

Article

Source-to-Tap Monitoring of *Legionella* in Drinking Water Distribution System: Comparison of Culture-Based and qPCR Detection Within a Risk-Based Framework

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Highlights

- *Legionella* prevalence increased towards indoor installations.
- qPCR demonstrated higher detection sensitivity than the culture method.
- The highest *Legionella* detection rate was observed in summer.
- *Legionella* presence correlated with water hardness and iron concentration.
- Indoor installations are the main reservoir of *Legionella*.
- Monitoring should encompass the entire source-to-tap chain.
- qPCR can support early detection of microbiological threats.
- Building installations require priority risk management.
- Physicochemical parameters can support *Legionella* risk assessment.
- The results support the implementation of EU Directive 2020/2184.

Abstract

The occurrence of *Legionella* bacteria in drinking water distribution systems poses an important public health concern, particularly in the context of opportunistic premise plumbing pathogens (OPPPs) and the implementation of EU Directive 2020/2184. This study evaluated the prevalence of *Legionella* using a “source-to-tap” approach and compared culture-based and molecular detection methods. A total of 120 water samples were collected between February and November 2025 from water intakes, treatment plants, distribution networks, and premise plumbing systems in Poland. Analyses were performed using the ISO 11731 culture method and quantitative PCR (qPCR) targeting the *ssrA*, *mip*, and *wzm* genetic markers. The highest prevalence of *Legionella* was observed in premise plumbing systems, with positivity rates of 64.0% by qPCR and 30.0% by culture. Overall, qPCR detected *Legionella* in 37.5% of samples compared with 14.2% using the culture method, demonstrating significantly higher sensitivity ($p < 0.001$). Seasonal variation was observed, with the highest detection frequencies during summer months. Positive samples were associated with higher water hardness as well as elevated iron and manganese concentrations. Significant differences in *Legionella* occurrence were found among sampling points and seasons ($p < 0.05$). The results indicate that premise plumbing systems constitute the primary reservoir of *Legionella* within drinking water distribution systems. Furthermore, qPCR proved to be a rapid and sensitive tool supporting microbiological risk assessment and monitoring within the “source-to-tap” framework recommended by EU Directive 2020/2184.

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1. Introduction

1.1. Overview and Characteristics of Opportunistic Premise Plumbing Pathogens (OPPPs)

Ensuring the microbiological safety of drinking water is a cornerstone of public health protection. In recent years, growing attention has focused on the risks associated with the presence of opportunistic premise plumbing pathogens (OPPPs), which are capable of surviving and proliferating within water distribution systems and internal building plumbing. Unlike classical fecal pathogens, OPPPs are typically not associated with a single contamination event at the water source; instead, they develop directly within the water supply infrastructure [1]. This growth is particularly favored by water stagnation, biofilm formation, and the limited efficacy of residual disinfection [2,3]. Among the most well-characterized opportunistic premise plumbing pathogens are bacteria belonging to the genus *Legionella*, *Pseudomonas aeruginosa*, and non-tuberculous mycobacteria (*Mycobacterium* spp.) [2,4]. Within this group, *Legionella pneumophila* holds particular epidemiological significance as the causative agent of Legionnaires' disease (legionellosis) and Pontiac fever. According to data from the European Centre for Disease Prevention and Control (ECDC), the number of reported legionellosis cases in Europe has been systematically increasing, with infections primarily linked to water aerosols generated by domestic plumbing installations, hot water supply systems, and aerosol-producing devices. Recent studies conducted in hotels, healthcare facilities and passenger ships have further demonstrated the widespread occurrence of *Legionella* spp. in engineered water systems and highlighted the importance of risk-based monitoring approaches [5].

1.2. Factors Determining Plumbing System Colonization by *Legionella* Bacteria

Contemporary research unequivocally indicates that the presence of *Legionella* bacteria in water supply systems is primarily determined by the conditions prevailing within distribution networks and internal building plumbing, rather than the quality of water at the intake or treatment stage. In many cases, water leaving the water treatment plants meets all microbiological requirements, whereas secondary colonization of the system occurs during the distribution and operational phases of premise plumbing networks [6]. Water stagnation is considered of paramount importance, as it leads to a depletion of residual disinfectant concentrations, temperature fluctuations, and increased contact time between microorganisms and the surfaces of plumbing materials [7]. Terminal sections of plumbing systems, infrequently used outlets, and oversized piping networks are particularly susceptible to colonization [6,8,9]. Recent studies conducted in building water systems have additionally demonstrated that stagnation, elevated water temperature, and biofilm formation are among the most important factors promoting *Legionella* colonization and persistence in premise plumbing systems [10]. Water temperature also plays a critical role. While *Legionella* bacteria can grow across a wide temperature range, the most intensive proliferation typically occurs between approximately 25 °C and 45 °C [11]. Furthermore, increasing attention is being paid to the impact of plumbing materials and physico-chemical water parameters on biofilm development and subsequent *Legionella* colonization. Research has demonstrated that the type of material can influence both microbial adhesion and biofilm stability, as well as disinfection efficacy. Corrosion processes and the accumulation of mineral scales are of particular significance, as they can create micro-environments that favor bacterial survival [12,13]. The growth of *Legionella* species is also

influenced by the physicochemical factors of the aquatic environment. Parameters such as iron and manganese concentrations, pH, electrical conductivity, and water hardness can modulate corrosion processes, biofilm stability, and the effectiveness of residual disinfection within distribution systems [9,14,15]. Corrosion products and mineral scales increase the surface area available for microbial colonization and can promote the long-term persistence of bacteria in water supply installations. Despite numerous studies, the correlation between physicochemical parameters of water and water supply distribution systems and the presence of *Legionella* has not yet been unequivocally defined and may vary depending on the characteristics of the specific water supply system [16].

1.3. Detection Methods: Classical Culture vs. Molecular Analysis

An essential element of microbiological risk management in water distribution systems is the appropriate selection of detection methods for *Legionella* bacteria. The classical culture-based method in accordance with the ISO 11731 standard remains the recognized reference method, enabling the detection of bacteria capable of growing on selective microbiological media and expressing results in colony-forming units (CFU) [17]. Although the culture method forms the basis of current legal regulations and water microbiological quality compliance assessments, its application is associated with a number of limitations [18,19].

One of the primary disadvantages of culture-based methods is the long analytical turnaround time, typically ranging from 7 to 10 days, which significantly hinders rapid response in the event of an epidemiological threat. Furthermore, detection efficiency can be compromised by the presence of competing background microbiota, bacterial environmental stress, and the occurrence of cells in the VBNC (viable but non-culturable) state. These cells retain metabolic activity but do not exhibit the ability to grow on standard microbiological media [20–22].

This issue is particularly critical for biofilm-associated bacteria, which may display increased resistance to environmental conditions and disinfectants. Consequently, classical culture methods can lead to an underestimation of the actual presence of *Legionella* in water supply networks, especially in internal plumbing installations and low-abundance samples [20].

