Temperature diagnostic to identify high risk areas and optimize *Legionella pneumophila* surveillance in hot water distribution systems

**Authors:** Emilie Bédard\textsuperscript{a,b}, Stéphanie Fey\textsuperscript{a}, Dominique Charron\textsuperscript{a}, Cindy Lalancette\textsuperscript{b}, Philippe Cantin\textsuperscript{c}, Patrick Dolcé\textsuperscript{d}, Céline Laferrière\textsuperscript{e}, Eric Déziel\textsuperscript{b}, Michèle Prévost\textsuperscript{a}

**Authors Affiliation:**
\textsuperscript{a}Department of Civil Engineering, Polytechnique Montréal, Montréal, QC, Canada
\textsuperscript{b}INRS-Institut Armand-Frappier, Laval, QC, Canada
\textsuperscript{c}Centre d’expertise en analyse environnementale du Québec, Québec, QC, Canada
\textsuperscript{d}Department of Medical Microbiology and Infectious Diseases, Centre Hospitalier Régional de Rimouski, Rimouski, QC, Canada
\textsuperscript{e}Department of Microbiology and Immunology (Infection control), CHU Ste-Justine, Université de Montréal, Montréal, QC, Canada

**Corresponding author:**
Emilie Bédard
NSERC Industrial Chair in Drinking Water
Polytechnique Montréal
P.O. Box 6079 Station Centre-ville
Montréal, QC, Canada
H3C 3A7
Tel: 514-340-4711 x3711
Email: emilie.bedard@polymtl.ca
Abstract

*Legionella pneumophila* is frequently detected in hot water distribution systems and thermal control is a common measure implemented by healthcare facilities. A risk assessment based on water temperature profiling and temperature distribution within the network is proposed, to guide effective monitoring strategies and allow the identification of high risk areas. Temperature and heat loss at control points (water heater, recirculation, representative points-of-use) were monitored in various sections of five healthcare facilities hot water distribution systems and results used to develop a temperature-based risk assessment tool. Detailed investigations show that defective return valves in faucets can cause widespread temperature losses because of hot and cold water mixing. Systems in which water temperature coming out of the water heaters was kept consistently above 60°C and maintained above 55°C across the network were negative for *Legionella* by culture or qPCR. For systems not meeting these temperature criteria, risk areas for *L. pneumophila* were identified using temperature profiling and system’s characterization; higher risk was confirmed by more frequent microbiological detection by culture and qPCR. Results confirmed that maintaining sufficiently high temperatures within hot water distribution systems suppressed *L. pneumophila* culturability. However, the risk remains as shown by the persistence of *L. pneumophila* by qPCR.

Key words

*Legionella pneumophila*, premise plumbing, viable but not culturable (VBNC), heat treatment, temperature profile, culturability.

Highlights

- Temperature profiles were generated for hot water distribution systems points-of-use
- Risk assessment based on temperature profile results at control points was developed
- *L. pneumophila* positive areas were predicted using the risk assessment tool
- A temperature diagnostic flowchart is proposed to identify *L. pneumophila* risk areas
1. INTRODUCTION

*Legionella pneumophila* (*Lp*) is an opportunistic pathogen that can proliferate in hot water distribution systems (HWDS) of large buildings, such as health care facilities (HCFs), where it can cause waterborne nosocomial pneumonias. Although its optimal growth temperature lies between 25 and 42°C (Yee and Wadowsky 1982), *Lp* has been isolated from water systems at temperatures up to 60°C (Martinelli et al. 2000), and in cold water systems with temperatures below 20°C (Arvand et al. 2011). The presence of *Lp* in HCFs water systems is well demonstrated, with reports of 10 to 50% positive hot water samples taken from taps and showers in Europe and the United States (Arvand et al. 2011, Bargellini et al. 2011, Martinelli et al. 2000, Serrano-Suarez et al. 2013, Stout et al. 2007). Risk characterization of water sources remains uncertain because of the lack of reliable dose-response models (Buse et al. 2012) and therefore the difficulty to define an acceptable level of *Lp* contamination that would minimize risk. While the level of positivity for *Legionella* in health care facilities (HCF) HWDS has been proposed as a reliable predictive risk factor (Best et al. 1983, Lin et al. 2011), the specificity and sensitivity of the 30% positivity cut-off point has been recently questioned (Allen et al. 2012, Allen et al. 2014, Pierre et al. 2014).

Control of *Legionella* risks in health care facilities (HCFs) is addressed and regulated through guidance documents (Bartram et al. 2007, BSR/ASHRAE 2013, CDC 2003, HSE 2013, République Française 2010a). System characterization and environmental monitoring are among the first steps to establish a water safety plan or to evaluate the
operational risk in hot water distribution systems (HWDSs), especially in HCFs (BSR/ASHRAE 2013, Department of Health (DH) and Estates and Facilities Division 2006, République Française 2010b, WHO 2011). Recent guidelines stress the need to properly manage hydraulics to ensure homogeneous temperature and biocidal control in all areas of the HWDS (CSTB 2012), and system balancing under varying demand should be verified.

Although a multitude of possible system architectures are encountered, a simplified schematic of a hot water distribution system (HWDS) can be established (Fig. 1) and should include: the number and characteristics of key systems components such as the calorifiers, reheating units and reservoirs, the distribution systems including principal, subordinate and tertiary flow and return loops and point-of-use devices (tertiary terminal end). A schematic and characterization of each HWDS within a premise must be established independently (BSR/ASHRAE 2013, HSE 2013, République Française 2010a). This data is the foundation for interpreting monitoring results and identifying high risk areas.

