 doi:10.20944/preprints201905.0186.v1

Colonization of *Legionella* spp. in Hot and Cold Water Networks of Residential Buildings in Pisa District (Tuscany, Italy): A Five-Years Survey

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Abstract

Literature data on *Legionella* spp. presence in houses water networks have been increasing during the last years, but epidemiological reports assert a high incidence of Legionnaires' disease infection in Italy. Updating our previously published data, we report a five-year survey on *Legionella* spp. colonization in 235 buildings with an independent hot water production (IB); 82 buildings with a central hot water production (CB); and 58 buildings with a solar thermal system for hot water production (TB). In all the 375 buildings *Legionella* spp. was researched in hot and cold water samples and microbiological potability standards of cold water were evaluated. *Legionella* spp. was detected in 27% of the water networks, mostly in CB and TB. We detected correlations between the presence of bacteria and some physical-chemical parameters (low chlorine level and optimal temperature for *Legionella* spp. growth). Cold water resulted free from microbiological hazards, except for coliform bacteria isolated in three separate cases, and *Legionella* spp., detected when cold water temperature was about 20°C. After a five-year survey we confirm the presence of a Legionnaires' disease risk and the need of training programs for all the workers involved in residential water systems management.

Keywords: *Legionella* spp.; residential buildings; waterborne pathogens, water safety plan.

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Introduction

The importance of opportunistic waterborne pathogens has been increasing in recent years, with *Legionella* spp. identified in several reports as the most common cause of waterborne infection outbreaks in Europe [1]. *Legionella* spp. is widely present in the environment and it may colonize water systems. This occurrence is mostly present in ancient buildings, into corroded pipelines and dead leg branches, allowing the biofilm growth in sites where the disinfectant can not be effective against microorganisms [2, 3, 4]. Legionnaires' disease is acquired by aspiration and inhalation of aerosol or water droplets carrying *Legionella* spp. Chronic diseases and immunodepression are predisposing factors for the development of the disease, mostly caused by *Legionella pneumophila* serogroup 1 [5]. The surveillance of Legionnaires' disease is performed, in Europe, by the European Centre for Disease Prevention and Control (ECDC), and in Italy by the Italian National Institute of Health.

Italian latest data, related to 2017, give 2014 cases of Legionnaires' disease in Italian population, corresponding to a notification rate of 33.2 cases per million inhabitants. However, it must be noted that 2017 has seen an increase of 26% in the cases when compared with the previous three years. Of the 2,014 notified cases, 124 (6.2%) were hospital acquired, while 1,580 (78.5%) were community acquired infections, highlighting the prevalence of Legionella colonization in community environment [6].

During the last three years an increase of literature data on *Legionella* spp. presence in water networks of residential buildings, has been observed [7, 8, 9, 10, 11]. Moreover, both an Italian guideline for Legionnaires' disease control in hospital and community settings, and the Drinking Water Directive identify the responsibilities for water quality control also in private residential buildings [12, 13].

In a three-year survey, performed from 2014 to 2017 on 220 residential buildings located in Pisa district (Italy), we found the colonization of *Legionella* spp. in 26% of the hot water networks. Following these published data, the purpose of this new study is the evaluation of *Legionella* spp. colonization in hot and cold water networks in private domestic apartments in Pisa during the whole period 2014-2019.

Materials and Methods

Setting and inspections

The survey was performed from April 2014 to April 2019 on 375 residential buildings located in Pisa (Italy). The buildings were mainly of small sizes with a range between four and thirty flats. 235 buildings had an independent hot water production (IB); 82 buildings had a central hot water production (CB); 58 buildings had a solar thermal system for hot water production (TB).

Inspection visits were performed with the aim of verify the adequate functioning of the thermal and water power plants. Inspections were performed following a checklist

 doi:10.20944/preprints201905.0186.v1

organized with some information such as name, address of the building, type of the hot water productions system, presence of devices (recirculation, boilers, softeners, autoclaves, ect), water disinfection system, periodicity and type of water system maintenance and cleaning, water supplying, and number of floor and apartments, as described elsewhere [9] .

Sampling and Laboratory tests

From each water system three cold water samples were taken, one at the inlet from the aqueduct network into the building pipework (Point I); one at the exit from pressure autoclave (point E); and one at the most remote tap from the autoclave (point T). Therefore, 1,125 cold water samples were analyzed for the determination of the microbiological potability requirements, as stated by the Council Directive 98/83/EC [13].