These limitations have driven growing interest in molecular methods, particularly quantitative polymerase chain reaction (qPCR), which enables the rapid and sensitive detection of bacterial genetic material in environmental samples. The qPCR technique provides results within a few hours and demonstrates high sensitivity, even in samples containing low amounts of template DNA. An additional advantage of molecular methods is the ability to utilize genetic markers with varying levels of specificity:

- the *ssrA* gene: widely used as a genus-specific marker for *Legionella* spp., enabling broad detection of bacteria belonging to this genus,
- the *mip* gene: recognized as a specific marker for *Legionella pneumophila*, which is the most frequently isolated species responsible for human cases of legionellosis. Recent reviews indicate that *L. pneumophila* is responsible for the vast majority of waterborne legionellosis cases, supporting its role as the primary target organism in routine monitoring programs [23].
- the *wzm* gene: associated with lipopolysaccharide biosynthesis, this gene is used to identify *L. pneumophila* serogroup 1, considered the most epidemiologically significant group responsible for the majority of infections [19,24].

Despite the numerous advantages of molecular methods, their interpretation remains more complex than that of classical culture. The qPCR techniques detect live, damaged, and dead cells alike, as well as free DNA present in the aquatic environment, which can lead to an overestimation of positive results (false positives in the context of viability as-

assessment). Consequently, there is an increasing emphasis on the necessity of concurrently applying both culture-based and molecular methods for a comprehensive microbiological risk assessment of water distribution systems [25,26].

1.4. Regulatory Framework and Aim of the Study

In response to the increasing significance of microbiological risks in water distribution systems, a risk-based assessment approach has been introduced, formally incorporated into Directive (EU) 2020/2184 of the European Parliament and of the Council on the quality of water intended for human consumption [27]. These new regulations expand safety assessments to encompass the entire water supply chain—from the catchment to the consumer's point of consumption (the “source-to-tap approach”)—and emphasize the necessity of identifying microenvironments that favor microbial proliferation within internal plumbing installations [28]. Despite a growing body of research on the occurrence of *Legionella* in water supply networks, there remains a limited number of studies that comprehensively combine the “source-to-tap” approach, classical culture methods, molecular analysis utilizing multiple genetic markers, and the evaluation of both physicochemical parameters and seasonal prevalence.

The aim of this study was to evaluate the prevalence of *Legionella* bacteria in drinking water distribution systems using a “source-to-tap” approach and to compare the culture-based and molecular methods employed for their detection. Additionally, the impact of selected physicochemical parameters and seasonality on the occurrence of *Legionella* within the analyzed water supply systems was investigated.

2. Materials and Methods

2.1. Study Area and Sampling Plan

The study was conducted from February to November 2025 in a single municipal water supply system located in the Silesian Voivodeship (Częstochowa County, Poland). The system included groundwater intakes, a water treatment plant, a distribution network, and the internal systems of single-family homes. The analyzed section of the distribution network had a total length of approximately 88.7 km and was constructed primarily of cast iron, polyethylene (PE), and polyvinyl chloride (PVC) pipes. Pipe diameters ranged from 118 to 144 mm, and the operating pressure was maintained at 0.38–0.48 MPa. The stability of hydraulic conditions during the study was confirmed based on operational data including flow and pressure in the network. Sampling points were designated using the “source-to-tap” concept and covered the various stages of the water supply system: groundwater intakes, treated water, the distribution network, and the internal systems of residential buildings. A total of 120 water samples were collected and analyzed in parallel using both the culture and qPCR methods. Sampling frequency accounted for seasonal variations during the study period.

Samples from indoor water systems were collected in the morning to capture the effect of nighttime water stagnation, which represents the most conservative consumer exposure scenario. However, the exact stagnation time for individual households was not recorded. All selected residential buildings were located at the terminus (endpoints) of the water distribution network within each designated zone. Information regarding the exact age of the water systems, specific pipe materials (e.g., iron, PE, PVC), and type of heating system (central vs. combi boilers) was not available.

Water Sampling Procedure

Water sampling for the determination of *Legionella* species was conducted in accordance with a procedure designed to evaluate actual user exposure (the “first-flush”

method). At each sampling point, the initial portion of water was collected immediately upon opening the tap, without prior flushing. Neither tap disinfection nor the removal of terminal plumbing devices (aerators) was performed, thereby maintaining conditions that reflected actual installation usage. Samples were collected in sterile containers, transported at 4 ± 2 °C, and analyzed within 24 h of collection. The sampling containers contained sodium thiosulfate to neutralize residual disinfectants, preventing further bactericidal effects on the microorganisms during sample transport and storage. The analyzed water originated primarily from supply systems relying on groundwater sources, which are characterized by a relatively stable physicochemical composition and low organic matter content. The water exhibited a neutral to slightly alkaline pH, moderate to high hardness, and relatively low iron and manganese concentrations, which are typical for groundwater-fed water supply networks [29].

2.2. Microbiological Analysis—Culture Method

Legionella spp. culture analysis was performed according to the ISO 11731:2017 standard for the enumeration of these microorganisms in environmental water samples. Filtration was performed using sterile 0.45 µm membrane filters. For samples from indoor installations and hot water systems with high suspected background flora, both thermal and acid treatments were applied in parallel to maximize selectivity. After filtration, the filters were acid-treated (with a solution of hydrochloric acid and potassium chloride at pH 2.2, introduced directly into the filter funnels for 5 min and then rinsed with sterile distilled water). After filtration and any required treatment, the material was inoculated onto selective GVPC agar (buffered charcoal yeast extract agar supplemented with glycine, vancomycin, polymyxin B, and cycloheximide) (POL-AURA, Zabrze, Poland). The plates were incubated at 36 ± 2 ° for up to 10 days. Colony growth monitoring and enumeration were performed on days 3, 5, and 10 of incubation. Characteristic colonies suspected of belonging to the genus *Legionella* were subjected to biochemical confirmation by parallel subculturing onto BCYE agar (with L-cysteine) and BCYE agar without L-cysteine (BCYE-Cys) (POL-AURA, Zabrze, Poland). Colonies capable of growing exclusively on the medium containing L-cysteine were confirmed as *Legionella* species. Colonies growing only in the presence of L-cysteine were further confirmed serologically using a commercial latex agglutination test (*Legionella* Latex Test, Oxoid, Basingstoke, UK). This test allowed the differentiation of *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14, and other *Legionella* species (*Legionella* spp.), ensuring that all 17 culture-positive isolates were formally confirmed and classified. The final results were expressed quantitatively as the bacterial count in colony-forming units per liter of water (CFU/L) and qualitatively as the presence or absence of the bacteria in the analyzed volume.