A summary of the key elements from selected regulations and guidelines to implement temperatures control of $L_p$ in large buildings, and when available, in HCFs is provided as supplementary material (Table S1). Approaches to control $L_p$ in hot water distribution systems (HWDSs) vary considerably, but all guides include objectives or obligations for optimal operating temperatures at critical points in the distribution systems. Also
commonly specified are construction and operational standards, such as minimizing stagnation (recirculation loops, elimination of hydraulic and physical dead ends, etc.), recommendations on the use of devices and materials not promoting bacterial proliferation (construction material, flow, temperature, etc.) and requirements for microbiological monitoring in relation to pre-established criteria that define corrective actions.

In France, recently strengthened regulations determine mandatory minimum temperature and *Legionella* monitoring at defined critical control points: 1) hot water outlet and reservoir when present; 2) return loop; and 3) representative points-of-use considered at risk (farthest from the water heater or serving vulnerable patients) but the number of sampling points to be monitored is not specified (République Française 2005, 2010a, b, Table S1). It is recommended that temperatures be monitored daily or continuously at hot water heater outlets and at each return loops, and weekly at service points in HCFs. Temperature measurements at points of use are conducted on flushed samples (2-3 min). In the United Kingdom, a risk management approach is proposed, with recommended preventive measures including system maintenance, elimination of stagnation or dead zones, reduction of aerosol formation, maintenance of adequate temperatures and use of materials unfavorable to biofilm development (Department of Health (DH) and Estates and Facilities Division 2006, HSE 2013). Temperature control regimen is presented as the preferred initial approach for *Legionella* control (Table S1). Minimal monthly temperature monitoring is specified at control points including water
heater outlet, return loops and sentinel taps. Sentinel taps include representative at-risk taps as well as the first and last taps of each return loops. The use of continuous temperature monitoring is recommended for the water heater outlets and the return loops. In addition, temperature at the tap should be monitored annually on a rotating basis covering 20% of taps yearly, to ensure the whole system is meeting required temperatures for *Legionella* control. It is not permissible to shut down pumped recirculation as it would lead to the loss of the required system temperatures. *Legionella* monitoring is not prescribed unless target temperatures cannot be achieved; however it is recommended in areas with highly vulnerable patients. Weekly flushing for several minutes is recommended for low usage taps.

Although all available regulations and guidelines provide information on various aspects of the implementation of a successful temperature control regimen, there is no consistent guidance on key elements such as the selection of sentinel points, the incorporation of *Lp* monitoring and the interpretation of the temperature monitoring results. Reports on the efficacy of the implementation of temperature control in health care facilities (HCFs) reveal limited success (Arvand et al. 2011, Bargellini et al. 2011, Blanc et al. 2005, Darelid et al. 2002, Hruba 2009, Lee et al. 2011, Serrano-Suarez et al. 2013). Nevertheless, adjusting the temperature at the heater outlet to ensure water temperatures greater than 50-55°C at distal outlets can be highly effective in reducing the proportion of positive swabs or water samples (Arvand et al. 2011, Blanc et al. 2005, Ezzeddine et al. 1989). Moreover, areas consistently positive for *Lp* were associated with
poor hot water recirculation leading to temperature losses (Blanc et al. 2005). In most
case studies, the actual conditions of application of the temperature control regimen
are poorly documented with some information on temperatures only available for the
water heater and return. The efficacy of temperature control regimens must be
assessed by its ability to suppress $L_p$ growth in the distal areas, as distal growth is highly
significant (Cristina et al. 2014, Serrano-Suarez et al. 2013). On the other hand, there is
increased risk of scalding for temperatures higher than 50°C at the tap (Moritz and
Henriques 1947). Some countries specify maximum temperatures at the point-of-use to
avoid scalding (Table S1), but newly updated regulation in United Kingdom require a risk
assessment comparison between the risk of scalding and the risk of infection before
limiting the hot water temperature below 50°C, a risk factor for $ Legionella$ proliferation.

Although the critical elements of temperature control in guidelines and regulations to
reduce $ Legionella$ risks in HWDSs rely on scientific evidence and application experience,
the detailed implementation, especially the selection of critical control points and
monitoring requirements, most often reflect economic constraints. In addition,
significant discrepancies exist between proposed modalities of implementation and
management. The objectives of the present study were to: (1) demonstrate the
potential of detailed temperature profiling to identify areas at risk of $L_p$ in the hot water
distribution systems (HWDSs) of five health care facilities (HCFs); (2) identify effective
monitoring strategies and guidance to conduct temperature profiling and interpret
monitoring results; (3) propose a risk characterisation approach based on temperature diagnostic at critical control points.

2. MATERIALS AND METHODS

2.1. Hot water system characterization

Five hot water distribution systems (HWDSs) were analyzed. Systems 1 to 4 are smaller systems within a 7-story general hospital facility of 255 beds using conventional electric water heater being fed chlorinated ground water. System 5 has a larger flash system feeding a ten-story 450 bed children’s hospital fed by surface filtered chlorinated water. A survey of the different HWDSs and connected units was first completed.