Total microbial count at 22°C and at 37°C, coliforms and enterococci counts were determined according to the Council Directive 98/83/EC, as described in our previous study [9].

Hot water samples were collected both on the first floor of the building (Point A) and on the last floor (Point B), for *Legionella* spp. detection. From April 2017, *Legionella* spp. was also researched in cold water collected at the most remote tap (point T).

Therefore, 750 hot water samples and 155 cold water samples were collected for *Legionella* spp. detection, as suggested by the Italian guidelines for legionellosis control (LG2015). Samples were tested for *Legionella* spp. presence according to ISO 11731:1998 (updated to 2017 version) (ISO11731:2017) [14, 15], as described elsewhere [9].

Water temperature and total chlorine concentration were determined in all the water samples, while pH and conductivity values were measured only in water samples collected for *Legionella* spp. detection.

Statistical analysis

The Shapiro-Wilk test was performed to verify normality of distributions. The Kruskall-Wallis test and the Dunn's test were performed to compare the total microbial counts at 22 and at 37°C detected in different sampling points. Power tests were used to estimate the sample sizes, the 1-β values of the significant variables were > 0.8, assuring a low risk of type II error and an appropriate sample size. Correlation tests were performed, and Pearson's coefficients were calculated with the aim to analyze the correlations between physical-chemical parameters of the samples (temperature, chlorine concentration and conductivity) and the presence of *Legionella* spp. These tests were independently applied for IB, CB and TB. 95% confidence levels were defined for the statistical tests. Therefore, we considered the following ranges of values: 0-0.3 (weak correlation); 0.3-0.7 (moderate correlation); 0.7-1 (strong correlation). All statistical analysis was carried out using the SPSS software package, version 17.0.1.

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Results

Inspections and physical-chemical results

All the 235 IB present an autoclave system which collects municipal water before being injected in water networks. Water is softened in 90 out of 235 (38%) buildings. Cold water (mean 19.8±2.6°C) is disinfected with sodium hypochlorite, which was detected at points of use (Point I, Point E and Point T) in a concentration range between 0 and 0.24 mg/L (mean 0.13±0.05 mg/L). In these buildings each flat has an independent boiler for hot water production. Hot water (50.1±10.6°C) is treated with sodium hypochlorite measured at concentration between 0 and 0.24 mg/L (mean 0.18±0.14 mg/L) at Point A and Point B. Physical-chemical data, measured in hot water samples, showed pH values ranging from 5.6 to 7.6 (mean 6.6±1.6) and conductivity values between 344 to 1101 µS (mean 824±245 µS). Maintenance and cleaning activities of the central water supplies are performed on a half-yearly basis.

Also all the 82 CB have an autoclave system, which distribute municipal water. Only 25 out of the 82 (30%) are provided with a softener. Sodium hypochlorite is the disinfectant used for cold and hot water treatment and its concentration, detected at points of use, ranged from 0 to 0.11 mg/L (mean 0.05±0.02 mg/L) in cold water (19.4±2.7°C), and from 0 to 0.12 mg/L (mean 0.06±0.03 mg/L) in hot water (43.4±8.3°C). All the buildings have a thermal central water system with a recirculation device, aimed to saving energy. Physical-chemical data, measured in hot water samples, showed pH values ranging from 5.9 to 7.5 (mean of 6.6±1.2) and conductivity values between 344 to 1086 µS (mean of 861±235 µS). Maintenance and cleaning activities of the cold and hot water systems are performed on a half-yearly basis.

Regarding the TB, all the 58 central water supplies have an autoclave system. 46 out of 58 (79%) water plumbing distribute municipal water, while 12 out of 58 (21%) of TB are fed by wells. No buildings have either softener or an adequate disinfection method. Therefore, all the cold water (19.1±2.2°C) and hot water (33.8±9.4°C) samples from TB were not chlorinated. All central water supplies have a boiler for water storage, heated by solar thermal systems. Physical-chemical data, measured in hot water samples, showed pH values ranging from 5.6 to 7.3 (mean 6.7±0.8) and conductivity values between 935 to 1156 µS (mean 1101±125 µS). Maintenance programs are absent in all the TB.

All physical-chemical value regarding the cold and hot water samples, collected at different points of use, are shown in Table 1.

