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Prevalence and distribution of *Legionella* in municipal drinking water supply systems in Madrid (Spain) and risk factors associated

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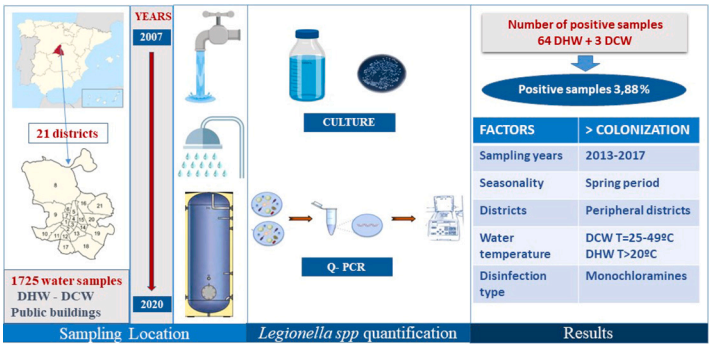
HIGHLIGHTS

- *Legionella* in Madrid drinking water distribution systems was evaluated for 14 years.
- In 96.1 % of the sampling points, *Legionella* was not detected during the study period.
- The greater presence was observed in older installations in peripheral districts.
- A seasonal distribution was found with the highest incidence in spring.
- The highest presence of *Legionella* was detected at water temperature between 25 and 49 °C.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence and concentration of *Legionella* in drinking water supply systems, in hot water (DHW) for human consumption in public buildings in Madrid with potential health risk was studied. Sampling covered a total of 1695 DHW samples and 30 cold water (DCW) as a control taken in the 21 districts of the city over a period of 14 years (2007–2020). The detection and quantification of *Legionella* was carried out by plate culture and quantitative qPCR. The study evaluated a series of variables including sampling year, districts, type of building, seasonality, sampling points (taps, tanks and showers), water temperature and type of disinfection used. The degree of compliance of *Legionella* in the water supply network of Madrid was very high (96.1 %). The degree of colonization of the positive samples ranged from 0.3×10^3 and 1.5×10^5 GU/L for a 97 % of the samples. A higher presence of this bacterium was detected in older facilities in the peripheral districts and end points able to produce aerosols such as showers. The highest number of samples with *Legionella* growth occurred in the 35–40 °C range. The strategies implemented have contributed to a remarkable decrease in the presence of *Legionella* in the last years of sampling.

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1. Introduction

Legionella is a ubiquitous environmental microorganism, distributed worldwide, living in all natural and anthropogenic aquatic habitats and in the sediments of watercourses in a symbiotic or parasitic way with amoebae or ciliate protozoa, along with other micro-organisms: bacteria *(e. g. Pseudomonas, Acinetobacter, Flavobacterium, Alcaligenes)* and algae *(cyanobacteria),* within biofilms (Donlan, 2002).

From these reservoirs the bacteria can colonize different facilities such as water supply and distribution systems (pipes, accumulators and interiors of refrigeration towers), mainly in domestic hot water (DHW) systems (showers or taps) where the bacteria can survive and multiply in biofilms attached to the walls of pipes and water tanks, from which they obtain nutrients (Rowbotham, 1980).

Biofilms consist of well-organized micro colonies of amoebae (Rożej et al., 2015) which along with other microorganisms, extracellular proteins, minerals (iron, copper, and manganese) and other compounds. *Legionella* proliferates in vacuoles containing 20–1000 bacteria and could be the infectious particles for humans (Lau and Ashbolt, 2009).

Interactions between *Legionella* and biofilm amoebae are the main regulator of L. *pneumophila* population, proliferation and spread (Ji et al., 2018; Abu Khweek and Amer, 2018; Sciuto et al., 2021). Therefore, the presence of a major thickness biofilm in the pipes together with the favorable water temperature may induce the proliferation and virulence of *Legionella* up to human-infectious water concentrations, if after aerosolization, they are inhaled. This relationship is crucial in the pathogenesis and ecology of the bacterium. The invasion and multiplication in amoebae can be considered as a pre-adaptation to the invasion of the human immune system (in alveolar macrophages) increasing its virulence and causing serious public health problems (Donlan et al., 2005; Escoll et al., 2017; Dey et al., 2020).

As a result of inhalation exposure to this microorganism from water contaminated in water systems and devices, legionellosis, a mandatory reportable disease of environmental origin, with high morbidity and mortality, can occur (Fields et al., 2002; Burillo et al., 2017).

Despite all the measures to prevent and control legionellosis, given its environmental origin, it is still far from being eradicated (ECDC, 2017). It is distributed worldwide, with a predominance in the most developed countries, as they have the largest number of big buildings with complex water supply circuits and cooling systems such as towers, able to produce aerosols and consequently respiratory infections by inhalation of airborne droplets with a sufficient concentration of virulent bacteria (ASHRAE, 2015; ESGLI, 2017). In Europe, the countries with the highest number of legionellosis notifications were France, Germany, Italy and Spain, accounting for 72 % of European cases. Currently in our country the number of reported cases and the incidence rate of legionellosis have increased since 2015. This increase seems to be due to a combination of factors, such as the improvement of surveillance systems, the increase in average age and population movements, together with insufficient maintenance of water distribution systems (Viñuela-Martínez et al., 2022). In 2018, Madrid was the 5th city in terms of the number of cases in Spain.

The city of Madrid spreads over 21 districts. It has an extensive and complex drinking water supply network that reaches up to the water intakes of the city's buildings and supplies a dense population of residents (3.33 million) and tourists (7.84 million/year).

The distribution systems include storage tanks, pumping stations, and control valves always maintain water pressure and flow. There is also an extensive network of pipes conducting water to reach the final consumer. In particular, domestic hot water installations, such as showers, taps and water storage systems, can provide a suitable habitat for the survival and multiplication of *Legionella*, a scene that justifies the need to study this bacterium in this environment. A recent study has attributed 25 % of *Legionella* disease cases to residential drinking water (Buchholz et al., 2020).

