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Assessment of monitoring approaches to control *Legionella pneumophila* within a complex cooling tower system

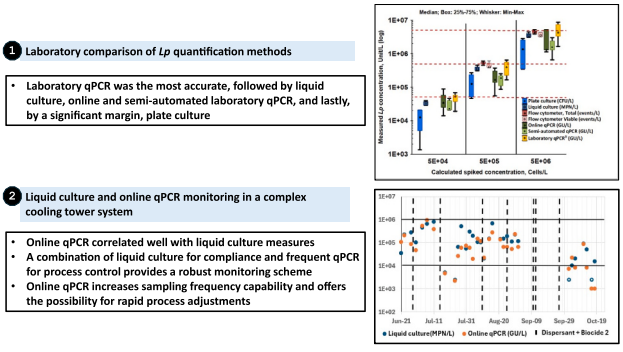
Hana Trigui [a], Sara Matthews [b], Emilie Bedard [a], Dominique Charron [a,*], Sakona Chea [c], Carole Fleury [c], Juan Francisco Guerra Maldonado [a], Mélanie Rivard [a], Sébastien P. Faucher [b], Michele Prevost [a]

[a] Polytechnique Montréal, Civil, Geological and Mining Engineering Dpt., P.O. Box 6079, Succ. Centre-ville, Montréal H3C 3A7, Québec, Canada
[b] Department of Natural Resource Sciences, McGill University, Sainte-Anne-de-Bellevue H9X 3V9, Québec, Canada
[c] Direction de l'épuration des eaux usées, Service de l'eau, Ville de Montréal, Montréal H1C 1V3, Québec, Canada

HIGHLIGHTS

- qPCR and liquid culture were most precise and accurate to quantify culturable *Lp* in laboratory conditions.
- qPCR results correlate with liquid culture in complex cooling tower samples.
- Daily changes in *Lp* levels observed suggest the need for increased monitoring frequency in this CT system.
- Online qPCR increases sampling frequency capability and offers the possibility for online process adjustment.
- Liquid culture is currently used for compliance, but frequent qPCR is better suited for process control.

GRAPHICAL ABSTRACT



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ABSTRACT

Precise and rapid methods are needed to improve monitoring approaches of L. *pneumophila* (*Lp*) in cooling towers (CTs) to allow timely operational adjustments and prevent outbreaks. The performance of liquid culture (ASTM D8429-21) and an online qPCR device were first compared to conventional filter plate culture (ISO 11731-2017), qPCR and semi-automated qPCR at three spiked concentrations of *Lp* (serogroup 1) validated by flow cytometry (total/viable cell count). The most accurate was qPCR, followed by liquid culture, online and semi-automated qPCR, and lastly, by a significant margin, filter plate culture. An industrial CT system was monitored using liquid and direct plate culture by the facility, qPCR and online qPCR. Direct plate and liquid culture results agreed at regulatory sampling point, supporting the use of the faster liquid culture for monitoring culturable *Lp*. During initial operation, qPCR and online qPCR results were within one log of culture at the primary pump before deviating after first cleaning. Other points revealed high spatial variability of *Lp*. The secondary pumps and chiller had the most positivity and highest concentrations by both qPCR and liquid culture compared to the basin and infeed tank. Altogether, this suggests that results from monthly compliance sampling at a single location with plate culture are not representative of *Lp* risks in this CT due to the high temporal and spatial

* Corresponding author.
E-mail address: dominique-2.charron@polymtl.ca (D. Charron).

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variability. The primary pump, rather than the CT basin, should be designated for sampling, as it is representative of the health risk. An annual multi point survey of the system should be conducted to identify and target *Lp* hot spots. Generally, a combination of liquid culture for compliance and frequent qPCR for process control provides a more agile and robust monitoring scheme than plate culture alone, enabling early treatment adjustments, due to lower limit of detection (LOD) and turnover time.