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Table 1: Mean values of physical-chemical (total chlorine, pH, conductivity and temperature) parameters detected in hot and cold water sampled at different point of use (Point A, Point B, Point I, Point E, and Point T) of the buildings IB, CB and TB. NA= Not Applied.

Building with independent hot water production (IB)					
PHYSICAL-CHEMICAL PARAMETERS	HOT WATER SAMPLES		COLD WATER SAMPLES		
	Point A	Point B	Point I	Point E	Point T
Total Chlorine (mg/L)	0.19±1.2	0.17±0.05	0.07±0.02	0.18±0.08	0.10±0.04
pH	6.5±0.7	6.6±1.1	NA	NA	NA
Conductivity (µS)	837±232	798±253	NA	NA	NA
Temperature (°C)	51.2±9.4	48.9±11.5	19.7±2.5	19.9±1.8	20.1±1.6
Building with central hot water production (CB)					
PHYSICAL-CHEMICAL PARAMETERS	HOT WATER SAMPLES		COLD WATER SAMPLES		
	Point A	Point B	Point I	Point E	Point T
Total Chlorine (mg/L)	0.07±0.05	0.06±0.04	0.04±0.01	0.05±0.02	0.05±0.03
pH	6.6±0.8	6.5±1.0	NA	NA	NA
Conductivity (µS)	854±224	866±287	NA	NA	NA
Temperature (°C)	48.1±7.8	39.7±8.8	18.6±2.1	19.3±2.2	20.6±1.7
Building with solar thermal system for hot water production (TB)					
PHYSICAL-CHEMICAL PARAMETERS	HOT WATER SAMPLES		COLD WATER SAMPLES		
	Point A	Point B	Point I	Point E	Point T
Total Chlorine (mg/L)	0	0	0	0	0
pH	6.6±0.7	6.7±1.0	NA	NA	NA
Conductivity (µS)	1011±135	1100±165	NA	NA	NA
Temperature (°C)	35.8±7.5	32.6±9.9	18.9±2.7	19.6±2.1	19.9±2.8

Drinking water parameters results

A variability in microbial growth at 22 and 37°C was observed among the cold water samples. No significant difference in the total microbial counts at 22 and 37°C was found between samples collected in IB, CB and TB. Overall, the total microbial counts at 22 and 37°C were between 1 and 400 CFU/ml. Bacterial counts higher than 100 CFU/ml) were detected in 12% (130 out of 1,125) of the samples, mostly at Point E and Point T.

The total microbial counts at 22 and 37°C detected at the inlet from the aqueduct (Point I) were significantly lower ($p<0.0001$) than those recorded in samples obtained at the most remove tap from the autoclave (Point T).
In CB and TB the cold water resulted free from microbiological hazards except for *Serratia liquefaciens* (3 CFU/100ml), *Enterobacter cloacae* (4 CFU/100ml) and *Escherichia coli* (7 CFU/100ml) isolated at the exit from three different pressure autoclave.

Legionella spp. results

Legionella spp. was detected in 198 out of the 750 (26%) hot water samples and in 17 out of the 155 (11%) cold water samples examined. Totally, 215 out of the 905 (26%) water samples resulted positive for *Legionella* spp.

In details, 46 out of the 235 (20%) IB, 33 out of the 82 (40%) CB, and 23 out of the 58 (40%) TB were colonized. Overall, 102 out of the 375 (27%) examined buildings had at least one sample positive for *Legionella* spp. In all the 17 buildings with the cold water network colonized by *Legionella* spp. also the hot water network resulted positive.

10 out of the 17 (59%) colonized cold water systems were placed in CB, while the last 7 (41%) were placed in TB.

In IB, *Legionella* spp. positive samples showed counts from $1x10^2$ to $5.2x10^4$ CFU/L (mean $9.4x10^3 \pm 4.8x10^3$ CFU/L) (Figure 1). *Legionella pneumophila* sg1, *Legionella pneumophila* sg2-16; and *Legionella* spp. were respectively recovered in 28 out of the 46 (61%), 8 out of the 46 (17%) and 10 out of the 46 (22%) water samples.

In IB samples, statistical results showed moderate correlations between the presence of *Legionella* spp. and the total chlorine concentration decrease ($r= - 0.63$; $p= 0.023$). No correlations were detected between the *Legionella* spp. concentration and the temperature and conductivity values.

