Rapid quantification of viable Legionella in nuclear cooling tower waters using filter cultivation, fluorescent in situ hybridization, and solid phase cytometry

Julia Baudart, C. Guillaume, Mercier A., P. Lebaron, M. Binet

Rapid quantification of viable *Legionella* in nuclear cooling tower waters using filter cultivation, fluorescent *in situ* hybridization, and solid phase cytometry

Baudart, J. [1,2], Guillaume, C.[3,4], Mercier, A.[1,2], Lebaron, P. [1,2], and Binet, M.[3]

[1]Sorbonne Universités, UPMC Univ Paris 06, USR 3579, LBBM, Observatoire Océanologique, F-66650, Banyuls/mer, France;

[2] CNRS, USR 3579, LBBM, Observatoire Océanologique, F-66650, Banyuls/mer, France;

[3] EDF, Laboratoire National d'Hydraulique et Environnement, 6 quai Watier, F-78401, Chatou cedex, France

[4] Euro Engineering, Énergie-Environnement, 22 terrasse Bellini, 92800 Puteaux, France

Running title: Rapid counting of viable *Legionella* in cooling tower water

Keywords: *Legionella spp.*, microcolonies, FISH, cooling tower water, solid phase cytometry

Corresponding author: Julia Baudart. Laboratoire ARAGO/ Laboratoire d'Océanographie Biologique de Banyuls, Avenue Fontaulé, F66650 Banyuls-sur-Mer, France

Tel: (33) 468 88 73 47

Fax: (33) 468 88 73 98

E-mail: **baudart@obs-banyuls.fr**

Abstract

Aims: to develop a rapid and sensitive method to quantify viable *Legionella* spp in cooling tower water samples.

Methods and Results: A rapid, culture-based method capable of quantifying as few as 600 *Legionella* microcolonies per liter within 2 days in industrial waters was developed. The method combines a short cultivation step of microcolonies on GVPC agar plate, specific detection of *Legionella* cells by a fluorescent *in situ* hybridization (FISH) approach, and a sensitive enumeration using a solid phase cytometer. Following optimization of the cultivation conditions, the qualitative and quantitative performance of the method was assessed and the method was applied to 262 nuclear power plant cooling water samples.

Conclusions: The performance of this method was in accordance with the culture method (NF-T 90-431) for *Legionella* enumeration.

Significance and Impact of Study: The rapid detection of viable *Legionella* in water is a major concern to the effective monitoring of this pathogenic bacterium in the main water sources involved in the transmission of legionellosis infection (Legionnaires' disease). The new method proposed here appears to be a robust, efficient and innovative means for rapidly quantifing cultivable *Legionella* in cooling tower water samples within 48h.

Introduction

The Legionellaceae family comprises a wide variety of species. To date, about 50 species and more than 60 serogroups have been described. The most pathogenic of the *Legionella* species, *L. pneumophila*, is responsible for the majority of cases of legionellosis infection (Legionnaires' disease), and serogroup 1 has been found in 70% of the isolates from patients suffering from this disease (Joseph, 2004). However, 19 other species have been involved in human infection (Olsen et al., 2010) or isolated from clinical cases (Muder and Yu, 2002). The main non *L. pneumophila* species isolated from legionellosis infections are *L. longbeachae*, *L. bozemanii*, *L. micdadei*, *L. feeleii*, *L. dumoffii*, *L. wadsworthii*, and *L. anisa*.

Human infection occurs through the inhalation of contaminated aerosols produced from aerosol-generating devices associated with various types of water system. Frequently, *Legionella* species have been isolated in cooling systems, and some cooling towers have also been shown to be implicated in outbreaks of legionellosis (Morton et al., 1986; Bentham, 2000; Kirrage et al., 2007). However, cooling tower associated outbreaks are mainly caused by *L. pneumophila* serogroup 1 strains. Although no cases of Legionnaires' disease have been linked to the presence of *Legionella* in nuclear power plant cooling circuits in France, an attention is also paid to monitor *Legionella* concentrations in the waters of these cooling towers by the authorities. Currently, the regulatory framework in France requires the monitoring of *Legionella* spp. in nuclear cooling tower water using culture-based methods (French guideline AFNOR T90-431, AFNOR, 2014). The results, which consider only cultivable cells, are expressed in colony forming units (CFUs) per liter. *Legionella* are fastidious bacteria with a slow *in vitro* growth rate, and as such they require some additional factors, such as L-cysteine and iron, to promote their growth on synthetic culture media. The visual macrocolonies that are counted on culture media contains about 10^6 cells, and usually appear after 2 to 7 cultivation days for *Legionella* cells isolated from an environmental water

sample. The French normalized method (AFNOR T90-431), which is culture-based, stipulates the use of the GVPC medium for *Legionella* recovery in water samples (AFNOR, 2014). However, this method has some well-recognized limitations, especially their low sensitivity, which is caused by the overgrowth of non target microorganisms. This overgrowth occurs because of the long cultivation time, despite the presence of inhibitors in the culture media, and the large number of confirmation steps required to identify a few of the species using latex agglutination tests which are time-consuming (another 2 days). There is clearly a need for a faster culture-based method for *Legionella* monitoring in cooling water systems.

Fluorescent *in situ* hybridization (FISH) is a detection technique that uses specific fluorescent-labeled DNA probes which target the rRNA of the cells of selected microorganisms to rapidly identify bacterial cells (Amann et al. 1995). When the rRNA in the cell is targeted, only metabolically active bacteria are detected. However, starved environmental bacteria often show low metabolic activity, and, as a result, a low rRNA content may not be detected (Amann et al., 1995). To overcome this limitation, bacteria can be concentrated from samples and incubated on a solid medium to increase their metabolic activity and generate *Legionella* microcolonies that can be detected by FISH (Wulling et al., 2002; Satoh et al., 2002). Reducing the growth time leading to microcolony formation will not only speed up the detection process, but will make it applicable to both the starved and active cells in water samples (Wulling et al., 2002). The *Legionella* cells that form microcolonies can be directly identified by FISH on a filter following filtration of the sample and a short time cultivation. The fluorescent microcolonies are then enumerated using a fluorescence detection device, such as digital imaging (London et al., 2010), an epifluorescence microscope (Baba et al., 2012), Scan VIT (Ditommaso et al., 2010), or a solid phase cytometer. The methods that use FISH in combination with a brief filter cultivation method have been developed to specifically enumerate viable pathogenic bacteria, such as

members of the Enterobacteriaceae family (Ootsubo et al., 2003), *Listeria* (Fuchizawa et al., 2008), and *Clostridium perfringens* (Shimizu et al., 2009). Recently, a combination of brief filter cultivation and FISH detection for *Legionella* enumeration in hospital water systems using a ScanVIT fluorescence detector was proposed by Ditommaso et al. (2010). However, no FISH method combined with the microcolonies enumeration for *Legionella* monitoring in industrial cooling tower systems as cooling tower of nuclear plants supplied with crude water has been proposed so far. This technique can constitute a favorable alternative for the monitoring of viable *Legionella*. Nevertheless, the quantitative performance of this method is mainly linked to the ability of the detector device to enumerate a large range of *Legionella* concentrations in a water sample. This ability is vital for *Legionella* monitoring in industrial cooling tower systems, because of the variability of the bacterial concentration, which is dependent on the quality of the water and the disinfection process used. Compared to other detectors, the main advantage of the solid phase cytometer is its very low detection limit and the large number of cells it can enumerate, as it scans the whole surface of the membrane filter. Moreover, it has a powerful ability to discriminate between fluorescent labeled cells and background fluorescence (Lemarchand et al., 2001, Baudart and Lebaron, 2010).

The main objective of this study is to develop a filter method of cultivation for microcolonies of *Legionella* combined with a FISH method for the rapid enumeration of viable *Legionella* spp. by solid phase cytometry applicable to the cooling waters of nuclear power plants, and to compare the trial method with the normalized culture method (NF-T 90-431) for *Legionella* spp. enumeration on a total of 262 cooling tower water samples.

Materials and methods

Oligonucleotide probes and FISH assay. Four 16S rRNA oligonucleotide probes targeting specific sequences located at different positions on 16S rRNA for *Legionella* spp. [Leg705,

CTGGTGTTCCTTCCGATC, and Leg226, TCGGACGCAGGCTAATCT (Manz et al., 1995); Legall11, CCTCCTCCCCACTGAAAGT, and Legall22, CACTGTATGTCAAGGGTAGG (Leskelä et al., 2005)] were selected from the literature. The probes were selected on their specificity, which was tested *in silico*, and on their closest Tm values. Specificity was first tested for each oligonucleotide individually in silico, using the probe match function of the Ribosomal database project software package (Maidak et al., 2001) and the BLAST function from the NCBI website (Altschul et al., 1997). Because of the use of multiple probes in the same mixture for the hybridization, the presence of secondary structures within a probe (self dimers) and between probes (cross dimers) was checked using the multiple primer analyzer module available online from the ThermoScientific website. No self dimers or cross dimers were detected in any of the four probes (http://www.thermoscientificbio.com/webtools/multipleprimer/). The oligonucleotides were synthesized and conjugated at their 5' end with 6-FAM (ThermoFisher Scientific, Ulm, Germany).

