Biomedicine & Prevention
An Open Access Transdisciplinary Journal

Legionella Detection in Hot Water Distribution Systems of Closed Community and Tourist Accommodation Facilities in the Lazio Region, Italy: Risk Assessment and Prevention

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Introduction

Some bacteria belonging to the genus *Legionella*, mostly *Legionella pneumophila*, are recognised as emerging water-borne pathogens in developed countries, able to cause two different human infections: Legionnaires' disease (LD), a community- or hospital-acquired, severe and potentially fatal atypical pneumonia,[1] and Pontiac Fever (PF), a febrile and generally benign non-pneumonic disease.[2] The underlying mechanisms responsible for either PF or LD have not yet been elucidated.[3,4]

What is known, however, is that chronic lung disease, diabetes and various conditions associated with immunodeficiency and also increasing age, male sex and smoking are all important risk factors for LD.[5,6]

All infections are acquired through inhalation of aerosols or aspiration of water containing *Legionella*,[7] and there is no evidence of person-to-person transmission: only one case has been described recently, although the scientific community is waiting for further evidence to confirm this.[8,9]

Legionella bacteria grow naturally in freshwater environments, but find optimal growth conditions in engineered water system, such as cooling towers, plumbing systems, water tanks, decorative fountains, whirlpool spas, mist machines, dental-unit water, hospital equipment and showerheads.[7,10-13] In these environments, many factors, such as aged plumbing, low flow rate or stagnation of the water, dead legs, surface materials and roughness, but also the chemical constituents of the water, warm temperatures and poor management, can promote the proliferation of *Legionella*. These conditions have been found to be conducive to the formation of biofilms, which often leads to the establishment and maintenance of chronic water system colonisation by *Legionella*.[14,15,16] Furthermore, the capacity of these bacteria to live in free-living protozoa such as amoebae, their natural host, can protect them from adverse conditions or biocide treatments.[17-22]

Several decontamination and/or disinfection techniques are available to control the risk of *Legionella* infections.

Decontamination treatments include:

- heat shock (maintaining temperatures 70°C – 80°C for three full days within the water system);[23]
- hyperchlorination (injecting chlorine into the plant to reach free residual chlorine concentrations of 20-50 mg/L throughout the whole water system).[23]

Non-continuous disinfection treatments include:

- non-continuous heat treatment (maintenance of a temperature of 55°C – 60°C[23]);
- chlorine dioxide, that has been shown to be effective if concentrations are > 0.5mg/L;[24-28]
- UV light;[29,30]
- Copper-silver ionisation, that has been shown to be effective if used in combination with free chlorine or chlorine dioxide;[31-34]
- Hydrogen peroxide and silver. Evidence of the efficacy of this treatment is taken mostly from in-vitro studies;[35,36]
- Monochloramine;[37-40]
- Ozone;[41]
- Peracetic acid.[41-46]

However, given the complexity of the environments in water systems, no 'gold standard' exists, and all the existing techniques have advantages and disadvantages.

Although *L. pneumophila* infections are still generally underestimated, notified cases have increased over the years and the most frequent source of infection is contaminated water from distribution systems.

There are numerous studies that have shown how the water supplies in hospitals have been directly linked to the occurrence of hospital-acquired legionellosis.[47,48,49,50]

There is also evidence of the widespread presence of *Legionella* in hot water distribution systems in hotels, schools, sport facilities, offices and private residences,[17,51-55] responsible for sporadic and community-acquired cases of LD.[6] The mortality rate for cases is much higher in health facilities than in the community, and these data are not surprising, as people affected by healthcare-associated legionellosis are probably more likely to suffer from underlying conditions.[56,57]

The number of LD cases in the United States reported by the Centers for Disease Control and Prevention (CDC) has been on the rise since 2000. Although a total of 6,000 cases of LD were reported in 2015, the true incidence may still be underestimated.[58]

The most recent ECDC 'Annual Epidemiological Report on Legionnaires' disease', based on data for 2016 retrieved from the European Surveillance System, reported 7,069 cases of LD, of which 6,560 (92.8%) were classified as confirmed. As in 2015, the number of notifications per 100,000 inhabitants was 1.4, which remains the highest number recorded. Of 5,404 cases with known outcomes, 441 were reported to have been fatal, with a fatality rate of 8.2%. *L. pneumophila serogroup 1* was the most commonly identified pathogen. Of all environmental sites testing positive, 411 were water systems, 22 cooling towers and 13 pools.[59]

Four countries (France, Germany, Italy, and Spain) accounted for 69% of all notified cases, and Italy was the country with the largest number.[59]

In fact, in the last ten years, the annual number of cases of legionellosis, primarily LD notified to the Italian National Surveillance System rose from 192 to 2,014, with an incidence of 33.2/1,000,000 cases/people-year.[57,60] In 2017, 78.5% of the LD cases reported were community-acquired, whereas 11.9% were travel-associated, 6.2% were nosocomial and 3.0% involved persons living in nursing homes, rehabilitation facilities or retirement homes. The case-fatality rate was 10.1% for community-acquired cases and 51.1% for hospital-acquired cases.[57]

In 2000, the Italian Institute of Health (ISS) produced the first guidelines on the control and prevention of legionellosis,[61] followed, in 2005, by instructions for tourist accommodation and spas.[62] In May 2015, a new document was approved with the aim of gathering and updating all the instructions reported in the previous national guidelines and regulations and integrating them in a single text.[23] The instructions recommend that factors critical for *Legionella* growth and diffusion must be taken into account during the design and maintenance of water systems. Although it cannot be guaranteed that the bacteria will be completely eradicated, such measures reduce possible contamination.

The aim of this study was to evaluate the frequency of colonisation by *Legionella* in some hot water systems of different facilities (recreational facilities, retirement homes and group homes) in order to assess possible risks of *Legionella* infection. The portion of the population using or living in these facilities could potentially be exposed to the risk of infection.

In recent years, the number of people attending sport facilities has greatly increased, with gymnastics courses being organised increasingly often for the elderly and for people undergoing rehabilitative treatments. The individual factors and concomitant diseases affecting these populations can represent a risk for developing legionellosis through the use of contaminated water, especially through showers.[53,63-65]

Where these were available, we also reported the results of the treatments carried out in some of the structures that tested positive for *Legionella* to eliminate or contain this contamination.