In CB, Legionella positive samples showed counts between $2x10^2$ and $3x10^4$ CFU/L (mean $4.8x10^3 \pm 7.1x10^2$ CFU/L) (Figure 2). *Legionella pneumophila* sg1, *Legionella pneumophila* sg2-16; and *Legionella* spp. were respectively recovered in 15 out of the 33 (46%), 11 out of the 33 (33%) and 7 out of the 33 (21%) water samples.

In water samples collected from CB a moderate correlation was detected between the *Legionella* spp. concentration and the reduction of total chlorine values ($r= - 0.50$; $p= 0.03$). Again, no correlations were detected between the *Legionella* spp. concentration and the temperature and conductivity values.

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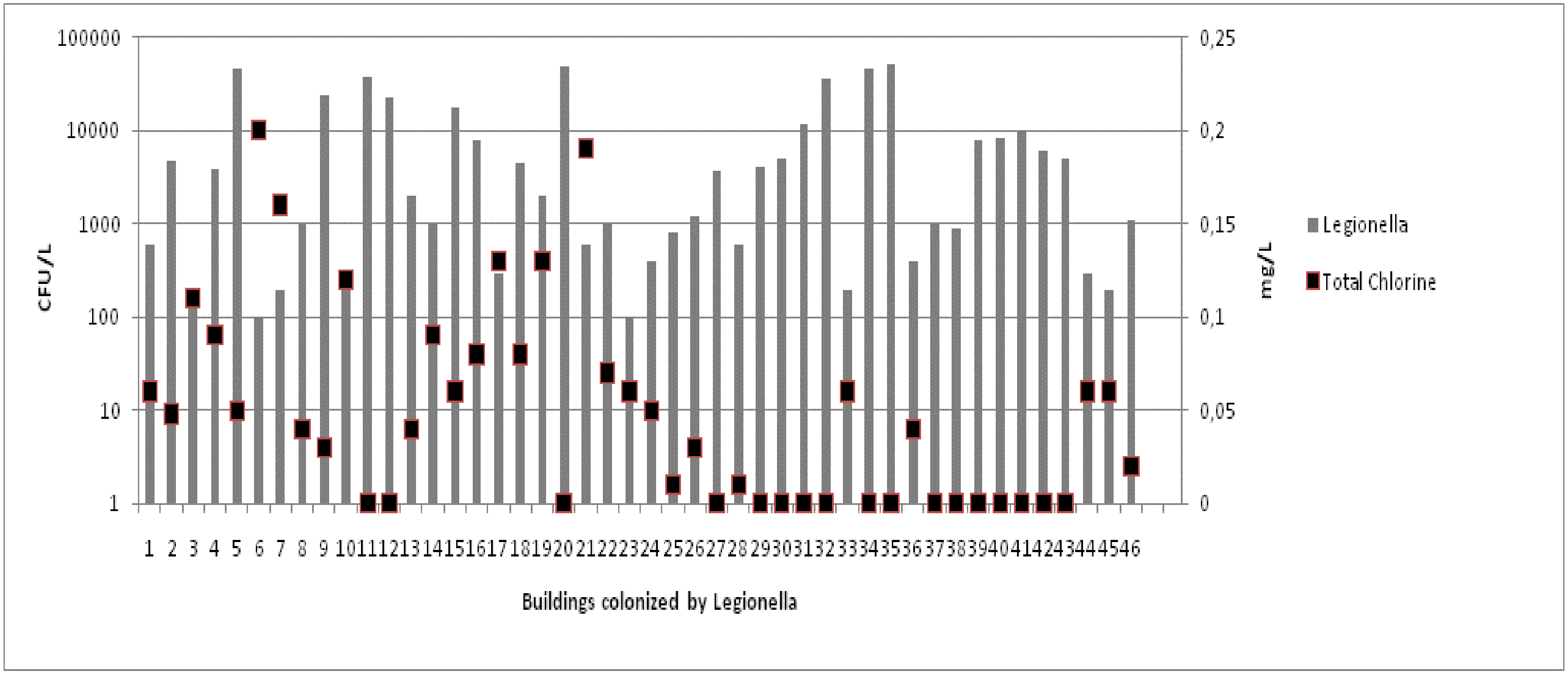

Figure 1: *Legionella* spp. counts and total chlorine concentration detected in 46 colonized IB.