The principal flow and return loop of each system was sampled at the water heater outlet, in the principal return loop and prior to the return point into the water heater. The sampling ports were seldom used and were flushed prior to sampling to ensure no stagnant water from the sampling port would be collected. The sampling port was cleaned with ethanol and sterilized MilliQ water. Two samples were collected at each point: 1) 2L in sterile polypropylene bottles with sodium thiosulfate (final concentration of 1.1mg/L) microbiological analysis and 2) 250 mL for pH, temperature, chlorine and conductivity measurements. Municipal water feeding the hot water systems was sampled following the same protocol. In addition continuous temperature monitoring was conducted on 3 subordinate return loops for system 5, using a Datalogger (RDXL4SD 4-Channel, Omega, Qc, Canada).
2.2. Temperature profiling and water sampling at points-of-use

Sentinel taps where sampling was performed were selected based on the following criteria: representative of different building levels, some at the far end and preferably in areas serving vulnerable patients such as intensive care units, surgical ward, transplant, infectious diseases. All sampling events were conducted between July 2012 and October 2013. The first part of the sampling campaign was conducted to establish temperature profiles at each selected sentinel point-of-use in hot water, across all 5 systems between July 2012 and March 2013. A temperature probe was inserted into the water to measure the temperature over a 20 minute period of continuous flow. Each system had a number of taps sampled proportional to the size of the system. Systems 1 to 5 had respectively 3, 6, 3, 7 and 36 taps sampled. The temperature profiles could not be generated for 1 mitigated tap in system 1, 3 in system 2 and 1 in system 3. In addition, three taps from system 5 were selected for a repeat temperature profile sampling. Residual chlorine was measured onsite (Pocket Colorimeter™ II, Hach, USA) for all samples.

The second part of the sampling campaign was conducted to evaluate the presence of *Lp* at the point-of-use. All sentinel points of systems 1 to 4 and 8 sentinel points from system 5 were sampled for microbiological analysis. Sentinel points from system 5 were selected based on temperature profile results. For each sampling point, 3L of hot water were collected without prior flush into sterile polypropylene bottles containing sodium...
thiosulfate (final concentration of 1.1 mg/L). Of the 3 liters collected, 1L was used for
culture, 1L for qPCR and 1L was collected as extra. This sampling was repeated 4 times
at 3 selected sampling points in 2 systems fed by the same source water: a system with
no positive sites for Lp (system 1) and a system with a high positivity rate (system 4).
The 3 control points selected were the water heater outlet, one representative tap and
the principal return loop.

2.3. Impact of stagnation

The third part of the sampling campaign was conducted on 2 taps of system 5 to study
the impact of stagnation on the detection of Lp. Stagnation is defined as the period
during which the tap is not used and water remains idle within the piping. One liter of
hot water was sampled in sterile propylene bottles with sodium thiosulfate (final
concentration 1.1 mg/L) after 1 hour, 1, 2, 3, 5 & 10 days of stagnation. These
stagnation times were chosen to represent various situations within a real system: 1h
for the time between usage in a patient room; 1-day for patient daily care; 2 and 3 days
for areas closed on the weekend (i.e. outpatients clinics); 5 and 10 days for an empty
room in between patients or a temporary ward closure. Lp concentrations were
measured by qPCR as described in section 2.4.

Heat losses during stagnation periods were evaluated in the laboratory, on 81 cm of
1.25 cm diameter copper pipes at room temperature (20°C) without insulation and with
insulation: Type 1, 2.54 cm thick fiberglass insulation with PVC jacket (Caltech Isolation,
Canada) and Type 2, 0.95 cm thick polyethylene foam insulation (Tundra, Industrial Thermo Polymers Limited, Canada).

2.4. Microbiological analyses

Water samples were mixed thoroughly and divided to perform isolation and quantification of *Legionella* spp. and *Lp* by culture and quantitative polymerase chain reaction (qPCR).

Culture was conducted according to the standard AFNOR NF T90-431 procedure (AFNOR 2006). Briefly, 1 liter was filtered on sterile 0.4 µm polycarbonate membranes (47 mm diameter; Maine Manufacturing, LLC), which were then sonicated in 5 ml sterile water at 47 kHz for 1 min (Branson, Danbury, USA). Heat treatment (50°C, 30 min), acid treatment (pH=2; 5 min) and combination of both were performed on 3 separate 1 ml aliquots. Samples were plated on GVPC selective agar (Innovation Diagnostics Inc.) and incubated at 36°C for 10 days. Typical colonies that developed after 4 to 10 days were sub cultured on confirmation plates for 2 to 4 days at 36°C. Resulting colonies that developed on BCYE agar, but neither on blood agar nor on BCYE without cysteine were considered as *Legionella* spp. Confirmation for *Lp* was conducted using the *Legionella* latex test (DR0800, OXOID Limited). The calculated detection limit for the culture method was 50 CFU/L for both *Legionella* spp. and *L. pneumophila*.
Quantification by qPCR was performed on a Corbett Rotorgene 6000 using the iQ-Check Quanti L. pneumophila kit (Bio-Rad, Mississauga, Canada) with the following protocol: 15 min initial denaturation at 95°C followed by 50 cycles with denaturation at 95°C for 15 s, annealing at 57°C for 30 s, elongation at 72°C for 30s and final elongation for 15 min at 72°C (Bonetta et al. 2010). An internal control and four DNA standards ranging between 19 and 3.9x10^4 genomic units (GU) were supplied with the kit. Sterilized water was used as negative control. DNA extraction was performed directly on filters using a bead beating method adapted from Yu and Mohn (1999). Briefly, 1L was filtered on 0.45 μm mixed cellulose ester and the filter was inserted into an extraction tube containing a garnet matrix and one 1/4-inch ceramic sphere (Lysing Matrix A, MP Biomedicals, Solon, USA). Lysing buffer was added to each tube prior to the bead beating step performed on a FastPrep MPBio-24, followed by ammonium acetate precipitation and successive ethanol washes.

