* cells were immunostained using the same primary antibodies as those used for the detection of total *L. pneumophila* cells, but using red fluorescence-conjugated secondary antibodies. Immunostaining was per-

formed after sample filtration (10 ml) through a black polyester membrane (0.45- μ m pore size and 25-mm diameter; Oxyphen AG, Lachen, Switzerland). Primary antibodies were revealed by a secondary antibody solution containing TriColor-conjugated goat anti-mouse IgG (Invitrogen, Molecular Probes Europe BV, Leiden, The Netherlands) (final concentration, 20 μ g ml⁻¹). The cellular viability was assessed after immunostaining by using the CV6 substrate (AES-Chemunex, Ivry-sur-Seine, France), a cellular esterase activity marker which is cleaved into a fluorescent green product inside viable cells (8, 11, 13, 21, 23). The viability of the immunostained cells was tested using a total viable count assay performed with the CV6 substrate and following the instructions provided by the manufacturer (AES-Chemunex). *L. pneumophila* hydrolyzing the substrate emitted both red and green fluorescence simultaneously, as shown in Fig. S1 in the supplemental material.

The analysis was performed in triplicate, and the results are expressed as viable *L. pneumophila* SPC counts liter⁻¹. The detection limit of the method was 34 viable *L. pneumophila* cells liter⁻¹.

SPC enumeration. The ChemScanRDI system (AES-Chemunex) scans the sample on the membrane support pad with an air-cooled argon laser emitting at 488 nm, and the fluorescence emission is collected in the green FL1 channel (500 to 540 nm), the orange FL2 channel (540 to 570 nm), and the red FL3 channel (655 to 705 nm). Total FITC-labeled *L. pneumophila* cells were detected with the FL1 channel, using the TVC Bioburden application provided by the manufacturer (AES-Chemunex). This application discriminated fluorescent events detected by SPC as targeted fluorescent bacterial signals or as particles (autofluorescent particles) by using a set of discriminant parameters (20).

The fluorescent secondary antibodies used for the viable *L. pneumophila* immunoassay are characterized by a 488-nm excitation wavelength and a 670-nm emission wavelength. Viable *L. pneumophila* cells corresponding to red immunostained cells and green cells hydrolyzing the enzyme substrate (CV6) were detected with both the FL1 and FL3 channels at the same time, using a modified TVC Bioburden application. After membrane scanning, a ratio of tertiary (FL3) to primary (FL1) signals (T/P) of 0.2 was activated to detect double-stained cells.

A final validation step for the discriminated fluorescence events was performed using a BH2 epifluorescence microscope (Olympus, Rungis, France) with an FITC block filter and a tetramethyl rhodamine isocyanate (TRITC) block filter installed on a motorized stage driven by the ChemScanRDI system.

Enumeration of *L. pneumophila* by the culture method. Plate counts were enumerated according to the French standard AFNOR T90-431 (2), which is in compliance with the international standard ISO 11731. One-hundred-milliliter to 1-liter water samples were concentrated by filtration through 0.45- μ m-pore-size polycarbonate filters (Sartorius SAS, Palaiseau, France). Each membrane was then transferred into 5 ml of sterile Milli-Q water and treated with ultrasonic energy twice for 1 min each with a Branson sonicator (Fisher Bioblock Scientific, Illkirch, France) operating at 42 kHz. One hundred microliters of the concentrate was spread onto a selective GVPC plate (BCYE α supplemented with L-cysteine and ferric pyrophosphate, 3 g of glycine, 100,000 U of polymyxin B, 80 mg of cycloheximide, and 1 mg of vancomycin per liter) (Oxoid). Samples (100 μ l) of the concentrate were also plated onto GVPC plates after heat treatment (50°C for 30 min) and acid treatment (0.2 M HCl, 0.2 M KCl, pH 2, for 5 min) to eliminate non-*Legionella* organisms. The inoculated plates were then incubated at 37 $\pm$ 2°C, and the colonies were counted after 3, 5, and 10 days. The colonies were then examined for fluorescence under a Wood lamp, and those exhibiting *Legionella* morphology were transferred to BCYE α medium, BCYE α medium without cysteine, and blood agar medium (bioMérieux, Marcy l'Etoile, France) as a control. At least five colonies per sample were identified by *Legionella*-specific immunolateral reagents (Oxoid). The selected colonies were also confirmed by IF staining. For filtration of 1 liter of water, the detection limit of the method was 1 colony per plate, equivalent to 50 CFU liter⁻¹, whereas the statistically significant quantification limit was based on the AFNOR recommendation of counting 5 colonies per plate, equivalent to 250 CFU liter⁻¹ (2).