 doi:10.20944/preprints201905.0186.v1

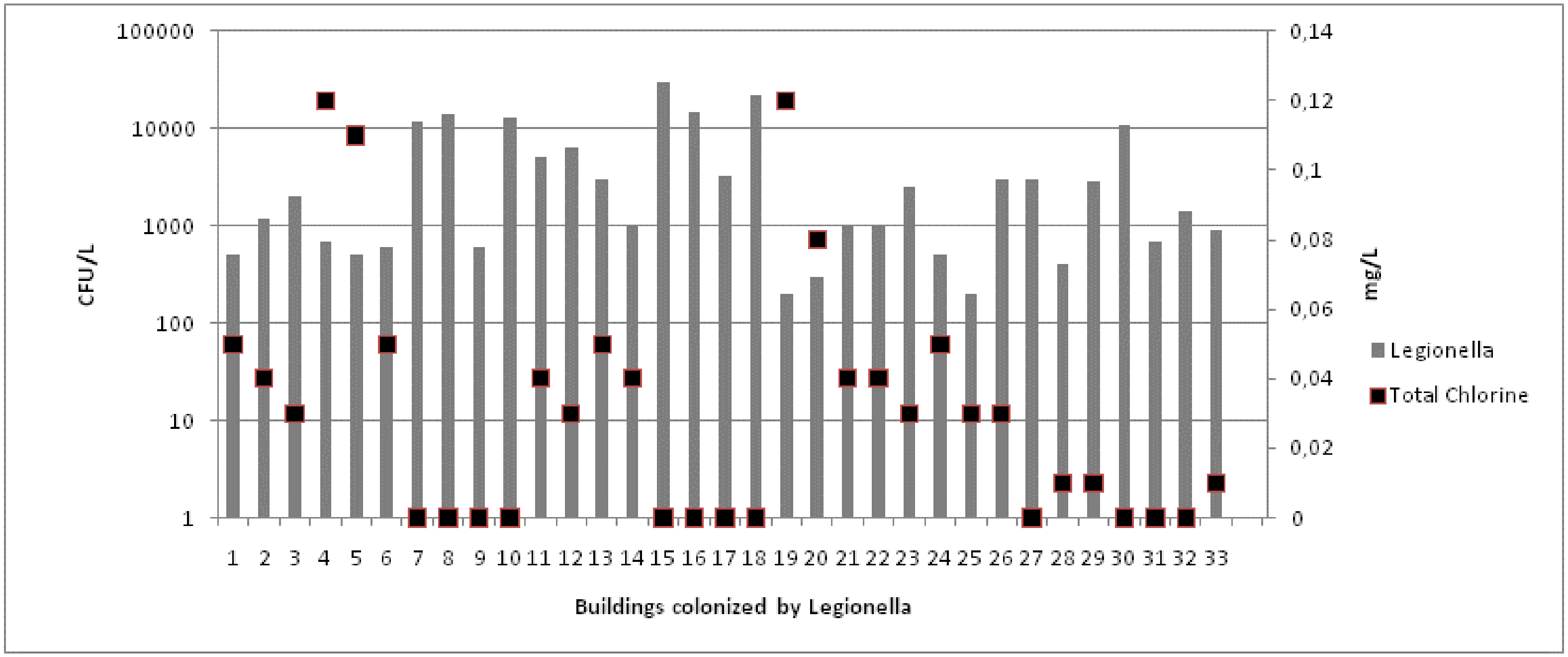

Figure 2: ***Legionella*** **spp. counts and total chlorine concentration detected in 33 colonized CB.**

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In TB, Legionella positive samples showed counts from $2x10^2$ to $7.6x10^5$ CFU/L (mean $3.8x10^4 \pm 1.6x10^4$ CFU/L) (Figure 3). *Legionella pneumophila* sg1, *Legionella pneumophila* sg2-16; and *Legionella* spp. were respectively recovered in 10 out of the 23 (44%), 9 out of the 23 (39%) and 4 out of the 23 (17%) water samples.

In water samples collected from TB no correlation was detected between the *Legionella* spp. concentration and the increase/decrease of the temperature and conductivity values.