Finally, we investigated the relationship between the presence of *Legionella* and heterotrophic plate count (HPC at 22°C and 37°C), parameters indicating general water quality within distribution systems,[9,66] to analyse the usefulness of these parameters in predicting the risk of *Legionella* contamination in hot water systems.

Materials and methods

Collection of samples

To recruit the structures, we were interested in for the survey, an information campaign under undertaken on the problems related to the presence of *Legionella* in water systems, and free checks on the terminal points of some water systems were offered. The samples were collected from the water systems of those structures whose administrators signed up to the initiative.

From May 2014 to June 2017, water samples were collected from hot water distribution systems of 36 recreational centres, 28 retirement homes and nine group homes located in the area around Rome (Lazio, Italy). All these buildings were supplied from the public network using groundwater. The water supplied by the municipality through the water network is drinkable, and this water is used by the facilities for all normal domestic uses. None of the facilities had a well connected to the domestic water system, and water gathered from wells was not therefore mixed with drinkable water and was used only for purposes other than domestic use (i.e. irrigation). All water samples were collected from showerheads.

Sampling method

All samples were taken without previously running the water and without flaming the outlet point, in accordance with the Italian Guidelines for water sampling in common use conditions, namely 'instantaneous sampling', to simulate theoretical user exposure.[23] The water temperature was also measured during sampling, and although all samples were collected by turning on the hot water, some of the samples did not exceed 30°C. *Legionella* standard sampling procedures for water were those provided in ISO 11731:1998 (Water quality – detection and enumeration of *Legionella*) and in the Italian guidelines for the prevention and control of legionellosis.[23]

The samples were collected in 1L sterile polyethylene bottles with 10% sodium thiosulphate to neutralise the chlorine (able to neutralise up to 5 mg/L of residual free and combined chlorine).[67,68]

The samples were transported to the laboratory in suitable containers, at room temperature and protected from direct light, for microbiological analysis.

Microbiological analysis

Legionella isolation was performed in accordance with ISO 11731:1998[69] and the Italian guidelines with minor modifications,[23] as described in our previous work.[65,70] The water sample was filtered through a 0.20 µm pore-sized cellulose nitrate filter (Sartorius). The filter was resuspended in 5 mL of the original water sample and shaken using a vortex for 2 minutes to detach the bacteria. In order to reduce contamination by interfering microorganisms, the sample was held at 50°C for 30 minutes. An aliquot of 0.5 mL was then applied to plates of *Legionella* CYE agar base (Oxoid) with the addition of BCYE growth supplement and GVPC selective supplement (Oxoid). The inoculated plates were incubated at 37±1°C in 2.5% CO_2 for ten days and read every day.

Suspected colonies were counted from each sampling plate. We then selected at least five suspected colonies, where available, for each plate,[23] and we subsequently confirmed *Legionella* positivity by their inability to grow on CYE agar base without BCYE growth supplement. Finally, we evaluated each suspected *Legionella* colony by final agglutination using a *Legionella* Latex Test Kit (Oxoid).[71]

The test allows a separate identification of *L. pneumophila* serogroup 1 and *L. pneumophila* serogroup 2-14, and detection of seven *Legionella* (polyvalent) species, which have been implicated in human disease: *L. longbeachae*, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, and *L. anisa*. Positive and negative control for *Legionella*, performed each time, was applied.

All positive colonies and a subset of negative colonies were also confirmed by PCR (see paragraph 2.4).

The results were expressed in Colony Forming Units per litre (CFU/L), and the detection limit, based on the concentration factor and the volume of the inoculum, was 10 CFU/L. The accuracy of the method was checked each month using internal titered controls.

An aliquot of each water sample was also taken to determine the load of the Heterotrophic Plate Count (HPC) at 22°C and 37°C. These bacteria were determined in duplicate using the pour plate method, with standard Plate Count Agar (Oxoid). The results are expressed in CFU/mL.[67,72]

PCR testing

All colonies that tested positive in the *Legionella* latex test and colonies showing morphological characteristics similar to those of *Legionella*, growing only on selective medium, but negative in the agglutination test, were also confirmed using polymerase chain reaction (PCR) assay, according to the Van der Zee et al. protocol.[73] The primer set used, LEG1 (50TACCTACCCTTG-ACATACAGTG-30) and LEG2 (50-CTTCCTCCGGTTTGT-CAC-30), was derived from the 16S rRNA gene sequence and used to amplify a 200 bp DNA fragment specific for all *Legionella* species. The PCR reaction mixture, 25 µL final volume, contained 10 pmol of each primer, LEG1 and LEG 2, 200 µM of each dNTP, 3 mM $MgCl_2$, and 2 U AmpliTaq Gold polymerase in 1 × PCR buffer (Promega). Samples were preheated for 10 minutes at 95 WC, followed by 40 cycles of 30 seconds at 94 WC, 30 seconds at 60 WC, and 30 seconds at 72 WC, with a final extension of 5 minutes at 72 WC. A negative and positive control was included in each PCR run. Amplified DNA was detected using agarose gel electrophoresis and ethidium bromide staining.

All colonies negative in the agglutination test were also negative for PCR.

Statistical analysis

All statistical tests were 2-sided, with statistical significance set at 0.05. Continuous variables were summarised using descriptive statistics and expressed as average and standard deviation (SD), as appropriate. Comparison between two means was undertaken using Student's t-test. Categorical data were expressed in percentages or summarised in contingency tables.

When necessary, continuous variables were categorised as follows:

- *Legionella* load was categorised for descriptive analyses in three group, based on the implications for the advices to be given on the decontamination procedures:[23] *Legionella* $<10^2$ CFU/L; *Legionella* $\geq 10^3$ CFU/L but $< 10^4$ CFU/L; *Legionella* $\geq 10^4$ CFU/L. Given the paucity of data, only two *Legionella* load categories were considered for statistical analyses and calculation of Odds Ratios (OR) and 95% confidence intervals (CI), when assessing decontamination treatments' effectiveness: *Legionella* $<10^2$ CFU/L; *Legionella* $\geq 10^3$ CFU/L;
- both HPCs at 22°C and at 37°C were categorised in five groups (HPC ≤ 10 CFU/mL; 10 CFU/mL < HPC ≤ 100 CFU/mL; 100 CFU/mL < HPC ≤ 300 CFU/mL; 300 CFU/mL < HPC ≤ 500 CFU/mL; HPC > 500 CFU/mL), consistently with our previous works,[65,70] in order to verify our hypotheses about a possible correlation between certain HPCs values and the presence/absence of *Legionella*. These HPCs groups were used both for descriptive analyses and for the construction of contingency tables.