As mentioned before, *Legionella* is airbonevia aerosols from drinking water distribution systems inside buildings under specific temperature conditions. Installations were classically classified according to the greater or lesser probability of risk of *Legionella* proliferation and dispersion. Higher risk facilities included refrigeration towers, DHW systems with storage and return circuit, spas, swimming pools, hydromassage bathtub and industrial humidification plants (Rivera et al., 2007). Lower risk facilities were domestic cold water for human consumption (DCW) and DHW storage systems without return and other facilities able to generate aerosols such as spraying irrigation systems, ornamental fountains and respiratory therapy equipment.

However, current Spanish legislation (Royal Decrete (RD) 487/2022) considers that all facilities can produce aerosols during their operation, service tests or maintenance and are considered as sources of risk to public health. Currently, DHW distribution systems are prioritized because they are the most frequently identified sources of *Legionella* infection, ahead of towers and other aerosol-producing water installations, at the same level and without ranking them as a greater or lesser risk to the health of the population. As a result the new Royal Decree 3/2023, which establishes the technical-sanitary criteria for the quality of drinking water, its control and supply, incorporates *Legionella* analysis as a mandatory control parameter.

In Spain, numerous studies have been conducted in cooling towers (Ordoñez-Iriarte et al., 2006; Ragull et al., 2007; Grúas et al., 2013; Cebrián et al., 2018), however, there are very limited studies in drinking water systems, which in most cases are restricted to hospital control (Rivera et al., 2007; Gavalda et al., 2019; Serrano-Suárez et al., 2013; Quero et al., 2021; Párraga-Niño et al., 2024). The present study is the first one carried on domestic hot water indoor facilities in the city of Madrid. And it is the first one that focuses mainly on sports centers in this City.

Regarding *Legionella* assays, plate culture is the only currently approved technique, but it needs >10 days to obtain results. However, it is interesting to propose a rapid test that reports the presence of *Legionella pneumophila* in water samples within hours that will improve the control of the colonization of this pathogen.

For all these reasons, this study focuses on evaluating the prevalence and distribution of *Legionella pneumophila* in the public hot water supply network in buildings in the city of Madrid, due to its greater relevance as a reservoir of this bacterium. The sampling was carried out over a period of 14 years, taking into account the problems of the different districts, type of building, seasonality, temperature and type of disinfection applied.

Furthermore, by covering such a long time period and looking at so many variables, we believe it is a pioneering, robust study and a starting point that provides valuable information for risk management decisions and for safeguarding water quality.

The aim is to gain a deeper understanding of the behavior of L. *pneumophila* in an urban environment and to establish effective control strategies adapted to the complex reality of water distribution in the city of Madrid to ensure the protection of consumers health.

2. Materials and methods

2.1. Sampling procedure

Prospective sampling of Madrid water supplied by Canal de Isabel II (CYII) in buildings of public health interest such as sports centers (1073 samples), hotels (256), public bodies (130), hostels (106), and schools, bars and hospitals (32). was carried out to analyze *Legionella.* This was a total of 1725 samples over a period of 14 years: 2007 to 2020 in the 21 different districts of Madrid (Fig. 1).

Water samples for *Legionella* detection were taken in the domestic distribution systems of the buildings, at points of highest proliferation risk or at representative points of systemic exposure, or both. Sampling in water tanks and at the end points of the network with diffusers that can produce aerosols (showers, taps or tanq) was emphasized. Almost all

Fig. 1. Districts of the city of Madrid.

the sampled domestic water systems comprise basically hot water distribution networks.

In the domestic hot water (DHW) network: 1695 water samples were taken, while in the domestic cold water network (DCW): 30 representative samples were collected in the same points of the system when a positive result was obtained from DHW, and were used as a control.

Two samples were collected in each sampling point. One of this was used for analysis of *Legionella* by the culture method and the other was set for q-PCR technique análisis. Samples were collected in clean and sterilized 1 L glass bottles with sodium tiosulphate to neutralize the chlorine and hermetic seal and easy opening. The samples were collected after overnight stagnation (at least 6 h). A volume of 1 L was collected, so that 100 mL was taken first and then the tap or shower was scraped with a sterile swab that was incorporated into the same bottle and then the rest of the water was collected until the 1 L was complete, dragging the remains of the scraping, to incorporate the biofilm according to ISO 11731 (1998).

Other parameters were recorded in situ, time of sampling, temperature of samples collected and presence of biocides (chlorine, etc.). The temperature was measured immediately after sample collection and the maximum achievable temperature at the sampling point was also documented according to RD 487/2022 (>60 °C in the water tank, $T >$ 55 °C in the return network and $T >$ 50 °C in showers or taps). The bottles were identified, sealed and the origin of the water and the exact sampling point were recorded.

Samples were taken to the laboratory for analysis protected from heat and sunlight. DHW samples were kept at room temperature directly after sampling. Samples with biocides were analyzed within 24 h of collection to avoid inhibition.

2.2. Methodology

Legionella analysis in the water samples was performed at the Public Health Laboratory of Madrid Salud accredited by ENAC (n° 215/LE 406 and n° 215/LE 1915) that recognizes its technical competence and the validity of its results. The procedures were validated and standardized according to ISO 17025 (2017). Two official techniques were applied as reference method and alternative method approved in RD 865/2022, respectively: counting by culture and detection by quantitative polymerase chain reaction (qPCR).

2.2.1. *Legionella* isolation, detection and quantification by culture

For *Legionella* growth and quantification, 1 L of water was centrifuged (10,000 rpm/5 min) and then resuspended and diluted 10-fold in water with peptone. Subsequently, 50 μL were seeded on plates with specific BCYEα (Buffered Charcoal Yeast Extract) medium with α-ketoglutarate and supplemented with amino acids such as L-cysteine and iron in the form of ferric pyrophosphate. To increase the selectivity of the culture, GVPC medium containing an amino acid (glycine) and antibiotics (vancomycin, polymyxin B and cycloheximide) was used. Incubation was then carried out at 35–37 °C, in aerobiosis with CO_2 (2.5–5 %). *Legionella* is visible from the 3rd day of incubation, although cultures must be kept for 10–12 days before they can be considered negative. (ISO 11731, 1998).