Bacterial strain and media for specificity test. The specificity of the fluorescent *in situ* hybridization (FISH) assay was tested on culture bacteria collections. A total of 39 *Legionella* strains and 21 non *Legionella* strains, provided from reference and environmental collections were used to check the specificity of the FISH assay (Table 1). The *Legionella* strains were grown on buffered charcoal-yeast extract supplemented with α-ketoglutarate (αBCYE) agar containing L-cysteine (Biomérieux) at 37°C for 48 to 72 h, and the non *Legionella* strains were grown at 30 or 37°C, depending on their optimum growth temperature, for 24 h on nutrient agar (Biomérieux). The tests were performed with freshly cultured cells in stationary growth phase. Cell suspensions were created in phosphate-buffered saline (PBS) adjusted to pH 6.9 (Sigma Aldrich), and then 100 µl of this cell suspension (diluted in PBS, several-fold if necessary) were filtered on a 0.40 µm black polyester membrane (CB04, Cycloblack, 25

147 mm, CHEMUNEX® - Biomérieux). Cells hybridization was performed directly on CB04
148 membrane as described in the fixation of cells and whole cells hybridization section. The cells
149 were observed by epifluorescence microscopy.

150 **Pre-treatment of environmental water samples before cultivation and hybridization**. The
151 environmental water samples were homogenized to disperse the bacterial cells and disrupt
152 aggregates by means of an optimized mechanical treatment prior to the filter cultivation of the
153 microcolonies. The homogenization treatment was performed as follows: 2 g of glass beads
154 (2-3 mm diameter, Dominique Dutscher, Brumath, France) were added to 35 ml of water
155 sample. This sample was then mixed using a vortex and treated with ultrasonic energy for 1
156 min with a Branson sonicator operating at 42 kHz (Fischer Bioblock Scientific, Illkirch,
157 France). This treatment was applied twice. The sample was mixed one final time using a
158 vortex shaker for 1 min, prefiltered on a polycarbonate membrane (3.0 µm pore size, 47 mm
159 diameter, Whatman, Dominique Dutscher), and then heat treated at 50°C for 30 min to slow
160 the development of non *Legionella* microorganisms during the cultivation time. Five
161 milliliters of the water sample were then filtered on a CB04 polyester membrane and acid
162 treated by placing the CB04 membrane on 200 µl of acid solution (0.2 mol l^{-1} HCl-KCl
163 buffer, pH 2.0) for 5 min at room temperature. Filter cultivation was then performed. The heat
164 and acid treatments were performed as recommended by the standard method (NF T 90-431).

165 **Optimization of cultivation conditions for microcolonies of *Legionella***. Cultivation time
166 and temperature were tested on the selective GVPC agar plate to define the optimal conditions
167 for growing microcolonies of *Legionella* on a filter from naturally contaminated cooling water
168 samples. The GVPC selective agar medium [αBCYE, supplemented with 3 g of glycine,
169 100,000 U of polymyxin B, 80 mg of cychloheximide, and 1 mg of vancomycin per liter]
170 (Biomérieux) is most often recommended for *Legionella* detection (ISO, 1998; CDC, 2005;

AFNOR, 2014), as it inhibits non *Legionella* microorganisms, which can be responsible for the lack of recovery of Legionella spp. Two cultivation temperatures, 30 and 37°C, were compared for microcolonies recovery and various incubation times (ranging from 20 to 52 h) were tested. Tests were performed on a naturally contaminated 5 ml water sample following previous pre-treatment (homogenization, heat treatment, filtration of water sample on CB04 membrane and acid treatment), as described above. The microcolonies of were hybridized using the protocol described below, and then enumerated with solid phase cytometer.

Fixation of cells and whole cells hybridization. The fixation of cells and hybridization were performed directly on the membrane as follows: the CB04 membrane was transferred onto a 25 mm cellulose pad (labeling pad, Millipore) soaked in 600 µl of 96.2% ethanol for fixation of cells. The membrane and pad were left in a Petri dish at room temperature for 4 ± 1 minutes, and then the membrane was dried at room temperature for 3 ± 1 minutes prior to being placed in 50 µl of hybridization buffer (900 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate, 20% deionized formamide, 10% dextran sulfate) containing the four probes (the final concentration of each probe being 5 ng μl^{-1}) in hybridization chambers and incubated at 46°C for 120 min in a covered water bath. Following hybridization, the membranes were placed on a labeling 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, and 5 mmol l^{-1} EDTA).

Cells and microcolonies detection. For the specificity tests, the hybridized cells were analyzed by epifluorescence microscopy. The membrane containing cells was air dried and mounted on an antibleaching medium (Citifluor AF1; Citifluor Ltd., Houdon, United Kingdom) for observation using a WIBA filter block for FITC detection (Olympus model AX70, Hamburg, Germany). A negative control (without oligonucleotides) was set up to test

195 the auto-fluorescence of the protocol for all the strains tested.

196 The microcolonies of *Legionella* spp. were counted using a solid phase cytometer, SPC
197 (ScanRDI^{TM}, CHEMUNEX® -Biomérieux) after: (i) a short cultivation time on a nutritive
198 medium; and (ii) the staining of cells using the FISH assay described above. The membrane
199 was then placed into the SPC's sample holder and on a 25 mm cellulose membrane (support
200 pad, 0.45 µm pore size), which had previously been saturated with 100 µl of washing buffer.
201 The SPC system scans the sample on the support pad with an argon laser beam (488 nm
202 emission wavelength), recording all the fluorescence events at 500-530 nm and 540-570 nm
203 with two photomultiplier tubes. Fluorescence events are discriminated as targeted fluorescent
204 bacterial signals or as false positives (autofluorescent particles) using a set of discriminant
205 parameters (Mignon-Godefroy et al., 1997). The selected discriminant parameters were: peak
206 intensity 100 to 20,000; secondary/primary ratio 0 to 0.9; number of lines 1 to 50; and number
207 of samples 1 to 250. The analytical results were plotted in 2 dimensions, where all the
208 discriminated fluorescence events are shown in terms of x and y coordinates on a schematic
209 diagram of the membrane. One membrane can be scanned in 3 minutes, including the
210 discrimination step. The validation step using epifluorescence microscopy was then
211 performed, using a BH2 epifluorescence microscope (Olympus) equipped with an FITC filter
212 block and a motorized stage driven by the ScanRDI^{TM} system. The microcolonies of
213 *Legionella* detected by the SPC were expressed as Microcolony Forming Unit (MicFU).
214
215 **Quantitative performances of the assay.** The sensitivity of the trial assay for enumerating
216 *Legionella* was tested on artificially contaminated cooling tower water samples. A different
217 aliquot of a sterilized (121°C for 15 min) cooling tower water sample was spiked with various
218 concentrations of *L. pneumophila* sg1 cells (ATCC 33152) and compared with theoretical
219 counts of the cells added. The cell suspension was made from a fresh colony resuspended

220 with milliQ water, and the cell concentrations were determined by SPC after staining of the
221 viable cells using the ChemChrome V6 assay (CHEMUNEX®-Biomérieux) (Parthuisot et al.,
222 2000). Serial decimal dilutions were then made in water samples to obtain concentrations
223 ranging from 10^2 to 10^7 cells l^{-1}. The repeatability of the method was then tested after 48 h of
224 filter cultivation time by determining the coefficient of variation (CV) from 11 replicates
225 performed by the same operator from the same cooling tower water sample. In this case, the
226 test was performed on a naturally contaminated cooling tower water sample.
227
228 **Naturally contaminated cooling tower water samples**. A total of 262 cooling tower water
229 samples were collected between 2009 and 2011 from 8 nuclear power plants in France. Fifty-
230 two water samples were collected during the process of disinfecting the cooling tower
231 systems, and, for these samples, the residual free chlorine was neutralized by the addition of
232 20 mg l^{-1} of sodium thiosulfate. Two liters of water were collected in a sterile bottle. The filter
233 cultivation was performed at 30°C for 24 and 48 h. The analysis was performed in triplicate.
234
235 **Quantification of *Legionella* by the reference culture method**. *Legionella* were enumerated
236 and identified according to the AFNOR T90-431 standard (AFNOR, 2014) in compliance
237 with the international standard ISO 11731 (ISO, 1998). Briefly, 200 µl of each cooling tower
238 water sample was placed directly onto a selective GVPC plate (Biomérieux). In addition,
239 water samples were concentrated by filtration through 0.45 µm pore size polycarbonate filters
240 (Sartorius SAS, Palaiseau, France). The filtered volume was dependent on the sample's
241 filterability, and ranged from 50 to 500 ml. The membrane was then transferred into 5 ml of
242 sterile milliQ water and treated with ultrasonic energy for 2 x 1 min with a Branson sonicator
243 (Fischer Bioblock Scientific, Illkirch, France) operating at 42 kHz. Volumes (100 µl) of the
244 concentrate were also placed on GVPC plates following heat treatment (50°C for 30 min) and

245 acid treatment (HCl 0.2 mol l^{-1}, KCl 0.2 mol l^{-1}, pH 2.0, for 5 min) to reduce the overgrowth
246 of the non *Legionella* organisms during the long incubation period. The inoculated plates
247 were incubated at 37 ± 2°C, and the colonies were counted after 3, 5, and 10 days. The
248 colonies were then examined for fluorescence under a Wood lamp, and those exhibiting
249 *Legionella* morphology were transferred to an αBCYE medium, an αBCYE medium without
250 cysteine, and a blood agar medium (Biomérieux) as a control. At least five colonies per
251 sample were identified by *Legionella*-specific immunolatex reagents (Oxoid). According to an
252 AFNOR recommendation for dirty and non filterable waters, *Legionella* quantifications
253 performed from 500 ml samples of cooling water leads to a limit of detection (LD) of the
254 method of 100 CFU l^{-1}, and a limit of quantification of 500 CFU l^{-1} (AFNOR, 2014).
255
256 **Mathematical model of variation and limit of detection (LD) determination.** The excess
257 of Poisson randomness was detected qualitatively by the Poisson index of dispersion, and is
258 called overdispersion. This additional random uncertainty was measured quantitatively by
259 estimating the overdispersion factor $u^2 = \frac{s^2 - m}{m^2}$ (s^2 = variance, m = mean value) (FD T90-
260 465-1, 2014). In overdispersed sample, a Negative Binomial model is appropriate to
261 determine the LD. The LD is the particle number x (per analytical portion), where the
262 probability p_0 of a negative result equals 5%. The particle number x is equal $x = \frac{(p_0{}^{-u^2} - 1)}{u^2}$.
263
264 **Statistical analysis**. Normally distributed data were tested using the Shapiro-Wilk test, and
265 the various cell concentrations were statistically compared using the Wilcoxon signed rank
266 test when normality was not confirmed. The relation between SPC counts and standard plate
267 counts was determined by regression models (Pearson test for the linear regression model).
268 All the statistics were calculated using XL stat software (Addinsoft, France).