2.3. Molecular Analysis—qPCR

2.3.1. Sample Concentration and DNA Extraction

Parallel 1 L water samples were used for molecular analyses. Samples were filtered through sterile 0.45 µm membrane filters to minimize the risk of filter clogging and improve process efficiency. After filtration, the membranes were aseptically transferred to sterile tubes. The extraction of template genetic material was performed using a commercial Environmental DNA & RNA Purification Kit (EURx, Gdańsk, Poland, Cat. No. E3580), strictly following the manufacturer's protocol designed for environmental samples. The quantity and purity of the obtained DNA eluate were assessed spectrophotometrically using a NanoDrop apparatus (Nabi, MicroDigital Co., Ltd., Pangyo, Republic of Korea). The absorbance ratios A260/A280 (as an indicator of protein contamination) and A260/A230 (as an indicator of the presence of salts and organic compounds) were analyzed. Values of A260/A280 within the range of 1.8–2.0 and A260/A230 above 1.8 were

considered acceptable, indicating high DNA purity. If PCR inhibitors were detected, the samples were appropriately diluted in nuclease-free water immediately prior to the qPCR analysis (EPPENDORF Mastercycler ep Realplex 2, Eppendorf, Hamburg, Germany).

2.3.2. qPCR Conditions and Genetic Marker Selection

The qPCR assays were performed using the SG qPCR Master Mix (2×) (EURx, Gdańsk, Poland) in a total reaction volume of 25 µL. The experimental strategy utilized a tiered approach based on three distinct genetic markers:

- Reaction A (Screening/Singleplex): Detection of the *ssrA* gene, which serves as a genus-specific marker for *Legionella* spp.
- Reaction B (Differentiation/Multiplex): Simultaneous detection of the *mip* gene (a species-specific marker for *Legionella pneumophila*) and the *wzm* gene (a specific marker for *L. pneumophila* serogroup 1).

The primers and probes used are presented in Table 1, while their nucleotide sequences and amplicon lengths are summarized in Table 2. The composition of the reaction mixtures for the singleplex (*ssrA*) and multiplex (*mip/wzm*) assays is detailed in Table 3 and Table 4, respectively. In each qPCR run, a positive control (template DNA extracted from the reference strain *Legionella pneumophila* ATCC® 33152) (Microbiologics, Saint Cloud, MN, USA) and a negative control (NTC—no-template control, where nuclease-free water was added instead of the template) were included. All reactions were performed in technical triplicates.

Table 1. Primers and probes used in the qPCR analysis.

Target Gene	Primers/Probe
<i>ssrA</i>	PanLeg-F/PanLeg-R/PanLeg-P1
<i>mip</i>	Lp-F/Lp-R/Lp-FAM
<i>wzm</i>	Lp1-F/Lp1-R/Lp1-P

Table 2. Sequences of primers and probes used in the qPCR analysis.

Target Gene	Oligonucleotide	Oligonucleotide Sequence (5'–3')	Product Length
<i>ssrA</i> (<i>Legionella</i> spp.)	PanLeg-F	GGCGACCTGGCTTC	101 bp
	PanLeg-R1	GGTCATCGTTTGCAATTTATATTTA	
	PanLeg-P1	CGTG GGTGCAA	
<i>mip</i> (<i>L. pneumophila</i>)	Lp-F	TTGTCTTATAGCATTGGTGCCG	115 bp
	Lp-R	CCAATTGAGCGCCACTCATAG	
	Lp-P	CGGAAGCAATGGCTAAAGGCATGCA	
<i>wzm</i> (<i>L. pneumophila</i> sg1)	Lp1-F	TGCCTCTGGCTTGCAGTTA	70 bp
	Lp1-R	CACACAGGCACAGCAGAAACA	
	Lp1-P	TTTATTACTCCACTCCAGCGAT	

Table 3. The qPCR reaction mixture composition for the *ssrA* gene qPCR assay.

Component	Volume
Master mix	12.5 µL
Primer Forward	1 µL
Primer Reverse	1 µL
Probe	0.5 µL
DNA	2.5 µL
UNG	0.25 µL
DNase- and RNase-free deionized water	7.25 µL
Final volume	25 µL

Table 4. Reaction mixture composition for the *wzm* and *mip* multiplex qPCR assay.

Component	Volume
Master mix	12.5 µL
Primer I—Forward	1 µL
Primer I—Reverse	1 µL
Probe I	0.5 µL
Primer II—Forward	1 µL
Primer II—Reverse	1 µL
Probe II	0.5 µL
DNA	2.5 µL
UNG	0.25 µL
DNase- and RNase-free deionized water	4.75 µL
Final volume	25 µL

2.3.3. Interpretation of qPCR Results

The qPCR assays were performed in a thermocycler under time-temperature conditions optimized for the specific primers and probes used (Tables 1–4). Samples exhibiting an amplification curve with a characteristic exponential phase below the established threshold were considered positive. Samples characterized by high cycle threshold values ($C_t > 30$) indicated the presence of low concentrations of *Legionella* spp. genomic DNA near the assay's limit of quantification (LOQ). Due to the technical limitations of standard qPCR in distinguishing between viable, damaged, or dead cells, these high C_t values were interpreted as qualitative indicators of low-abundance target DNA rather than precise quantitative measurements of viable cells. The final quantitative results were expressed as mean values along with the standard deviation (SD). The selection of genetic markers and amplification conditions was based on previously published and validated protocols utilized in environmental *Legionella* spp. detection, including procedures described by Benitez and Winchell [30] as well as guidelines specified in the ISO/TS 12869:2019 [31] standard for the detection and quantification of *Legionella* species by qPCR.

Amplification conditions included initial denaturation at 95 °C for 3 min, followed by 45 cycles consisting of a denaturation step (95 °C, 15 s) and a combined primer annealing and extension step (60 °C, 60 s).

2.3.4. qPCR Assay Performance, Detection Limits, and Inhibition Control

To ensure the analytical reliability of the qPCR assays, standard curves were constructed using serial dilutions of genomic DNA from *Legionella pneumophila* ATCC® 33152. The amplification efficiency (E) for all three assays (*ssrA*, *mip* and *wzm*) ranged between 94.3% and 98.5%, with determination coefficients (R^2) exceeding 0.992 (Table 5).

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined as follows:

For the *ssrA* assay: LOD = 3 genomic copies/reaction, LOQ = 10 genomic copies/reaction. For the *mip* and *wzm* assays: LOD = 5 genomic copies/reaction, LOQ = 15 genomic copies/reaction.

To monitor potential PCR inhibition, an exogenous Internal Amplification Control (IAC) (non-target DNA target fragment with a specific primer/probe set) was spiked into each reaction mixture. No significant shifts in the cycle threshold (C_t) of the IAC (<0.5 cycles compared to the negative control) were observed, confirming the absence of significant PCR inhibitors in the extracted DNA samples.