2.2.2. *Legionella* detection and quantification by qPCR

This detection technique combines DNA amplification with simultaneous gene quantification, measured by fluorescence emission. The water samples were analyzed according to a protocol based on the publication of Yáñez et al. (2005) modified and adapted to the updated ISO 12869, 2017.

The procedure followed to determine *Legionella* in the drinking water samples consists of a series of steps described below:

2.2.2.1. Isolation of Legionella from water samples. 1 L water samples were filtered using an automatic vacuum system through a 0.45 μm polycarbonate membrane. Total bacterial cells free of contaminants were retained on the membrane and recovered by resuspension in deionized water, followed by shaking and centrifugation (9000 rpm/3 min).

2.2.2.1.1. DNA extraction. An aliquot of water from the filtrate containing the *Legionella* cells (5 mL) were collected and resuspended in 200 μL of Chelex 20 % resin (Bio-Rad Laboratories, Richmond, CA) in order to avoid sample inhibitors. For DNA extraction, 180 μL of extraction buffer (VK SB) and 25 μL of reconstituted proteinase K were added and incubated at 56 °C/1 h after shaking. Finally, it was centrifuged at 13000 rpm/10 min and the supernatant with the DNA was collected and transferred to an Eppendorf tube where the reagents for amplification were added (Macherey Nagel, Düren, Germany).

2.2.2.1.2. DNA amplification and quantification. Amplification reactions were performed in optical microplates using a commercial kit for the detection and quantification of *Legionella* (Applied Biosystems, Foster City, CA, USA) that provides the appropriate reagents in a total volume of 50 μL. The kit contains a TaqMan universal PCR master mix, a primer set for specific of *L. pneumophila* dotAF (ATTGTCTCGCGCGATTGC) and dotAR (CCGGATCATTATTAACCATCACC) reported by Yáñez et al. (2005), the buffer contains $MgCl_2$, dNTPs, AmpliTaq Gold DNA polymerase, 6-carboxy-x-rhodamine (ROX), uracil N-glycosylase (UNG) essential to avoid any contamination and TaqMan Minor Grove Binding (MGB) labeled with 6-carboxy fluorescein (FAM) probes for specific detection of *L. pneumophila* The kit also provides an internal inhibition control (gyrB recombinant plasmid from *Aeromonas hydrophila* DNA), as well as the appropriate positive and negative controls.

The amplification reaction was performed in the thermal cycler, ABI Prism 7500 (Thermofisher Diagnostic) programmed with the following thermal conditions: 50 °C/2 min for UNG activation, 95 °C/10 min for Taq Gold Polymerase activation, followed by 42 cycles at 95 °C/15 s and 60 °C/1 min for hybridization and elongation.

The ABI Prism 7500 analyzer measures the fluorescence emission whose exponential increase is proportional to the amount of DNA present in the water samples. The reaction can be followed in real time, by exponential increase of the fluorescence of the sample, obtaining threshold cycles (Ct) that are inversely related to the concentration of *Legionella* in the sample expressed in genomic units. The Ct number is considered positive if it is between 25 and 40 cycles. To determine the DNA concentration in the samples as a function of Ct, a standard

calibration curve is prepared with serial dilutions of known DNA concentrations of pure *L pneumophila* cultures. The standard curve correlation coefficient was $R^2 \geq 0.980$. The validation tests of the *Legionella* analysis by the qPCR method (LOD, LOQ, accuracy, precision, reproducibility) were regularly checked through an internal control protocol throughout the study period by the Laboratory according to ISO 17025, 2017.

2.3. Statistical analysis

The variables analyzed correspond to two types: categorical (qualitative) variables and non-categorical (quantitative) variables. In all cases, the frequency and individualized percentage for each variable was calculated, as well as the degree of compliance with respect to the prevalence of *Legionella.*

To analyze the presence or absence of this bacterium in relation to the different categorical variables, Pearson's Chi-square test was applied. Spearman's correlation analysis was also used between the *Legionella*-positive samples and the factors of influence studied with a significance of $p < 0.05$.

3. Results and discussion

To assess the presence and prevalence of *Legionella* at critical points in the sanitary water distribution systems of various buildings in Madrid and their risk factors some variables were evaluated. The variables considered include year, type of building, season, district of Madrid, type of water disinfection, water temperature and sampling point.

The present study has been carried out over 14 years in the period 2007–2020, on a total of 1725 sample DHW sampling, used as a control, was the majority with a number of 1695 samples, which represents 98 % of the total. DCW samples were a total of 30 over the entire study period (2 % of the total).

3.1. Legionella detection during the study period

Legionella pneumophila was isolated by the two methods applied in 67 sampling points, 64 of them in DHW systems, representing 3.8 % of the DHW samples. Of the DCW samples, used as a control, only 3 were positive for *Legionella,* which corresponds to 10 % of the total of this type of samples.

The level of contamination in the cultures ranged from 10^2 to 10^5 CFU/L in final consumption points (showers and DHW taps) and from 2×10^2 to 5×10^3 CFU/L in intermediate points (accumulators and central systems). The DHW samples were those that reached the highest contamination levels and were mainly located in buildings at risk such as sports centers where values of 10^3–10^5 CFU/L were always found. A total of 22.4 % of the samples presented values $\geq 100 < 1000$ CFU/L and a very high percentage of the samples presented levels ≥ 1000 CFU/L, which led to corrective measures being taken in accordance with the stipulations of RD 487/2022.