**2.5. Statistical analysis**

Statistical analyses were performed with Statistica10 (StatSoft). A one-way analysis of variance (one-way ANOVA) was used to evaluate differences between the 3 control points sampled in systems 1 and 4 during the repeat sampling. A t-test was used to detect differences between the two taps sampled at various stagnation times in system 5. Significance level was set at p = 0.05.
3. RESULTS & DISCUSSION

3.1. General system characterization

Systems 1, 3, 4 & 5 presented a multiple vertical subordinate flow and return loop configuration feeding in average three devices per story. System 2 was a simplified horizontal architecture with only few vertical pipes feeding water to horizontal subordinate flow and return loops (Fig. 1). There is no reported evidence showing that the vertical or horizontal configuration is a determining factor for the risk of contamination. Other factors including hot water temperature, effective recirculation in the subordinate loop, the presence of dead-ends, piping material and water velocity have been identified as risk factors (Health and Safety Executive (HSE) 2013). Nevertheless, it is important to know and document the configuration of a studied system to interpret temperature data collected. With information on the pipe diameter and configuration, the location and relative importance of recirculating and stagnating volumes can be determined providing information to guide monitoring and control strategies. For example, the recirculated volume was approximately 900L, of which 600L in the principal flow and return loop (50 mm diameter) and 300L in the subordinate flow and return loops (10 vertical risers of 25 mm mean diameter). The distal volume in the tertiary terminal end was about 300L (Fig. 1). However, this volume can be minimized if a tertiary return loop is added, leaving only the small connecting volume of less than 150mL per device accounting for a total of 90L of stagnant volume (Fig. 1).
For systems 1 to 4, incoming water had chlorine residual of 0.30±0.03 mg Cl₂/L, pH of 7.77±0.05 and conductivity of 307±29 μS. For system 5, residual chlorine was higher, at 0.5±0.1 mg Cl₂/L, pH of 7.82±0.07 and conductivity of 288±13 μS. There was no additional disinfection treatment in any of the hot water systems studied and mean residual chlorine was 0.04±0.02 mg Cl₂/L for all systems.

3.2. Temperature monitoring

3.2.1. Water heater outlet

Most guidelines specify that target temperatures must be maintained at all times, but seldom do they specify the monitoring requirements of measurement frequency. Periodic temperature readings, even daily measurements, do not provide insurance of temperature maintenance in the hot water distribution system (HWDS), unless the stability of the system’s performance has been fully established. Systems seemingly providing water above 60°C based on daily measurements can actually produce lower temperature water for extended periods of time. In fact, the mean temperatures at the water heater outlet for four of the five systems studied were above 60°C, but online temperature monitoring revealed that production temperature was repeatedly below 60°C and reached down to 43°C in some cases (Table 1 and Fig. 2). System 1 consistently produced water above 60°C while systems 2, 3 and 5 regularly produced water below 60°C at certain periods of the day (Fig. 2). For system 3, temperature was monitored weekly by the operators on Saturday mornings during low water demand.
providing an average of 62.5°C over a period of 24 months (Table 1). Nonetheless, when online monitoring was performed during a typical weekday, mean temperature was lower (57.8°C). It is also interesting to point out that even a very recently installed system (2011) equipped with a flash heating unit was also subject to periodic temperature drops (System 5, Fig. 2). These observations demonstrate the need to use online monitoring to assess the temperature compliance of a HWDS compared to periodic manual readings of temperature. Daily variations in hot water demand in large HCFs with typical peak flow factors of > 6 (Bujak 2010) can influence the temperature at the water heater outlet depending on the system’s capacity. The extent and duration of the non-compliance of the hot water outlet temperature set point is important to consider and has been limited to the sporadic short duration (minutes) events in the German technical rules (Table S1).

3.2.2. Return loops

The return loop at the point closest to the water heater is designated as the furthest point from the water heater and continuous temperature monitoring is often recommended (Fig. 1). It is considered as an indicator of the system’s capacity to maintain temperatures throughout the hot water distribution system (HWDS). In the five systems studied, the principal return loop temperatures ranged between 50.4 and 58.9°C with varying levels of blending from multiple return loops occurring upstream of the principal return control point (Table 1). Continuous monitoring for 2 months at the return loop manifold for combined returns of units 3&5 (45.7°C), units 1&2 (48.0°C),
single return for the kitchen (58.1°C) and for unit 3 prior to merging with unit 5 (46.6°C) revealed wide differences compared to the overall combined return loop (53.9°C).

Although a regulated control location (Table S1), temperature at the principal return loop is not indicative of the conditions in all subordinate loops within a complex HWDS if the system is not balanced for all water demand conditions. In such cases, it merely represents the mean temperature of the blended recirculated hot water from various sectors of the HWDS. More specifically, it does not provide any information on the actual levels of recirculation and temperature losses in the various sectors of the HWDS and does not in any way confirm efficient recirculation in all subordinate loops. These results suggest the temperature monitoring of subordinate return loops together with the principal return loop as a tool to identify imbalances within a system and as an ongoing system validation measure.