The qualitative analysis of categorical data was performed by constructing contingency tables and applying Fisher's exact test or Chi-square test, where appropriate.

Wilcoxon-Mann-Whitney tests were performed in order to compare concentrations in two populations: precisely, this statistical test was used to compare the following pairs of variables: HPCs at 22°C and at 37°C; *L. pneumophila* sg 1 and *L. pneumophila* sg 2-14; *L. pneumophila* sg 1 and *Legionella* spp.; *L. pneumophila* sg 2-14 and *Legionella* spp.

Statistical analysis was performed using STATA version 13.[74]

Results

Legionella prevalence

A total of 370 water samples were collected, from 370 sampling points (showerheads), from hot water distribution systems in 73 buildings, during 93 sampling operations performed from May 2014 to June 2017. The collected samples are divided as follows:

- 216 samples from 36 recreational and tourist accommodation facilities. Specifically, 17 were sports centres, eight campsites, five hotels, three bathing establishments, two holiday farms and one amusement park.
- 154 samples from 37 social-assistance structures. Specifically, 28 were retirement homes and nine group homes.

On average, 3.98 samples (SD=2.75) were collected during each sampling procedure, based on the number of showers provided with hot water in the facilities.

Legionella was found in 97 samples (26.2%) collected from 21 (28.8%) of the structures inspected. Specifically:

- 39 samples were found to be positive for *L. pneumophila* sg 1 alone;
- 38 samples were found to be positive for *L. pneumophila* sg 2-14 alone;
- 13 samples were found to be positive for *Legionella* spp alone;
- five samples were found to be positive for both *L. pneumophila* sg 1 and sg 2-14;
- one sample was found to be positive for both *L. pneumophila* sg 1 and *Legionella* spp;
- one sample was found to be positive for both *L. pneumophila* sg 1 and sg 2-14 and *Legionella* spp.

With regard to the structures, *Legionella* was found in the water systems of 12 (33.3%) recreational and tourist accommodation facilities and nine (24.3%) social-assistance facilities, respectively. However, this difference was not statistically significant in the Fisher's exact test (p = 0.4459).

Legionella prevalence according to the type of structure is shown in detail in **Table 1**.

Legionella load

Overall, in 53 (54.6%) samples, *Legionella* load was $\geq 10^3$ CFU/L, while in 27 (27.8%) it was $\geq 10^4$ CFU/L.

The median *Legionella* load in the positive samples was 2,000 CFU/L (IQR: 260-12,000 CFU/L). Regarding the differences between isolated serogroups, *Legionella* load in samples

Table 1. Legionella prevalence according to the type of structure.

	Hotels and holiday farmhouses	Sports Centres	Retirement homes	Camping sites	Group homes	Beach resorts and amusement parks
Water systems positive for Legionella, n (%)	4/7 (57.1%)	7/17 (41.2%)	8/28 (28.6%)	1/8 (12.5%)	1/9 (11.1%)	0/4 (0%)

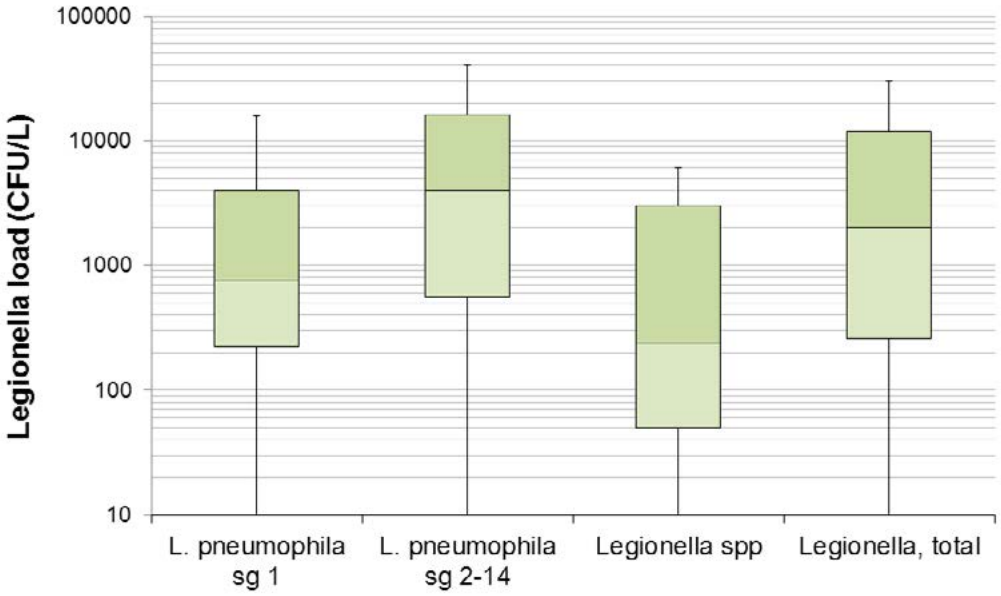

Figure 1. Legionella load in samples found positive for Legionella based on the serogroup or species.

positive for *L. pneumophila* sg 2-14 alone appeared significantly higher in the Wilcoxon-Mann-Whitney test compared to the samples positive for *L. pneumophila* sg 1 alone (p = 0.0147) and for *Legionella* spp. (p=0.0013). The difference in *Legionella* load between samples positive for *L. pneumophila* sg 1 and *Legionella* spp., however, was not significant (p = 0.1236) (see also **Figure 1**).

General water quality: Heterotrophic Plate Counts (HPCs)

Data on HPC at 22°C and 37°C are available for 291 samples belonging to 66 water systems, with 145 samples collected from 31 sports centres or tourist accommodation facilities, and 146 from 35 social assistance facilities. Samples collected from the latter facilities were significantly more contaminated in the Wilcoxon-Mann-Whitney tests, both for HPC at 22°C ($p < 0.00001$) and at 37°C ($p < 0.00001$) (see also **Figure 2**).

