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Risk factors of *Legionella* occurrence in nursing homes hot-water systems

M. Deloge[1*], S. Oberti[2], N. Dhome[1], D. Zmirou-Navier[3], L. Mathieu[4]
[1]*Biotechnologies and Environment Research Team, Faculté Polydisciplinaire de Taza, BP1323, Taza, Morocco.*
[2]*Anjou-Recherche, Chemin de la digue, BP 76, 78603 Maisons-Laffitte cedex, France.*
[3]*DESP, Faculté de Médecine, 9 avenue de la Forêt de Haye, BP 184, 54 505 Vandoeuvre-lès-Nancy, France.*
[4]*EPHE, Faculté de Médecine, 9 avenue de la Forêt de Haye, BP 184, 54 505 Vandoeuvre-lès-Nancy, France.*

Received 10 June 2014; Revised 10 January 2015; Accepted 19 January 2015.
[*]*Corresponding Author: m_deloge@yahoo.fr ; Phone: +212.660.254.892.*

Abstract
Legionella contamination was studied in 34 nursing homes hot-water systems. A critical points analysis was conducted in each home in order to evaluate risk factors for occurrence of *Legionella* bacteria across four components of the water systems: *cold water system feeding the hot-water production*, *hot-water production*, *hot-water distribution system* and *points-of-use*. Risk factors were scaled according to criticity levels (1 to 4) and related to the prevalence and intensity of *Legionella* contamination. Hot-water of the nursing homes was contaminated by culturable *Legionella* (24.6%) and by *Legionella* detected using the *in situ* hybridization (FISH) technique (48.5%). Logistic regressions showed that criticity levels of hot-water system were predictive of hot-water contamination by culturable *Legionella* for two major components: "*hot-water production*" and "*hot-water distribution system*", and to a lesser extent for the "*points-of-use*" component. These results also hold true for the "*points-of-use*" criticity levels according to *Legionella* bacteria detected by FISH. In conclusion, because it shows a strong relationship between *Legionella* prevalence and critical points analysis of the water system, this study provides some tools for Legionella risk management.

Keywords: *Legionella*, hot-water system, nursing homes, critical points analysis, risk factors.

1. Introduction
Outbreaks of Legionnaires'disease in French nursing homes have been reported [1]. The causal agents are *Legionella* bacteria inhaled with aerosols generated by contaminated water systems [2]. Artificial aquatic environments such as hot-water systems are generally thought to be major sources of *Legionella* bacteria.
Several favourable factors for *Legionella* colonization have been described [3], among which water temperature lower than 60°C [4-5], state of the hot-water tanks [5], or usage of certain metals and materials for water pipes [6]. However, it is difficult to eradicate *Legionella* in water systems, because presence of amoebae [7] and biofilms create a protective shield against disinfection processes [8].
Since, the standard culture method [9] systematically underestimate the levels of *Legionella* in hot-water samples [10], new technologies allow to quickly identify pathogenic bacteria: PCR [11] or the technique of *in situ* hyridization (FISH) [12].
Legionellae risk management is not harmonized at an international level and it lacks specific criteria. The level of *Legionella* bacteria concentration justifying corrective actions remains discussed. In Germany and France, action levels are 10^4 CFU/l and 10^3 CFU/l respectively; in the Netherlands, the threshold is set at 10^2 CFU/l while a zero concentration is aimed at in the United States, with no maximum threshold being established [13-16]. But risk management cannot be based solely on routine measurements because culture results are delayed (10 days lag) and very dependent upon the choice of the sampling points.
To overcome this difficulty, some national environment or health authorities [14-15;17] have set reference frameworks for critical points on the water systems with a view to preventing legionellae prevalence.
Within the Legion' Air research project [18], whose objective are to assess risks related to *Legionella* exposure among elderly subjects living in nursing homes in the north of France, we studied the predictive value of an

evaluation grid of the water system quality, as to the degree of *Legionella* contamination in the nursing homes hot-water systems at the showers. This grid, registred mark, has been designed by the Research and Development department Veolia Environment (France) and is based on guidelines from the French ministry of health regarding a series of critical points across water systems.

2. Materials and methods

Samplings of shower water were performed between October 2003 and November 2005 in 34 nursing homes in North-East of France (Fig. 1). The level of *Legionella* contamination in these homes had not been checked prior to this study, not to introduce a selection bias.