Heat losses between the water heater outlet and a remote point will occur during stagnation (if recirculation is not effective or shut down for energy conservation purposes) or during circulation in the principal and subordinate flow and return loops. During low demand conditions, recirculation will dictate residence time and drive heat losses. Mean system heat losses were evaluated for each of the five studied systems (Table 1). For three of the five systems, temperature losses between the water heater and the principal return loop mean temperatures exceeded the target of ≤5°C set in several guidelines (Table S1). Heat losses during circulation can be minimized by reducing residence time. Water velocity can be set to meet desired maximum heat...
losses and general recommendations suggest maintaining a minimal velocity of 0.2 m/s (Blokker et al. 2010, CSTB 2012), which would result in approximately 30 min residence time and 5°C heat losses in large health care facilities (HCF) insulated HWDS. Although insulation minimizes heat losses under flowing conditions, it is not sufficient to maintain high temperatures over prolonged periods of stagnation. Actually, slower heat losses during stagnation may lead to sustained optimal temperatures for *L. pneumophila* growth. Figure 3 shows that temperature decreased from 60°C to below 50°C within 30 min in fully insulated copper pipes and within 10 min for non-insulated pipe, both reaching room temperature after 3.5 hours. Periods of stagnation of 30 min or more are expected in the connecting piping upflow of points-of-use and in areas of inefficient recirculation.

Existing standards and guidelines set design and operational obligations to control heat losses in hot water distribution systems (HWDS) to maintain at minimum target temperatures throughout the HWDS and to meet energy conservation goals, but these are generally only compulsory for new buildings. Recirculation flow rates should be calculated to maintain a <5°C system heat loss or to ensure a minimum temperature of 50-55°C at the end of the return loop assuming adequate recirculation throughout the system (ASPE 2008). The control points results required to evaluate heat loss goals compliance include the principal and subordinate return loops, the most distant point of the flow loop or all points of the system (Table S1). Monitoring results from the five HWDSs clearly show that the selection of the return loop reference point is critical. Heat
loss evaluation from the principal return loop may mask major heat losses in subordinate flow and return loops, as we observed in system 5 with losses ranging from 3.5 to 16.3°C when evaluated for single or dual subordinate return loops (Table 1). Indeed, wide differences in temperature can occur between secondary return loops, and thus all return loops should be considered individually. The overwhelming importance given to temperature maintenance has also led to the specific banning of recirculation shutdown in Austria and United Kingdom (Table S1). The nightly shutdown of recirculation for energy conservation purposes is only allowed in two rules (CMMQ/RBQ 2013, DVGW German Technical and Scientific Association for Gas and Water 2004) and only with the demonstration of unobjectionable hygienic conditions. Our results point out that the temperature losses of isolated subordinate loops during stagnation resulting from such shutdowns would quickly generate durable temperature conditions favorable to the growth of \( Lp \). More importantly, such shutdowns during low or nil demand conditions expose the whole HWDS, instead of a relatively small volume \( (1,200\text{L versus 90 to 300L in System 5}) \) to these undesirable temperature conditions.

3.2.3. Temperature distribution at point-of-use

Sequential volume profiling results identify in which sections of the HWDS the heat losses take place, namely the tap and its connecting piping, the secondary piping, the distribution columns and/or the main feeder pipes. Profile variability for a given sampling point at different times and days was found to be small, with overall profile and maximum temperature reached being consistent over time despite variable
temperature in the first liter (Fig S1). Temperature profiles obtained on the studied
systems are summarized in three groups (Fig. 4), with detailed profiles presented in Fig.
S2. Systems 1, 2 and 3 (Fig. 4a) met recommendations for water heater outlet and
return loop temperatures, with 86% of points reaching 55°C and all points being above
50°C after 2 minutes of flow, indicative of limited stagnant water volumes and effective
recirculation. Ideal systems should have no or very little transition and reach equilibrium
at recommended temperatures in order to maintain sufficient temperatures within the
whole system. Despite reaching equilibrium temperature rapidly (<60s), system 4 could
not achieve recommended temperature at the points-of-use with 57% of points never
reaching 55°C although all above 50°C, mainly due to the insufficient water temperature
at the water heater outlet (Fig. 4b). System 5 shows a longer transition period before
reaching temperature equilibrium and is unable to meet 55°C for 47% and 50°C for 19%
of points, despite water heater and principal return loop temperatures meeting
recommendations (Fig. 4c).

Additional temperature monitoring using surface thermocouples on subordinate flow
and return pipes were conducted on system 5 (Fig. S3). The ongoing temperature
monitoring in subordinate flow and return loops in addition to the principal flow and
return loop provided helpful information to identify local issues. For instance, broken
valves in a shower faucet resulted in cold water entering the hot water feed pipe and
riser. Fixing the device increased the minimal temperature by an average 5°C in all 10
subordinate risers in this wing (Fig. S3, a-c). A second example was insufficient
recirculation causing a significant heat loss during night flow, which was corrected by the addition of a local pump on the subordinate return loop, after the furthest pair of risers (Fig. S3, d-g). These examples show the importance of characterizing local conditions and the potential of single faulty devices to influence temperature maintenance in large sections of hot water distribution systems (HWDS). Again, we conclude that relying on temperature maintenance in the principal return loop is not sufficient to identify such risk areas.