The distribution of the degree of colonization by this bacterium expressed in GU/L is shown in Fig. 2. The results obtained show that the non-compliant samples were detected in the range of 10^3 and 10^5 GU/L. 56.3 % of the non-compliant samples have low colonization, in the range of 0.7 and 0.1×10^3 GU/L. However, 28.1 % of the non-compliant samples have a higher concentration in the range of 0.7 and 0.3×10^5 GU/L and 15.6 % of the positive samples are at a higher detection level between 3.1 and 1.5×10^5 GU/L.

Given the rapidity of detection of *Legionella* in water distribution systems by the q-PCR method compared to the culture method, we suggest that it would be convenient to implement the q-PCR method as an additional routine method for the inspection and control of this type of samples.

In the majority of the *Legionella*-positive samples, the most significant locations were found to be in the biofilm in the return circuits and adhering to the seals and three-way valves, as well as in the accumulators, mainly on the bottom and on surfaces with obvious signs of corrosion.

3.2. Analysis of the distribution of Legionella according to sampling years

The annual case distribution for all samples (DHW and DCW) is detailed in Table 1. It shows that sampling has not been homogeneous, as it is a regulatory sampling, according to Madrid water quality sanitary plan. Thus, in the first 6 years the number of samples was higher, ranging between 143 and 185 samples, while in the period 2013–2020, the number of samples was close to or <100, ranging between 65 and 101 total samples.

The degree of compliance was 96.1 % throughout the study period, although some differences can be observed. There was a lower percentage of positives between 2007 and 2012, coinciding with a higher number of samples analyzed and lower sampling selectivity. From 2013 to 2017, there was an increase with maximum levels in 2014, 2015 and

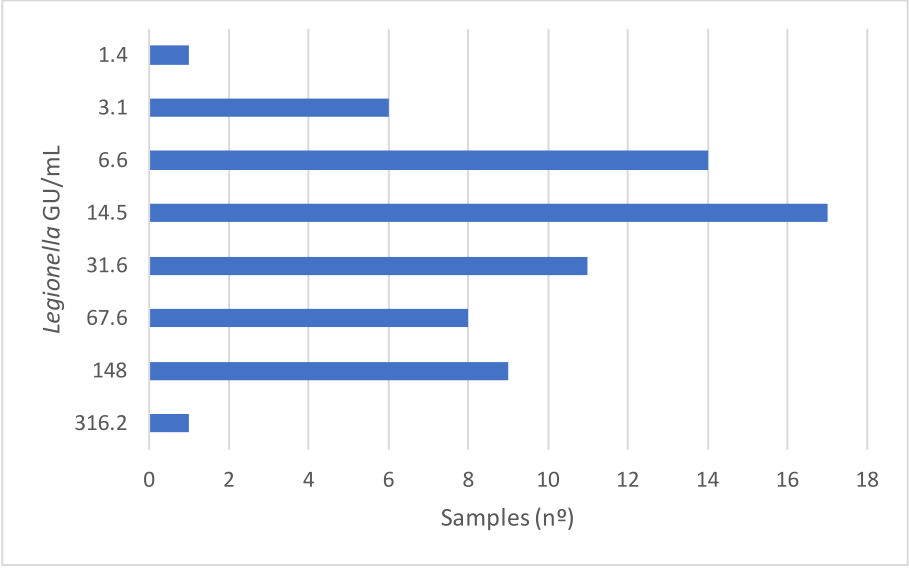

Fig. 2. Positive samples: distribution of the degree of colonization by *Legionella* by qPCR (GU/mL).

Table 1
Distribution of sampling and detection of *Legionella* in the study period.

Year	Total samples/year		Positive samples	
	N°	%	N°	%
2007	185	10.7	7	3.8
2008	272	15.8	5	1.8
2009	167	9.7	1	0.6
2010	184	10.7	5	2.7
2011	143	8.3	1	0.7
2012	152	8.8	2	1.3
2013	101	5.9	7	6.9
2014	74	4.3	8	10.8
2015	69	4.0	12	17.4
2016	75	4.3	6	8.0
2017	65	3.8	10	15.4
2018	74	4.3	1	1.4
2019	82	4.8	1	1.2
2020	73	4.2	0	0.0

2017 (11–17 % detected) attributable to the greater selectivity in sampling, as an inspection criteria based on the risk factors of the facilities was followed. On the other hand, in the last 3 years, a lower incidence was detected with levels of 1.4 %, 1.6 % and even 0 % in 2020. This decrease is probably due to the effective strategy followed to minimize its presence, which will be discussed below, and it is consistent with the decrease in cases of legionellosis reported by the Carlos III Institute in the Community of Madrid in recent years (ISCIII, 2021).

Regarding DCW, the 3 non-compliant samples were found in the years 2015 (2) and 2016 (1), a period that corresponds with the highest overall non-compliance for *Legionella*, as mentioned above.

3.3. Presence of Legionella according to building type

When the presence of the bacteria in the different buildings of the city of Madrid was evaluated, it was found that the sports centers have the highest incidence, as shown in Table 2, when compared with the other type of buildings ($p < 0.001$) Although to a lesser extent, the percentages detected in public bodies and educational centers are also noteworthy.

In the control of *Legionella* in sports centers, a percentage of 5.31 % of *Legionella* positives was found, with a higher incidence in the years 2013 to 2016 with a seasonal component. One of the centers in D17 in which the presence of this bacterium was repeatedly detected in the DHW installation stood out, because once *Legionella* colonizes an installation it is very difficult to eradicate because of the insidiousness of the biofilm, especially when the installation is old. In this case, preventive measures were immediately adopted consisting of closing the showers of the DHW circuit to avoid the dissemination of contaminated aerosols to the rest of the users of the sports center, as well as cleaning and disinfection and total renovation of the water circuit.

Following the indications of the Technical Guide for the prevention and control of legionellosis in facilities of the Spanish Ministry of Health (MS, 2006), the structural risk (SR) referring to the characteristics of the facility itself, the operational risk (OR) associated with the operation and maintenance risk (MR) referring to the treatments carried out for this purpose were calculated. Based on these results, the overall index was evaluated.