Table 5. qPCR validation and calibration curve parameters for *ssrA*, *mip*, and *wzm* gene assays.

Target Gene	Slope	R ²	Efficiency (E, %)	LOD (Copies/Reaction)	LOQ (Copies/Reaction)
<i>ssrA</i>	−3.42	0.995	96.1%	3	10
<i>mip</i>	−3.39	0.993	97.2%	5	15
<i>wzm</i>	−3.46	0.992	94.6%	5	15

2.4. Physicochemical Parameters of Water

To evaluate the impact of environmental conditions on the survival and proliferation of *Legionella* species, monitoring of selected physicochemical water parameters was conducted in parallel with microbiological sampling. The analysis included the determination of temperature, pH [32], electrical conductivity [33], total hardness [34], and the concentrations of total iron and manganese [35], in order to determine the correlations between these environmental parameters and both the presence and abundance of *Legionella* bacteria.

2.5. Statistical Analysis

Statistical analyses were performed using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). Differences in the frequency of positive samples between water system compartments were evaluated using the chi-square test. Agreement between the culture-based method and qPCR results was assessed using Cohen's kappa coefficient, while differences in paired detection frequencies between methods were evaluated using McNemar's test. Statistical significance was assumed at $p < 0.05$.

To evaluate seasonal variations, monthly data were grouped into four seasonal clusters: winter (February–March, $n = 24$), spring (April–May, $n = 24$), summer (June–August, $n = 36$), and autumn (September–November, $n = 36$). Differences in the frequency of positive samples among these seasonal clusters were evaluated using the chi-square test.

3. Results

A total of 120 water samples were analyzed, yielding 120 culture-based assays and 120 qPCR assays (amounting to 240 determinations in total). Comparative results were evaluated using a paired design ($n = 120$ samples).

3.1. Occurrence of *Legionella* Bacteria Across Different Sampling Points of the Water System

The analysis of 120 water samples revealed varying frequencies of *Legionella* bacteria occurrence depending on the sampling point. Detailed data regarding the number and percentage of positive results obtained using the culture-based method and individual molecular markers (*ssrA*, *mip*, *wzm*) are presented in Table 5. In raw water (intakes) and after the treatment process (WTP), no viable bacterial cells were detected using the culture-based method. However, molecular assays revealed the presence of *Legionella* genus genetic material (*ssrA* marker) in 10.0% of the intake samples and 15.0% of the WTP samples. The *mip* and *wzm* markers were not detected at these sampling points. In the distribution network, the detection frequency of the *ssrA* marker increased to 26.7%. At this stage, the *mip* and *wzm* genes were also identified by qPCR in 13.3% and 6.7% of the samples, respectively, while the culture-based method allowed for the isolation of bacteria in 6.7% of the tested samples. The highest colonization rates were recorded in premise plumbing systems. The presence of *Legionella* spp. was confirmed in 30.0% of the samples using the culture-based method. In the qPCR analysis, positive results were obtained for 64.0% of the samples for the *ssrA* marker, 44.0% for the *mip* marker, and 24.0% for the *wzm* marker (Table 6). A summary comparison of the detection differences between the conventional and molecular methods (for the *ssrA* marker) is presented in Figure 1.

Table 6. Detection frequency of *Legionella* depending on the water system sampling point.

Sampling Point	Number of Samples	Plate Culture (+)	Plate Culture (%)	qPCR <i>ssrA</i> (+)	<i>ssrA</i> (%)	qPCR <i>mip</i> (+)	<i>mip</i> (%)	qPCR <i>wzm</i> (+)	<i>wzm</i> (%)
Water intake	20	0	0.0	2	10.0	0	0.0	0	0.0
Water treatment plant (WTP)	20	0	0.0	3	15.0	1	5.0	0	0.0
Distribution network	30	2	6.7	8	26.7	4	13.3	2	6.7
Premise plumbing installations	50	15	30.0	32	64.0	22	44.0	12	24.0
Total	120	17	14.2	45	37.5	27	22.5	14	11.7

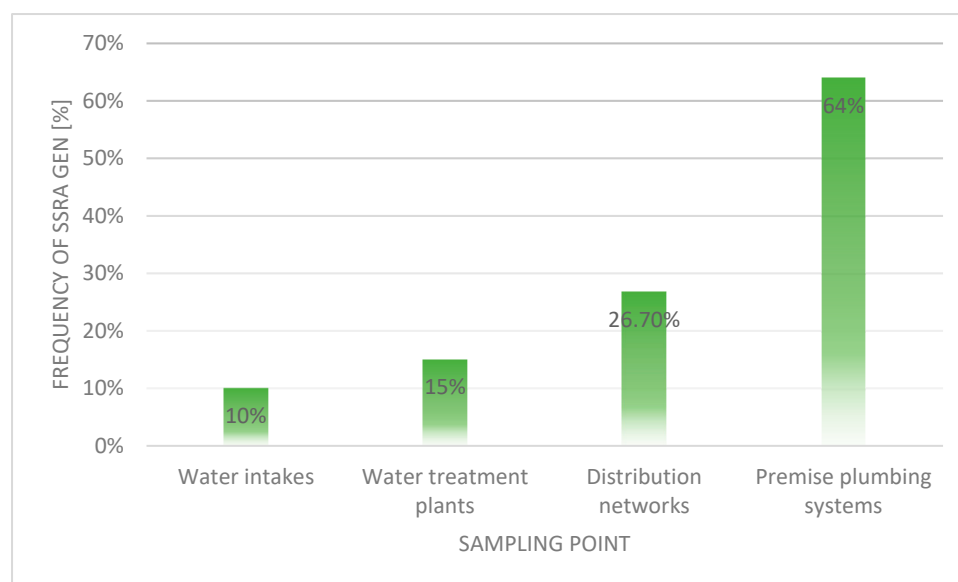


Figure 1. Prevalence of *Legionella* detected by qPCR (*ssrA*) in different parts of the drinking water distribution system.

Statistical analysis confirmed significant differences in the frequency of *Legionella* occurrence depending on the sampling point. For the qPCR assay targeting the *ssrA* marker, detection frequency differed significantly among the analyzed sections of the water supply system (chi-square test, $\chi^2 = 27.26$, $p < 0.001$). Similarly, significant differences were observed for the culture-based method ($\chi^2 = 18.30$, $p < 0.001$), confirming a progressive increase in *Legionella* prevalence toward terminal sections of the water supply chain, particularly premise plumbing systems.