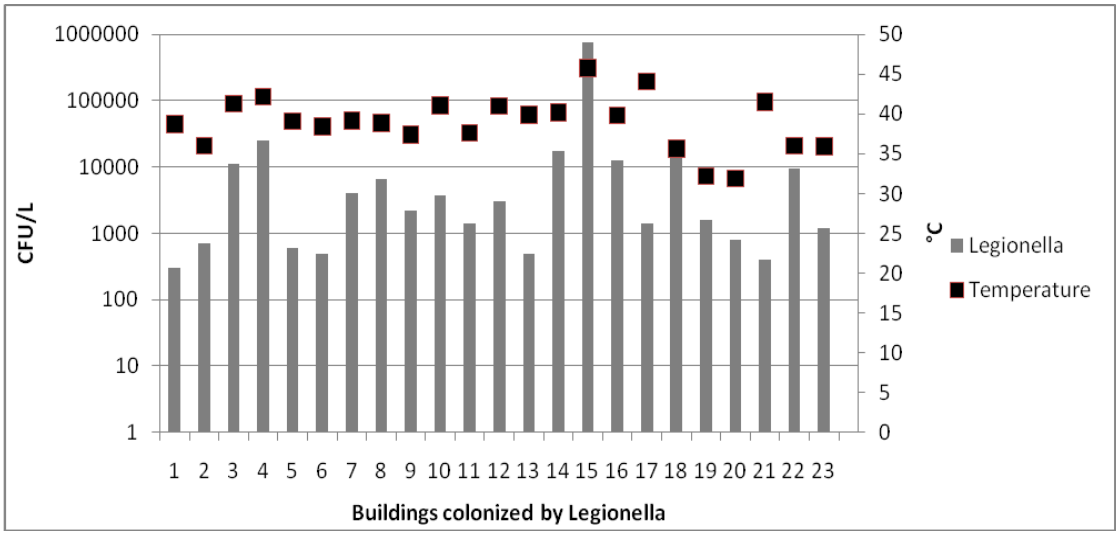

Figure 3: *Legionella* spp. counts and total chlorine concentration detected in 23 colonized TB.

At last, *Legionella* spp. was detected in the cold water systems of 10 CB and 7 TB. With counts between $2x10^2$ and $1.2x10^4$ CFU/L (mean $1.3x10^3 \pm 2.8x10^2$ CFU/L) (Figure 4). *Legionella pneumophila* sg1, *Legionella pneumophila* sg2-16; and *Legionella* spp. were respectively recovered in 6 out of the 17 (35%), 10 out of the 17 (59%) and 1 out of the 17 (6%) water samples. In cold water samples statistical results showed moderate correlations between the presence of *Legionella* spp. and the increase of temperature values ($r= 0.46$; $p= 0.035$). No correlation was detected between the *Legionella* spp. concentration and the chlorine values.