The MicFU-FISH-SPC and reference culture methods were compared using the ISO 17994 criteria for establishing equivalence between microbiological methods (ISO, 2014). Briefly, the methods are considered as quantitatively equivalent (not different) if the mean relative difference of the paired confirmed counts does not vary significantly from zero.

Results

Oligonucleotide specificity for the FISH assay. The probe specificity was checked *in silico* using the probe matching function of the Ribosomal database project software package and the BLAST function from the NCBI website. According to the Blast results, all the probes are highly specific for the genus *Legionella*. In fact, of the 46 species of *Legionella* used, the probes matched with all 46 species for the Leg705 probe, and with 31 species, 33 species, and 39 species for the Leg226, Legall11, and Legall22 probes, respectively. Moreover, all the *Legionella* species tested were detected with at least 2 probes (Table S1). The specificity of the probe set (consisting of the four probes: Leg705, Leg226, Legall11, and Legall22) was then evaluated for a wide spectrum of bacterial strains obtained from reference collections or isolated from various environments. Thirty-nine *Legionella* strains and 21 non *Legionella* strains were used to assess the specificity of the FISH protocol. All the *Legionella* strains (100%) yielded a strong positive fluorescent signal with the probe set (Figure S1), and no fluorescent signal was generated with the non *Legionella* strains. No autofluorescent signal (without oligonucleotides) was detected for any of the bacterial strains tested (Table 1).

Influence of microcolonies cultivation conditions. The effect of cultivation temperature (30 and 37°C) performed on GVPC for the recovery of microcolonies by SPC was tested after 48 h of incubation. Analyses were performed on 11 cooling tower water samples collected from 3 different industrial sites. The microcolonies were counted by SPC from duplicate water

294 samples, and the results were expressed as the number of *Legionella* spp. MicFUs per liter.
295 The MicFU concentrations were significantly higher for cultivation at 30°C (signed Wilcoxon
296 test, p =0.015), compared to those measured at 37°C (Figure 1). In addition, a few
297 microcolonies were detected in two water samples (g and h, collected from site 2) at 30°C,
298 while none were detected at 37°C. According to these results, the optimal temperature for
299 microcolonies cultivation on GVPC agar is 30°C. A comparison of MicFUs measured at
300 different cultivation times and CFUs enumerated using the standard method is presented in
301 Figure 2. The standard plate count is 2.5 10^4 CFU l^{-1}, and MicFU counts measured in the same
302 water sample increased gradually with cultivation time up to 48 h of incubation time (2.7
303 (±4.6) 10^2 l^{-1} for 20 h of incubation time up to 2.5 (±0.12) 10^4 l^{-1} for 48 h of incubation time).
304 After this incubation time, the MicFU counts remained constant and similar to the standard
305 plate count. Consequently, the maximum number of recoveries on GVPC was obtained after
306 48 h at 30°C. In conclusion, for the cooling tower sample analyzed, the GVPC plate showed a
307 better recovery rate for microcolonies of *Legionella* enumeration cultivated at 30°C. These
308 cultivation conditions were applied to compare the MicFUs and the standard plate counts in
309 other cooling tower water samples. However, because a shorter cultivation time could indicate
310 the contamination level of the sample and be useful for faster circuit monitoring, we also
311 enumerated the microcolonies after 24 of incubation time (Methods S1).
312
313 **Quantitative performance of the MicFU-FISH-SPC method**. The quantitative performance
314 of the method for enumerating viable *Legionella* from cooling tower water samples was first
315 evaluated on an artificially contaminated water sample with known log-decimal
316 concentrations of *L. pneumophila* sg 1 to define the linearity domain, and the detection limit
317 of the method. The water sample was analyzed prior to artificial contamination, and showed
318 an absence of *Legionella* (equivalent to < 200 *Legionella* MicFU l^{-1}). The quantification was

performed on five replicates with a cell density level ranging from 10^2 to 10^7 viable cells added. The microcolonies were enumerated by SPC after 48 h, at 30°C. The theoretical counts and the MicFU counts were linearly correlated with the number of *L. pneumophila* cells added as the inoculums in a log-log plot ($n = 30$; $r = 0.980$, $P < 0.0001$; Spearman test correlation). Consequently, we estimated the linearity domain of the trial method to range between 10^2 and 10^7 MicFU l^{-1}. The repeatability expressed as the precision under the same operating conditions was carried out on 11 analyses performed on the same day from the same natural water sample and analyzed by the same operator, as recommended by the French standard (ENV ISO 13843, Guidance on validation of microbiological methods) (AFNOR, 2001). The microcolonies were enumerated in the water sample after it was physically and chemically treated and filtered on a CB04 membrane of 5 ml of the sample. Enumerations were performed by SPC after 48 h of incubation at 30°C on GVPC. The concentration of *Legionella* spp. measured by both methods were similar and were 2.5 10^4 CFUs per liter for standard plate count and 2.1 10^4 MicFUs per liter. The repeatability of the trial method was investigated by determining the coefficient of variation (CV = 24.1%) measured from the number of microcolonies detected by SPC from each water sample (Table 2). The LD of the trial method was determined according to the French standardization procedure, set out in the document (ENV ISO 13843, AFNOR 2001). The theoretical LD of the method is 1 microcolony per membrane, corresponding to 200 MicFU l^{-1} when a 5 ml water sample is analyzed. Nevertheless, as expected in an environmental sample, the repeatability assay demonstrated that parallel determinations vary even more than is explained by the Poisson distribution. The samples are clearly overdispersed with an overdispersion factor of 0,049 (estimated with the repeatability assays). Because of this overdispersion, the Negative binomial model was applied to determine the LD of the method and was estimated to be 600 MicFU l^{-1} (3 microcolonies per analytical portion of 5 ml).

***Legionella* spp. quantification in naturally contaminated cooling tower water samples by MicFU-FISH-SPC and comparison with standard plate counts**. A total of 262 cooling tower water samples, collected between July 2009 and March 2011 from 8 different power plants located in France, were analyzed by both the trial and standard culture methods. According to nuclear plant officials, the cooling circuits use freshwater water inputs from the larger rivers in France, such as the Seine, the Rhône, the Moselle, and the Loire. So, the physico-chemical qualities of the cooling tower water samples tested in this study varied, depending on the freshwater source and on temporal changes, such as the season. The microcolonies of *Legionella* from all the water samples were enumerated following application of the physical and chemical treatments described previously, and following cultivation performed 48 h at 30°C on GVPC agar. As reported in Table 3, 80.1% and 78.6% of the water samples were positive using the standard culture method and the trial method, respectively: *Legionella* CFU concentrations ranged between 5.10^2 and $8.5\ 10^5$ CFU l^{-1} and the microcolonies concentrations ranged between 6.10^2 and $3.8\ 10^6$ MicFU l^{-1}. The percentages of water samples for which the results of the two methods were in agreement (MicFU+/CFU+ and MicFU-/CFU- pair results) was 95% (n = 248) and the MicFU and CFU counts were linearly correlated (Pearson test, $P<0.0001$ for $\alpha=5\%$) (Figure 3).

The equivalence between the trial assay and the standardized culture method was tested according to ISO 17994 (ISO, 2014) to determine whether or not the trial method is 'at least as reliable' as the standardized culture method. Of the 262 water samples tested, 46 were found to be negative by both methods and excluded from the calculation, as recommended by ISO 17994. The 'two-sided' comparison performed on 216 water samples was considered appropriate for the acceptance of the trial method (Bargellini et al., 2010), and the results showed that the performance of the FISH method did not differ from that of the culture

369 method (NF T 90-431), because the two values of the confidence interval (x_L= -22.71, x_H =
370 4.22) were on one side of zero and the second was on the other side of zero.
371
372 **Discussion**
373 The plate count methods for *Legionella* isolation and counting are currently standard ways to
374 monitor *Legionella* in cooling tower water samples. Although they are widely used, these
375 methods are time-consuming, because a long cultivation time is required to visualize the
376 colonies, and because the procedure includes re-isolation of the strains and serological
377 identification of microcolonies of *Legionella*. As a result of these limitations, the standard
378 culture methods are not compatible with a rapid management of *Legionella* proliferation.
379 However, they are still valuable detection methods for the information they provide on cell
380 viability, because they are based on growth activity. Other methods have been proposed for
381 the rapid detection of viable *Legionella* in water samples, such as the real-time PCR-based
382 methods using ethidium monoazide or propidium monoazide (Inoue et al., 2008; Chen and
383 Chang, 2010), direct fluorescent antibody analysis (DFA), which includes a vital staining step
384 to detect esterase activity (Parthuisot et al., 2011) or FISH-based techniques (Kirschner et al.,
385 2012). The real-time PCR-based methods have been successfully applied to *Legionella*
386 monitoring in hot sanitary water (which can be described as ‘clean water’). However, PCR-
387 based methods have some drawbacks when applied to a dirty, turbid, and complex water, such
388 as the water that characterizes industrial cooling tower systems. The occurrence of PCR
389 inhibitor agents in water has been extensively documented and reported by several authors in
390 cases where PCR was applied to environmental water samples, such as cooling tower waters
391 (Koide et al., 1993; Yamamoto et al., 1993; Catalan et al., 1997; Villari et al., 1998; Behets et
392 al., 2007; Edagawa et al., 2008; Morio et al., 2008). These inhibitor agents are frequently
393 present in turbid water, such as cooling tower water, as well as in untreated freshwater. In