Heterotrophic Plate Counts (HPCs) related to *Legionella* presence

The distribution of *Legionella* presence appears to be significantly correlated to both HPC at 22°C (p = 0.0035) and HPC at 37°C (p = 0.0023) using Chi-square tests (see **Table 2** and **Figure 3**). It would seem that intermediate values of HPC at 22°C ($10 < HPC \leq 300$) favour the presence of *Legionella*, while very high HPC 22°C (> 500 CFU/mL) have a deterrent effect. Regarding HPC at 37°C, on the other hand, data show that very low HPCs (< 10 CFU/mL) correlate negatively with the presence of *Legionella*, while medium-low ($10 < HPC \leq 100$) or medium-high HPC ($300 < HPC \leq 500$) correlate positively.

Repeated samplings

At least a second sampling was performed in seven out of 12 (58.3%) sports centres or tourist accommodation facilities, and

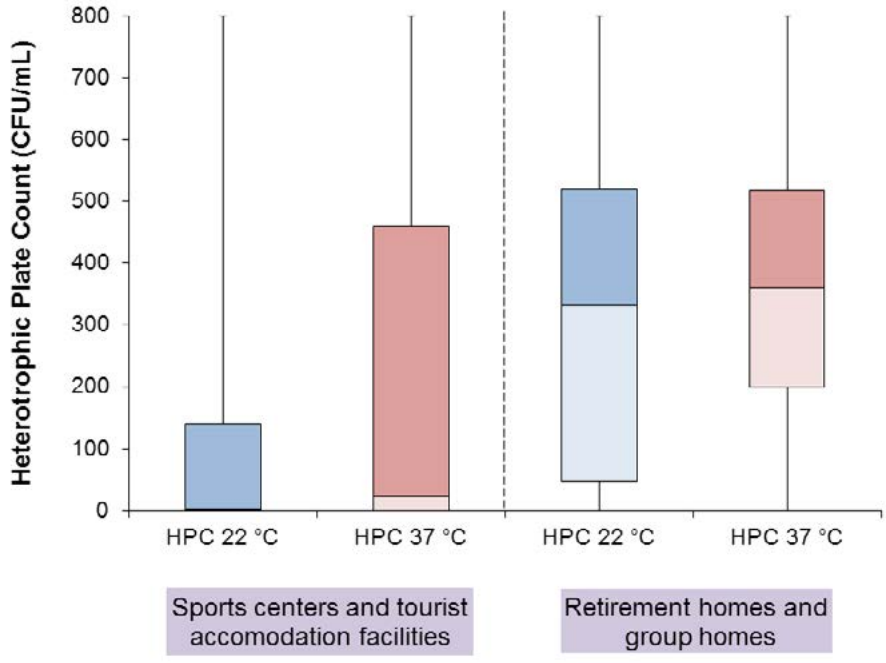

Figure 2. Heterotrophic Plate Counts (HPCs) at 22°C and at 37°C observed in sports centres/tourist accommodation facilities and social-assistance facilities.

Table 2. Heterotrophic Plate Count (HPC) related to the presence of Legionella.

	HPC 22°C			HPC 37°C		
HPC class (CFU/mL)	Samples	Legionella Positive, n	Legionella Positive, %	Samples	Legionella Positive, n	Legionella Positive, %
HPC ≤ 10	110	27	24.5%	85	12	14.1%
10 < HPC ≤ 100	45	19	42.2%	32	15	46.9%
100 < HPC ≤ 300	26	11	42.3%	27	7	25.9%
300 < HPC ≤ 500	41	11	26.8%	71	25	35.2%
HPC > 500	69	9	13.0%	76	18	23.7%
HPC not assessed	79	20	25.3%	79	20	25.3%
Total	370	97	26.2%	370	97	26.2%

in two out of nine social-assistance facilities positive for the presence of *Legionella* during the first sampling. The second and any subsequent sampling operations were performed in all cases following decontamination intervention, carried out at the initiative of the managers of the structures to eliminate *Legionella* from the water system. In one of the structures, following post-decontamination negativisation, a new sampling operation (third sampling), which again revealed the presence of *Legionella*, was carried out after one year. The structure was therefore resampled a fourth, a fifth and a sixth time: taking into account the extensive period between negativisation and the third sampling, the sampling operations following the first negativisation were considered in the analyses as the first second and third sampling operations, respectively. Ultimately, ten, six and three structures were subjected to second, third and fourth sampling operations, respectively.

Overall, in 60% (6/10) of the structures analysed, *Legionella* was still present on the second sampling operation following intervention to disinfect the water system. In 83% (5/6) of the structures in which a third sampling operation was undertaken, *Legionella* was still present, while 67% (2/3) of the structures were also positive for the presence of the bacterium at the fourth sampling (**Figure 4**).

In five (55.5%) of the resampled structures, a *Legionella* load equal to or greater than 10^3 CFU/L was found in at least one sample after disinfection, and in four of these (44.4%), the concentration was even higher than 10^4 CFU/L. In all cases but one (SPA Hotel, ID 36 in **Table 3**), the *Legionella* class of serogroups or species identified in the sampling operations performed after disinfection was the same one identified during the first sampling operation (**Table 3**).

***Legionella* persistence after water system disinfection**

We performed 19 sampling operations in the facilities in which *Legionella* was found a few days after water system disinfection had been carried out.

With regard to the treatments carried out before sampling, in ten cases only shock hyperchlorination was performed, in seven cases this treatment was combined with heat shock, and in one case the use of hydrogen peroxide was combined with the above two treatments. Finally, in only one case, the manager relied solely on the heat shock method (**Table 3 and Table 4**).