1. Introduction

Legionnaires' disease (LD) is a severe pneumonia caused by the inhalation or aspiration of water contaminated with infectious *Legionella* (Fields et al., 2002; National Academies of Sciences, 2019). In Europe and North America, *Legionella pneumophila* (*Lp*) is responsible for 95–99 % of all cases of LD and can be nosocomial or community-acquired, causing sporadic and outbreak-associated cases (Adams et al., 2017; ECDC, 2017; Garrison et al., 2016; Hamilton et al., 2018; Jain and Krygowska, 2023). LD is a growing concern worldwide, with the number of reported cases increasing nine-fold between 2000 and 2018, and with nearly 10,000 reported cases in the US in 2018 alone (U. S. CDC, 2022). Furthermore, it is estimated that there are 2.3 unreported cases of legionellosis for each reported case due to underdiagnosis (Collier et al., 2021).

Cooling towers (CTs) are of particular concern due to their design and operating characteristics (van Heijnsbergen et al., 2015). They are large semi-open water systems that operate at warm temperatures and generate large quantity of aerosols. They are particularly suitable environments for microbial growth throughout the year and can expose large numbers of people to contaminated aerosols over long distances (up to 10 km) (Addiss et al., 1989; Nhu Nguyen et al., 2006). Recent outbreaks due to CTs have been making headlines in North America (Fitzhenry et al., 2017; Gallagher, 2017; Grossman et al., 2023; Rebellato et al., 2023; Lévesque et al., 2014). As a result, different organizations worldwide have reinforced their water management programs, policies, communications, and regulations to outline proper maintenance and safe practices for CTs (Bartram et al., 2007; ASHRAE, 2023; CDC & HHS, 2017; HSE, 2024; NYS Department of Health, 2016; Walker and McDermott, 2021). In some jurisdictions, CTs are required to be registered, have a maintenance program and a *Legionella* control plan. In Canada, France, Spain, Germany and Australia, specifically monthly measures of *Lp* or Legionella species (*Lspp*) are required (PWGSC, 2013; GNB, 2024; RF, 2013; Spain, 2022; Germany, 2017; QLD, 2018). Methods considered acceptable by at least one regulation are summarized in Table 1. Such practices have been suggested in an effort to reduce *Lp* levels and incidences of *Legionella* positivity in CTs in the jurisdictions where they have been implemented (Health and Commission, 1991; Quebec Government, 2013a, 2013b, 2014; Racine et al., 2019).

The most common method in the industry for monitoring *Legionella* is selective plate culture (McCoy et al., 2012; World Health Organisation, 2007). Although isolates can easily be retained to investigate the link between environmental and clinical isolates (Walker and McDermott, 2021), this method is time-consuming, producing results in 10–14 days, and requires technical skills for sample handling and colony identification due to interference of non-*Legionella* flora (Diaz-Flores et al., 2015; Leoni and Legnani, 2001; Lucas et al., 2011; Roberts et al., 1987; Scaturro et al., 2020). Plate culture methods also underestimate the true concentration of *Lp* (Barrette, 2019) and false negative rates are variable between laboratories (HSE, 2013; Lucas et al., 2011). The additional steps required to discriminate *Lp* from other *Legionella* species on a plate, such as filtration, centrifugation, heat treatment and/or acid washes, also ultimately lower its accuracy and affect the limit of detection (LOD) (Boulanger and Edelstein, 1995; Roberts et al., 1987; Lee et al., 2011; Leoni and Legnani, 2001).

Legiolert, a liquid culture method developed by IDEXX (ASTM D8429, Legiolert, IDEXX Laboratories, Westbrook, ME, USA), is a promising alternative method based on bacterial enzyme detection (AFNOR, 2019; ASTM, 2021). This approach streamlines liquid culture and determines the most probable number (MPN) of *Lp* present in water samples in 7 days, without additional steps for species confirmation (Rech et al., 2018). Studies have found Legiolert to be more sensitive and effective than standard plate culture for monitoring *Lp* (Barrette, 2019; Inoue et al., 2020; Petrisek and Hall, 2017; Rech et al., 2018; Sartory et al., 2017; Checa et al., 2021). As CFU and MPN results are considered equivalent, MPN can be interpreted using the existing guidelines for plate culture (AFNOR, 2019; ISO6107:2021, 2021; Sartory et al., 2017; Walker and McDermott, 2021). In fact, it was recently accepted by the

Table 1
Methods used in this study and their analytical characteristics. The limit of detection was defined as the level at which the target (DNA or bacterial cell) remains detectable and produces a stable signal that exceeds background noise as determined by the manufacturer; GU, genomic unit; MPN, most probable number; CFU, colony forming unit.