Water samples. The performances of IF methods for *L. pneumophila* quantification were first evaluated with hot sanitary water artificially contaminated with various concentrations (10³ to 10⁷ cells liter⁻¹) of *L. pneumophila* serogroup 1 cells (ATCC 33152), and the results were compared to those of the culture method.

The cell suspension was created from a fresh colony resuspended in PBS, and viable cell concentrations were measured after CV6 staining.

Next, the occurrence of *L. pneumophila* was investigated in 46 hot shower water samples collected from May to December 2005 from shower water of the bathroom outlets of private homes representative of communities in the Paris area and more widespread areas in France.

In addition, 8 non-hot-water samples, comprising thermal spring waters (3 samples), cooling tower waters (3 samples), and freshwaters (2 samples), were

also tested to investigate the robustness of IF assays for the detection of total and viable *L. pneumophila* cells in naturally contaminated water samples. Cooling tower samples were collected from an industrial site in Spain during the spring of 2007. Thermal spring waters and freshwaters were collected from sites located in the Pyrénées (France) in the summer of 2007.

For hot shower (including spiking experiment samples) and cooling water samples, residual free chlorine was neutralized by the addition of 20 mg liter⁻¹ of sodium thiosulfate before the analysis was performed.

Additional treatments were applied on the non-hot-water samples before application of the viable *L. pneumophila* SPC protocol. A preliminary study showed that the associated microflora present in these samples interfered with the detection of viable *L. pneumophila* cells by SPC (data not shown). These water samples were characterized by the presence of abundant photosynthetic microeukaryotes showing an autofluorescent signal detected in channel FL3 by SPC. To eliminate these microeukaryotes, the samples were prefiltered through a polycarbonate membrane (0.8- μ m pore size and 47-mm diameter) (Whatman, Dominique Dutscher, Brumath, France) before application of the viable *L. pneumophila* test. Two approaches were performed for the esterase activity measurement of *L. pneumophila*. The first consisted of the direct detection of cellular activity immediately after sample filtration (direct activity), and the second consisted of detecting cellular activity after the incubation of cells on a selective nutritive plate (activity after GVPC incubation). For the latter, activity was measured after incubation of the membrane on a GVPC plate for 4 h and 15 h at 37°C. In addition, acid treatment before GVPC incubation was applied to reduce the overgrowth of nontarget microorganisms, as recommended by the French guidelines for the standard culture method (2). The acid treatment of the sample was performed immediately after water sample filtration and before incubation on the GVPC agar plate. The membrane was placed on 200 μ l of acid solution (0.2 M HCl-KCl buffer, pH 2.0) for 5 min at room temperature. The viable *L. pneumophila* cells were then immunostained as described previously.

Statistical tests. The normal distribution of data was tested using the Shapiro-Wilk test, and statistical comparisons between the various cell concentrations were performed using the Wilcoxon test. Correlation tests were performed (Spearman test and Pearson test with an α level of 0.05), and the relations between SPC counts and standard plate counts were determined by linear regression models. All statistics were calculated using XL Stat software (Addinsoft, France).

RESULTS

Specificity tests. The specificity of the monoclonal anti-*L. pneumophila* antibody cocktail used in this study has already been published (3, 17). The specificity list was completed in our study with 52 *Legionella* and non-*Legionella* strains, including 15 *L. pneumophila* (serogroups 1 to 15) and 24 *Legionella* non-*pneumophila* strains, as reported in Table S1 in the supplemental material. Specificity tests were performed by applying the IF protocol using the FITC-conjugated anti-*L. pneumophila* cocktail, as described above. All serogroups of *L. pneumophila* strains, except for serogroup 7 and serogroup 11, which are not targeted by the antibody cocktail, were recognized by the monoclonal antibodies, and no cross-reaction was observed for the non-*L. pneumophila* strains.

Accuracy of immunofluorescence assays for enumeration of *L. pneumophila* cells in artificially contaminated hot waters. The accuracies of the IF-based assays for enumerating total and viable *L. pneumophila* cells were evaluated with hot waters artificially contaminated with known log decimal concentrations of *L. pneumophila* serogroup 1. The reproducibility of both methods for quantifying *L. pneumophila* was evaluated based on two independent assays and using a wide range of *L. pneumophila* concentrations. The recovery rate of viable *L. pneumophila* cells was measured and compared to the standard plate counts from a third experiment.