Given that maintenance of the terminals (showerheads) should be a routine hygiene practice, even if declared by the operator during sampling as a decontaminating method implement-

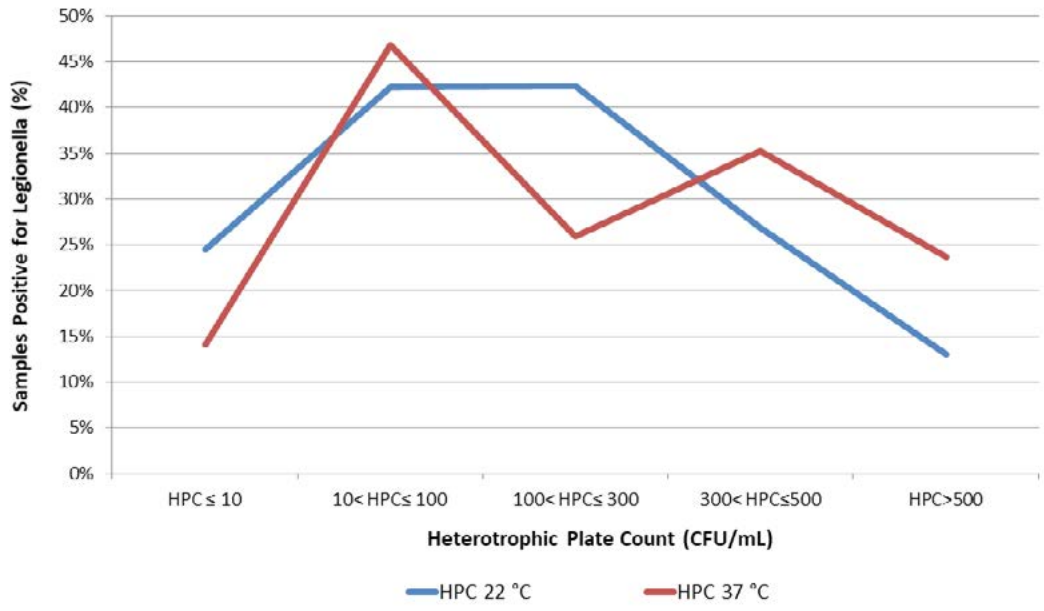

Figure 3. Percentage of Legionella positive samples related to Heterotrophic Plate Count (HPC, CFU/mL) at 22°C and 37°C.

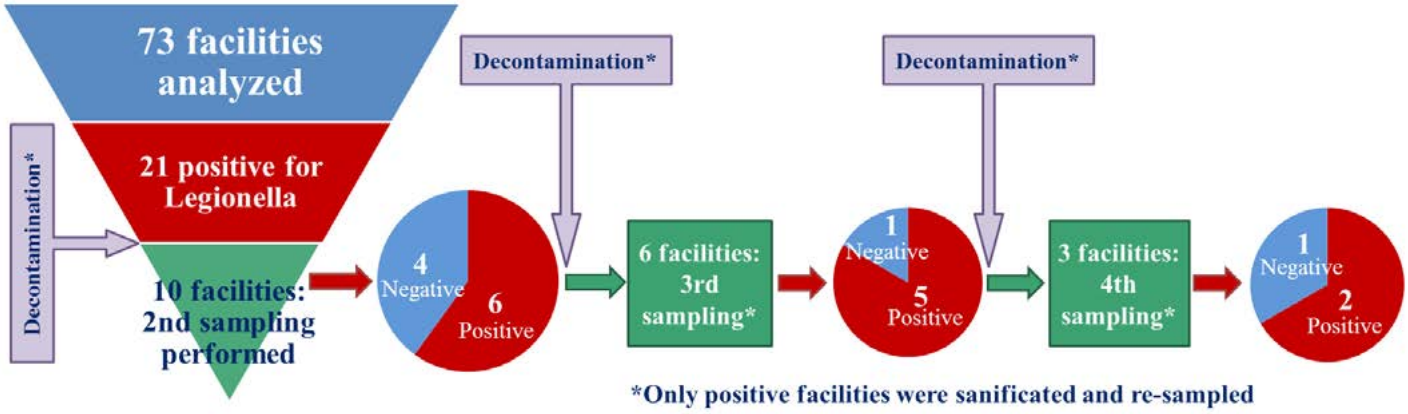

Figure 4. Repeated sampling of water systems and Legionella persistence.

Table 3. Structures in which Legionella was isolated and in which at least a second sampling operation was performed: summary of samplings' results.

Structures analysed: type, ID	Sampling #	Declared disinfection treatment carried out before sampling	Sampling result (isolated *Legionella*, % positive samples)	Maximum *Legionella* load detected (UFC / L)
Sports Centre, 3	1	/	*L. pneumophila* sg1, 67%	$8x10^3$
	2	Shock hyperchlorination		< 10
Sports Centre, 6	1	/	*L. pneumophila* sg2-14, 33%	$6x10^3$
	2	Heat shock every day, showerhead management		< 10
Sports Centre, 10	1	/	*L. pneumophila* sg2-14, 100%	$1.2x10^4$
	2	Shock hyperchlorination, heat shock and showerhead management		< 10
Sports Centre, 11	1	/	*L. pneumophila* sg1 and 2-14, 67%	$3x10^4$
	2	Shock hyperchlorination and showerhead management	*L. pneumophila* sg1 and 2-14: 50%;	$1.4x10^5$
	3	Shock hyperchlorination	*L. pneumophila* sg2-14: 25%	< 10
Sports Centre, 12	1	/	*L. pneumophila* sg2-14, 100%	$6.2x10^4$
	2	Shock hyperchlorination[1]	*L. pneumophila* sg2-14, 75%	$5.7x10^4$
	3	Shock hyperchlorination[1]	*L. pneumophila* sg2-14, 83%	$3.0x10^4$
Sports Centre, 17	1	/	*L. pneumophila* sg1, 25%; *L. pneumophila* sg 1 and 2-14, 25%	$1.6x10^4$
	2	Shock hyperchlorination[2]	*L. pneumophila* sg2, 67%; *L. pneumophila* sg 1 and 2-14, 17%	$3.2x10^4$
	3	Shock hyperchlorination, heat shock every day, hydrogen peroxide	*L. pneumophila* sg1, 13%	$2x10^3$

(Continued)

Table 3. Structures in which Legionella was isolated and in which at least a second sampling operation was performed: summary of samplings' results. *(Continuation)*