3.2. Comparison of Culture-Based and Molecular Methods

A comparison of the detection results for *Legionella* bacteria obtained concurrently via the culture-based method and qPCR (*ssrA* marker) is presented in Table 7. In the molecular analysis, the presence of bacteria was detected in 37.5% of the samples ($n = 45$), whereas the culture-based method demonstrated their presence in 14.2% of the samples ($n = 17$). All samples that tested positive by the culture-based method also yielded a positive result in the qPCR analysis (100% agreement for the positive results of the conventional method). The largest group among the discrepant results consisted of samples that were positive exclusively in the qPCR analysis ($n = 28$; 23.3%). Concordance of negative results for both methods was recorded in 62.5% of the cases ($n = 75$). The aggregated data confirm the higher diagnostic sensitivity of the molecular method. The absence of samples testing positive solely by the culture-based method indicates high DNA extraction efficiency and the lack of a significant impact of PCR inhibitors on the final analytical outcome.

Table 7. Comparison of culture vs. qPCR results (*ssrA*, $n = 120$).

	qPCR (+)	qPCR (−)	Total
Culture (+)	17	0	17
Culture (−)	28	75	103
Total	45	75	120

A statistically significant difference was observed between the detection efficiency of the culture-based method and qPCR (McNemar test, $p < 0.001$). The molecular assay demonstrated significantly higher sensitivity for the detection of *Legionella* spp. compared to the conventional culture method. Complete agreement was observed for all culture-positive samples, whereas 28 samples yielded positive qPCR results despite negative culture outcomes. Rather than definitively indicating missed active colonization, these discrepancies reflect the inherent differences between the two methods; while they may point to the presence of low bacterial abundance near the limit of detection or cells in a viable but non-culturable (VBNC) state, they can also be attributed to the detection of DNA from dead or damaged cells, or extracellular DNA persisting in the system. Agreement between the culture-based method and qPCR was classified as moderate to substantial (Cohen's $\kappa = 0.49$), reflecting complete concordance for culture-positive samples but frequent additional detections obtained exclusively by qPCR.

3.3. Structure of Genetic Markers Among *ssrA*-Positive Samples

The detection frequency of individual genetic markers determined by qPCR is presented in Table 8. The molecular analysis allowed for a precise determination of the structure of the detected microorganisms. The *ssrA* marker (*Legionella* spp.) was detected in a total of 45 samples (37.5% of all analyzed samples). The *mip* marker (*L. pneumophila*) was found in 27 samples (22.5%), whereas the *wzm* marker (*L. pneumophila* sg1) was detected in 14 samples, accounting for 11.7% of the total assays. All samples positive for the *mip* and *wzm* genes constituted a subset of the samples that were positive for the *ssrA* marker. As demonstrated in Table 7, among the *ssrA*+ samples, the *L. pneumophila* species (*mip*) accounted for 60.0% of the results, while serogroup 1 (*wzm*) was responsible for 31.1% of all positive samples for the genus *Legionella*. The distribution of the markers was closely associated with the sampling point. The highest occurrence frequency for all analyzed genes was observed in internal building plumbing systems. In samples originating from water intakes and water treatment plants (WTP), only the *ssrA* marker was detected, with a complete absence of markers specific to *L. pneumophila* and serogroup 1 *mip* (−)/*wzm* (−).

Table 8. Share of *mip* and *wzm* among samples positive for *ssrA* ($n = 45$; *ssrA* = 100%).

Category	Number of Samples	% Relative to <i>ssrA</i>
<i>ssrA</i> (<i>Legionella</i> spp.)	45	100
<i>mip</i> (<i>L. pneumophila</i>)	27	60.0
<i>wzm</i> (<i>L. pneumophila</i> sg1)	14	31.1

3.4. Seasonality of *Legionella* Occurrence

To improve statistical power and better visualize the seasonal trend, the monthly data were grouped into seasonal clusters (Table 9). The lowest prevalence of *Legionella* was recorded during the winter cluster (February–March). During this period, the presence of *Legionella* genus genetic material (*ssrA*) was detected in only 8.3% of the samples (2/24), with a concurrent absence of specific markers (*mip*, *wzm*) and no bacterial growth observed via the culture-based method. A progressive increase in positive results was observed during spring (April–May), reaching a detection frequency of 25.0% for *ssrA* and 8.3% for the culture method (Figure 2). The peak of system colonization occurred during

the summer cluster (June–August), which exhibited the highest detection rates across all markers. In summer, the *ssrA* marker was detected in 58.3% of the samples (21/36), the *mip* marker in 38.9% (14/36), and the *wzm* marker in 19.4% (7/36), while the culture-based method yielded viable bacteria in 25.0% of samples (9/36). During the autumn period (September–November), a gradual decrease in the bacterial occurrence frequency was noted (*ssrA*: 44.4%, culture: 16.7%), though detection rates remained higher than those in the winter and spring periods. Statistical analysis confirmed a highly significant seasonal effect on *Legionella* occurrence for qPCR detection ($\chi^2 = 17.72$, $df = 3$, $p < 0.001$) and a significant effect for the culture-based method ($\chi^2 = 8.30$, $df = 3$, $p = 0.040$).

Table 9. *Legionella* detection frequency across seasonal clusters (n = 120 samples total).

Season (Months)	n	Culture (+) n (%)	qPCR <i>ssrA</i> (+) n (%)	qPCR <i>mip</i> (+) n (%)	qPCR <i>wzm</i> (+) n (%)
Winter (Feb–Mar)	24	0 (0.0%)	2 (8.3%)	0 (0.0%)	0 (0.0%)
Spring (Apr–May)	24	2 (8.3%)	6 (25.0%)	3 (12.5%)	1 (4.2%)
Summer (Jun–Aug)	36	9 (25.0%)	21 (58.3%)	14 (38.9%)	7 (19.4%)
Autumn (Sep–Nov)	36	6 (16.7%)	16 (44.4%)	10 (27.8%)	6 (16.7%)
Total	120	17 (14.2%)	45 (37.5%)	27 (22.5%)	14 (11.7%)