Method	Type	Detection target	Theoretical limit of detection	Response time	Notes
Plate culture: filter plating (ISO 11731:2004, CFU/L)*	Culture	Culturable *Lp* for lab assay	10 CFU/L	3 to 7 days	*Lp* spiked in 0.45 µm filtered water (no GVPC), dilution needed
Plate culture: Direct plating (NF T90-431, CFU/L)*	Culture	Culturable *Lp* for CT samples	2200 CFU/L	10 to 14 days	Possible interfering flora, dilution needed, GVPC antibiotics
Liquid culture (MPN/L)*	Culture-based enzymatic	Culturable *Lp* for lab assay (potable) & CT samples (non potable)	100 MPN/L (potable) 1000–10,000 MPN/L (non potable)	7 days	Possible interfering flora, dilution needed for CT water
FCM (events/L)	Individual cell detection	Total, Viable, Dead cells	1×10^6 events/L	1 h	*Lp* can only be quantified by cytometry in pure cultures
Online qPCR (GU/L)	Molecular	Culturable, VBNC, Dead *Lp*	100 GU/L (potable) 750 UG/L (non potable)	4 h	Online equipment, water line connected to the device
Semi-automated qPCR (GU/L)*	Molecular	Culturable, VBNC, Dead *Lp*	190 GU/L	>24 h	Lab DNA extraction + qPCR
Laboratory qPCR[B] (GU/L)*	Molecular	Culturable, VBNC, Dead *Lp*	100 GU/L	>24 h	Lab DNA extraction + qPCR

* Considered acceptable for *Legionella* monitoring by at least one regulation.

UK's Standing Committee of Analysts (SCA), the New Brunswick government (GNB), and the Hungarian Public Health Center (NNK) for testing *Lp* in CTs (Bernadett et al., 2021; GNB, 2024; SCA, 2020). However, the lack of formal protocols for recovering viable *Lp* from positive Legiolert tests for strain identification has made other public health agencies cautious in adopting it. Recent work resolved this barrier by developing a simple protocol to preserve strains from positive Legiolert tests (Matthews et al., 2022).

Quantitative PCR (qPCR) is also widely used to monitor *Legionella* and *Lp*, providing detection and quantification in <24 h (Lucas and Fields, 2016; UNE 100030:2017, 2017; Whiley and Taylor, 2016), and an excellent negative predictive value (Collins et al., 2015; Toplitsch et al., 2021). qPCR is also significantly more sensitive than plate culture and liquid culture (Walker and McDermott, 2021), as it can detect very low amounts of target DNA. It is considered suitable as a complementary method to culture to monitor *Lp* trends or rapid increase and to implement corrective actions in a timely manner (Young et al., 2021). However, it cannot distinguish between live or dead cells, extracellular DNA, and viable but non-culturable cells (VBNC) (Collins et al., 2017; Grúas et al., 2014; Lee et al., 2011; Whiley and Taylor, 2016). Therefore, results do not directly relate to LD risk, but qPCR trends are good indicators of the effectiveness of *Lp* control measures in CTs. Cooling towers are dynamic systems with a steady turnover of water overtime and harsh chemicals that can degrade nucleic acid (Moradinejad et al., 2021). And so high levels of DNA cannot occur without an actively growing, viable source of *Legionella* to continually input DNA into the system. Lee et al. (2011) similarly noted how tracking these trends can reveal potentially growing sources of *Lp*, and offer crucial information even when culture methods yield negative results, thus enabling more comprehensive risk assessment and management. This has also been shown in a hot water system by Bédard et al. (2016). Laboratory qPCR techniques require expensive reagents and a high level of expertise for their execution and data interpretation. As all methods, the results are also susceptible to the effects of delays between sample collection and processing in the laboratory. A delay of 24 h between collection and processing led to issues such as *Legionella* growth and/or degradation during transportation (Ahmed et al., 2019). To circumvent this issue and to provide on-demand analysis capacity for CT managers, a commercial on-site qPCR option has been developed. It provides near real-time quantification of *Lp* DNA and is accompanied by a chart that gives a risk level classification based on the value, prompting proactive interventions from operators.