3.3. Legionella monitoring

Results of microbiological measurements for the five studied systems are presented in Table 1. Detection by qPCR was used in complement of culture detection as it has been shown to be efficient in monitoring changes in the bacterial numbers (Krojgaard et al. 2011, Lee et al. 2011). Culture positive samples for Lp were detected in systems 4 and 5 with 22 and 27% positivity respectively (detection limit = 50 CFU/L; quantification limit = 250 CFU/L). Culture positive samples results were low, with only one count above quantification limit at 600 CFU/L, located at a tap in system 5. Positivity increased above 80% for both systems when measured by qPCR and remained below detection limit for systems 1-3, except for one sample in system 2 (Table 1, Table S2). Systems in which water temperature was kept consistently above 60°C coming out of the water heater and maintained above 55°C across the network were below detection limit for Legionella by culture or qPCR. Such results strongly suggest that satisfactory management of temperature at control points in the studied systems resulted in lower
prevalence. However, these results represent a water quality snapshot at a point in time and are not necessarily representative of microbial quality over time or at other locations in the HWDS. Several factors affecting *Lp* densities at a given point have been identified including intrinsic biological system heterogeneity, culturability, prior stagnation and sample volume. Napoli et al. showed variation of ≤ 20% concentrations of CFU/ml from one day to the next within a ward during repeated sampling over five consecutive days across eight units within a hospital (Napoli et al. 2009). In the present study, confirmation sampling was conducted in two of the five HWDSs to investigate the temporal variability. Fig. 5a shows results from repeated sampling conducted at three control points (water heater outlet, principal return loop and a point-of-use) in systems 3 & 4. All samples were negative in qPCR and culture for system 1, whereas samples from system 4 were consistently positive in qPCR and to a lesser degree in culture (Fig. 5a). Mean levels of *Lp* detected in system 4 were not significantly different between the 3 control points (*p* > 0.05). These findings are in agreement with recent reports of discrepancies between trends in *Lp* by qPCR and culture in suboptimal conditions for inactivation of viable but not culturable (VBNC) cells (Krojgaard et al. 2011, Lee et al. 2011). Krojgaard et al. showed that qPCR levels can be used to verify the impact of corrective actions such as thermal shock and demonstrated non-detects qPCR results as a predictor of low risk.

Another factor that may influence levels of *Legionella* in water is the duration of stagnation prior to sampling. Recent evidence reported an increase in bacterial
concentrations after various stagnation times (overnight to 14 days) in the cold water distribution system of a large building (Lautenschlager et al. 2010, Lipphaus et al. 2014). A steady increase was observed in the first 12 hours of stagnation whereas longer stagnation time did not lead to further increase (Lautenschlager et al. 2010). In the present study, hot water was sampled from two taps at different stagnation times and \( Lp \) concentration was evaluated by qPCR (Fig. 5b). The taps were not found to be statistically different when comparing mean results and no correlation was established between the mean \( Lp \) concentration and the stagnation time. However, the stagnation times were longer than 12 hours, except for the 1h stagnation and samples were taken from the hot water systems. To our knowledge, there is no reported data on the impact of stagnation on bacterial concentrations in hot water. These results suggest that \( Lp \) concentrations in the first liter of hot water at the tap may not be affected by stagnation time.

The volume of sample determines the source of the water within the HWDS. \( Lp \) monitoring can be performed to assess the risk associated with 1) the water heater and primary distribution network using flushed samples, and 2) the distal system, including the tap and its connection to the main distribution system, using samples collected without prior flushing. Cristina et al. (2014) reported that distal stagnation increased the number of positive sites from 2.63 % to 15.79% and mean concentration from 7 vs 637 vs CFU/L for \( Lp \) sg1. Such distal amplification was not as clearly observed by these authors for \( Lp \) sg2-14 with 40.79 % to 42.11% positive and mean concentration from
19,455 vs 26,746 CFU/L. Similar trends were observed for *Legionella* spp in HWDS taps with increased concentration from 45 CFU/L (23% positivity) after a 3 minute flush to 226 CFU/L (35% positivity) in the first liter (Serrano-Suarez et al. 2013).

Although post-flush samples provide insight into systemic hot water distribution system (HWDS) contamination, results from the first volume to flow are indicative of the acute concentrations to which patients may be more readily exposed. In the first volume to flow from the tap, water temperature will often be lower due to previous stagnation and disinfectant will be absent, favoring culturability of cells. On the other hand, higher copper concentration present after prolonged stagnation could impact culturability. Non-detection of *Lp* by culture at a given sampling point and time doesn’t necessarily equate to absence of risk for the system.

Volume sampled, typically 1L or more for *Lp*, plays an important role in data interpretation, either for temperature measurements or microbiological detection where the detection limit of the method improves with the use of higher volume of samples. As illustrated on Fig. 1, sampling the first liter will collect water from the tap and connecting pipes, and might reach water from the subordinate return and flow pipes depending on the configuration. For example, 8 meters of a 13 mm diameter pipe are required to reach 1L. If a larger sample volume is required to do multiple analyses (i.e. culture and qPCR or simultaneous detection of other waterborne opportunistic
pathogens), it should be kept in mind that water will become less representative of the point-of-use.

3.4. Value of Temperature Control in Lp Risk Management

The implementation of a water safety plan is the recommended approach for preventive risk-management related to drinking water (WHO 2011) and temperature control is widely recognized as the first risk mitigation measure for Legionella control in hot water distribution systems (HWDS) (Table S1).