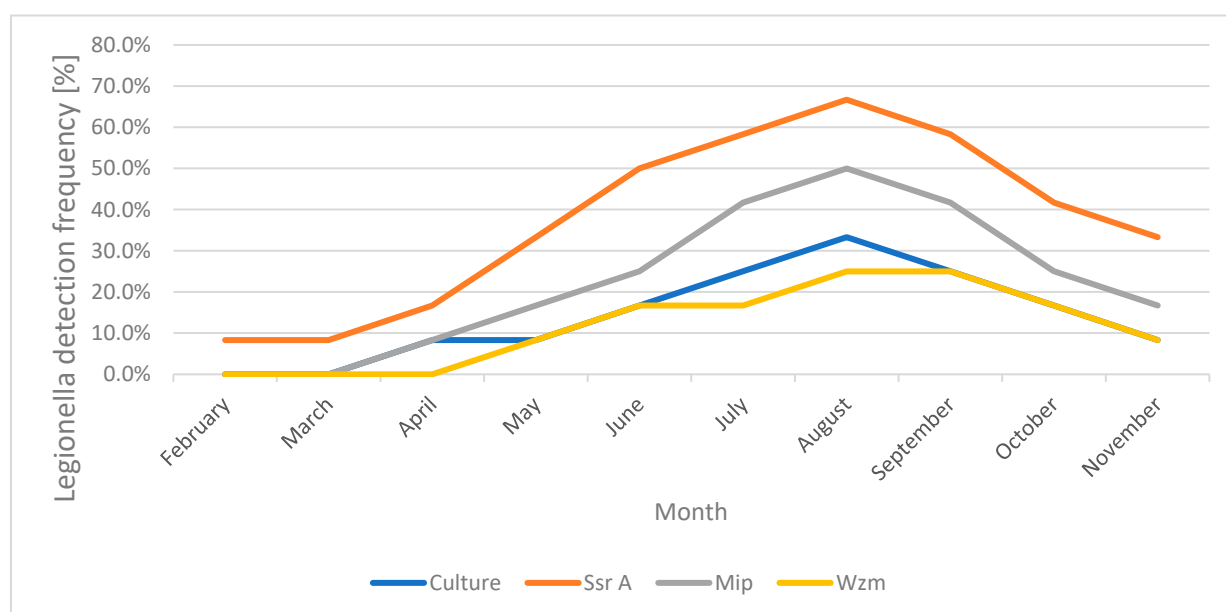


Figure 2. Seasonal variation in the frequency of *Legionella* detection in the analyzed water samples based on the culture method and qPCR (*ssrA*, *mip*, *wzm*).

3.5. Influence of Physicochemical Parameters

Statistical comparison of physicochemical parameters between *Legionella*-positive and *Legionella*-negative samples revealed significant differences for selected indicators (Table 10). Positive samples were characterized by significantly higher concentrations of total iron ($p < 0.001$), manganese ($p = 0.034$), increased water hardness ($p = 0.021$), and, in premise plumbing systems of residential buildings, higher water temperatures compared to negative samples (27.4 ± 7.2 °C vs 18.8 ± 5.3 °C), with the highest values observed during the summer months. In contrast, no statistically significant differences were observed for pH, conductivity, turbidity ($p > 0.05$), or water temperature at sampling points encompassing the water intake, water treatment plant (WTP), and distribution network, where temperatures remained relatively stable and differences between positive and negative samples were not statistically significant (Table 11). Statistical analysis additionally

demonstrated significant correlations between the occurrence of *Legionella* bacteria, water hardness, and total iron concentration, with higher values recorded in samples where the presence of either genetic material or viable bacterial cells was confirmed (Table 11). These findings suggest that corrosion-related parameters, mineral deposition, and elevated temperatures in premise plumbing systems may promote microbial persistence and system colonization by increasing the available adhesion surface for microorganisms and stabilizing biofilm structure.

Table 10. Other physicochemical parameters in *Legionella* positive and negative samples.

Parameter	<i>Legionella</i> (–) Mean ± SD	<i>Legionella</i> (+) Mean ± SD	<i>p</i> -Value
pH	7.39 ± 0.14	7.50 ± 0.17	0.081
Conductivity [µS/cm]	642 ± 41	703 ± 56	0.063
Hardness [mg CaCO ₃ /L]	190 ± 19	203 ± 23	0.021
Iron [mg/L]	0.021 ± 0.010	0.056 ± 0.028	<0.001
Manganese [mg/L]	0.006 ± 0.003	0.016 ± 0.008	0.034
Turbidity [NTU]	0.33 ± 0.09	0.44 ± 0.13	0.072

Table 11. Water temperature depending on the sampling point and the presence of *Legionella*.

Sampling Point	<i>Legionella</i> (–) [°C] Mean ± SD	<i>Legionella</i> (+) [°C] Mean ± SD
Water intake	11.4 ± 0.6	12.1 ± 0.5
Water treatment plant	12.0 ± 0.7	13.2 ± 0.9
Distribution network	14.1 ± 1.9	17.2 ± 2.4
Premise plumbing installations	18.8 ± 5.3	27.4 ± 7.2

3.6. Ct Values Depending on the Sampling Point (qPCR)

The range of Ct values obtained for individual genetic markers depending on the sampling point is presented in Table 12. The *ssrA* marker, utilized for the broad detection of *Legionella* spp., was characterized by the highest number of positive results and the widest range of Ct values. The highest Ct values were observed in samples originating from water intakes and water treatment plants (WTP), where the mean values were 33.2 ± 0.9 and 32.8 ± 1.0, respectively. In samples originating from the distribution network, Ct values were lower (29.9 ± 1.3), whereas the lowest values were recorded in the internal plumbing systems of residential buildings (27.4 ± 1.5), indicating a higher level of *Legionella* genetic material in the terminal sections of the distribution system. A similar trend was observed for the *mip* marker, which is specific to *Legionella pneumophila*. The highest Ct values were recorded in samples originating from the WTP (34.1 ± 0.8), while lower values occurred in the distribution network (31.8 ± 1.2) and internal plumbing systems (29.7 ± 1.6). The differences between the sampling points were statistically significant. The *wzm* marker, associated with the identification of *L. pneumophila* serogroup 1, was detected primarily in samples originating from the distribution network and internal plumbing systems. The mean Ct values were 33.6 ± 1.1 and 31.2 ± 1.7, respectively, with lower values observed in the terminal sections of the water supply system.

Table 12. Ct values (mean $\pm$ SD) for qPCR markers depending on the system point.

Marker	Sample Point	Ct Min	Ct Max	Average Ct $\pm$ SD
<i>ssrA</i>	Water intake	32.5	33.9	33.2 $\pm$ 0.9
	Water treatment plant (WTP)	32.2	33.7	32.8 $\pm$ 1.0
	Distribution network	28.2	32.0	29.9 $\pm$ 1.3
	Premise plumbing installations	24.2	31.1	27.4 $\pm$ 1.5
<i>mip</i>	Water treatment plant (WTP)	32.9	35.3	34.1 $\pm$ 0.8
	Distribution network	30.1	33.6	31.8 $\pm$ 1.2
	Premise plumbing installations	26.4	32.9	29.7 $\pm$ 1.6
<i>wzm</i>	Distribution network	32.1	35.1	33.6 $\pm$ 1.1
	Premise plumbing installations	28.5	34.0	31.2 $\pm$ 1.7

Statistical analysis demonstrated significant differences in Ct values depending on the sampling point for all analyzed genetic markers (Kruskal–Wallis test, $p < 0.01$). Lower Ct values observed in premise plumbing systems indicate a significantly higher abundance of *Legionella* genetic material in terminal sections of the water distribution system. Conversely, higher Ct values detected at water intake and treatment stages suggest lower bacterial loads and possible occurrence of trace DNA or viable but non-culturable (VBNC) cells.