The goal of this study was to evaluate liquid culture and a novel online qPCR system for monitoring a complex CT system. Their efficacy was first compared to plate culture, laboratory qPCR (qPCR) and semi-automated qPCR with *Lp* suspensions of various known concentrations in laboratory conditions. Next, qPCR and online qPCR were used to monitor water from one point in a complex industrial CT system and compared to existing culture-based monitoring that was practiced at the facility. Sampling was also performed in multiple locations across the system to observe how liquid culture and qPCR correlate and if they would detect changes in the distribution and dynamics of *Lp* within this system. These results will help improve *Lp* management through optimised detection methods and sampling locations.

2. Material and methods

2.1. Bacterial strains and culture for laboratory comparison of Lp quantification methods

Lp strain ID120292 was provided by the Laboratoire de Santé Publique du Québec. It is a serogroup 1, sequence type 62 strain isolated from a CT during an outbreak in Quebec in 2012 (Lévesque et al., 2014). It was stored in ACES Yeast Extract media (pH 6.88) with 60 % glycerol at −80 °C and grown on a BCYE-Oxoid agar plate for 3 days at 36 °C. A 5 mL pre-culture was grown for 12 h at 36 °C in AYE with the growth supplement SR0110-Oxoid and used to inoculate 5 mL of fresh growth medium ($OD_{600} = 0.05$). Cells were harvested by centrifugation (3000*g* for 30 min) after 48 h at 36 °C ($OD_{600} = 3$), washed twice with 5 mL of sterile tap water, and resuspended in 5 mL of filtered (0.22 μm) tap water (estimated concentration = 10^{10} cells/mL). To more closely stimulate their state in environmental conditions, cells were starved for 7 days at room temperature (22 °C) (Bédard et al., 2021). The total and viable *Lp* concentration were measured by flow cytometry (FCM) before and after starvation following the procedure described previously by Bédard et al. (2015) and Prest et al. (2013). Briefly, 500 μL of Lp suspension were heated to 37 °C for 3 min, stained with 5 μL SYBR Green (total count), or combined with 5 μL propidium iodide (dead cell count), and incubated for another 10 min in duplicate. Data analysis was performed using the BD Accuri CFlow® software. Quantification of this stock was also done on BCYE to ensure correct Lp levels in our trials. This concentrated stock was 10^{13} cells/L and diluted to inoculate, by the same volume, 3 L of sterile tap water to produce the final three concentrations respectively: 5×10^6, 5×10^5 and 5×10^4 cells/L. For every concentration, three replicates of 3 L final suspension were prepared from the same stock and their concentrations re-confirmed by FCM. Every 3 L final suspension was then well homogenized and aliquoted for quantification by qPCR-based methods (semi-automated system: Pall GeneDisc® *Legionella pneumophila* and laboratory qPCR: Bio-Rad iQ-Check *Legionella* Real-Time PCR), filter plate culture (ISO 11731:2017), liquid culture (Legiolert®-IDEXX, ASTM D8429), and online qPCR (BioAlert). Their attributes are summarized in Table 1. Sterile tap water was used as a negative control. Except the semi-automated qPCR, which was done by the Centre d'expertise en analyse environnementale du Québec (CEAEQ) 24 h after preparing aliquots for quantification, the methods were performed at Polytechnique immediately after preparing aliquots. The entire experiment was performed twice on two different days. The coefficient of preparation (due to DNA extraction) was calculated for laboratory qPCR and results were adjusted accordingly, whereas, for semi-automated and online qPCR, the coefficient of preparation was already included in the final results.

2.2. Lp quantification methods

Viable and total *Lp* were measured by FCM with the BD Accuri™ C6 cytometer equipped with an argon laser (488 nm) as described in the previous section. As such, it can only quantify *Lp* in the pure cultures of the laboratory experiment at the two highest concentrations. The lowest concentration (5×10^4 cells/L) falls below the LOD and so only the level in the concentrated stock used to prepare it could be confirmed by FCM.