dirty water samples, even if purification DNA processes are applied during DNA extraction and before DNA amplification, they usually are too drastic to preserve all the DNA or too inefficient to remove PCR inhibitors. Currently, a tenfold serial dilution of DNA extract samples before amplification is proposed to reduce the inhibitor effect. In the case of industrial cooling tower systems, the presence of PCR inhibitors co-extracted with DNA can involve the application of *a posteriori* dilution factors of 5-100, which considerably decreases the sensitivity of the method, especially when the abundance of *Legionella* in the water is low, and leads to false-negative results (Touron-Bodilis et al., 2011). Another concern is the diversified nature of the inhibitors. Their presence in water samples, lead to the success or failure of the PCR based methods applied for *Legionella* monitoring in these systems (Chen and Chang, 2010; Touron Bodilis et al., 2011). Moreover, it has been demonstrated that viable PCR is not suitable for monitoring highly contaminated water samples, such as cooling tower water, especially following decontamination treatment. In the presence of high levels of suspended solids or biomass, the dye could undergo chemical adsorption onto other compounds, and the cross linking step by light could be inhibited, since the radiation probably would not be able to penetrate the liquid (Fittipaldi et al., 2011, Slimani et al., 2012). Recently, a new DFA method combining a cellular test based on the detection of the esterase activity of bacteria cells and the specific detection of *L. pneumophila* cells by specific monoclonal antibodies has been proposed for the detection of active *L. pneumophila* in hot sanitary water and freshwaters (Parthuisot et al., 2011). This method offers an efficient means for detecting the Viable But Non Culturable (VBNC) *L. pneumophila* cells in water samples. Nevertheless, its application to the detection of active *Legionella* spp. is currently still limited, because of the absence of highly specific antibodies able to directly target all *Legionella* species in a complex bacterial community, like those of cooling tower systems.

Finding a highly conserved nucleic acid region in *Legionella* species for its detection using the FISH technique can overcome this problem. The four probes that we selected target four distinct 16S rRNA sequences, which hybridize with targets in the same hybridization conditions. The specificity test performed on a large number of *Legionella* and non *Legionella* strains has revealed the high degree of specificity of the FISH technique, which has been extensively applied to the direct detection, identification, and enumeration of specific bacteria in an environmental water sample in combination with a fluorescence detection device. However, the majority of studies using these methods for cell detection in environmental samples have limited application because of the very low fluorescence signals given out following hybridization. These low fluorescence signals are mostly due to a low ribosomal content of individual and starved cells (Amann et al., 1995). To overcome the ribosomal content limitations, treatments like the catalyzed reporter deposition (CARD)-FISH technique have been proposed. This technique increases the fluorescent signal of hybridized cells using horseradish peroxydase-labeled probes (Lebaron et al,. 1997; Baudart et al., 2002). A CARD-FISH technique was recently proposed by Kirchner et al. (2012) for the direct enumeration of *L. pneumophila* in two hospital cooling tower systems in combination with epifluorescence microscopy. With CARD-FISH, the authors measured concentrations of *L. pneumophila* that were higher than with standard plate counts by two to three orders, and up to six orders of magnitude, depending on the hospital investigated. The results obtained by Kirchner et al. (2012) are closer to the counts usually measured by real-time PCR based on total DNA detection (included DNA extracted from dead cells, cultivable cells, and VBNC cells) than to the culture results. Some care should be taken in interpreting CARD-FISH results, particularly in the physiological interpretation of CARD-FISH-targeted cells. CARD does, in fact, efficiently increase the fluorescent signal of hybridized cells, leading to the detection of cells with a low ribosomal content, as is the case with starving or non viable cells (Pernthaler et al.,

2002). Consequently, and depending on the amplification signal emitted by the CARD process, viable cells, but also the non viable ones, can be detected by the CARD-FISH technique, providing counts that are similar to those in real-time PCR performed on the total DNA extracted. In addition, in assessing the performance of a rapid trial relative to that of a standard culture method, a major concern is that viability be measured using a widely accepted test in both methods which targets the same physiological function, such as the ability of a cell to grow

The microcolony-based method developed in this study offers the following advantages: cell viability, as determined by the cultivation, is based on the same physiological function, like cell growth ability, as in the standard method. The time of analysis can be reduced to 48 h, because microcolonies can be detected in their early stage of development when combined with solid phase cytometry. In addition, decreasing the cultivation time reduces the risk of the overgrowth of contaminating microorganisms, which can inhibit or mask *Legionella* growth. The overgrowth of contaminating microorganisms is an important issue, as it leads to a decrease in the sensitivity of the culture method, especially for cooling tower water samples where microorganisms, other than *Legionella*, are abundant and diversified, which can make the results unusable.

For *Legionella*, the formation of the microcolonies depends on the cultivation conditions, as reported in our results. On the basis of ecological knowledge, *Legionella* growth occurs in an environment with temperatures in the 20 to 45°C range. An incubation temperature of 36°C ± 1 is advocated for *Legionella* isolation in the normalized culture method, but this temperature was selected because it corresponds to the optimal temperature of a human host, and allows better recovery of *Legionella* involved in human infections. Outside a human host, for example, *Legionella* species living in aquatic environments are able to grow over a large temperature range. A study performed on the isolation rate of *Legionella* species in

groundwater have demonstrated that the rate of formation of *Legionella* isolates increases when the isolation process occurs at 30°C instead of 37°C (Riffard et al., 2002). Our study confirms that culture at 30°C improves the recovery rate of the microcolonies of *Legionella* from the cooling tower water samples analyzed.

Our objective was to determine a suitable cultivation time for naturally contaminated water samples for use with the trial method. Detectable microcolonies were counted by SPC after an incubation time as short as 20 h, but the maximum number was counted for an incubation time ranging between 44 h and 48 h, which is similar to the count yielded by the standard culture method. The increase in the recovery rate of the method is highly dependent on the cultivation time, as well as the cultivation temperature. This is explained by a hybridization signal intensity that may be affected by the ribosomal content of *Legionella* and the fact that the number of rRNA in cells is dependent on the incubation time (Kerkhof and Kemp, 1999). In our study, the conditions of microcolonies cultivation were determined to be optimal at 30°C for 48 h on GVPC. Even though some studies have demonstrated that some non *L. pneumophila* grows poorly on GVPC medium (Ta et al., 1995), this medium remains currently used and suggested for the isolation of *Legionella* spp. from environmental water samples. Under these conditions, the repeatability of the trial method measured by the standard deviation calculated from 11 replicates was acceptable (<25%) for the quantification of *Legionella* in cooling tower water and the detection limit was 600 MicFUs per liter.

In light of these results, MicFU-FISH-SCP appears to be an efficient method in accordance with the standard method to perform a rapid monitoring (within 2 days) of cultivable *Legionella* in industrial cooling tower systems, independently of the quality of the input freshwater and the presence of a biocide treatment. In addition, the analysis performed within 1 day (24 h test results shown in supplementary material) could be used as a rapid test to indicate whether or not a high level of *Legionella* is contaminating the cooling systems (semi

quantitative test). The rapid analysis is performed on a small volume (5 ml *vs* 1 l), and a membrane sample can be stored for one month after cultivation, and ethanol treatment prior to hybridizing the microcolonies. These storage conditions can lead to wide-scale sampling, which is not always realizable with the standard method. The manipulation of a small volume of contaminated water could be another advantage, which would reduce the risk of exposure in laboratories and among healthcare employees.

Some studies are needed before the industrialization of this method in order to make it more applicable in industrial surroundings and daily use.

Acknowledgments

We gratefully acknowledge the help of Marion Mottier from the Observatoire Océanologique of Banyuls-sur-Mer and of Gaëlle Le Meur from Euro Engineering with sample collection and *Legionella* culture.

Conflict of interest: All authors declare that they do not have any conflict of interest.

References

AFNOR (Association Française de Normalisation) (2001) Qualité de l'eau-lignes directives pour la validation des méthodes microbiologiques. ENV ISO 13843.

AFNOR (Association Française de Normalisation) (2014) Water quality - Detection and enumeration of *Legionella* spp. and *L. pneumophila*. Method by direct inoculation and after concentration by membrane filtration or centrifugation. NF T90-431.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Amann, R. I., Ludwig, W., Schleifer, K. H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**, 143-169.

Baba, T., Inoue, N., Yamaguchi, N., Nasu, M. (2012) Rapid enumeration of active *Legionella pneumophila* in freshwater environments by the microcolony method combined with direct fluorescent antibody staining. *Microbes Environ* **27**, 324-326.

Bargellini, A., Marchesi, I., Leoni, E., Mansi, A., Cristino, S., Marcelloni, A.M., Borella, P. (2010) Inter-laboratory validation of a rapid assay for the detection and quantification of *Legionella* spp. in water samples. *Lett Appl Microbiol* **51**, 421-427.

Baudart, J., Coallier, J., Laurent, P., Prévost, M. (2002) Rapid and sensitive enumeration of viable diluted cells of members of the family *Enterobacteriaceae* cells in freshwater and drinking waters. *Appl Environ Microbiol* **68**, 5057-5063.

Baudart, J. Lebaron, P. (2010) Rapid detection of *Escherichia coli* in waters using fluorescent in situ hybridization, direct viable counting and solid phase cytometry. *J Appl Microbiol* **109**, 1253-1264.