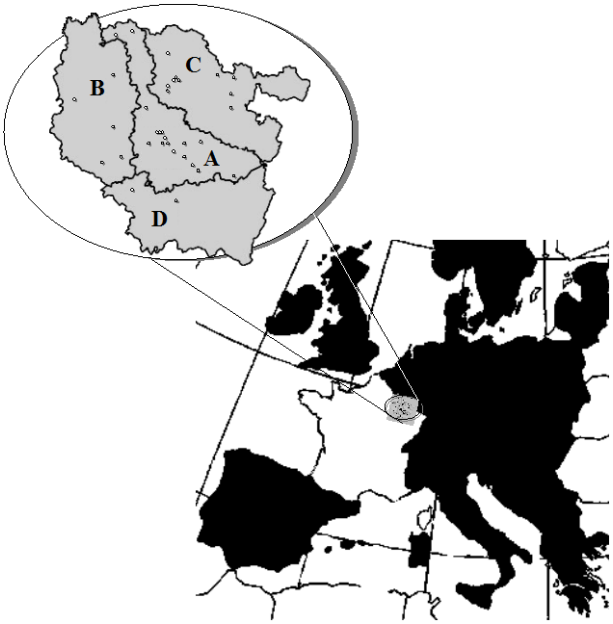

Figure 1: Location of the 34 study nursing homes in Lorraine, an administrative area of France, composed of four departments (A-D)

2.1. Critical points analysis

Information on the hot-water system of each nursing home was collected by a dedicated professional according to a predefined protocol. The points to be evaluated are divided into four components: (i) *the cold water system that feeds the hot-water production*, (ii) *the hot-water production*, (iii) *the hot-water distribution system* and (iv) *the points-of-use*. Within each of these components, critical points of *Legionella* contamination had been determined in accordance with the recommendations of the French Health authotities [14] (Table 1). On the basis of these guidelines, a grid for analysis of these critical points was developed, so as to scale criticity levels (from 1 to 4), following an increasing risk of *Legionella* contamination: level 1 corresponds to a "total control of the *Legionella* risk of contamination"; level 2 refers to "insufficient control of *Legionella* contamination"; level 3 qualifies "a deficient control of the *Legionella* risk of contamination"; and level 4 characterizes a "total absence of *Legionella* contamination risk". This paper does not intend to validate this software (patent rights); but merely, it aims at analyzing the predictive value of the criticity levels compared to the degree of *Legionella* bacteria in the hot water.

2.2 Determination of the water sampling points

This critical control point analysis of the hot-water system was used to select samplings points of showers associated with a risk of contamination by *Legionella*, because all showers could not be sampled, for practical and financial reasons. The identification of these points was based on the configuration of the hot-water distribution network: design (dead ends, balancing, plumbing materials), mode of hot-water production, maintenance of point-of-use, water temperature and treatment. One to eight showers were sampled according to the nursing home (median of 5).

2.3 Water sampling procedure

The methodology and detection techniques used to assess exposure are detailed elsewhere [10]. Sampling of each shower point selected was performed twice, two days apart; only the hot-water faucet was opened to its mawimum flow. For each run, two water samples of 1 liter were collected in sterile bottles after 7 minutes of water flushing and placed at 4-10°C during 24h maximum until analysis. The water temperature was measured throughout the 7 minutes of water flushing.

Table 1: Critical points associated with a risk of contamination by *Legionella* (adapted from [14]).

Water system components	Parameters observed	Critical points
Cold water system feeding the hot-water production		
	Dead ends	Presence
	Heat insulation	Absence
Hot-water production		
	Temperature	<55°C
	Maintenance	no cleaning, no disinfection
	Dead ends	Presence
	Water treatments	Bad adjustment
	General mixing valve	Presence
Hot-water distribution system		
	Plumbing materials	Both metal and plastic
	Balancing of the network	Bad balancing
	Heat insulation	Absence
	Dead ends	Presence
Points-of-use		
	Maintenance	no cleaning, no disinfection
	Dead ends	Presence or very few used (<1/week)
	Temperature	<50°C

2.4 Microbiological analysis

(i) Bacterial counts. Total number of bacteria was determined with the DAPI (4', 6-diamidino-2-phenylindole) staining, which enables detection of whole bacterial population, both alive and dead, irrespective of its physiological state or a specific bacterial target. This detection method was adapted from the protocol described by Saby et al. [19]. Briefly, 20 ml of water were filtered through 0.2 µm pore black polycarbonate membranes and were overlaid with 3 ml of a 3 µg/ml sterile DAPI solution for 15 min. After washing and air drying, the membranes were mounted on a microscope slide with a drip of Citifluor (Biovalley AF87-10) and a cover slip. Slides were examined with an epifluorescence microscope (Olympus, BX41), equipped with filter sets appropriate for DAPI staining detection (Olympus, U-MNU2). Thirty randomly chosen microscopic fields were counted for each sample. The results were expressed in cells per liter of water and the detection limit was 6.7×10^4 bacteria cell/l.