Total and viable *L. pneumophila* SPC counts were linearly correlated with the number of *L. pneumophila* cells added as

FIG. 1. Total (A) and viable (B) SPC counts of *L. pneumophila* in artificially contaminated hot water samples at different log concentrations.

the inoculum in a log-log plot, as reported in Fig. 1A for total counts ($n = 15$, $r^2 = 0.994$, $P < 0.0001$; Pearson test) and in Fig. 1B for viable counts (direct activity measurements) ($n = 14$, $r^2 = 0.990$, $P < 0.0001$; Pearson test). The cell recoveries were 102.4% for total SPC counts and 73.4% for viable SPC counts (Fig. 1).

The reproducibility of the IF methods was investigated by determining the coefficient of variation (CV) for three replicates and was found to be globally lower than 30%. CVs ranged from 0.7 to 25.0% for total counts and from 3.5 to 29.8% for viable SPC counts, except for the lowest concentration (10^2 cells liter⁻¹), where they increased considerably, up to 100%.

The viable *L. pneumophila* SPC counts and plate counts were compared, and the results are reported in Table 1. Counts measured by both approaches were positively correlated ($r^2 = 0.974$ and $P < 0.0001$; Pearson test), and the recovery rates of SPC counts were higher than the plate counts for all cell concentrations.

Occurrence of *L. pneumophila* in hot waters. The occurrence of total and viable *L. pneumophila* cells was investigated in 46 hot water samples, and SPC counts were compared to the standard plate counts (Table 2).

Although the counts for all samples were below the detection limit of the culture method, *L. pneumophila* cells were detected in 91.3% of the water samples (42/46 samples) for measuring total cells and in 36.9% of the water samples (17/46 samples) for measuring viable cells. Total cell concentrations were globally higher than those of viable cells and ranged from 3.3×10^1 to 8.9×10^5 cells per liter. For comparison purposes,

TABLE 1. Viable *L. pneumophila* SPC counts and standard plate counts quantified in artificially contaminated hot sanitary water samples

Theoretical count (log ₁₀ cells liter ⁻¹)	Direct plate count (log ₁₀ CFU liter ⁻¹)	Direct viable SPC count (log ₁₀ cells liter ⁻¹)
7.30	5.02	6.64
	5.14	6.63
	5.08	6.61
6.30	3.50	5.64
	3.64	5.65
	3.80	5.60
5.30	2.06	4.52
	2.39	4.65
	<1.67	4.41
4.30	<1.67	3.53
	1.76	3.46
	2.24	3.41
3.30	<1.67	2.60
	<1.67	2.40
	<1.67	2.30

the number of viable *L. pneumophila* cells per liter ranged from 3.3×10^1 to 1.3×10^4 cells per liter. Eleven of the 17 samples testing positive for viable cells showed very low viable SPC concentrations, and 6/17 samples (samples 3, 5, 6, 8, 13, and 34) showed higher viable SPC counts (up to 1.3×10^4 cells per liter). All viable *L. pneumophila* SPC-positive samples were also positive for total SPC counts, except for samples 1 and 2. For these samples, the counts measured by the viable method were close to the detection limit of the immunofluorescence assays.

The recovery rates of viable *L. pneumophila* cells detected with and without GVPC incubation were compared for 9 hot water samples (samples 38 to 46). For both conditions, viable SPC counts were below the detection limit, except for sample 39, in which viable *L. pneumophila* cells were counted only after a revivification step on a GVPC plate.

When total and viable *L. pneumophila* cells were detected in the same sample, the ratio of viable to total *L. pneumophila* counts varied between 0.04% (sample 16) and 100% (sample 28).

The reproducibility of the IF methods was investigated by determining the CVs measured for three replicates for all hot water samples. CVs were clearly dependent on cell concentrations, as reported in Fig. 2, and the relationships between cell concentration and CV were similar for the total and viable SPC counts. The lowest CV values were measured for cell concentrations above 5×10^2 cells per liter, but the values of CV increased at the lowest concentrations.