532 Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. (2007) Development and
533 evaluation of a Taqman duplex real-time PCR quantification for reliable enumeration of
534 *Legionella pneumophila* in water samples. *J Microbiol Methods* **68**, 137-144.
535 Bentham, R. H. (2000) Routing sampling and control of *Legionella* spp. in cooling tower
536 water systems. *Curr Microbiol* **41**, 271-275.
537 Catalan, V., Garcia, F., Moreno, C., Vila, M.J., Apraiz, D. (1997) Detection of *Legionella*
538 *pneumophila* in wastewater by nested polymerase chain reaction. *Res Microbiol* **148**, 71-78.
539 Centers for Disease Control (CDC) US Department of Health and Human Service (2005)
540 Procedures for the recovery of *Legionella* from the Environment. Atlanta, USA: Laboratory.
541 Chen, N. T., Chang, C. W. (2010) Rapid quantification of viable *legionellae* in water and
542 biofilm using ethidium monoazide coupled with real-time quantitative PCR. *J Appl Microbiol*
543 **109**, 623-34.
544 Ditommaso, S., Giacomuzzi, M., Gentile, M., Zotti, C.M. (2010) Evaluation of the usefulness
545 of a new direct immunofluorescence assay (ScanVIT-*Legionella*^{TM}) for monitoring hospital
546 water systems contaminated with *Legionella* spp. *Lett. Appl. Microbiol*. **50**, 341-346.
547 Edagawa, A., Kimura, A., Doi, H., Tanaka, H., Tomioka, K., Sakabe, K., Nakajima, C.,
548 Suzuki, Y. (2008) Detection of culturable and nonculturable *Legionella* species from hot
549 water systems of public building in Japan. *J App Microbiol* **105**, 2104-2114.
550 FD T90-465-1 (2014) Water quality - Protocol for the estimation of the uncertainty of
551 measurement associated with an analysis result for microbiological enumeration methods -
552 Part 1 : references, definition and general principles
553 Fittipaldi, M., Codony, F., Adrados, B., Camper, A. K., Morató, J. (2011) Viable real-time
554 PCR in environmental samples: can all data be interpreted directly? *Microb Ecol* **61**, 7–12.

555 Fuchizawa, I., Shimizu, S., Kawai, Y., Yamazaki, K. (2008) Specific detection and
556 quantitative enumeration of *Listeria* spp. using fluorescent *in situ* hybridization in
557 combination with filter cultivation (FISHFC). *J Appl Microbiol* **105**, 502-9.
558 Inoue, D., Tsutsui, H., Yamazaki, Y., Sei, K., Soda, S., Fujita, M., Ike, M. (2008) Application
559 of real-time polymerase chain reaction (PCR) coupled with ethidium monoazide treatment for
560 selective quantification of viable bacteria in aquatic environment. *Water Sci Technol* **58**,
561 1107–1112.
562 International Organisation for Standardisation, ISO (1998) *Water quality* – Detection and
563 enumeration of *Legionella*. Geneva, Switzerland: ISO 11731.
564 International Organisation for Standardisation, ISO (2014) Water quality – Requirements for
565 the comparison of the relative recovery of microorganisms by two quantitative methods: ISO
566 17994.
567 Joseph, C.A. (2004) Legionnaire's disease in Europe 2000-2002. *Epidemiol Infect* **132**, 417-
568 424.
569 Kirrage, D., Reynolds, G., Smith, G. E., Olowokure, B. (2007) Investigation of an outbreak of
570 Legionnaires' disease: Hereford, UK 2003. *Respi Med* **101**, 1639-1644.
571 Kerkhof, L., Kemp, P. (1999) Small ribosomal RNA content in marine *Proteobacteria* during
572 non-steady-state growth. *FEMS Microbiol Ecol* **30**, 253-260.
573 Kirschner, A. K., Rameder, A., Schrammel, B., Indra, A., Farnleitner, A. H., Sommer, R.
574 (2012) Development of a new CARD-FISH protocol for quantification of *Legionella*
575 *pneumophila* and its application in two hospital cooling towers. *J Appl Microbiol* **112**, 1244-
576 56.
577 Koide, M., Saito, A., Kusano, N., Higa, F. (1993) Detection of *Legionella* spp. in cooling
578 tower water by the polymerase chain reaction method. *Appl Environ Microbiol* **59**, 1943-
579 1946.

580 Lebaron, P., Catala, P., Fajon, C., Joux, F., Baudart, J., Bernard, L. (1997) A new sensitive,
581 whole-cell hybridization technique for detection of bacteria involving a biotinylated
582 oligonucleotide probe targeting rRNA and tyramide signal amplification. *Appl Environ*
583 *Microbiol* **63**, 3274-3278.
584 Lemarchand, K., Parthuisot, N., Catala, P., Lebaron, P. (2001) Comparison assessment of
585 epifluorescence microscopy, flow cytometry and solid-phase cytometry used in the
586 enumeration of specific bacteria in water. *Aqua Microb Ecol* **25**, 301-309.
587 Leskelä, T., Tilsala-Timisjärvi, A., Kusnetsov, J., Neubauer, P., Breitenstein, A. (2005)
588 Sensitive genus-specific detection of *Legionella* by a 16S rRNA based sandwich
589 hybridization assay. *J Microbiol Methods* **62**, 167-79.
590 London, R., Schwedock, J., Sage, A., Valley, H., Meadows, J., Waddington, M., Straus, D.
591 (2010) An automated system for rapid non-destructive enumeration of growing microbes.
592 *PLoS One* **5**(1).
593 Maidak, B.L., Cole, J.R., Lilburn, T. G., Parker Jr, C.T., Saxman, P.R., Farris, R.J., Garrity,
594 G.M., Olsen, G.J., Schmidt, T.M., Tiedje, J.M. (2001) The RDP-II (Ribosomal database
595 project). *Nucleic Acids Res* **29**, 173–174.
596 Manz, W., Amann, R., Szewzyk, R., Szewzyk, U., Stenström, T.A., Hutzler, P., Schleifer,
597 K.H. (1995) *In situ* identification of *Legionellaceae* using 16S rRNA-targeted oligonucleotide
598 probes and confocal laser scanning microscopy. *Microbiology* **141**, 29-39.
599 Mignon-Godefroy, K., Guillet, J.G., Butor, C. (1997) Solid phase cytometry for detection of
600 rare events. *Cytometry* **27**, 336-344.
601 Morio, F., Corvec, S., Caroff, N., Le Gallou, F., Drugeon, H., Reynaud, A. (2008) Real-time
602 PCR assay for the detection and quantification of *Legionella pneumophila* in environmental
603 water samples: Utility for daily practice. *Int J Hyg Environ Health* **211**, 403-411.

Morton, S., Bartlett, C. L. R., Bibby, L. F., Hutchinson, D. N., Dyer, J. V., Dennis, P. J. (1986) Outbreak of legionnaires' disease from a cooling water system in a power station. *British J Industrial Medicine* **43**, 630-635.

Muder, R. R., Yu, V. L. (2002) Infection due to *Legionella* species other than *L. pneumophila*. *Clin Infect Dis* **35**, 990-998.

Olsen, J. S., Aarskaug, T., Thrane, I., Pourcel, C., Ask, E., Johansen, G., Waagen, V., Blatny, J. M. (2010) Alternative routes for dissemination of *Legionella pneumophila* causing three outbreaks in Norway. *Environ Sci Technol* **4422**, 8712-871.

Ootsubo, M., Shimizu, T., Tanaka, R., Sawabe, T., Tajima, K., Ezura, Y. (2003) Seven-hour fluorescence *in situ* hybridization technique for enumeration of *Enterobacteriaceae* in food and environmental water sample. *J Appl Microbiol* **95**, 1182-90.

Parthuisot, N., Catala, P., Lemarchand, K., Baudart, J., Lebaron, P. (2000) Evaluation of ChemChrome V6 for bacterial viability assessment in waters. *J Appl Microbiol* **89**, 370-380.

Parthuisot, N., Binet M., Touron-Bodilis A., Pougnard, C., Lebaron, P., Baudart, J. (2011) Occurrence of total and viable *L. pneumophila* cells in hot and natural waters measured by immunofluorescence-based assays and solid phase cytometry. *Appl Environ Microbiol* **77**, 6225-6232.

Pernthaler, A., Pernthaler, J., Amann, R. (2002) Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Environ Microbiol* **68**, 3094-4101.

Riffard, S., Springthorpe, S., Filion, L. (2002) Occurrence of *Legionella* in Groundwater (AWWA Research Foundation Reports).

Satoh, W., Nakata, M., Yamamoto, H., Ezaki, T., Hiramatsu, K. (2002) Enumeration of *Legionella* CFU by colony hybridization using specific DNA probes. *Appl Environ Microbiol* **68**, 6466-6470.