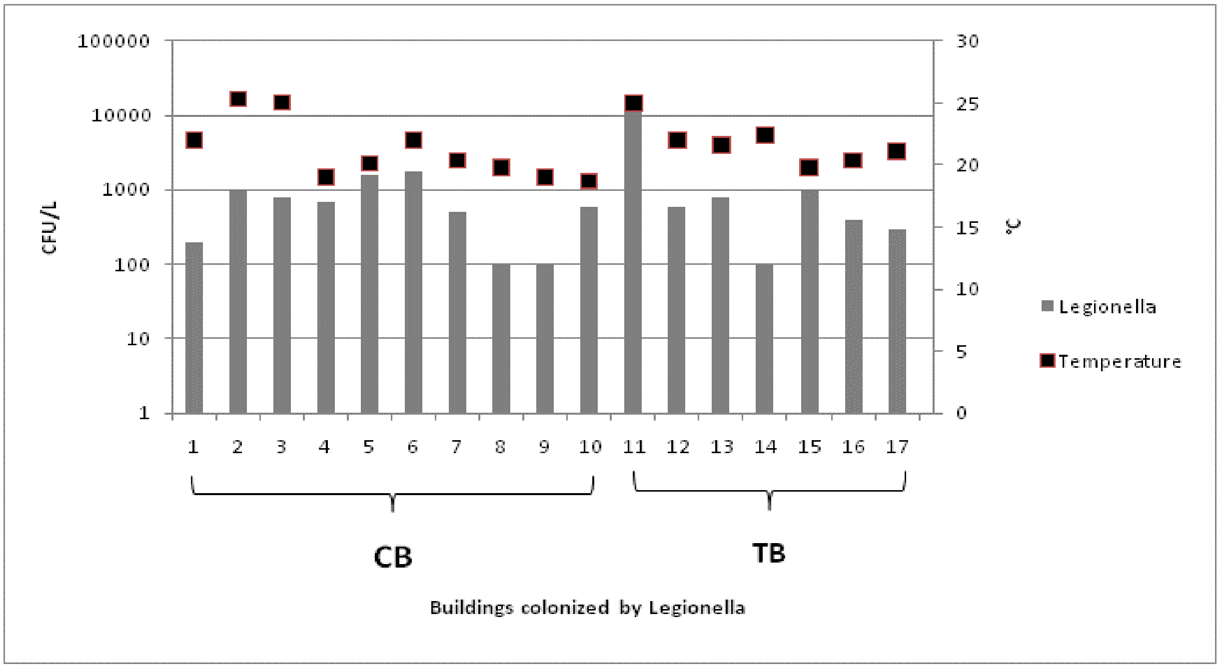

Figure 4: *Legionella* spp. counts and temperature values detected in 17 cold water systems (CB and TB).

Discussion

Although literature data regarding the risk of *Legionella* spp. in community settings (residential buildings, touristic and sport facilities, ect) increased during the last three years, epidemiological reports regarding the incidence of Legionnaires' disease in Italy and Europe have not changed during the same period [16, 17, 18, 19].

Furthermore, in our previous studies [9, 10], we highlighted the need of water safety plans applied to residential buildings, aimed to manage the underestimated *Legionella* spp. risk in these facilities.

Moreover, we described the responsibility of the building administrators in ensuring the water hygienic control from the point of delivery by the water supplier up to the points of use.

After two years from our latest published manuscript, these updated data highlight the colonization by *Legionella* spp. in 26% and in 11% of the hot and cold water samples, respectively.

Considering all the 375 investigated water networks, we observed a higher incidence of colonization in CB and TB (40%) compared to IB (20%).

This may be due to the lack of maintenance activities, often highlighted during the inspections. In fact, in all the TB we observed absence of disinfections and low temperature values (usually less then 40°C).

Moreover, in CB, despite we detected chlorine presence and a mean temperature value of 43.4°C in hot water samples, the high rate of *Legionella* spp. presence may be due to the discontinuous activity of electric boilers inside the flats, which may cause a progressive water heating and cooling, as described elsewhere [20].

Another issue evidenced by this study is the high temperature of cold water (higher than 20°C in several cases), which may represent an optimum condition for *Legionella* spp. growth [21, 22].

All these drawbacks are considered risk factors for *Legionella* spp. proliferation in water plants, as described by Italian Guidelines for Legionnaires' disease prevention and control [12].

In fact, in almost all the type of water plants, we detected correlations between the increase of *Legionella* spp. counts and the reduction of total chlorine concentration. Further correlations were obtained between the increase of *Legionella* spp. concentration and the decrease and increase of temperature in hot and cold water samples, respectively.

As previously described [9], we confirm higher microbial counts in water samples collected at Point T. Although microbiological potability of cold water was almost always observed, a statistically significant increase ($p<0.0001$) in total microbial counts at 22 and 37°C between the samples collected at Point I and the ones collected at Point T was observed. This evidence may be due to biofilm formation in pipeworks, as described elsewhere [4, 23, 24].

Conclusions

In conclusion, our work confirms *Legionella* spp. risk in residential buildings of Pisa district (Italy). Considering the epidemiological data regarding the incidence of Legionnaires' disease in Italy, in a five-years survey we assert the need of water safety plans for these type of facilities, highlighting the responsibility of building administrators for appropriate hot and water hygienic control, through monitoring schemes of water networks, at least on a yearly-basis.

Through this study we may state the need of training programs for all the workers involved in residential water systems management (building administrators, plumbers). These may be the tools needed to disseminate the knowledges regarding Legionnaires' disease risk in community facilities improving public health safety.

Acknowledgments: The study was not funded.
Authors contributions: Angelo Baggiani and Gaetano Privitera conceived and designed the experiments. Michele Totaro, Beatrice Casini, Paola Valentini and Anna Laura Costa performed the experiments and wrote the paper. Lorenzo Frendo performed the water samplings.
Conflicts of interest: The authors declare that they have no competing interests.

 doi:10.20944/preprints201905.0186.v1

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