Enumeration of total and viable *L. pneumophila* cells in cooling tower water and natural waters by SPC and comparison with standard plate counts. The performances of the IF methods in detecting and enumerating *L. pneumophila* cells by SPC were evaluated with cooling tower water samples and natural water samples, such as thermal spring waters and freshwaters (Table 3). These kinds of water samples are known as complex waters because of their more abundant and different

bacterial communities compared to those found in hot sanitary waters. All of the samples tested positive with both the IF methods and the standard method. The mean total *L. pneumophila* SPC counts were systematically higher than the standard plate counts, and cultivable *L. pneumophila* cells accounted for 0.1% to 16% of the total cells. Viable SPC counts measured without GVPC incubation were either similar to the standard plate counts or lower. Nevertheless, for some water samples, the viable SPC counts increased after a short incubation time on GVPC. Taking into account the counts with and without GVPC incubation, viable SPC counts and plate counts were positively correlated ($r^2 = 0.862$ and $P = 0.002$; Spearman test) (Fig. 3), and viable cell recovery was 103.9% for the IF assay.

DISCUSSION

The monitoring of *L. pneumophila* concentrations in water is a major goal for stakeholders and industries if they are to improve the quality assessment of their water systems. The culture method is currently used for monitoring *Legionella* in water systems. However, culturing is too time-consuming (taking up to 10 days) to be used for diagnostic purposes and has some limitations in terms of detecting all viable *L. pneumophila* cells present in a water sample (27). The risk management strategy for *Legionella* detection requires more rapid methods for the real-time monitoring of all *L. pneumophila* cells. Moreover, it should include VBNC cells in water systems, as they can be resuscitated under some environmental conditions (1, 11, 25). This strategy may be considered a validation of quality assurance and quality control procedures (16). Various tests have already been proposed to detect directly bacterial viability or activity at the cellular level without a culture stage and to quickly provide information on the physiological state of cells. Most of the tests used in microbial ecology are enzyme activity tests (18). The esterase activity test offers some advantages for rapid cell viability measurement, because enzymatic activity and cell membrane integrity can be measured simultaneously in only a few minutes. Another advantage is the possible combination of the esterase test, which stains viable cells green, with immunofluorescence assays using red fluorescence-conjugated antibodies. The proposed test allows the simultaneous detection of *L. pneumophila* cells by SPC by combining taxonomical and physiological information in the same assay by use of appropriate discriminants (optimized FL3-to-FL1 ratio). The mix of monoclonal antibodies used was highly specific to the majority of *L. pneumophila* serogroups but did not recognize serogroups 7 and 11. However, the role of these serogroups in legionellosis outbreaks is minor (15).

Results obtained with spiking experiments in hot sanitary water clearly showed that immunofluorescence methods combined with SPC quantified *L. pneumophila* cells with accuracy and reproducibility for a wide range of concentrations (10^3 to 10^7 cells liter⁻¹). The viable counts were positively correlated with plate counts and were systematically higher, with ratios ranging from 25 to 192. The results confirm that the viability of *L. pneumophila* can be tested at the cellular level by using a culture-independent method, such as the esterase activity test, which can be combined with an immunofluorescence method

TABLE 2. Comparison of total and viable *L. pneumophila* counts in hot water samples obtained by the reference culture method and IF assays