629 Shimizu, S., Ootsubo, M., Kubosawa, Y., Fuchizawa, I., Kawai, Y., Yamazaki, K. (2009)
630 Fluorescent *in situ* hybridization in combination with filter cultivation (FISHFC) method for
631 specific detection and enumeration of viable *Clostridium perfringens*. *Food Microbiol* **26**,
632 425-431.
633 Slimani, S., Robyns, A., Jarraud, S., Molmeret, M., Dusserre, E., Mazure, C., Facon, J. P.,
634 Lina, G., Etienne, J., Ginevra, C. (2012) Evaluation of propidium monoazide (PMA)
635 treatment directly on membrane filter for the enumeration of viable but non cultivable
636 *Legionella* by qPCR. *J Microbiol Methods* **88**, 319-321.
637 Ta, A. C., Stout, J. E., Yu, V. L., Wagener, M. M. (1995) Comparison of culture methods for
638 monitoring *Legionella* species in hospital potable water systems and recommendations for
639 standardization of such methods. *J Clin Microbiol* **33**, 2118-2123.
640 Touron-Bodilis, A., Pougnard, C., Frenkiel-Lebossé, H., Hallier-Soulier, S. (2011) Usefulness
641 of real-time PCR as a complementary tool to the monitoring of *Legionella* spp. and
642 *Legionella pneumophila* by culture in industrial cooling systems. *J Appl Microbiol* **111**, 499-
643 510.
644 Villari, P., Motti, E., Farullo, C., Torre, I. (1998) Comparison of conventional culture and
645 PCR methods for the detection of *Legionella pneumophila* in water. *Lett Appl Microbiol* **27**,
646 106-110.
647 Wulling, B., Voogt, R., Veenendaal, H., van der Kooij, D. (2002) The fluorescent *in situ*
648 hybridization test in comparison with culture for detection of *Legionella pneumophila* in
649 water samples. In Legionella. Marre, R. et al. (eds). ASM press. Washington, DC, USA. 263-
650 266.
651 Yamamoto, H., Hashimoto, Y., Ezaki, T. (1993) Comparison of detection methods for
652 *Legionella* species in environmental water by colony isolation, fluorescent antibody staining,
653 and polymerase chain reaction. *Microbiol Immunol* **37**, 617-622.

Table 1 Bacterial strains used for specificity control of the fluorescent *in situ* hybridization assay.

Table 2 Repeatability of the MicFU counts measured from 11 replicates from the same cooling tower water sample.

Table 3 Comparison of the number of sample (percentage in parentheses) positive or negative with MicFU-FISH-SPC (MicFU) assay performed for 48 h of incubation time and the standard culture method (CFU) for detecting *Legionella* spp. in 262 cooling tower water samples.

Fig. 1 *Legionella* MicFU concentration measured by solid phase cytometry for 48 h at 37°C (black bars) and 30°C (grey bars) on GVPC agar in water samples collected from 3 different power plants.

Fig. 2 *Legionella* MicFU concentration measured on GVPC agar for cultivation times ranging from 20 h to 52 h at 30°C, and standard plate count measured in the same water sample.

Fig. 3 Relationship between *Legionella* counts measured from naturally contaminated cooling tower water samples by the standard method and the trial method.

Table 1

Bacterial strain	Origin*	With the set of probes	Without probes
Legionella anisa	ATCC 35292	+	-
Legionella birminghamensis	CIP 103871	+	-
Legionella bozemanae sg1	CIP 103872	+	-
Legionella bozemanae sg2	CIP 103873	+	-
Legionella cherii	ATCC 35252	+	-
Legionella cincinnatiensis	CIP 103875	+	-
Legionella dumoffi	ATCC 33279	+	-
Legionella erythra	CIP 103843	+	-
Legionella feeleii sg1	CIP 103877	+	-
Legionella feeleii sg2	CIP 103878	+	-
Legionella hackeliae sg1	CIP 103844	+	-
Legionella hackeliae	CIP 105112	+	-
Legionella jordanis	CIP 105268	+	-
Legionella longbeachae sg1	CIP 103880	+	-
Legionella longbeachae sg2	CIP 103881	+	-
Legionella lansingensis	ATCC 49751	+	-
Legionella gormanii	CIP 104724	+	-
Legionella micdadei	ATCC 33218	+	-
Legionella maceachernii	CIP 103846	+	-
Legionella oakridgensis	CIP 103884	+	-
Legionella parisiensis	CIP 103847T	+	-
L, pneumophila sg 1	ATCC 33152	+	-
L. pneumophila sg 2	ATCC 33154	+	-
L. pneumophila sg 3	CNRL	+	-
L. pneumophila sg 4	ATCC 33156	+	-
L. pneumophila sg 5	ATCC 33216	+	-
L. pneumophila sg 6	CNRL	+	-
L. pneumophila sg 7	CIP 103861	+	-
L. pneumophila sg 8	ATCC 35096	+	-
L. pneumophila sg 9	CIP 103863	+	-
L. pneumophila sg 10	ATCC 42283	+	-
L. pneumophila sg 11	CIP 103865	+	-
L. pneumophila sg 12	CIP 103866	+	-
L. pneumophila sg 13	ATCC 43256	+	-
L. pneumophila sg 14	ATCC 43703	+	-
L. pneumophila sg 15	ATCC32251	+	-
Legionella sainthelensi	CIP 103885	+	-
Legionella tucsonensis	CIP 105113	+	-
Legionella wadsworthii	CIP 103886	+	-
Aeromonas hydrophila	AER 2.4	-	-
Alcaligene faecalis	ALC 1.1	-	-
Bacillus subtilis	BA 5.1	-	-
Burkholderia cepacia	ATCC 24416	-	-
Enterobacter aerogenes	CIP 60.86T	-	-
Escherichia coli	ATCC11775	-	-
Flavobacterium spp	FLA 1.1	-	-
Flavobacterium spp	FLA 2.1	-	-
Klebsiella oxytoca	CIP 103134 T	-	-
Listeria monocytogenes	ATCC 15313	-	-
Mucor bacilliformis	UMIP 2557.01	-	-
Proteus vulgaris	CIP A232	-	-
Pseudomonas aeruginosa	PSE 1.5	-	-
Pseudomonas fluorescens	PSE 5.1	-	-
Pseudomonas fluorescens	PSE 5.3	-	-
Pseudomonas putida	B494	-	-
Serratia marcescens	B513	-	-
Serratia marcescens	SER2.1	-	-
Stenotrophomonas maltophila	STE1.1	-	-
Xanthomonas campestris	CIP 100069T	-	-
Vibrio parahaemolyticus	M3110B	-	-

*ATCC, American Type Culture Collection; CIP, Institut Pasteur Collection; other strains provided by the Microbial Observatory of the Laboratoire ARAGO (MOLA) Strain Collection (Banyuls/mer, France).

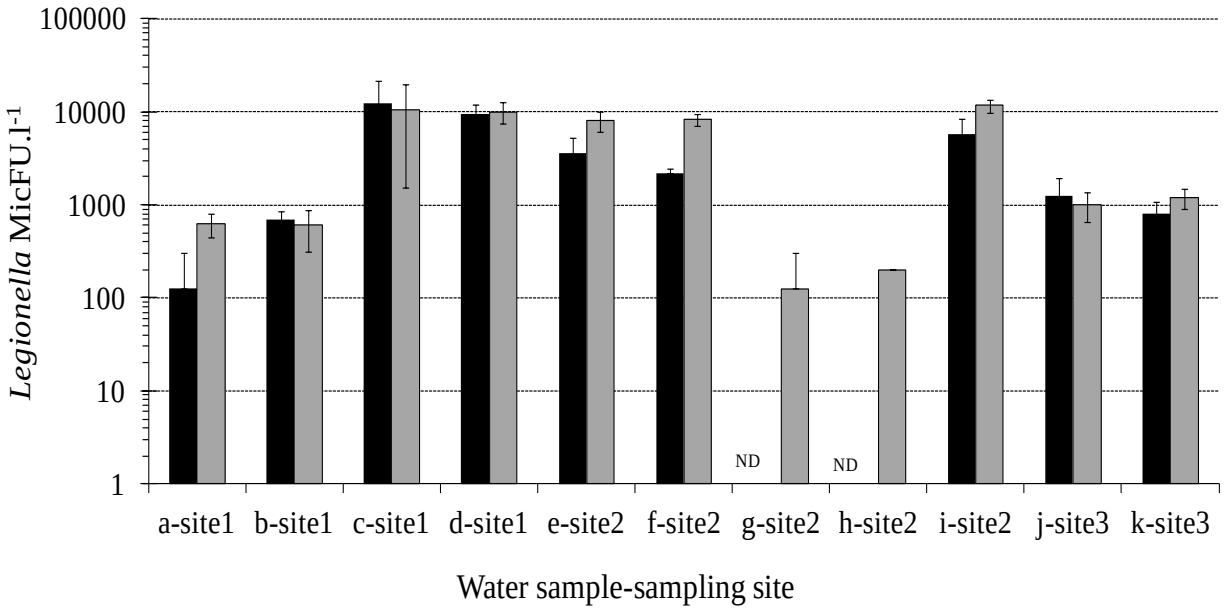

Figure 1

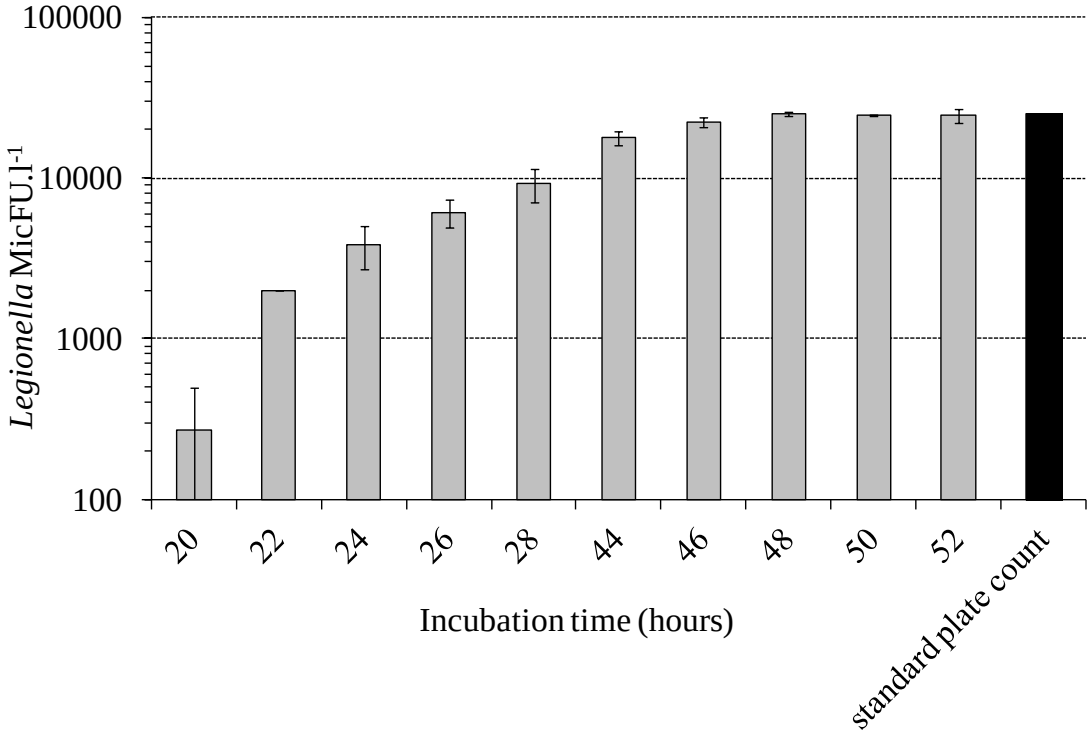

Figure 2

Table 2

Replicat	Numbre of MicFUs detected by SPC from 5ml of water sample	MicFu concentration (l^{-1})
R1	75	15000
R2	138	27600
R3	119	23800
R4	99	19800
R5	126	25200
R6	99	19800
R7	75	15000
R8	72	14400
R9	145	29000
R10	108	21600
R11	128	25600
Mean	107.64	21527.3
Standard deviation	25.98	5195.6
Coefficient of variation	**24.1%**	