Table 2
Detection of *Legionella* in the different types of buildings studied.

Buildind type	Number of samples		Non detected		Detected	
	N°	%	N°	%	N°	%
Hostels	106	6,25	106	100	0	0
Bar	13	0,77	13	100	0	0
Schools	42	2,48	42	97,61	0	0
Fountains	44	2,6	44	100	0	0
Hotels	255	15,04	253	99,22	2	0,78
Hospitals	32	1,89	32	100	0	0
Public Bodies	130	7,67	125	96,15	5	3,85
Sport centers	1073	63,3	1016	94,69	57	5,31
Total	1695	100	1631		64	

Two of the sports centres, both located in D17, one of them mentioned above, had a very high overall rating, which required a complete overhaul of their DHW network. In these cases, as they were very old installations, they were completely renovated and the accumulator elements and sections of pipes in poor condition were replaced in their entirety.

The poor effect of the disinfection methods found in these two centres could be partly due to having several areas of piping where water could stagnate and faulty valves in taps and showers can cause widespread temperature losses due to the mixing of hot and cold water and consequently the development of biofilm.

Improved disinfection in these sports centres, based on chlorine level control, along with other measures such as regular draining of water from the networks, regular flushing of accumulators and partial removal of corroded pipe sections, succeeded in reducing the levels of this bacterium.

In recent years, the presence of *Legionella* in sports centers has been decreasing and can be attributed to the strategies followed for prevention. Thus, this fact can be mainly related to the gradual increase of set point temperatures mainly of end points (>55 °C) and accumulators (>65 °C) in these facilities. Thus, studies of *Legionella* occurrence in sport centers which until now have not been monitored in Madrid, may elucidate household factors which may promote *Legionella* growth and evaluate actions which can be taken to reduce risks of *Legionella* growth in this environments such as increasing hot water temperatures.

Colonization by *Legionella* has also been detected in public bodies, although in a much lower proportion, only 5 cases corresponding to 3.85 % of the total sampled for this type of building. Two of the cases were detected in 2010 in the district of Fuencarral-El Pardo (D8) and the rest were found in the years 2017 to 2019 in the district of Retiro (D3). It is noteworthy that in all samples the temperature was at the optimum for *Legionella* growth.

In hotels, two positive cases were detected in districts belonging to the central area, one of them in 2008, corresponding to a sample from a water tank with signs of corrosion, poorly sized, with little demand for use, which caused thermal stratification and therefore the growth of *Legionella.* The other was detected in 2017, it was in a corroded shower of the staff locker room located in a basement, with little maintenance and low use. In both cases, the set point temperature was lower than regulation and the water quality was poor.

In the rest of the studied buildings (hostels or residences, senior centers, bars and restaurants, drinking fountains and hospitals) no positive samples were found.

Also, it is important to comment the absence of *Legionella* in the samples from hospitals. In contrast to the data from our work, the studies in hospitals carried out by different authors (Kruse et al., 2016; Barna et al., 2016; Napoli et al., 2019) highlight a high incidence in this type of building.

In the most prominent studies on prevalence of *Legionella* in drinking water, carried out in different European countries, it can be observed that they focus on different types of buildings: hotels, hospitals, public buildings, schools and homes, in which different levels of detection are obtained. The more moderate data are found in homes (5–38 %) whole they are higher in hotels (49–75 %) and hospitals (34–92 %), although a wide spectrum of ranges is denoted, in any case. (Napoli et al., 2019, Barna et al., 2016; Mouchtouri and Rudge, 2015; Valcina et al., 2016; Kruse et al., 2016; De Filippis et al., 2018; Dilger et al., 2018, Collins et al., 2017, Kyritsi et al., 2018).

In relation to the data on *Legionella* in sports centers, the low prevalence found in gyms and locker rooms by Schiavano et al. (2021) compared to those found in the present work is remarkable. However, De Filippis et al. (2017) in showers or taps located in locker rooms of

sports centers detected an incidence of *Legionella* of 41.2 %.

In general, in the urban environment, the disease is usually associated with poor building designs, inappropriate construction and negligent maintenance of water facilities.

3.4. Legionella control according to seasonality

Environmental conditions influence water quality and the presence of *Legionella*. The sampling in this study was not homogeneous in the different months of the year. There was a decrease in August and December as they coincide with holiday periods and a greater number of maintenance checks of the entire network.

The results from the DHW samples tested have a clearly seasonal prevalence. The distribution shows higher and significant ($p < 0.05$) percentages of *Legionella* detection in the spring period (40 %) compared to the other seasons. In DCW samples, two of the three positive samples were taken in spring and the third in autumn.

A detailed analysis by month showed a higher number of positive samples for *Legionella* in the quarter of April, May and June, when the ambient temperature was high and the supply water entering the distribution system frequently exceeds 20 °C, which is the consensus temperature of the drinking water entering the system. A very high rate was also detected in October, in this case attributable to the reopening of courses in the sports centers. It is significant that there were no cases in July, which could be due in part to the opening of the summer swimming pools, coinciding with the decrease in the use of indoor swimming pools in sport centers, and the lower use of DHW in showers in all the facilities.

3.5. Presence of Legionella according to the district of Madrid

A comparative study was carried out comparing the districts of the periphery and those of the central core of Madrid in relation to positive cases of *Legionella*. The sample size was much higher in peripheral districts ($n = 1132$) than in central districts ($n = 593$). In DHW, a higher prevalence was found in peripheral districts (4.32 %) compared to central area (2.74 %).

In DCW, the 3 positive samples were detected in peripheral districts D13, D17 and D18. The positive sample in D-13 was taken due to the detection of a case of Legionellosis in personnel working in the building. The positive samples in D17 and D18 were taken due to the detection of *Legionella* in the hot water system of the same buildings.