Maintaining sufficient temperatures at all critical points, including the subordinate return loops, and minimizing volumes of uncontrolled temperature in the terminal ends appear essential to a successful system wide thermal control of culturable and VBNC Legionella. Most studies report on the results of temperature control based on prevalence measured by culture-based detection methods. Although lower prevalence is generally observed after temperatures are increased, limited efficacies are often reported. An early study observed 50% reduction of tap positivity following an increase in temperature from 45 to 60°C at the water heater outlet, although an elevated number of taps located in patient rooms remained positive (Ezzeddine et al. 1989). Water temperature at the tap ranged between 30 and 56°C after a few minutes of flushing, demonstrating the system’s inability to provide elevated temperatures in all areas. A similar reduction in % positive taps from 60-90% to 30-40% was reported in a hospital when water heater temperature was raised from 50 to 65°C, in that case
providing temperatures >50°C at most outlets (Blanc et al. 2005). Importantly, the remaining positive outlets were situated in an area with inadequate recirculation. A third field study documented a successful reduction of *Legionella* positive taps from 100% to a mean value of 12% maintained over 10 years following the hot water temperature increase from 45 to 65°C (Darelid et al. 2002). This temperature regimen was implemented following an outbreak and resulted in water temperatures between 56 and 61°C at the tap after 5 minutes flushing. Recent field studies support the importance of maintaining elevated temperatures at distal locations (estimated by the temperature after 1 minute of flushing), with 4–11% of positive at T≥55°C vs 14-82% for T<55°C (Arvand et al. 2011, Bargellini et al. 2011, Hruba 2009). Those observations show that the efficiency of thermal inactivation in complex recirculated full scale HWDS is enhanced when temperature exposure is sufficient in all areas of the HWDS. However, significant distal amplification of *Legionella* can occur as evidenced by long term full scale sampling results (Cristina et al. 2014, Serrano-Suarez et al. 2013) and a number of taps may remain positive for *Legionella*.

The limitations of thermal control in HWDS raise questions on the validity of the existing threshold temperatures of 50-60°C. Pioneer work evidenced the consistent susceptibility of 40 *Lp* isolates to temperature, with 1 log reduction achieved in 2.3-5 min at 60°C and 8 log reduction after 25 min as estimated by culturability (Stout et al. 1986). Recent findings show that elevated temperatures between 55 and 70°C will produce VBNC cells that cannot be detected by culture methods. Laboratory studies
conducted on HWDS samples confirm the suppression of culturability at T≥55°C as evidenced by the presence of \textit{Lp} when measured by qPCR and viable qPCR (Lee et al. 2011, Mansi et al. 2014). Despite a rapid loss of culturability at temperatures >55°C, some \textit{Lp} strains can resist in the VBNC state for periods of 30-60 minutes at temperatures between 55 and 70°C (Allegra et al. 2008, Allegra et al. 2011, Epalle et al. 2014). Furthermore, the development of heat resistant \textit{Lp} strains was observed over time for groups of strains isolated in hospital water systems submitted to periodic extreme temperature (24h @ 65°C a few times a year), while no such resistance was observed for strains isolated from the system where heat shock treatments (70°C 30 minutes) were sparingly applied. Finally, the efficacy of thermal disinfection on biofilm, the main reservoir of \textit{Lp} in HWDS (Buse et al. 2014), is at best scarce and reports limited and non-lasting efficacy of 70°C for 2 hours on culturable \textit{Legionella} spp (Saby et al. 2005). These findings stress that high temperature regimen provide \textit{Lp} control not \textit{Lp} eradication and the importance of maintaining a constant temperature regimen throughout the system to provide adequate contact time and avoid growth.

We propose a system wide risk classification to assess risk in a HWDS based on published reports and our findings (Table 2). In addition to monitoring temperature at critical control points, the evaluation criteria also include the percentage of time that temperature is maintained at the hot water production unit or return loops. Indeed, exposure to temperature should be considered instead of temperature alone, as regulated for chemical disinfection (Concentration X Time concept). Subordinate return
loop temperatures are used to evaluate the system’s heat loss within each sector of the building. Temperature exposure in the subordinate flow and return loop is estimated based on temperatures measured after 1 minute of flushing and serves to determine risk in specific areas. When evaluating the five systems against the proposed risk classification (Table 2), results from the characterization of the HWDS combined with the temperature profiles at point-of-use were good predictors of areas at risk for \( L_p \) detection (Table 1). In light of these findings and considering the presence of VBNC Legionella at temperature ranging between 55-70°C (Epalle et al. 2014), the set points proposed in existing regulations and guidelines and selected for the proposed risk classification approach appear minimal and should be met at all times. The development of heat resistant strains following periodic heat shock also supports the maintenance of a steady thermal preventative inactivation regimen instead of relying on periodic curative thermal shock (Allegra et al. 2011). The apparent limited success of HWDS in large buildings may have been caused by inconsistent maintenance of sufficiently elevated temperatures in all areas of the building because of inadequate recirculation and/or low set-points.