For laboratory plate culture, filtration was required to cover the whole range of concentrations. A defined volume was filtered through 0.45 μm membrane (S-Pak black gridded 47 mm, catalog #HABG047S6) and plated on BCYE agar media according to ISO 11731:2017 (ISO 11731, 2017) (Scaturro et al., 2020; Spies et al., 2018). Field samples were already monitored via plate culture with monthly compliance monitoring performed at certified laboratory (Barrette, 2019) and bi-weekly in-facility using AFNOR NF T90-431 direct standard culture method (AFNOR, 2014).

Liquid culture was done with the 10 mL-Legiolert protocol for potable water (ASTM D8429) for laboratory spiked suspensions (Inoue et al., 2020). For field samples, the water was analysed using the 1 mL-Legiolert protocol for non-potable water (ASTM D8429) according to manufacturer instructions.

Final spiked suspensions were also analysed by two laboratory qPCR methods for *Lp* quantification. For semi-automated qPCR, samples were shipped at room temperature to CEAEQ within 2 h after aliquoting the final suspensions and qPCR was performed on the following day based on Pall's manufacturer's instructions (see SI. 2). Laboratory qPCR was done with iQ-Check Quanti L. *pneumophila* kit (Bio-Rad, Mississauga, Canada) at Polytechnique using a Corbett Rotorgene 6000 with DNA extractions produced from filters using a bead beating method adapted

from Yu and Mohn (1999) as described by Bédard et al., 2015, (see SI. 2).

BioAlert (Sherbrooke, Canada) has recently commercialized an on-line qPCR detection system for near real time quantification of *Lp* in CT water circuits. In a laboratory setting at Polytechnique, a bottle containing 1 L of *Lp* suspensions was connected to the device and processed automatically according to standard procedure; blanks were periodically carried out with Milli-Q water. For investigative on-site monitoring at the industrial plant, a BioAlert system was connected directly to the piping of the three pumps. Measurements were automatically taken every 24 h or 48 h or conducted manually for split samples purposes in paired analysis with liquid culture. Factory calibrated cartridges allow quantification of *Lp* sg1–15 at the same efficiency rate. Periodic cleaning was performed when needed under manufacturer's instructions.

2.3. Investigative monitoring of complex CT system

The system comprises three CTs on the roof, two water retention basins split into hot and cold, and three circulating water pumps (60 hp) (Fig. 1). The regular treatment in the CT was bromine with the addition of a biodispersant and corrosion inhibitor. Physico-chemicals parameters such as temperature (°C), conductivity (S/m), pH, free chlorine, anticorrosion debit, accumulated biocide, oxido-reduction potential (ORP), cycles of concentration, dissolved oxygen (DO) were measured on-line by a calibrated TrueSense multiparameter device. The operation condition of this system is as follow: one CT is always in operation (primary, circulating water 24 h/day), if additional cooling is needed a second CT will begin running (secondary), and one CT remains off-line. All 3 pumps recirculate water for at least 1 h/day.

2.3.1. Monitoring 1

During this study period, the *Lp* levels exceeded an action threshold of 10^4 cells/L on different occasion and the site operators had to perform interventions such as cleaning, which involved completly emptying the towers, or disinfection, which is the addition of dispersants and/or biocides while CTs were running. Biodispersant BD1501E and biocide NX1102 (Spectrus (US)) were used on July 21, August 27 and September 7, 2021. A new chemical (biocide NX1100, Spectrus (US)) was used in combination with the usual biodispersant on September 9, 2021.

The facility already conducted liquid and direct plate culture testing at the primary circulation pump every 2 weeks and sent a monthly compliance sample to external accredited laboratories for plate culture. We supplemented this with two periods of in-situ monitoring: (1) from May 27 to September 13, 2021, using an online qPCR device (BioAlert) installed at the three pumps which can take on a primary (constantly circulating water), or secondary (auxiliary and off-line) role as described, and (2) from April 7 to July 14, 2021, across the circuit (pumps, chillers, basins, and infeed to tanks) with paired samples of qPCR and liquid culture. After 5 min of rinsing, samples were collected in a 1 L sterile plastic bottle containing sodium thiosulphate (10 % w/v). Since blowdown water accounted for 30 to 45 % of water volume each day, with an even higher blowdown rate when there was an intervention, qPCR and culture analysis were performed at least 24 h after treatment asprescribed by guidance and regulation (RBQ, 2014; PWGSC, 2013). This sampling delay allows time for DNA from dead cells to clear, and so permits the evaluation of the short-term impact of disinfection treatments.