(ii) Fluorescent *in situ* hybridization (FISH). The FISH protocol applied for *Legionella* detection was described elsewhere [10]. It used a mix of three specific oligonucleotide probes, validated to detect *Legionella* bacteria: LEG705, LEG226 and LEGPNE1, labelled with a carbocyanine at the 5' end. 150 ml of water samples were filtered through 0.2 µm pore white polycarbonate membrane. As previously described, the filter was fixed with a 3.7 % formaldehyde solution for 30 min, then washed with phosphate-buffered saline (pH 7.4), air-dried and dehydrated by serial exposure to 50, 80, and 95% ethanol for 3 min each. Then, the hybridization steps were performed during 2 h at 46±1°C in a moisture chamber as follow: 50 µl of hybridization solution (20% formamide, 900mM NaCl, 0.1% sodium dodecyl sulfate, 20mM Tris-HCl [pH 7.2]), containing 50 ng of each labelled probe, were applied to the membrane. The membrane was washed twice with preheated wash solution at pH 7.2 (20 mM Tris HCl, 215 mM NaCl, 0.1% sodium dodecyl sulfate) for 30 min at 46°C±1°C, and with preheated distilled water at 46°C and air dried. Fluorescence microscopy conditions were the same as described above with filter sets appropriate for carbocyanine (Olympus, U-MWG2) detection. Slides were counterstained with DAPI as previously detailed. The results were expressed in *Legionella* cells/l of water and the detection limit was 9.0×10^3 cells/l. The hybridization procedure quality was checked systematically by using species known to hybridize (or not) with the *Legionella* probes used.

(iii) Culture method. The detection of the culturable *Legionella* was adapted from the French standard method [9], using the selective nutrient agar BCYE (Buffer Charcoal Yeast Extract) (Oxoïd, France).Characteristic *Legionella* spp. colonies were enumerated after 7 days' incubation at 37±1°C. They were tested fot their cysteine dependence (CD) by inoculation onto BCYE agar with cysteine (0.4g/l) and without cysteine, incubated at at 37°C ±1°C. Colonies that grew on both media were considered CD negative and were reported as non-Legionella bacteria. Colonies that grew only on BCYE with cysteine (CD positive colonies) were identified by latex agglutination (kit Oxoïd, DR800M) allowing thereafter the identification between *L. pneumophila* and *L. species* as previously described [20]. The results were expressed in CFU/l of water.

2.5 Statistical analysis

The objective was to assess the association between the criticity levels of the hot-water networks and *Legionella* contamination of the hot-water-showers. To qualify as "contaminated" a water shower, the positivity threshold of *Legionella* was set at 10^2 CFU/l for the culture method (i.e. its detection limit), and at 10^4 cells/l for the *in situ*

hybridization method. As the distribution of the variables was not Gaussian, nonparametric tests were used (Spearman correlation coefficient, Mann-Whitney rank sum test, Kruskall-Wallis test; StatView 5.0, SAS Institute Inc., Cary, NC), in order to study the association between *Legionella* presence and the criticity of the water network
Bivariate Logistic regression (Stata 7, Stata Corporation – USA) was also used to quantify the relationship between presence-absence of *Legionella* in the shower hot-waters according to increasing levels of concentrations; the reference category being below the aforementioned concentration for the culturable and FISH detection techniques.
The independent variables were the points-of-use hot-water temperature and the criticity levels of the four components of the hot-water systems (cold water system, hot-water production, hot-water distribution system and points-of-use).

3. Results

3.1. General characteristics of the nursing homes water systems

Table 2 exhibits the main characteristics of the study hot-water systems. The nursing homes were equipped with metallic material pipes (88.2%), mainly with copper (70.6%). Hot-water tanks were found in 86.3% of the production systems; hot-water temperature at the outlet of production was lower than 60°C in half the homes (median temperature: 55.0°C; interquartile range: 9.0°C). The dead ends, located either in the production chain, or in the water distribution system, or at points-of-use, were observed in 64.7% of the nursing homes. Lastly, hot-water temperature at the points-of-use ranged from 38°C to 60°C (median: 50.2°C; interquartile range: 7.4°C).