Table 3 shows a high incidence of cases in the peripheral districts of D17 Villaverde (15.8 %), D10 Latina (7 %), D18 Villa de Vallecas (5.9 %), D9 Moncloa-Aravaca (5.4 %), and D8 Fuencarral-El Pardo (3 %) and the presence of *Legionella* in some central districts: D7 Chamberí (6.1 %), D4 Salamanca (5.9 %) D2 Arganzuela (4.3 %) and D3 Retiro (3.6 %). The high incidence in districts D17, D10 and D18 is due to the age and poor upkeep of their facilities, which is why structural and maintenance reforms were carried out. Regarding the incidence in districts D8 and D9, the percentages of positives respond to the more disaggregated population, with more extensive distribution networks, so that a loss of water temperature is more easily caused, which along with the oversizing of facilities, produces the consequent stagnation of water. In relation to the central districts, the reasons for colonization by *Legionella* are due to the age of the buildings, poor maintenance, the complexity of the installations with the presence of corrosion in the pipes, even with the shorter water flow. In these districts, the material was subsequently changed to polyethylene, by means of a major structural reform, as evidenced by the reduction of cases over the years.

Table 3
Detected cases of *Legionella* in DHW systems in Madrid: Distribution by district.

Distrit	Samples		Detected cases	
	N°	%	N°	%
1	100	5.9	1	1
2	90	5.31	4	4.44
3	136	8.02	5	3.68
4	65	3.83	4	6.15
5	66	3.89	0	0
6	93	5.49	0	0
7	33	1.95	2	6.06
8	97	5.72	3	3.09
9	148	8.73	8	5.41
10	122	7.2	9	7.38
11	71	4.19	0	0
12	85	5.01	2	2.35
13	102	6.02	3	2.94
14	43	2.54	0	0
15	42	2.48	0	0
16	49	2.89	0	0
17	136	8.02	21	15.44
18	50	2.95	2	4
19	38	2.24	0	0
20	54	3.19	0	0
21	75	4.42	0	0

In view of the obtained results, it can be deduced that other factors such as the year and complexity of the installations in the buildings sampled or the size of the networks in the sampling area have greater effect on Legionella proliferation. Similarly, Valcina et al. (2016) in their study carried out in Latvia, when comparing rural peripheral districts with central districts in Riga, concluded that the higher level of detection in the latter is justified by the complexity of the interior installation of buildings in the central areas of large cities.

3.6. Presence of Legionella depending on water temperature and sampling point

The water temperature in the indoor DHW installation must be above 60 °C measured in the storage tank, directly from the thermometer integrated in the tank, in the return network it must be at $T > 55$ °C and at the final sampling point (tap or shower) above 50 °C to limit the possibility of colonization by *Legionella*. The DHW facility must allow the water to reach a temperature of 70 °C in case a thermal disinfection treatment is required. Regarding the water temperature, the cold-water temperature should preferably be <20 °C.

The experimental data on the distribution of samples according to the different sampling temperature ranges are shown in Fig. 3, taking into account that the possible range of *Legionella* growth is very wide since it ranges from 25 to 49 °C. The highest number of samples with *Legionella* growth (24 %) occurred in the 35–40 °C range which is the temperature close to that of the human body and corresponds to the optimum T for Legionella growth. Regarding other temperature ranges, positive samples detected in the 30–35 °C and 40–49 °C ranges were significantly lower at around 12 % in both cases, and a percentage of 9 % were found at temperatures between 25 and 30 %. Finally, positive samples in hot water installations found at more extreme temperatures such as 20–25 °C and even 49–60 °C were similar between 3 and 4 % respectively.

In fact, as *Legionella* grows optimally in a range between 25 and 50 °C, the increase in water temperature above this value is essential to minimize the survival and multiplication of *Legionella* in indoor installations, although there are other factors of influence such as dirt, the presence of biofilm or corrosion, among others, which also condition its presence.

In relation to the different sampling points, Table 4 shows the distribution of *Legionella* detection according to the range of water temperature, with the results for taps, showers and tanks in particular. The temperature range of DHW samples ranged widely from a minimum of 15 °C to temperatures >60 °C. Most of the samples were taken in showers (96.2 %) because this is where aerosols are produced, which contribute to a higher risk of contracting the disease by users.

The results obtained for DHW samples from showers analyzed show that the degree of non-compliance with temperature was very high (82.6 %), as the temperature of most of the samples was between 15 and

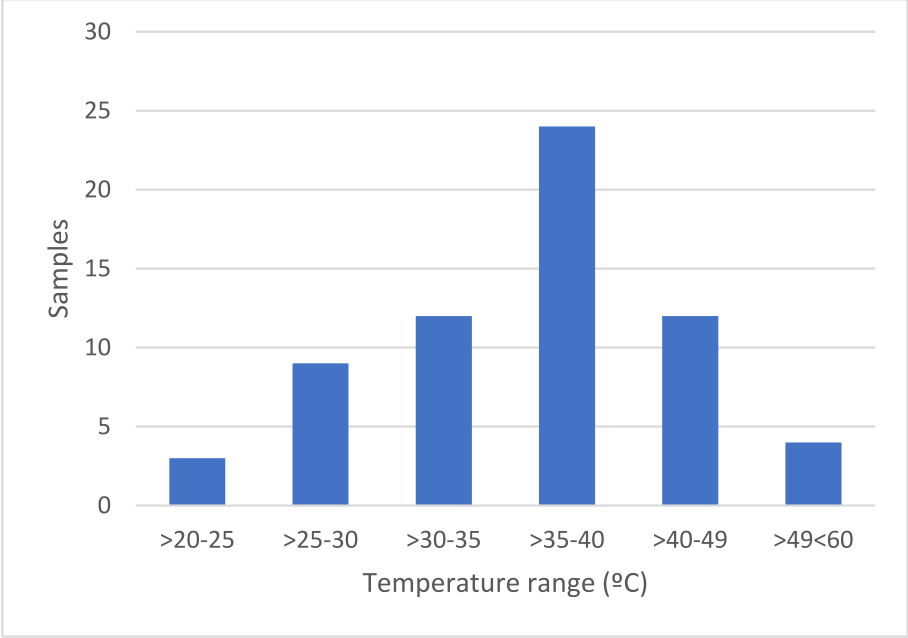

Fig. 3. Distribution of *Legionella* positive samples as a function of temperature.