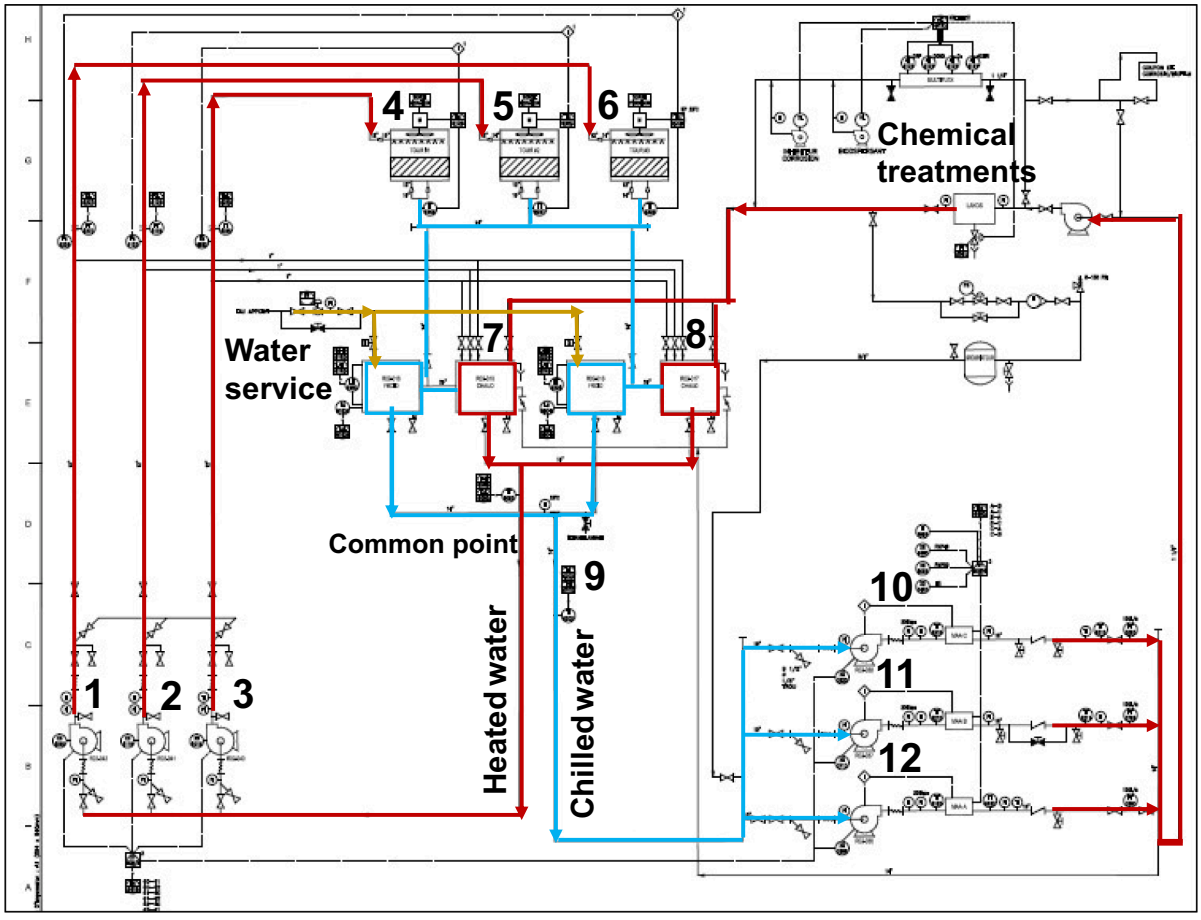

Fig. 1. Industrial plant characteristics. Heated circuit is represented by red arrows and chilled water circuit by blue. Numbers represent sampling points that were taken across the circuit: (1) Pump 3, (2) Pump 2, (3) Pump 1, (4) Basin of CT 1, (5) Basin of CT 2, (6) Basin of CT 3, (7) Infeed to tank 19, (8) Infeed to hot tank 17, (9) cold tanks outflow, (10) Chiller C, (11) Chiller B, (12) Chiller A.