Table 3

		MicFU		Total
		+	-	
CFU	+	201 (76.7)	9 (3,4)	210
	-	5 (1,9)	47 (17,9)	52
total		206	56	262

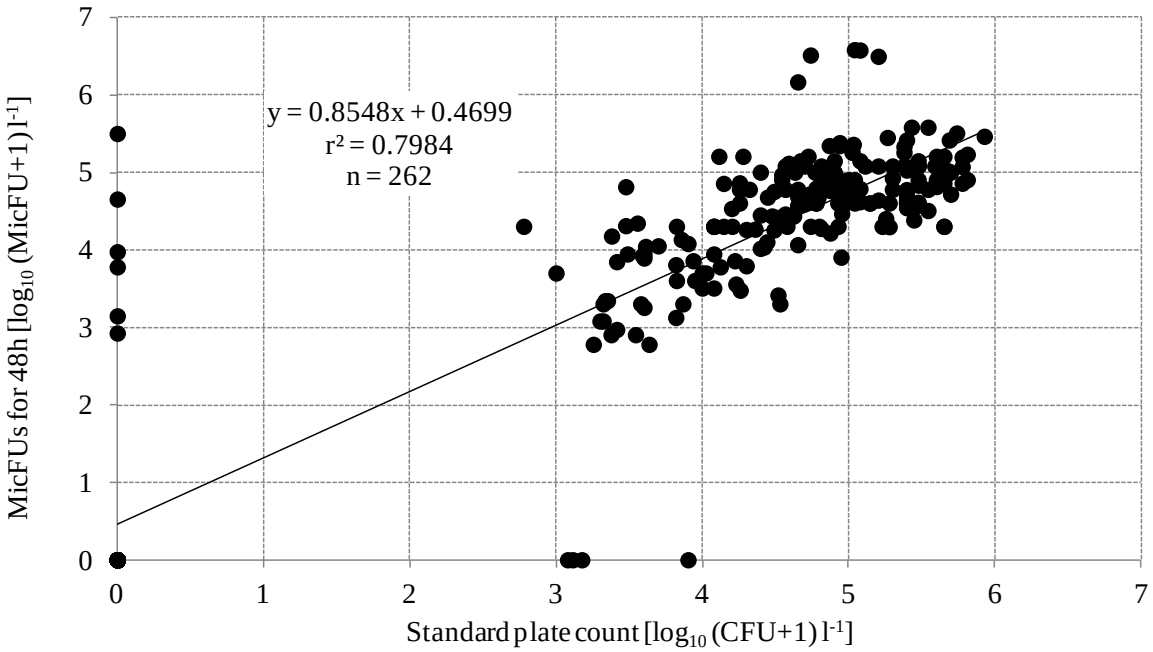
y = 0.8548x + 0.4699
r² = 0.7984
n = 262
MicFUs for 48h [log10 (MicFU+1) l-1]
Standard plate count [log10 (CFU+1) l-1]
0
1
2
3
4
5
6
7

Figure 3

Table S1. *In silico* specificity test of the four oligoprobes (Leg705, Leg226, Legall11, and Legall22) using the probe matching function of the Ribosomal database project software package (Maidak et al., 2001) and the BLAST function on the NCBI website (Altschul et al., 1997).

Leg705	Leg 226	Legall11	Legall22	Number of matching probes
L. dumoffii	*L. dumoffii*	*L. dumoffii*	*L. dumoffii*	4
L wadsworthii	*L wadsworthii*	*L wadsworthii*	*L wadsworthii*	4
L. gormanii	*L. gormanii*	*L. gormanii*	*L. gormanii*	4
L. cherrii	*L. cherrii*		*L. cherrii*	3
L. parisiensis	*L. parisiensis*	*L. parisiensis*	*L. parisiensis*	4
L. anisa	*L. anisa*	*L. anisa*	*L. anisa*	4
L. bozemanae	*L. bozemanae*	*L. bozemanae*	*L. bozemanae*	4
L. cincinnatiensis	*L. cincinnatiensis*	*L. cincinnatiensis*	*L. cincinnatiensis*	4
L. sainthelensi	*L. sainthelensi*	*L. sainthelensi*	*L. sainthelensi*	4
L. gratiana	*L. gratiana*	*L. gratiana*	*L. gratiana*	4
L. longbeaches		*L. longbeaches*		2
L. santicrucis	*L. santicrucis*	*L. santicrucis*		3
L. lytica	*L. lytica*	*L. lytica*	*L. lytica*	4
L. steigerwaltii	*L. steigerwaltii*		*L. steigerwaltii*	3
L. tucsonensis	*L. tucsonensis*	*L. tucsonensis*	*L. tucsonensis*	4
L. lyticum		*L. lyticum*	*L. lyticum*	3
L. shakespearei		*L. shakespearei*	*L. shakespearei*	3
L. moravica		*L. moravica*	*L. moravica*	3
L. worsleiensis		*L. worsleiensis*	*L. worsleiensis*	3
L. quateirensis		*L. quateirensis*	*L. quateirensis*	3
L. pneumophila	*L. pneumophila*	*L. pneumophila*	*L. pneumophila*	4
L gresilensis	*L gresilensis*		*L gresilensis*	3
L. waltersii		*L. waltersii*	*L. waltersii*	3
L. londiniensis	*L. londiniensis*	*L. londiniensis*	*L. londiniensis*	4
L. israelensis			*L. israelensis*	2
L. fairfieldensis	*L. fairfieldensis*	*L. fairfieldensis*	*L. fairfieldensis*	4
L. lansisgensis			*L. lansisgensis*	2
L. birminghamensis		*L. birminghamensis*	*L. birminghamensis*	3
L. quinlivanii		*L. quinlivanii*	*L. quinlivanii*	3
L. geestiana		*L. geestiana*		2
L. adelaidensis		*L. adelaidensis*	*L. adelaidensis*	3
L. oakridgensis			*L. oakridgensis*	2
L. nautarum			*L. nautarum*	2
L. micdadei	*L. micdadei*	*L. micdadei*	*L. micdadei*	4
L. maceacherni	*L. maceacherni*	*L. maceacherni*	*L. maceacherni*	4
L. donaldsonii	*L. donaldsonii*	*L. donaldsonii*	*L. donaldsonii*	4
L. feeleii	*L. feeleii*	*L. feeleii*	*L. feeleii*	4
L. erythra	*L. erythra*	*L. erythra*	*L. erythra*	4
L. spiritensis	*L. spiritensis*			2
L. rubrilucens	*L. rubrilucens*	*L. rubrilucens*	*L. rubrilucens*	4
L. taurisensis	*L. taurisensis*	*L. taurisensis*	*L. taurisensis*	4
L. brunensis	*L. brunensis*			2
L. beliardensis	*L. beliardensis*			2
L. jamestowniensis	*L. jamestowniensis*		*L. jamestowniensis*	3
L. hackeliae	*L. hackeliae*			2
L. jordanis	*L. jordanis*		*L. jordanis*	3
46 species	**31 species**	**33 species**	**39 species**	