4. Discussion

The introduction of the new Directive (EU) 2020/2184 of the European Parliament and of the Council on the quality of water intended for human consumption has necessitated the implementation of a risk assessment-based approach throughout the entire water supply chain. The results obtained in this study confirm the validity of the “source-to-tap” model, indicating significant differences in the level of *Legionella* bacteria colonization across the individual stages of the distribution system.

4.1. Effectiveness of Detection Methods in Risk Monitoring

A key aspect of the conducted research was the assessment of the applicability of molecular methods (qPCR) in routine monitoring. The higher detection frequency of *Legionella* spp. genetic material (37.5%) compared to the culture-based method (14.2%), as demonstrated in Section 3.2, highlights the inherent differences in what these methods measure. The 28 samples that were qPCR-positive but culture-negative require a highly balanced interpretation, as these discrepant results do not automatically imply active, ongoing water system colonization that was missed by traditional culturing. Samples with high cycle threshold values ($Ct > 30$) collected at the water intake and treatment stages are of particular significance. Because conventional qPCR amplifies DNA from both living and dead bacteria, as well as free extracellular DNA, these 28 positive results may represent residual genetic material from non-viable cells, past disinfection events, or low-abundance targets near the limit of detection. However, while the literature suggests that viable but non-culturable (VBNC) states are common under environmental stress, we acknowledge that standard qPCR without viability dyes (such as PMA) cannot differentiate between these biological states. Therefore, these results should be interpreted cautiously as the presence of low bacterial DNA loads rather than active colonization, and further viability-qPCR studies are required to confirm the actual proportion of viable cells. Nevertheless, in the context of a risk-based approach, such molecular signals can serve as early warning indicators of potential system vulnerability, warranting closer monitoring even if the immediate biological risk remains unconfirmed.

4.2. Influence of Environmental Conditions and Physicochemical Parameters on System Colonization

The analysis of physicochemical parameters allowed for the identification of key factors favoring the development of *Legionella* bacteria in the investigated system. Water temperature emerged as the most significant factor, which is particularly evident in internal building plumbing systems. The temperature difference recorded in this study between positive and negative samples (27.4 ± 7.2 °C vs. 18.8 ± 5.3 °C) clearly indicates that exceeding the 20–25 °C threshold creates optimal conditions for the proliferation of these microorganisms. These findings are consistent with the observed seasonal trend—the peak detection frequency in August directly corresponds to the highest water temperatures at the sampling points. This confirms the hypothesis that internal plumbing systems, due to water heating and the potential for stagnation, constitute the most critical stage in the water supply chain. Another equally important factor, as indicated by the positive correlation shown in Table 10, is the total iron concentration and water hardness. The statistically significant association observed between *Legionella* occurrence and iron concentration supports previous reports indicating that corrosion products may facilitate microbial adhesion and biofilm development within drinking water systems. The obtained results confirm that water temperature, corrosion processes, and mineralization are key determinants of *Legionella* colonization in installations. The positive relationship between iron concentration, water hardness, and the presence of the pathogen demonstrated in the study correlates with literature reports. These findings are additionally supported by previous research conducted in internal water supply installations, where pipeline material significantly influenced microbial abundance and biofilm development, with polymeric materials showing the highest microbial growth [36]. Corrosion products, especially iron, increase microorganism adhesion and stabilize the biofilm structure [13], while mineral deposits and the type of installation material determine the composition of local microbiomes [12]. Furthermore, elevated temperatures in the final sections of the network, combined with a decrease in disinfection efficiency, drastically increase bacterial survival in biofilm [15]. This indicates that the physicochemical properties of water and the technical condition of the installation create a microenvironment conducive to the secondary proliferation of *Legionella*. Similarly, higher water hardness observed in positive samples suggests that mineral deposition may contribute to the formation of protective microenvironments favorable for bacterial persistence. Although pH and conductivity exhibited slight increases in positive samples, these differences did not reach statistical significance, suggesting a secondary role in system colonization. According to the obtained results, higher concentrations of corrosion products in building systems favored the occurrence of *Legionella*. This mechanism can be explained in two ways. First, iron ions represent an essential micronutrient for the growth of *Legionella* bacteria. Second, the presence of mineral deposits and corrosion products may increase the surface roughness of internal pipe walls, which is thought to facilitate microbial adhesion and biofilm stabilization based on the literature cited above, although biofilm was not directly sampled in this study. Such conditions could plausibly protect bacteria from disinfectant action, which may help explain their high prevalence in terminal plumbing sections despite the absence of viable cell detection at the water intake and water treatment plant (WTP) stages; direct biofilm characterization would be required to confirm this mechanism.

4.3. Genetic Diversity and Pathogenic Potential of the Detected Strains

The analysis of specific markers allowed for a more precise assessment of the pathogenic potential of the bacteria present within the system. While the *ssrA* marker confirmed the presence of the genus *Legionella* in 37.5% of the samples, the *mip* marker and the *wzm* marker were detected less frequently—in 22.5% and 11.7% of the samples, respectively

(Table 8). This phenomenon carries significant clinical relevance. The detection of the *wzm* gene indicates the presence of *L. pneumophila* serogroup 1, which, according to epidemiological data, is responsible for the majority of Legionnaires' disease cases worldwide. The fact that this marker was most frequently detected in internal plumbing systems during the summer period suggests that the conditions prevailing at these points (high temperature, presence of biofilm, and corrosion products) favor the selection of strains with the highest virulence potential. The obtained results are consistent with previous studies indicating that indoor water systems constitute the main reservoir of strains of greatest epidemiological importance. Petzold et al. [24], analyzing drinking water systems using PCR methods, demonstrated that *L. pneumophila*-specific markers were more frequently identified in the final sections of the distribution system, especially under conditions favoring stagnation and biofilm formation. Similar observations were reported by Arrigo et al. [19], indicating that the use of molecular methods enables more effective identification of potentially pathogenic strains, including serogroup 1, even at low bacterial counts. The particular epidemiological significance of the detection of the *wzm* gene stems from the fact that *L. pneumophila* serogroup 1 is responsible for the majority of legionellosis cases reported in Europe and worldwide, which emphasizes the importance of early detection of this marker within microbiological risk management systems. Concurrently, the discrepancies between the results for the *ssrA*, *mip*, and *wzm* markers indicate the presence of other, potentially less pathogenic species of the genus *Legionella* in the investigated water. From the perspective of the new EU Directive, monitoring the genus alone (*Legionella* spp.) constitutes a safer approach in terms of public health, as it enables the early detection of system colonization, even if the most virulent strains of *L. pneumophila* sg1 do not dominate at that particular moment.