Table 4
Distribution of *Legionella* detection as a function of water temperature in DHW samples.

Range °C	Taps		Showers		Tanks	
	Cases	Detected	Cases	Detected	Cases	Detected
15–25	3	0	44	5	2	0
25–49	13	0	1302	47	36	7
≥ 50 < 60	4	0	276	3	4	2
>60	0	0	8	0	3	0
Total	20	0	1630	55	45	9

49 °C. The level of *Legionella* positives for this sampling point was 3.4 %. In relation to the detection of *Legionella* at this sampling point, the majority was in the optimum growth range for this microorganism: 25–49 °C (85.4 %), followed by the lower temperature range of 15 to 25 °C (9.1 %). In the temperature range > 50–60 °C only 3 positive cases were detected, representing 5.4 % of the total.

These data agree with those found by De Filippis et al. (2018) in water distribution systems of Italian nursing homes and hotels, regarding the higher frequency of *Legionella* positives when samples reached $T \geq 30$ °C compared to those with $T < 30$ °C.

As the aerosol particle size of 1–8 μm in diameter is small enough to penetrate the lower human respiratory system, changing shower heads with a larger droplet output and lower aerosolization has been found to decrease the possibility that showers and taps in the sampled buildings may be implicated as a means of *Legionella* transmission from drinking water (Schoen and Ashbolt, 2011; Prussin et al., 2017).

In relation to water tanks, the percentage of non-compliance with respect to temperature was 93.3 % and the level of *Legionella* positives was 20 %, with samples with temperatures in the range of 20–49 °C contributing the most, as expected. However, 2 positive samples were detected at $T > 50$ °C, both taken in storage tanks for solar panel systems with a high degree of thermal stratification. The importance of the presence of *Legionella* in tanks is crucial for the study of the indoor installation because it means a greater dissemination of this bacterium to the rest of the pipes of the DHW networks and to the end points.

On the other hand, samples taken at the tap were the fewest (1.2 %). The percentage of non-compliance in relation to temperature was 80 %, although *Legionella* was not detected in any case, probably due to its greater frequency of use.

The results obtained for positive samples of *Legionella* in both showers and tanks indicate that reaching the set point temperature > 50 °C and >60 °C in each of these points of the installations helps to prevent its presence in them.

Although the optimum T for growth is between 25 and 49 °C, a small proportion of the positive samples (7.8 %) have been isolated in a higher range (50 > 60 °C), in agreement with those reported by the authors. Dilger et al. (2018) identified *Legionella* in water at a temperature range between 50 °C and 59 °C. The authors showed that the risk of isolating *Legionella* at a terminal point is 2.46 times higher when the temperature is <55 °C compared to >55 °C. In this sense, the study by Gavalda et al. (2019) corroborates the need to increase the endpoint DHW temperature in order to minimize the risk of this bacterium in installations.

In addition, frequency of use must be considered as a key factor for the establishment of *Legionella.* Thus, according to the study by Collins et al. (2017) carried out in 82 showers in homes in the south of England, *Legionella* was detected in 8 % of the samples, with positivity being mainly associated with lower frequency of use, cleanliness of the shower head and with the age of the building and the shower itself. Dilger et al. (2018) reported detection levels 2.84 times higher when the frequency of use of the endpoints is occasional compared to daily use.

The DCW samples were also taken mainly from showers (87 %), but also from some taps (10 %) and a tank (3 %) in different properties. Of the 3 positive samples, two were taken from taps and the third from a tank. It should be noted that in all of them the temperature exceeded the regulatory threshold ($T \geq 20$ °C), which favors colonization by *Legionella*. The *Legionella* detected in the DCW networks were found fixed in the biofilm on taps that were not used much, in tanks with corroded valves or joints and inside corroded pipes. In this sense, the research by Valcina et al. (2019) on biofilm in drinking water supply systems supports the lower presence of *Legionella* in cold water samples with an average of 12.5 % versus 54 % in hot water.

We have found that dirty and poorly maintained showerheads and taps in both hot and cold water deposits containing *L. pneumophila* In this cases, several amounts of the microorganism could be aerosolized during routine use resulting in a serious public health hazard.

3.7. Legionella control as a function of disinfection type

A comparison of the detected *Legionella* positives has been made according to the type of biocide used in the sampled water: chloramines or free chlorine (Table 5).

Disinfection using chloramines or chloramination is commonly used by CYII in Madrid in drinking water treatment plants (DWTP). Monochloramines are formed by adding ammonia to water containing free residual chlorine. Monochloramines are therefore found at the inlet to the water distribution system in 81.5 % of the sampled buildings.

In cold water tanks, cisterns, or cisterns of large water installations such as those in hotels, hospitals or sports centers that are supplied with CYII chloramines, preventive chlorination is carried out. The control of this treatment is very important, as the level of total free chlorine added to the installation must be sufficient to overcome the break point of the residual combined chlorine and prevent the growth of *Legionella*.

In the study in the DHW samples analyzed, the application of chloramines was very extensive (82 %), and only the remaining 18 % were disinfected with other biocides: chlorine or different hypochlorites, from automated systems (automatic chlorinators located in intermediate tanks upstream or downstream of the DHW storage tanks). In the DCW samples, the application of chloramines accounted for 43 % and chlorine or hypochlorite was applied to a greater extent (57 %).

In all the samples, the levels of both combined residual chlorine and free residual chlorine obtained in the inspections were always within the range of 0.2–0.4 mg/L combined residual chlorine and 0.2 mg/L free residual chlorine, in accordance with the legislated Paramentric Value (2 mg/L and 1 mg/L) respectively, so that no corrective measures had to be applied (RD 3/2023).