Sample	Standard plate count (CFU liter ⁻¹)	Mean (SD) SPC count (cells liter ⁻¹)			Ratio of viable to total SPC counts (%)
		Total <i>L. pneumophila</i>	Viable <i>L. pneumophila</i>		
			Direct activity measurement	With GVPC incubation ^a	
1	<2.5 × 10 ²	<3.4 × 10 ¹	5.0 × 10 ¹ (10.0 × 10 ¹)	ND	
2	<2.5 × 10 ²	<3.4 × 10 ¹	3.3 × 10 ¹ (5.8 × 10 ¹)	ND	
3	<2.5 × 10 ²	5.8 × 10 ³ (1.3 × 10 ³)	3.4 × 10 ³ (1.3 × 10 ³)	ND	58.8
4	<2.5 × 10 ²	4.5 × 10 ³ (0.9 × 10 ³)	1.0 × 10 ² (1.0 × 10 ²)	ND	2.2
5	<2.5 × 10 ²	7.3 × 10 ³ (1.1 × 10 ³)	1.2 × 10 ³ (0.06 × 10 ³)	ND	16.9
6	<2.5 × 10 ²	9.8 × 10 ³ (0.8 × 10 ³)	4.7 × 10 ² (0.6 × 10 ²)	ND	4.7
7	<2.5 × 10 ²	3.0 × 10 ² (0.0)	<3.4 × 10 ¹	ND	
8	<2.5 × 10 ²	4.6 × 10 ³ (0.8 × 10 ³)	1.1 × 10 ³ (0.3 × 10 ³)	ND	23.2
9	<2.5 × 10 ²	1.2 × 10 ³ (0.1 × 10 ³)	<3.4 × 10 ¹	ND	
10	<2.5 × 10 ²	1.7 × 10 ² (1.1 × 10 ²)	<3.4 × 10 ¹	ND	
11	<2.5 × 10 ²	1.0 × 10 ³ (0.4 × 10 ³)	3.3 × 10 ¹ (5.8 × 10 ¹)	ND	3.2
12	<2.5 × 10 ²	9.0 × 10 ³ (1.2 × 10 ³)	6.7 × 10 ¹ (11.5 × 10 ¹)	ND	0.7
13	<2.5 × 10 ²	1.9 × 10 ³ (0.6 × 10 ³)	1.7 × 10 ³ (0.3 × 10 ³)	ND	90.9
14	<2.5 × 10 ²	2.0 × 10 ² (1.0 × 10 ²)	<3.4 × 10 ¹	ND	
15	<2.5 × 10 ²	4.3 × 10 ² (1.5 × 10 ²)	3.3 × 10 ¹ (5.8 × 10 ¹)	ND	7.7
16	<2.5 × 10 ²	7.3 × 10 ⁴ (0.1 × 10 ⁴)	3.3 × 10 ¹ (5.8 × 10 ¹)	ND	0.04
17	<2.5 × 10 ²	3.3 × 10 ¹ (5.8 × 10 ¹)	<3.4 × 10 ¹	ND	
18	<2.5 × 10 ²	4.1 × 10 ⁴ (0.4 × 10 ⁴)	1.0 × 10 ² (1.0 × 10 ²)	ND	0.24
19	<2.5 × 10 ²	<3.4 × 10 ¹	<3.4 × 10 ¹	ND	
20	<2.5 × 10 ²	6.5 × 10 ² (0.7 × 10 ²)	3.3 × 10 ¹ (5.8 × 10 ¹)	ND	5.1
21	<2.5 × 10 ²	1.3 × 10 ⁴ (0.3 × 10 ⁴)	3.3 × 10 ¹ (5.8 × 10 ¹)	ND	0.26
22	<2.5 × 10 ²	<3.4 × 10 ¹	<3.4 × 10 ¹	ND	
23	<2.5 × 10 ²	2.5 × 10 ² (0.7 × 10 ²)	<3.4 × 10 ¹	ND	
24	<2.5 × 10 ²	5.1 × 10 ³ (1.9 × 10 ²)	<3.4 × 10 ¹	ND	
25	<2.5 × 10 ²	8.7 × 10 ² (1.5 × 10 ²)	<3.4 × 10 ¹	ND	
26	<2.5 × 10 ²	6.7 × 10 ¹ (11.5 × 10 ¹)	<3.4 × 10 ¹	ND	
27	<2.5 × 10 ²	1.0 × 10 ² (1.0 × 10 ²)	<3.4 × 10 ¹	ND	
28	<2.5 × 10 ²	6.7 × 10 ¹ (0.6 × 10 ¹)	6.7 × 10 ¹ (11.5 × 10 ¹)	ND	100.0
29	<2.5 × 10 ²	8.5 × 10 ³ (0.7 × 10 ³)	<3.4 × 10 ¹	ND	
30	<2.5 × 10 ²	8.0 × 10 ³ (2.0 × 10 ³)	<3.4 × 10 ¹	ND	
31	<2.5 × 10 ²	1.9 × 10 ⁴ (0.4 × 10 ⁴)	<3.4 × 10 ¹	ND	
32	<2.5 × 10 ²	1.3 × 10 ² (2.3 × 10 ²)	<3.4 × 10 ¹	ND	
33	<2.5 × 10 ²	3.3 × 10 ¹ (5.8 × 10 ¹)	<3.4 × 10 ¹	ND	
34	<1.0 × 10 ⁴	8.9 × 10 ⁵ (1.3 × 10 ⁵)	1.3 × 10 ⁴ (1.2 × 10 ⁴)	ND	1.5
35	<5.0 × 10 ²	4.3 × 10 ² (0.6 × 10 ²)	<3.4 × 10 ¹	ND	
36	<2.5 × 10 ²	2.6 × 10 ³ (0.5 × 10 ³)	<3.4 × 10 ¹	ND	
37	<2.5 × 10 ²	2.6 × 10 ⁴ (0.6 × 10 ⁴)	<3.4 × 10 ¹	ND	
38	<2.5 × 10 ²	2.9 × 10 ⁵ (0.6 × 10 ⁵)	<3.4 × 10 ¹	<3.4 × 10 ¹	
39	<5.0 × 10 ²	6.8 × 10 ³ (1.6 × 10 ³)	<3.4 × 10 ¹	6.7 × 10 ¹ (11.5 × 10 ¹)	0.98
40	<2.5 × 10 ²	1.5 × 10 ⁵ (0.3 × 10 ⁵)	<3.4 × 10 ¹	<3.4 × 10 ¹	
41	<2.5 × 10 ²	3.7 × 10 ⁵ (0.2 × 10 ⁵)	<3.4 × 10 ¹	<3.4 × 10 ¹	
42	<2.5 × 10 ²	1.4 × 10 ⁴ (1.1 × 10 ⁴)	<3.4 × 10 ¹	<3.4 × 10 ¹	
43	<2.5 × 10 ²	4.7 × 10 ³ (0.3 × 10 ³)	<3.4 × 10 ¹	<3.4 × 10 ¹	
44	<2.5 × 10 ²	3.8 × 10 ³ (0.4 × 10 ³)	<3.4 × 10 ¹	<3.4 × 10 ¹	
45	<2.5 × 10 ²	2.6 × 10 ³ (0.2 × 10 ³)	<3.4 × 10 ¹	<3.4 × 10 ¹	
46	<2.5 × 10 ²	4.5 × 10 ² (0.7 × 10 ²)	<3.4 × 10 ¹	<3.4 × 10 ¹	