Regulations and guidelines all recommend the identification of representative sampling points for \( L_p \) sampling and temperature monitoring at designated control points. However, the rationale for frequency and number of sites for temperature monitoring is not evident and the limited number of proposed control points implies that the HWDS is well balanced. Furthermore, there is little guidance for follow-up action to identify the
cause of temperature losses. To remediate this shortfall, a diagnostic flowchart for the initial assessment of _Legionella_ risk within an existing HWDS is proposed using temperature measurements and profiles at the water heating unit, return loops and critical points (Fig. 6). We propose a step approach starting from the principal return and flow loop system that indicates the overall system risk level, then moving progressively to the subordinate flow and return loops to identify large building areas or sectors at risk, and finally to the tertiary terminal ends, to identify local issues with defective faucets or showers. The diagnostic flowchart also proposes a staged response in terms of corrective and preventative actions, including _Lp_ monitoring. Critical control points, defined as the water heater outlet, the principal return loop and representative at risk points-of-use (not reaching control temperature, farthest from the water heater or serving vulnerable patients) are prioritized for sectors or systems identified at risk by the initial risk assessment (Fig. 6). This step approach can help direct efforts towards high risk areas and optimize resource allocation, especially costly _Lp_ monitoring. Nevertheless, an ongoing _Lp_ monitoring strategy and schedule should be put into place through a water safety plan once initial assessment is completed and corrective measures have been completed.

Although temperature control is a central element of risk mitigation, other factors affecting the persistence of _Legionella_ in HWDS should be considered in the water safety plan such as: the susceptibility of environmental strains to heat inactivation; the relative importance of terminal volumes not subject to recirculation; the sampling
protocol used for *Legionella* monitoring (first volume, flushing, etc.); the presence of biofilm & amoeba and the use of chemical disinfection. The limitations of traditional culture-based methods to detect the presence of *Lp* when in presence of environmental stressors should also be considered when determining a sampling strategy. Although culture is the gold standard and helpful to isolate strains, qPCR can be a valuable tool to monitor changes in a system. An increase in qPCR signal compared to a baseline is indicative of cell growth, even if there is no distinction between viable and dead cells. The relation between *Lp* culture and qPCR results is still not clear, but a recent study by Lee et al. showed qPCR results following the trends of culture in a hot water system, with exceptions for temperatures above 50°C and in the presence of additional disinfection (Lee et al. 2011). These exceptions may be attributed to the impact of temperature on culturability.

4. CONCLUSIONS

- A step approach combining temperature monitoring of the hot water distribution system (HWDS) main components and temperature profiling at points-of-use can be used to determine the susceptibility of overall hot water distribution system and specific areas of large buildings to *Legionella* proliferation. When multiple subordinate return loops are present, temperature should be monitored at each subordinate return loop prior to the principal return loop. Monitoring temperature representative points or even all points on
a rotating basis (e.g. 20%/year) is time consuming and yet insufficient for rapidly detecting faulty equipment such as defective valves.

- The impact of faulty thermostatic devices extends far beyond the terminal connecting piping and can affect large areas of buildings, placing significant volumes of hot water at risk. Faulty return valves should be rapidly identified and repaired or replaced. A change observed in results from continuous temperature monitoring of the subordinate return loop can provide useful information to identify the occurrence of a faulty device.

- Temperature monitoring will help understand the hydraulics, quantify the thermal losses of the recirculating system and identify the distribution columns that need balancing. A systematic diagnostic is necessary to identify areas most at risk in hydraulically unbalanced HWDS or in older buildings where original plans and drawings may not be available or renovations and rearrangements have occurred.

- Systems assessment and monitoring should also take into account area specific hydraulic conditions within the building, including closed units, low usage and configuration of the overall system.

- Temperature profiling should be performed at a large number of points confirming the extent (volume) and nature (systemic or distal) of undesirable temperatures in HWDS, guiding $L_p$ monitoring decisions. The staged approach based on inexpensive and easily implemented temperature profiling can
optimize resources and funds allocation by directing efforts towards high risk areas.

• Although necessary, \textit{Lp} monitoring is costly and time-consuming, and should be targeted to enable decision making for infection control. Our staged approach can guide corrective system interventions and serve as a basis to justify preventive risk reduction actions and select sampling points for \textit{Lp} monitoring.

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Figure legends:

Fig. 1: Hot water distribution system general schematic including temperature control points. Three different types of vertical and horizontal distribution systems are represented: ① recirculation before the last tap; ② recirculation connected after each device; ③ recirculation connected after the last device.

Fig. 2: Examples of continuous temperature monitoring at water heater outlet for each studied system (black line) and at the return loop for system 4 and 5 (blue line). No continuous monitoring data was available for systems 1 to 3. Date format is MM/DD/YY.

Fig. 3: Heat loss during stagnation of hot water in 1.25 cm diameter copper pipes with and without insulation at room temperature.

Fig. 4: Hot water temperature profiles at points-of-use as a function of volume for a) systems 1, 2 and 3 grouped, n = 7; b) system 4, n = 7; c) system 5, n = 36. Mean temperature at the hot water production unit outlet and at the return loop are shown for each system.
Fig. 5: Variability of *L. pneumophila* concentration measured by qPCR (a) in system 4, for repeat sampling events without prior stagnation (*n* = 5, Jan-Oct 2013) (b) in system 5, after different water stagnation times for tap A (light gray) and tap B (dark gray) (*n* = 2, Nov-Oct 2012).

Fig. 6: Diagnostic flowchart for the initial assessment of *Legionella* risk in an existing HWDS.

Table 1: HWDS Systems characterization through control points temperature and microbiological measurements

Table 2: Proposed risk classification based on temperature control points