2.3.2. Monitoring 2

Biodispersant BD1507 and biocide NX1100 (Spectrus (US)) were used on June 29, July 14, August 9–25 and September 11–13–20, 2023.

A paired sample analysis of online qPCR and liquid culture was carried out from June 21 to October 18, 2023, in the facility laboratory. A volume of 2 L was collected in a sterile plastic bottle and separated, 1 L sterile for online qPCR and 250 mL with thiosulphate (10 % w/v) and ethylenediaminetetraacetic acid (EDTA) for liquid culture.

2.4. Statistical analyses

Differences between the data series for laboratory comparison were assessed by Wilcoxon test and the percent coefficient of variation (%CV) was calculated as an indication of their precision from the total 6 replicate measurements. Correlations between liquid culture and qPCR in the field were investigated with Pearson's product correlation coefficient. All statistical tests were performed using the R statistical software (version 3.6.2) and R Studio (version 1.3.959). *P* values of 0.05 or less were deemed to be statistically significant.

3. Results and discussion

3.1. Laboratory comparison of Lp quantification methods

The accuracy and precision of liquid culture and the novel online qPCR method were evaluated using 0.22 μm filtered tap water spiked with three concentrations of starved *Lp*: 5×10^4, $5 \pm 0.5 \times 10^5$ and $5 \pm 0.47 \times 10^6$ cells/L. The total and viable cell counts were re-confirmed by the reference method, FCM, for 5×10^5 and 5×10^6 cells/L spiked samples, and viable cells represented 85.6 % and 83 % of total *Lp* (Fig. 2). Results were compared to plate culture, laboratory qPCR and semi-automated laboratory qPCR (Fig. 2). Pairwise comparisons between all methods are summarized in Table SI.1.

Accuracy was defined as the closeness between a test result and the accepted reference value (U. S. EPA-FEM, 2009). Filter plate culture was by far the least accurate method with <30 % of the expected concentration detected on average at all spiked concentrations. Liquid culture had far better accuracy overall, detecting 68–72 % of the expected concentrations (Fig. 2, Table SI. 1). Online qPCR had comparable accuracy to semi-automated qPCR at the two highest concentrations, detecting respectively, 35 % and 40–51 % on average. Online qPCR performed better at 5×10^4 cells/L, with 85 % expected concentration

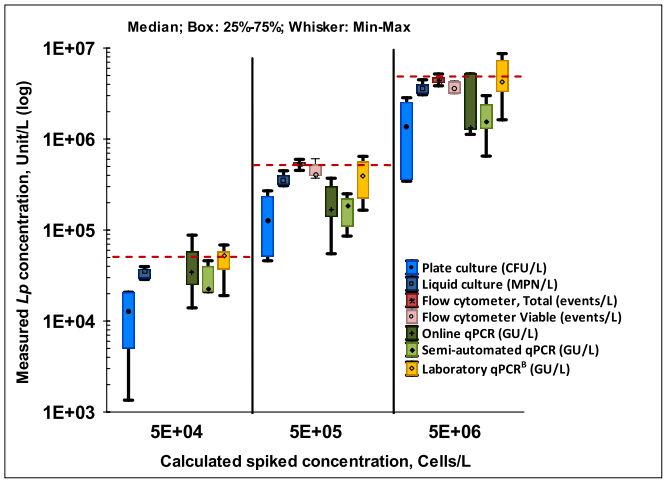

Fig. 2. Comparison of *Lp* quantification methods. Box plots show the median value (black centre geometric forms) of six replicates; Box: 25 %–75 %; Whisker: Min-Max. The red dashed lines represent the targeted spiked concentration. FCM was unavailable at 5×10^4 CFU/L since that concentration is below the limit of detection (LOD).

compared with 58 % measured by semi-automated qPCR. However, no significant difference was observed between online qPCR and both laboratory qPCR (Table SI. 1), while semi-automated qPCR was significantly lower ($p = 0.005$) than laboratory qPCR (Fig. 2, Table SI. 1). The most accurate method was laboratory qPCR, achieving 95 %, 79 % and 98 % of *Lp* GU/L for ascending spiked *Lp* concentrations respectively.