Table 2: Main characteristics of the water systems in the 34 study nursing homes

Characteristics	Value (%)
Plumbing materials of hot-water distribution systems	
Metal	30 (88.2)
Plastic	1 (2.9)
Both metal and plastic	3 (8.8)
Heater type	
Hot-water tank	10 (29.4)
Instantaneous heater	5 (14.7)
Semi-instantaneous (=instantaneous + tank)	19 (55.9)
Heater hot-water temperature	
<60°C	16 (47)
≥60°C	9 (26.5)
Unknown data	9 (26.5)
Dead ends	
Absent	12 (35.3)
Present	22 (64.7)
Water treatment	
Chlorination	6 (17.6)
Softener	19 (55.9)
Anti-corrosion	15 (44.1)
Point-of-use hot-water temperature	
<50°C	17 (50)
≥50°C	17 (50)

3.2 Water systems criticity

The grid of the critical points diagnosis (Table 1) allowed to characterize the criticity of four components of the water systems (Table 3). No home exhibited a total control of the *Legionella* bacteria risk across all components of the water system. Moreover 76.4% had a water system with a *Legionella* risk presence (level >=2) for at least a component.
Regarding the «*hot-water production*» component, the critical points that were most frequently met relate to water treatment and temperature at the production system exit, which was <60°C among 47% of the sites. Regarding water treatment, critical points observed were absence of checking of the consumed products quantities (salt quantity in the softener, chlorine), or an absence of cleaning of the various elements (valves,

tanks, heater) composing the water treatment(s). As to the «*hot-water distribution system*» component, presence of dead end (65% of sites) and absence of an integral heat insulation of the hot-water system explained cases of "insufficient control" of the *Legionella* risk (level 2) or of "deficient control" (level 3). For the «*points-of-use*» component, critical points were : (1) presence of dead ends and absence of maintenance and disinfection, a situation prone to deposits and tartar, and also (2) hot-water temperature below 50°C in 50% of nursing homes, contrary to French regulations. Total absence of *Legionella* control risk was identified only for this component of the system, and in only one site.

Table 3: Criticity levels water systems in the nursing homes across the four components (n=34)

	Cold water system	**Hot-water production**	**Hot-water distribution system**	**Points-of-use**
Criticality levels		**no (%)**		
1	16 (47.0)	7 (20.6)	4 (11.8)	5 (14.7)
2	17 (50.0)	18 (52.9)	25 (73.5)	11 (32.3)
3	1 (3.0)	9 (26.5)	5 (14.7)	17 (50.0)
4	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.0)

3.3 Legionella contamination of hot-water systems

From October 2003 through November 2005, 338 hot-water samples were collected from 169 showers used by the patients (Table 4). Among these, 83 water samples (24.6%), in 15 nursing homes (44.1%) were contaminated by at least one culturable *Legionella* (CFU) per liter. On average, 78.3% of culturable *Legionella* bacteria present in hot-water showers were *L. pneumophila*. Concentrations of culturable *Legionella* $\geq 10^3$ CFU/l and 10^4 CFU/l were reached for 59% and 30.1% of the positive samples, respectively. The FISH technique allowed to detect *Legionella* in twice more (48.5%) hot-water samples, with a maximum concentration of 7.3×10^6 *Legionella spp.*/l.

Table 4: Microbiological characteristics of water systems in the study nursing homes

	Positive samples number (%)	Geometric mean[a]	Median[a]	Minimum concentration[a]	Maximum concentration[a]
***Legionella spp.* (CFU/l)**	83/338 (24.6)	3.91×10^3	1.40×10^3	2.00×10^2	2.30×10^6
$\geq 10^3$	49 (14.5)				
$\geq 10^4$	25 (7.4)				
***L. pneumophila* (CFU/l)**	65/338 (19.2)	2.07×10^3	1.40×10^3	1.00×10^2	2.47×10^5
$\geq 10^3$	36 (10.6)				
$\geq 10^4$	17 (5.0)				
Hybridized *Legionella* (Cells/l)	164/338 (48.5)	1.05×10^5	9.02×10^4	9.02×10^3	7.28×10^6
$\geq 10^4$	149 (44.1)				
$\geq 10^5$	75 (22.2)				
Total Bacteria (Cells/l)	338	5.65×10^7	7.17×10^7	4.94×10^5	1.19×10^9

[a]Only positive samples were included: CFU$\geq 1.0 \times 10^2$ CFU/l; Hybridized *Legionella* $\geq 9.0 \times 10^3$ cells/l.