Structures analysed: type, ID	Sampling #	Declared disinfection treatment carried out before sampling	Sampling result (isolated *Legionella*, % positive samples)	Maximum *Legionella* load detected (UFC / L)
Spa hotel, 36	1	/	*L. pneumophila* sg1, 22.2%; *L. pneumophila* sg1 and spp., 11.1%; *Legionella* spp., 11.1%	$1.6x10^4$
	2	Shock hyperchlorination every day		< 10
	3[3]	/	*Legionella* spp., 18%; *L pneumophila* sg1: 10%	$4x10^3$
	4[3]	Shock hyperchlorination every day	*Legionella* spp.,71% *L pneumophila* sg1 e 2-14 and *Legionella* spp, 14%	$1x10^3$
	5[3]	Shock hyperchlorination every day	*Legionella* spp., 17%	90
	6[3]	Shock hyperchlorination every day	*L. pneumophila* sg2-14, 20%; *Legionella* spp., 20%; *L. pneumophila* sg1, 10%	$1.9x10^3$
Retirement home, 48	1	/	*L. pneumophila* sg1, 100%	850
	2	Shock hyperchlorination, heat shock every day	*L. pneumophila* sg1, 63%	$4.1x10^4$
	3	Shock hyperchlorination, heat shock every day	*L. pneumophila* sg1, 25%	150
	4	Shock hyperchlorination, heat shock every day	*L. pneumophila* sg1, 25%	40
Retirement home, 49	1	/	*L. pneumophila* sg1, 100%	750
	2	Shock hyperchlorination, heat shock every day	*L. pneumophila* sg1, 75%	470
	3	Shock hyperchlorination, heat shock every day	*L. pneumophila* sg1, 50%	10
	4	Shock hyperchlorination, heat shock every day		< 10

[1] Carried out by the operator, not by a specialised technician
[2] Performed only in sections of water systems leading to the distal points that were positive for Legionella
[3] Taking into account the negativisation of the structure in the previous sampling operation, the third, fourth, fifth and sixth samplings were considered in the analyses as the first, second, third and fourth sampling operations, respectively.

Table 4. Result of the sampling operations following the treatment of the water system, based on the type of disinfection treatment performed.

	Sampling after treatment, n	Legionella isolated (%)	Legionella load > 10^3 CFU/L (%)	Legionella load > 10^4 CFU/L (%)
Shock hyperchlorination (alone or combined)	18	13 (72%)	8 (44%)	5 (28%)
Shock hyperchlorination alone	10	7 (70%)	6 (60%)	4 (40%)
Heat shock (alone or combined)	9	6 (67%)	2 (22%)	1 (11%)
Heat shock alone	1	0 (0%)	0 (0%)	0 (0%)
Shock hyperchlorination plus heat shock	7	5 (71%)	1 (14%)	1 (13%)
Shock hyperchlorination plus heat shock plus hydrogen peroxide (continuous)	1	1 (100%)	1 (100%)	0 (0%)

ed, this process has not been included in the analysis as a 'combined treatment' if associated with one of the abovementioned methods.

Given the limited number of sampling operations performed after disinfection, and the almost total overlap between the cases in which the shock hyperchlorination was performed in combination and those in which this combination consisted of the addition of the process to heat shock, we decided to analyse only the difference in the efficacy of shock hyperchlorination alone vs shock hyperchlorination combined with heat shock, or of other treatments vs shock hyperchlorination alone. Although the data seem to indicate a greater efficacy of the combination of shock hyperchlorination and heat shock in maintaining the *Legionella* load < 10^3 CFU/L, this correlation does not reach statistical significance (p = 0.081) (**Figure 5, third column**). Moreover, the addition of heat shock to shock hyperchlorination does not seem to have any effect on the presence of *Legionella* in the water system (p = 0.949) (**see Figure 5, first column**). We have also tried to establish a correlation between *Legionella* serogroup and disinfection efficacy, but, although the data seem to indicate a greater efficacy of the combined treatments against *L. pneumophila* sg 2-14 compared to *Legionella* sg 1, at least in terms of the presence of the bacterium in the water system, the results are never statistically significant.

Discussion

We found a high prevalence of *Legionella* in the water systems of the buildings analysed. This prevalence was higher among tourist accommodation facilities than retirement homes and group homes, but *Legionella* prevalence was highest in hotels, holiday farmhouses and sports centres (57.1% to 41.2%) and lowest in camping sites, group homes, beach resorts and amusement parks (12.5% to 0%) (**Table 1**).

Legionella prevalence in the water systems of hotels

Our findings are in line with several previous reports in the literature. In fact, Fragou et al.[75] detected *L. pneumophila* in 36% of the water distribution systems of hotels in Greece, while in another extensive study performed in the same country,[55] the authors found *Legionella* in only 20.8% (80/385) of hotel water systems. However, the detection limit they used was far higher (≥ 500 CFU/L) than in our study (≥ 10 CFU/L), so the difference Mouchtouri et al (2007) found in the same country could be due to this parameter. Erdogan et al.[76] recently found lower *Legionella* prevalence (21.2%) in water systems in Turkish baths in Turkish hotels. Conversely, higher prevalence levels (71%) in hotel water systems were recently identified in Hungary,[77] using the same detection limit as our study, and similar results were reported by Leoni et al.[46] and Borella et al.,[52] who discovered a *Legionella* prevalence of 63.6% and 75% respectively in Italian hotel water systems analysed, using a detection limit of 25 CFU/L.

Bonetta et al.[78] found a significant difference between isolation of *Legionella* in hotel water systems using the culture method (42% water systems positivity, 100 CFU/L detection limit) and using RT-PCR (74%). A similar difference (10% vs 38%) was found by Edagawa et al. in hotels,[79] and by Collins et al. (6% vs 48%) in households.[80] However, this difference could be due to viable but non-culturable cells (VBNC) of *L. pneumophila*, which are detectable only using sensitive molecular techniques[81] and whose ability to cause LD or PF epidemics is still debated.[66,82,83] On the other hand, some authors hypothesise that these forms can become vital and proliferate when the concentration of the disinfectant used to control contamination in the water system falls below a certain threshold value (e.g. monochloramine < 1.5 mg/L).[83-85]

Legionella prevalence in recreational facilities

There are limited data in the literature about the prevalence of *Legionella* in spas, and these vary: for example, Donati et al. never found *Legionella* in their environmental samples,[86] although they collected water samples from the pools and not from the terminals of the water system. Conversely, Brousseau et al. found widespread contamination in spa pools, as *Legionella* was detected in 26% of spas.[87] Similar results were found by Guillemet