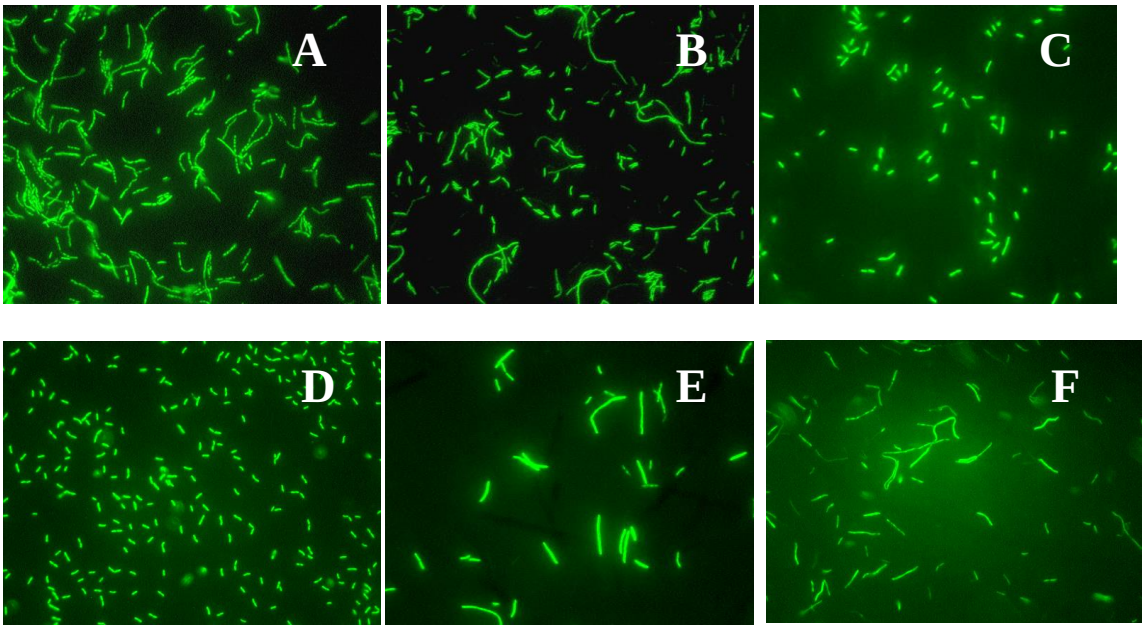

Figure S1. Examples of epifluorescent micrographs of hybridized cultured *Legionella* cells obtained using the fluorescent *in situ* hybridization method. (A) *L. longbeaches* CIP 103881 matching with 2 probes; (B) *L. jordanis* CIP 105268 matching with 3 probes, and strains matching with the set of 4 probes; (C) *L. anisa* ATCC 35292; (D) *L. pneumophila* sg1 ATCC 4370; (E) *L. pneumophila* sg15 ATCC 32251; (F) *L. sainthelensi* CIP 103885.

Method S1

Quantitative performances of the assay performed for 24h of cultivation time

The quantitative performance of the method for enumerating microcolonies of *Legionella* from cooling tower water samples was evaluated on an artificially contaminated water sample with known log-decimal concentrations of *L. pneumophila* sg 1 to define the linearity domain, and the detection limit of the method. The water sample was analyzed prior to artificial contamination, and showed an absence of *Legionella* (equivalent to < 200 *Legionella* MicFU l^{-1}). The quantification was performed on five replicates with a cell density level ranging from 10^2 to 10^7 viable cells added. The microcolonies were enumerated by SPC after 24 h at 30°C. The theoretical counts and the *Legionella* MicFU counts were linearly correlated with the number of *L. pneumophila* cells added as the inoculums in a log-log plot for both cultivation times (n = 20, r = 0.656, P = 0.002; Spearman test). Twenty out 30 water samples (66.7%) were positive using the approach for 24 h. For this shorter incubation time, no microcolony was detected for the theoretical cells added at 380 cells l^{-1}, and MicFUs were detected in 2 replicates of the 5 analyzed for theoretical cells added at 3800 cells l^{-1}. Consequently, we estimated the linearity domain of the trial method to range between 10^4 and 10^7 MicFU l^{-1} for the shorter incubation time (24 h).

Enumerations were performed by SPC after 24 h of incubation at 30°C on GVPC. The concentration of *Legionella* spp. measured by the standard culture method was 2.5 10^4 CFUs per liter. The mean concentrations were 1.3 10^3 and 2.1 10^4 *Legionella* MicFUs per liter after 24 h of cultivation. The repeatability of the trial method was investigated by determining the coefficient of variation (CV) measured from the number of microcolonies detected by SPC from each water sample. The CV was 44.5% for enumeration performed after 24 h of cultivation.

***Legionella* spp. quantification in naturally contaminated cooling tower water samples by MicFU-FISH-SPC after 24h of cultivation and comparison with standard plate counts.**

The microcolonies of *Legionella* were enumerated from all the water samples following application of the physical and chemical treatments described previously, and following cultivation performed for 24 h at 30°C on GVPC agar. About 80% of the water samples were positive using the standard culture method (Table A). *Legionella* CFU concentrations ranged between 5.10^2 and $8.5\ 10^5$ CFU l^{-1}. In contrast, 62.7 % of the water samples were positive using the trial method for *Legionella* enumerated after 24 h. The MicFU concentrations ranged between 6.10^2 and $3.2\ 10^5$ MicFU l^{-1}. The percentage of water samples for which the results of the two methods are in agreement (MicFU+/CFU+ and MicFU-/CFU- pair results) was 81% (n = 176) for cultivation of microcolonies performed for 24 h. If the results were not in agreement, this was mainly due to the MicFU-/CFU+ pair result for 24 h of cultivation.

Table A. Comparison of the number of sample (percentage in parentheses) positive or negative with MicFU-FISH-SPC (MicFU) assay performed for 24 h of incubation time and the standard culture method (CFU) for detecting *Legionella* spp. in 217 cooling tower water samples.

		MicFU		Total
		+	-	
CFU	+	135 (62.2)	40 (18,4)	175
	-	1 (0.5)	41 (18,9)	42
total		136	81	217

In total, 217 water samples were analyzed with both methods. The MicFU counts were linearly correlated in a log-log plot, as reported in Figure B ($P<0.0001$; Pearson test). The counts enumerated after 48 h were significantly higher than those measured for the shorter cultivation time (Wilcoxon signed rank test, $P<0.0001$ for $\alpha=5\%$).

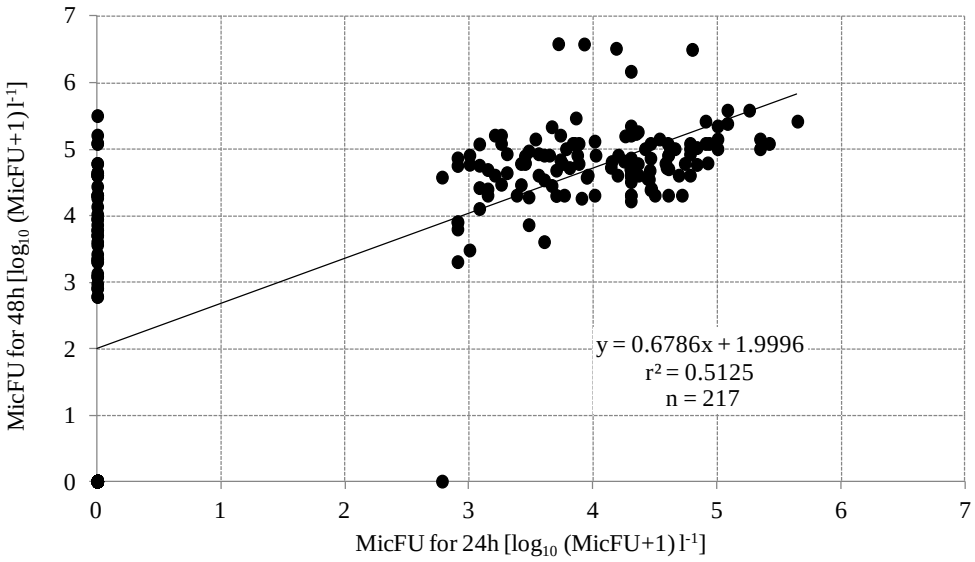

Figure B. Relationship between MicFU counts of *Legionella* measured after 24 h and 48 h on GVPC at 30°C.

The MicFU measured after 24h of cultivation and CFU counts were linearly correlated (Pearson test, $P<0.0001$ for $\alpha=5\%$) (Figure C).

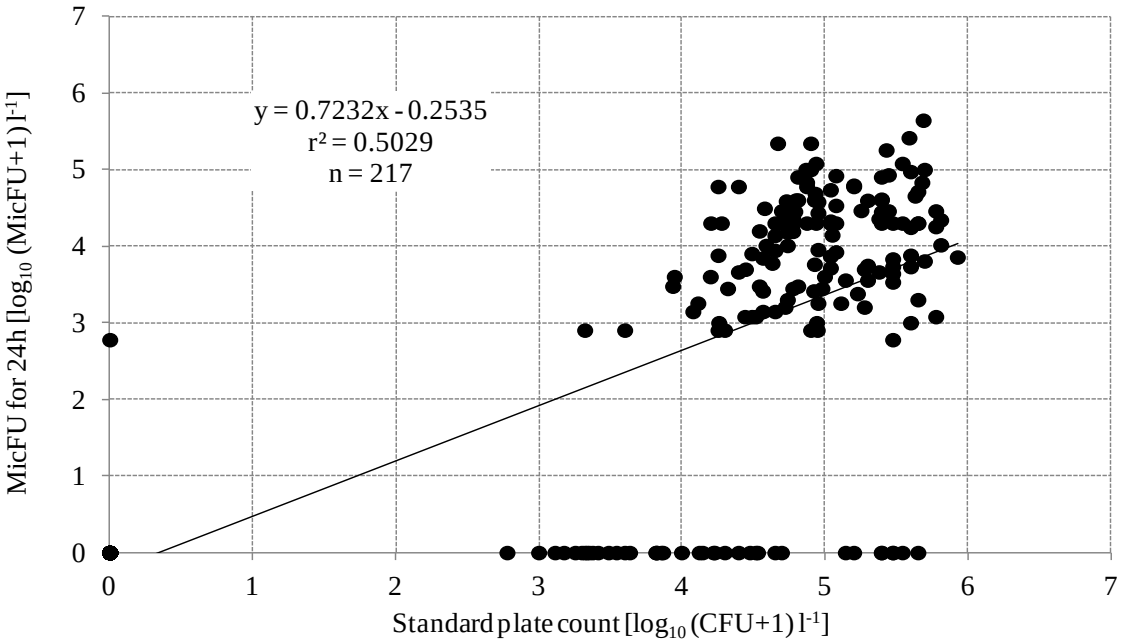

Figure C. Relationship between *Legionella* counts measured from naturally contaminated cooling tower water samples by the standard method and the trial method after: (A) 24 h; and (B) 48 h of cultivation on GVPC at 30°C.