^a ND, not determined.

for more sensitive detection of viable *L. pneumophila* cells in waters (21).

Applied to naturally contaminated hot water samples, the methods showed the weakest accuracies for the lowest concentrations (below 5 × 10² cells liter⁻¹). This can be explained by the heterogeneous distribution of *L. pneumophila* cells in drinking water distribution systems, which can be influenced by the presence of biofilms or free amoebas providing favorable ecological niches supporting the survival of *Legionella* (1, 5). Moreover, on spiked hot sanitary waters, the CV was the high-

est for concentrations above 10² cells liter⁻¹. Thus, for these waters, the precision of the methods can be improved by increasing the volume of water analyzed or by increasing the number of filtered membranes analyzed.

The investigation of 46 hot sanitary water samples revealed a more frequent occurrence of *L. pneumophila* by IF methods than that obtained by culture, since 91% of samples were positive by the total *L. pneumophila* assay and 37% of them showed the presence of viable *L. pneumophila* cells, whereas all samples were negative by culture. Ours results confirmed those

FIG. 3. Logarithms of concentrations of *L. pneumophila* per liter in water samples (thermal spring water, cooling tower water, and fresh-water), determined by the viable IF test and the standard culture method.

mulation. While these were probably not lethal, they may have temporarily reduced the enzymatic activity of the cells. This activity recovered after a short incubation step on a nutritive medium, which provided the nutrients and energy required for enzyme synthesis and/or maintenance of the membrane potential (18). Consequently, in the absence of any information on the physiological status of cells before the test is performed, the protocol for detecting viable *L. pneumophila* cells should include an analysis performed directly and with an incubation step on GVPC varying from 4 h to 15 h.

To conclude, the IF methods combined with SPC made it possible to quantify accurately and rapidly the numbers of total and viable *L. pneumophila* serogroup 1 and serogroup 2 to 6, 8 to 10, and 12 to 15 cells present in diverse water samples.

Combined with a viability test, the technique showed a higher sensitivity than the standard culture method for counting viable cells and offered the possibility of detecting viable but nonculturable *L. pneumophila*. Since VBNC *L. pneumophila* may constitute a fairly significant proportion of *Legionella* cells in water systems, and because these cells may be infectious (26), the proposed methods could be a useful technique for assessing the health risks associated with these microorganisms. In fact, such techniques could be powerful tools, similar to what is now proposed for real-time PCR, for high-frequency monitoring of hot waters for routine investigation. Moreover, they could help to provide more information on natural reservoirs and ecological niches of *Legionella* in aquatic environments and consequently lead to a better understanding of their ecology and how to prevent their transfer to water systems.

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