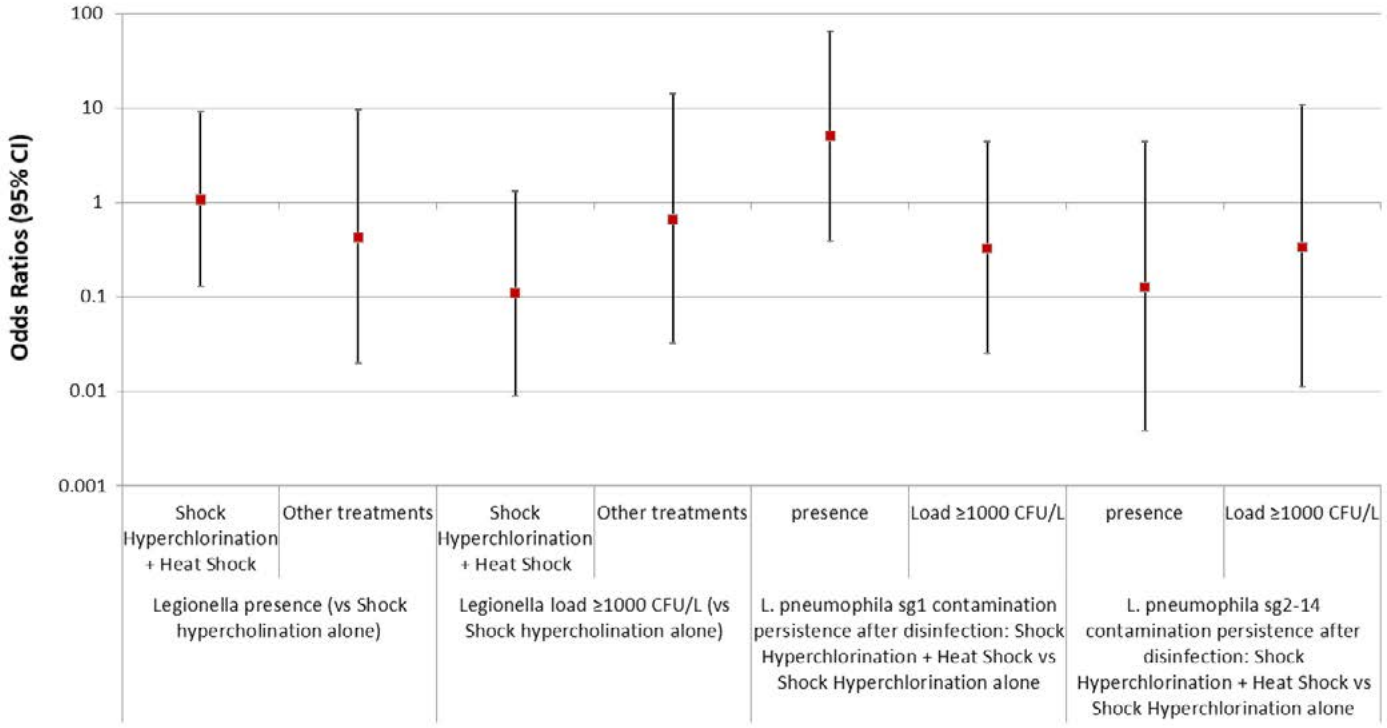

Figure 5. ORs for Legionella presence and load based on the disinfection treatments carried out.

et al., who identified the bacterium in 27% of samples using the culture method, while *Legionella* prevalence reached 62% on analysis of the same samples using real-time PCR.[88] However, cases of legionellosis related to recreational facilities are far from rare: a recent literature review reports 42 events, including sporadic cases and outbreaks, and 1079 legionellosis cases, of which 42.5% were LD, causing 29 deaths.[89] The fatality rate for legionellosis cases related to recreational facilities such as spa pools appears to be the lowest (3.6%) when compared to other sources of disease.[90]

Legionella prevalence in domestic water systems

The prevalence of *Legionella* we have identified in retirement homes and group homes, where water system complexity can be compared to domestic systems, is consistent with previous reports in the literature. In fact, in Italy *Legionella* sample positivity in the hot water of domestic water systems ranges from 22.6% to 33%, although one study reports a higher prevalence (53.1%) of the bacterium in water systems.[51,54,91,92,93] However, further confirmation of our data, with *Legionella* prevalence between 6% and 35% in home water systems, is provided by studies conducted in the US and other European countries.[77,80,94-97]

Legionella prevalence and buildings size

As already shown in our previous studies,[53,65,70] *Legionella* has been found more frequently in larger buildings. In fact, we found *Legionella* in 45.8% of the water systems of hotels and sports centres compared to 10% of beach resorts and camping sites.[53] Though not statistically significant (Fisher's exact test: $p = 0.0607$), these data, combined with data on the correlation in retirement homes and group homes between number of storeys and presence of *Legionella* ($p = 0.0514$),[65,70] seem to confirm the link already identified in the literature between the complexity of the water system and *Legionella* colonisation and persistence.[52,63,77,95,98]

Legionella load

Our data on *Legionella* load are consistent with the previous literature. In fact, considering studies examining buildings similar to the ones we investigated, the percentage of positive samples with a *Legionella* load ≥ 10^3 CFU/L usually ranges from 58% and 83%, while the percentage falls to 9%-38% when considering only samples with a *Legionella* load ≥ 10^4 CFU/L.[51,54,55,77,78,92,93,99] Considerably lower contamination values were found in three studies conducted in Turkey, Greece and Italy, with only 17%, 11%, and 5%, respectively, of positive samples with a *Legionella* load ≥ 10^3 CFU/L.[75,76,100]

Results for drinking water parameters

Our HPC values are higher when compared to the results of extensive monitoring in warm, in-building distribution systems in Germany[101] and, for example, the works by Totaro et al. and Baggiani et al. recently conducted in Italy, in which the HPC ranged from 1 to 400 and had a mean value at the distal point of 41.2 ± 59 CFU/mL, respectively.[91,93] Both works show that HPC were significantly lower at the distal point than at the inlet. Bargellini et al. (2001) reported a geometric mean value for HPC at 22°C and at 37°C of 35 and 101 CFU/mL, respectively.[99] Conversely, Moutchouri et al. found widespread contamination of hotel water systems, with 72% of samples characterised by an HPC ≥ 400 CFU/mL. They also found a statistically significant ($p < 0.001$) increase in *Legionella* prevalence among samples with HPC ≥ 400 CFU/mL,[55] and proposed the HPC as an indicator of the presence of *Legionella* spp. Similarly, Bargellini et al. (2001) showed a significant correlation between values of HPC at 37°C > 150 CFU/mL and presence of Legionella by performing univariate regression (OR 2.31, 95%CI 1.55-3.43), and a significant correlation between values of HPC at 22°C > 27 CFU/mL both by univariate regression (OR 2.68, 95%CI 1.80 to 4.00) and by multiple logistic regression (OR 2.24, 95%CI 1.47 to 3.42).[99] Solimini et al. (2014) in their *in-vitro* experiments showed that the presence of other bacteria, such as the heterotrophs, sustain *L. pneumophila* growth, being probably the source of other essential nutrients. In fact, although iron was found to be linked to *L. pneumophila* growth, this metal was unable to enhance *L. pneumophila* growth without the contemporary presence of other microrganisms.[102] In our previous papers[65,70] we found a statistically significant peak of *Legionella* prevalence for HPC at 22°C between 10 and 300 CFU/mL. Yet, despite their relationship with the presence of *Legionella*, HPC are not considered an indicator of health risk in themselves.[103] Accordingly, they are not considered suitable for public health target-setting as part of a Water Safety and Risk Management Plan for a potable water supply.[9,104,105] Conversely, monitoring the changes in HPC between water entering a facility and distal points can help in identifying where stagnation in a water distribution system may be occurring, leading to microbial growth. This is the reason why, although there is no precise threshold, the 'Reference Analytical Methods for Water Intended for Human Consumption According to Italian Legislative Decree No 31/2001' provide for the mandatory assessment of HPC at 22°C. This must not experience 'anomalous variations'.[67]