4.4. Implications for Monitoring and Risk Management in Water Supply Systems

The results obtained in this study provide strong arguments for including molecular methods in routine water quality surveillance. The demonstrated discrepancy between qPCR results and the culture-based method suggests that traditional monitoring based solely on culturing may not reflect the actual degree of risk, particularly during transitional periods (autumn, spring) or at the initial stages of the distribution system. Implementing a "source-to-tap" approach allows for the identification of "hotspots" within the network. The obtained results fit into the paradigm of risk-based water safety management, with particular emphasis on identifying local foci of secondary colonization. Water quality monitoring should encompass the entire distribution system, with particular emphasis on premise plumbing systems, where specific operating conditions favor microbial proliferation [28]. This requires a systems approach that considers the interactions of environmental parameters, material parameters, and biofilm structure [6].

In this context, the implementation of molecular methods provides significant support for classical monitoring, enabling the detection of microbiological changes at the pre-analytical stage for culture methods. The presented conclusions directly support the assumptions of Directive (EU) 2020/2184 regarding the implementation of risk-based water safety plans. In the investigated system, internal building plumbing systems emerged as such points, where a combination of factors (elevated temperature, corrosion, water stagnation) led to the intensification of the colonization process. From the perspective of the new EU Directive 2020/2184, a key action should therefore involve not only monitoring water at the outlet of the treatment plant but, above all, educating building managers and regularly controlling physicochemical parameters at the sampling points. Furthermore, the application of genetic differentiation (*mip* and *wzm* markers) enables risk gradation. Under Directive (EU) 2020/2184, member states must ensure that risk assessments of domestic distribution systems are established, particularly focusing on priority premises.

According to Annex I, Part D of the Directive, the parametric value for *Legionella* is set at <1000 CFU/L. In this context, our results demonstrate how qPCR can serve as an operational “action trigger” within Water Safety Plans. Specifically, the detection of the *wzm* gene (representing the highly pathogenic *L. pneumophila* sg1), even in the presence of low bacterial titers or negative outcomes in culture-based assays, should serve as a signal for the immediate initiation of localized corrective actions. Under the risk-based framework of the Directive, a *wzm*-positive signal in critical areas (such as hospital wards or senior care facilities) should temporarily bypass the 10-day culture waiting period to trigger immediate control measures, followed by culture-based confirmation. When such a trigger is met, thermal disinfection (thermal shock) remains one of the most widely accepted physical corrective measures for premise plumbing. To be biologically effective, the hot water circulation temperature must be raised to 70 °C to 75 °C at the heat source, ensuring that water reaching every single terminal outlet (taps and showerheads) is maintained at a minimum of 60 °C (ideally 65 °C) for at least 20 to 30 min. While highly effective at rapidly flushing out planktonic *Legionella* and surface-associated biofilms, the cost-effectiveness and overall utility of thermal disinfection must be evaluated realistically. The process is energy-intensive, requiring substantial fuel or electrical consumption to elevate system-wide temperatures. Furthermore, frequent thermal shocks significantly accelerate material degradation (particularly in plastic PE-Xa, polybutylene, or older galvanized iron pipes), increasing the long-term maintenance costs and risk of hydraulic failures. Additionally, thermal disinfection provides only temporary suppression; if systemic issues such as dead legs, low circulation flow, or inadequate water heater temperatures are not structurally resolved, *Legionella* recolonization from deep biofilm layers typically occurs within a few weeks. Therefore, while thermal flushing is highly cost-effective as an immediate, short-term emergency response to a *wzm*-positive qPCR trigger, long-term risk management must rely on continuous temperature control (maintaining hot water distribution consistently above 55 °C and cold water below 20 °C as mandated by the proactive safety philosophy of Directive 2020/2184).

4.5. Study Limitations and Future Perspectives

The present study has several limitations that should be acknowledged. First, the conventional qPCR assays applied here do not discriminate between live, injured, and dead cells or extracellular DNA, and may therefore overestimate the truly viable *Legionella* burden, particularly at sampling points characterized by low bacterial abundance. Coupling qPCR with a viability marker such as propidium monoazide (PMA), which selectively excludes DNA originating from membrane-compromised cells prior to amplification, was beyond the scope of the present study but represents a valuable next step toward a more accurate estimation of the viable, and thus epidemiologically relevant, fraction of the population; its incorporation is recommended for future monitoring campaigns. Second, the sampling strategy employed here relied exclusively on first-flush water samples, collected without tap disinfection or removal of aerators, in order to capture realistic consumer exposure. While this approach is well suited to exposure assessment, it does not permit direct characterization of biofilm communities. Consequently, the associations reported between physicochemical parameters (e.g., iron concentration, water hardness) and *Legionella* occurrence, as well as the proposed role of biofilm stabilization and the formation of protective microenvironments, are inferred from indirect evidence rather than confirmed through direct biofilm sampling (e.g., pipe coupons or swabs), and should be interpreted with appropriate caution. Future studies combining bulk water and biofilm sampling with viability-based molecular assays would allow for a more mechanistic understanding of *Legionella* persistence within premise plumbing systems.

Although pipe material, system age, and heating type are well-known factors contributing to *Legionella* colonization, these parameters could not be individually controlled or analyzed in this study due to the lack of technical documentation from private households. While we acknowledge this as a limitation of our field study, the location of sampling points at the network ends and the focus on morning first-flush samples allowed us to monitor the system under high-risk conditions.

5. Conclusions

A comparative statistical analysis confirmed that the qPCR method demonstrates significantly higher sensitivity for the detection of *Legionella* bacteria compared to the conventional culture-based approach (McNemar test, $p < 0.001$), supporting its application as a complementary tool in microbiological risk assessment. The occurrence of *Legionella* bacteria in the investigated system exhibits a distinct and statistically significant seasonal trend ($\chi^2 = 17.72$, $p < 0.001$ for qPCR; $\chi^2 = 8.30$, $p = 0.040$ for culture). The highest frequency of microorganism detection was recorded during the summer months (June–August), with *ssrA* genetic material detected in 58.3% of the samples and viable cells isolated in 25.0%, which directly corresponded to the seasonal increase in water temperature within internal plumbing systems. Implementing the “source-to-tap” approach allowed for the identification of internal building plumbing systems as critical control points. These specific locations exhibited the lowest cycle threshold (Ct) values, indicating the highest concentration of genetic material, as well as the highest rate of viable cell isolation via the culture-based method. Ct values above 30, recorded at the water intake and water treatment plant (WTP) stages, suggest the presence of only trace amounts of DNA or bacterial cells in a viable but non-culturable (VBNC) state. This confirms the capability of the qPCR method for the early detection of colonization, even when the bacterial concentration is below the detection limit of the culture-based method. Physicochemical factors, such as water temperature, total iron concentration, and water hardness, are significantly correlated with the presence of *Legionella* bacteria. Corrosion products and mineral deposits in internal plumbing systems favor the stabilization of the biofilm structure, creating favorable conditions for the survival and proliferation of these pathogens. Genetic marker differentiation revealed the presence of the most pathogenic serogroup in 11.7% of all analyzed samples. The detection of this marker, particularly when correlated with low Ct values, should serve as a priority signal for the initiation of corrective actions within water safety plans.

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