In the *Legionella* positive DHW samples the type of biocide associated was monochloramines from CYII and there was no additional chlorination of the water. These cases were mostly from DHW samples where the temperature was <50 °C. However, in DCW one positive was detected in a chlorinated sample, where inadequate cleaning and maintenance of the sampled installation was observed. In drinking water distribution systems, inactivation of *Legionella* in the biofilm by different disinfectants is costly because the free-living amoeba cysts in the biofilm can be carriers of *Legionella*, which is able to survive inside the biofilm and resist disinfection treatments.

For water systems preservation, chlorination is carried out with automatic continuous chlorinators to always obtain a free chlorine concentration < 1 mg/L, whereas chloramination produces monochloramines which are much more stable and do not dissipate before reaching the consumers. Disinfection with chlorine is highly efficient and inexpensive. Chlorine reduces and controls populations of this bacterium in outbreaks, if residual concentrations are maintained, although it does not completely eradicate *Legionella* from the system. In this sense, Amado-González et al. (2019) alluded to the advantages of chloramination compared to chlorination. The disadvantage is that it can lead to the growth of mycobacteria and can attack rubber and plastic components of the pipes.

Valcina et al. (2016) considered that to continuously control this microorganism, much higher chlorine concentrations than those typically found in drinking water are needed. When hyperchlorination is performed in emergency situations, in cases of outbreaks the treatment is effective in eliminating the bacteria, with the advantage that the residual free chlorine can be removed by exposure to sunlight for about 4 h.

In the study by Buse et al. (2019), the efficacy of two common disinfectants, free chlorine and monochloramine, was evaluated on mature *Legionella pneumophila* colonizing drinking water biofilms established on copper and PVC surfaces. The results showed that inactivation depended on the type of disinfectant and the biofilm substrate, with chloramine being more effective in inactivating *Legionella* present in biofilms on copper surfaces and free chlorine causing faster inactivation of the bacteria on PVC surfaces.

Table 5
Relationship between the biocide used in water disinfection and the presence of *Legionella*.

	Biocide	Sample		Legionella negative		Legionella positive	
		N°	%	N°	%	N°	%
DHW	Chlorine/hypochlorite	313	18	313	100	0	0
	Monochloramines	1382	82	1318	95	64	5
DCW	Chlorine/hypochlorite	17	57	16	94	1	6
	Monochloramines	13	43	11	85	2	15
TOTAL		1725	100	1658	96	67	4

3.8. Strategies for Legionella control

The most effective strategy used over the years, in indoor installations in public buildings to eradicate *Legionella,* was to carry out measures based on the control and monitoring of water quality at all critical points in the systems, in accordance with a sanitary plan to reduce the potential risk to people's health.

The increase in temperature to 70–80 °C in accumulation was also promoted by ensuring that this water was recirculated under these conditions throughout the distribution system for two or three consecutive days to guarantee that the temperature at the furthest points was not <60 °C. In these cases, in addition to a thermal shock or heat treatment depending on the characteristics of the pipelines, continuous chlorine disinfection was carried out using chlorine dosing units equipped with alarm systems. In the buildings with the highest risk in DHW systems, an increase of 5 °C was applied above what is set by legislation for the end points of the installations and for the storage tanks, and the frequency of use was increased to avoid stagnant water in the most distal end sections.

On the other hand, it is essential to clean, maintain and check the materials of tanks and pipes to limit the formation of biofilm and corrosion, which play a crucial role in the survival and propagation of the bacteria in the installations.

To minimize the effect of heat loss of water in long networks and to eradicate *Legionella* at the end points of taps and showers, the correct sizing of networks was checked. Pumps and return networks were installed to increase the recirculation of water and the water was insulated by means of thermal shells. In relation to the design of the water storage tanks, it was proposed that their capacity should be reconsidered to allow the daily and total renewal of the water inside them and with better access to the far end for monitoring purposes.

In the case of sports centres, the actions were aimed at reducing the structural risk in the facilities, with the replacement of deteriorated elements and reducing the maintenance risk, by means of the following actions: Increasing the DHW temperature in accumulation to >60 °C, in the return network to above 55 °C and at terminal points to above 50 °C. Controlling and assessing of the hygienic state with microbiological control after maintenance of the installation, increasing the frequency of cleaning and disinfection and reviewing of the structural condition and renovation of altered elements, as well as replacement of shower diffusers with larger pore widths to allow thicker water droplet jets.

4. Conclusions

The degree of compliance of *Legionella* in the water supply network of Madrid during the period studied was very high, although a greater presence of the bacteria was observed in older installations in peripheral districts, and it presented a seasonal distribution with a higher incidence in spring and autumn. Regarding the type of building sampled, the highest incidence has occurred in sports centers compared to hotels or public centers, with very few cases detected in the rest of the buildings sampled. The positive cases are associated with non-efficient designs,

construction of large networks of indoor DHW installations with areas of low use and negligent maintenance of water installations. The degree of non-compliance with respect to the set point temperature was very high and the highest presence of *Legionella* was detected at water temperatures between 35 and 40 °C. The presence of *Legionella* was found in the samples taken in the most distal and final networks of the DHW circuit with little use, in the return circuit and fixed on the inner surface in the biofilm. 97 % of the positive samples were found in showers and in bottom drain taps with obvious signs of corrosion. The strategies followed to minimize its presence based on temperature control, network sizing, maintenance, cleaning and disinfection have proved to be very effective in achieving the eradication of this bacterium.

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CRediT authorship contribution statement

María Concepción Almonacid Garrido: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **María José Villanueva-Suárez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Data curation, Conceptualization. **María Jesús Montes Martín:** Methodology. **Alejandra Garcia-Alonso:** Writing – review & editing, Writing – original draft, Visualization, Supervision. **María Dolores Tenorio Sanz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during and/or analyzed during the current study will be available from the corresponding author on reasonable request.

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