Efficacy of *Legionella* disinfection systems

Despite a clear trend indicating greater effectiveness of combining heat shock with shock hyperchlorination, at least in reducing *Legionella* at high concentrations (> 10^3 CFU/L) but not on *Legionella* presence, we did not find any significant correlation between the disinfection treatment applied and the presence/load of *Legionella* in the water systems analysed.

However, these results could have been due to the limited number of facilities analysed and to the fact that often the same treatment was repeated several times in the same structure, making the data redundant. Another limitation of the study is that few types of disinfection treatment were performed by the building administrator, and the fact that we have no information about non-continuous disinfection treatments carried out before the first sampling operation. Moreover, shock hyperchlorination sometimes carried out by the operator and not by a specialised technician or performed only in sections of water systems leading to the distal points that were positive for *Legionella* (see **Table 3**) may have contributed to increasing heterogeneity in the effectiveness of this type of treatment.

Considering the facilities vertically (**Figure 4** and **Table 3**), we can see how frequently the sanitisation interventions performed were ineffective. Indeed, according to the available data, *Legionella* was still found in the water systems after decontamination in 68% of facilities inspected.

Based on their experience, Marchesi et al. proposed the adoption of electric boilers and chlorine dioxide to prevent and/or reduce *Legionella* contamination, while monthly shock hyperchlorination and heat shock were considered less effective, and filter installation at the distal point, though effective, was far too expensive and is therefore used primarily to prevent nosocomial infections.[106,107] Despite its short-term effectiveness and low cost, heat shock is widely considered in the literature to be a temporary method to reduce *Legionella* contamination in water systems, be-

cause recontamination reoccurs within one to two months after the intervention is performed.[106-108] Slightly greater long-term effectiveness is associated with shock hyperchlorination, but only when it is followed by non-continuous water chlorination.[108,110] In our work, as in the paper by Marchesi et al.,[106] there were no facilities in which a copper-silver ionisation system was installed as a non-continuous disinfection method after decontamination was performed, so we could not establish the effectiveness of this process in reducing *Legionella* presence as others have done. In particular, Lin et al. found this treatment to be effective both in the short and long term,[107-109] while Ortolano et al. found that its efficacy is strongly correlated with the content of dissolved solids in the water.[110] At the same time, we did not have any data about the effectiveness of other significant, well studied treatments such as chlorine dioxide and monochloramine, which have frequently been shown to be effective in reducing *Legionella* contamination.[24-28,38-40,111] Recently, Totaro et al. (2019) reported that both chlorine dioxide and anolyte (hypochlorous acid) had been able to avoid Legionella spp. growth in previously contaminated water networks of nursing homes in a long-term period.[111]

Along with efficacy, safety must be taken into account when choosing the disinfection method to be implemented. Many disinfection methods use disinfectants and can create by-products that can be harmful to human health. For example, copper is the most widely investigated metal because of its role in the formation of amyloid plaques in Alzheimer's disease, with 36 studies assessing concentrations of this metal in different biological matrices, and the successful treatment of this disorder in rodent models using copper chelating is a very interesting development.[112,113] However, the concentrations of copper and silver effective for disinfection are 0.2 to 0.6 mg/L and 0.02 to 0.06 mg/L, respectively.[114] In 2004, the WHO established a threshold of 2 mg/L for copper concentrations in drinking water:[115] this threshold should permit consumption of 2-3 litres of water per day, without exceeding the tolerable upper intake level of 10 mg/day.[116] With regard to chlorination, high residual chlorine could react with organic materials, accelerating the production of trihalomethanes, which are known carcinogens,[110] and an increased risk of scalding through heat shock has been reported by some authors.[103,117] By-product formation in the presence of bromide (bromate) and chlorine (chlorate) can also occur when ozonation is performed.[118] Finally, there are damaging effects on the rubber, lead or copper components of the plant and nitrogen products can be produced in the water following non-continuous disinfection using monochloramine.[37]

Conclusions

LD cases diagnosed in Italy are increasing slowly but constantly.[57] The Italian situation is in line with the picture in Europe as a whole, where legionellosis cases have been increasing since 2011. This increasing trend is probably driven by several factors, such as improved surveillance, travel patterns and climate change, since aging can only partially explain the trend: indeed, the age-standardised notification rate has also increased over the same period. In fact, weather conditions such as temperature, humidity and rainfall have been associated with a higher LD incidence, as they have an effect in terms of increased use of aerosol-producing devices or installations in the environment, such as cooling towers.[59] The attention given to *Legionella* must remain high if we want to prevent epidemic clusters of disease. As evidence of this, we need only look at the most recent epidemic, probably derived from cooling towers in Lombardy, which caused the second largest epidemic of all time in terms of number of cases of Legionnaires' disease due to pollution of the cooling towers.[119,120] The high prevalence of Legionella we have identified, and in general the low quality of water we detected, together with epidemiological data, suggest that there is still significant work to be done to improve the management of the water systems of private facilities. Our work included the efficacy of disinfection systems only as a secondary outcome, and was not powered for evidencing statistically significant differences in the efficacy of the various treatments carried out. More research is warranted to assess the comparative effectiveness of decontamination methods.

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