About half of the samples presented hybridized *Legionella* concentrations equal or greater than 10^5/l. This technique could detect more *Legionella* in hot-water-shower than the culture method (Table 4) ($p<10^{-4}$).
Legionella represented only a small proportion of the total bacterial population in hot-water systems: hybridized *Legionella* population (FISH/DAPI) amounted to 0.23%, the culturable fraction (CFU/DAPI) only 0.02%, and the *Legionella* culturable fraction among the hybridized *Legionella* population (CFU/FISH) represented 8.3%.
Presence of culturable *Legionella* in the hot-water-showers of nursing homes decreased over the study-time ($p<10^{-4}$): culturable *Legionella* were detected among 54.8% of the water samples in 2003, 26.1% in 2004 and

0.8% in 2005. The proportion of *Legionella*-positive nursing homes was divided by seven in three years from 75% to 11%.

3.4 Factors associated with the hot-water system Legionella contamination

Presence of *Legionella* (by culture or by FISH) in the hot-water-shower was studied according to the criticity level of water systems components (Table 5). Characteristics of *cold water system* were not related to presence /absence of *Legionella*. Significant associations were found between culturable *Legionella* bacteria and criticity levels of components «*hot-water production*» ($p<10^{-2}$), «*hot-water distribution system*» ($p<10^{-4}$) and «*points-of-use*» (p=0.01). These relations exhibited a trend : increasing prevalence of *Legionella* was found with higher criticity levels (i.e. greater number of observed risk factors).

Similarly, a trend was found with presence of *Legionella* measured by FISH for criticity levels of the «*hot-water distribution system*» (p=0.04) and «*points-of-use*» (p=0.05) components. *Legionella* presence is inversely correlated with the hot-water temperature for both detection techniques (p=0.05).

Table 5: Association between criticity levels and prevalence of *Legionella* bacteria in shower water, as measured by culture or FISH, according to water system components (p values*)

Water system components	Criticity levels	Culturable *Legionella*	Hybridized-*Legionella*
Cold water system feeding hot-water production	from 1 to 3	0.58	0.42
Hot-water production	from 1 to 3	$<10^{-2}$	0.62
Hot-water distribution system	from 1 to 3	$<10^{-4}$	0.04
Points-of-use	from 1 to 4	0.01	0.05
Point-of-use water temperature	Measure (°C)	0.05	0.05

*Kruskall-Wallis test

Quantitative relationships between criticity levels and prevalence of *Legionella* were assessed by logistic regression models. Only risk factors statistically related to presence of *Legionella* after the previous step (p<0.10) were introduced into the multivariate model (Table 6).

Table 6: Quantitative association between criticity levels of water systems and *Legionella* prevalence

Water system components	Criticity levels	O.R. (95% I.C.)	
		culturable *Legionella*	Hybridized *Legionella*
Hot-water production	2	5.72 (0.60 – 53.99) *	ND
	3	26.00 (1.34 – 502.99) **	ND
Hot-water distribution system	2	6.86 (0.37 – 127.20)	2.00 (0.36 – 11.20)
	3	224.00 (3.91 – 12845.76) **	18.00 (0.57 – 571.02)
Points-of-use	2	2.64 (0.25 – 27.97)	6.23 (0.57 – 67.77)
	≥3	8.47 (0.82 – 87.44) *	10.80 (0.99 – 116.68) **

* p<0.10; ** p≤0.01; ND=Non Determinated

High criticity levels (≥3) were associated with a high risk to detect culturable *Legionella* in hot-water, for the «*hot-water production*», the «*hot-water distribution system*» and, to a lesser extent, the «*point-of-use*» components (Table 6). Moreover, greater hot-water temperatures were inversely related to occurrence of culturable *Legionella* (O.R. =0.84 [I.C.0.74 – 0.95], p<0.01). These results were not found using the FISH technique, except for component «*point-of-use*».

4. Discussion

Contamination of hot-water systems *with Legionella* was described in typical nursing homes in North-East of France. Key characteristics of the water systems, termed as 'critical points' were shown strongly associated with prevalence of *Legionella* in hot-water samples.

4.1 Legionella occurrence in the hot-water systems

Culturable *Legionella* were detected in one out of four hot-water samples. This result is in agreement with studies conducted in collective facilities in Italy (private residences: 22.6%), in Finland (apartments: 31%) and in the United States (hospitals: 34%) [21-23]. However, this hot-water systems contamination has decreased since 2003 and appears lower than that observed from 1999 to 2001 by [24] in the same area in health facilities (hospitals and nursing homes altogether), where 61% of the water-showers were shown contaminated. Because inclusion of nursing homes in the present study was unrelated to prior knowledge of water contamination, the 2002 French regulations relating to *Legionella* in health facilities [14] is likely to be the main explanation of this improvement.
Concentrations of culturable *Legionella* reached a maximum of 2.3 x 10^6 CFU/l, much higher than the few published studies in collective water systems where a maximum of 10^5 CFU/l had been observed [21-22]. The high *L. pneumophila* prevalence, *i.e.* in 78% of positive samples, is corroborated by other studies where prevalence of 69 and 100% had been found [21-22;25]. These results underline the health risk incurred by elderly populations residing in nursing homes, since *L. pneumophila* is the species responsible for most of the legionellosis cases in the world [26].
Culture underestimates the true presence of *Legionella* [10] and usage of FISH seems an adequate way to overcome this caveat since it is a faster technique, more sensitive and capable to detect non culturable *Legionella*. In our study, culturable *Legionella* represented only 8% of the FISH *Legionella* in the water systems. FISH detection is not a bacterial viability technique (appreciated by cellular multiplication, but it is recognized as a physiological marker of bacteria activity [27].

4.2 Predictive value of the critical points analysis
For the 34 nursing homes studied, the water systems were analyzed for their *Legionella* contamination risk from cold water system feeding the production up to the points-of-use. Borella et al. [21] did a similar analysis in hotels hot-water system with observation of critical points characteristics and of chemical parameters. They concluded that the risk of hot-water *Legionella* contamination increased with age of hotels and decreased with dissolved oxygen contents greater than 3.0 mg l^{-1} and total hardness greater than 20°F, but did not consider presence of dead ends, the state of the pipes nor points-of-use. The present study is an effort to appraise the hot-water system as a whole.
High criticity levels ($\geq$3) for «*hot-water production*» component were associated with an increased risk to detect culturable *Legionella* in hot-water. Among the risk factors of «*hot-water production*» (Table 1), two main critical points had already been described in the literature: temperature and hot-water tanks.
Hot-water temperature was in 47% of the production system lower than 60°C, which favours *Legionella* colonization [4]. Our data clearly showed that a high hot-water temperature at the point-of-use restricted *Legionella* colonization with concentrations below the detection limit of the methods. This result backs the current French regulations, whereby a points-of-use temperature between 50°C and 55°C is recommended [14].
A hot-water tank was present in 29 nursing homes. Other authors tied *Legionella* presence with age, lack of maintenance and presence of sediments in bottom of these tanks [5].
This study also showed that critical points found in the «*hot-water distribution system*» would make it vulnerable to *Legionella* contamination. Presence of dead ends (found in 64% of the study nursing homes) yields water stagnation and is known to favour *Legionella* presence. Pipes corrosion was not directly taken into account by the critical point analysis grid; now it could also enhance discharge of ions, such as iron, a described risk factor for *Legionella* contamination [6]. Presence of tartar and humidity-related organisms (fungi) at «*points-of-use*» have been related with presence of *Legionella* [5].
The critical points grid used in this work is the result of long lasting experience of heating professionals in association with biologists and hygienists, and stem from confrontation of engineers' observations and bacterial analyses of hot water. Today, usage of the FISH technique enhances speed and sensitivity. Hence, we thought interesting to compare characteristics of *Legionella* risk prediction using the two detection techniques. A greater relationship was found between *Legionell*a contamination and criticity levels for culturable *Legionella* that for hybridized *Legionella* (FISH) population. The critical points grid used to assess criticity levels might explain this result because it assigns strong coefficients to key parameters of *Legionella* survival, like water temperature which directly influences *Legionella* culturability. Further analyses that would modify the coefficients of such parameters, would allow to check the validity of this assumption. In the meanwhile, the grid does what it was made for, that is predicting the risk of culturable *Legionella* presence.

Conclusion

1. This study demonstrated that nursing homes are an environment where residents incur exposure to *Legionella* contamination of the water systems is frequent and may exhibit high concentrations, with presence of *L. pneumophila* species.
2. Under our study setting, the main risk factors for *Legionella* contamination are critical points found at the points-of-use, but more so in the hot-water distribution system.
3. The critical point analysis grid used in the work, along the other grids based on the same principles, is relevant for its object which is to predict the risk of culturable *Legionella* presence and it prove to be good tools for *Legionella* risk management.
4. This work also underlines that risk control of *Legionella* is multiparametric and should comprehend these critical points in the whole hot-water system.

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