Precision can be defined as the closeness in agreement between independent test results obtained under stipulated conditions (ASTM, 2014; U. S. EPA-FEM, 2009). The %CV was calculated using the average and standard deviation from two triplicate experiments (Table 2). Filter plate culture always had the greatest variance (73.4–83.2 %) while liquid culture was the most precise (12.6–14.5%CV). Laboratory qPCR-based methods were comparable, with %CVs increasing across concentrations ranging from approximately 36–54 %CV, while online qPCR was slightly higher with a range of 58–79 %CV.

Our results are in accordance with other field and laboratory studies reporting that liquid culture detects higher counts and has a higher accuracy for *Lp* when compared to the ISO 11731 standard method (Barrette, 2019; Monteiro et al., 2021; Rech et al., 2018; Scaturro et al., 2020; Boczek et al., 2021; Niu et al., 2022; Spies et al., 2018). Culture methods are generally limited by the sample volume used and cannot detect or differentiate other developmental forms and physiological states, such as filamentous forms, VBNC, and slow-growing strains (Diaz-Flores et al., 2015). This would contribute to the underestimation by both culture methods, but filter plate culture would incur additional viability loss due to sample filtration and pre-treatments (Walker and McDermott, 2021). The <30 % detection of the expected concentration with the filter plate culture observed in our laboratory experiment was consistent with the 13–53 % recovery by filtration observed by Boulanger and Edelstein (1995). The stability of this ~30 % detection across spikes also supports the presence of this systematic effect on quantitative accuracy. The absence of filtration or centrifugation steps for sample preparation prior to liquid culture reduces the loss or destruction of bacteria compared to plate culture which can include both. Finally, bacteria grown in liquid broth often leads to higher recovery rates than on an agar medium (Ahn et al., 2014). In addition to the increased detection potential, enzymatic culture offers an alternative streamlined process for detecting and quantifying *L. pneumophila* which is less expensive and labor-intensive than standard plate culture (Matthews et al., 2022). The accurate detection of a positive signal is easier to acquire for untrained personnel as it is based on color change observation of turbidity.

The three qPCR methods tested showed higher precision and accuracy than filter plate culture, consistent with past studies that found plate culture to be less sensitive than qPCR-based methods (Behets et al., 2007; Donohue, 2021; Morio et al., 2008; Toplitsch et al., 2021; Yaradou et al., 2007). Based on Wilcoxon test, the accuracy results were significantly different between both laboratory qPCR methods (Table SI. 1). As already shown for CT water samples in Ahmed et al. (2019), this may be due to sample processing differences between both methods and the 24 h shipping delay experienced by the samples evaluated by semi-automated qPCR. Indeed, Ahmed et al. (2019) found a consistent shipping effect for samples evaluated by qPCR, reporting that approximately 72 % of samples displayed degradation, 15 % showed no change, and 13 % showed growth. Altogether, in terms of accuracy and precision our results confirm the reliability of liquid culture, laboratory qPCR and online qPCR for *Lp* quantification under laboratory conditions. However, these analyses were performed in a control environment, with clean municipal water, and without flora. Direct extrapolation to CT systems should be cautioned.

3.2. Investigative monitoring of CT

3.2.1. Monitoring 1 - routine culture monitoring and qPCR methods at primary pumps

A complex CT system consisting of three interconnected tower

Table 2
Precision of the six methods at 5×10^4, 5×10^5 and 5×10^6 CFU/L as described by the percent coefficient of variation (%CV). Six replicates were performed for each method at each concentration. The expected *Lp* concentration was based on FCM (total cell) of a concentrated stock suspension.

Expected *Lp* concentration (event/L)	Coefficient of variation (%)						
	Reference flow cytometry		Culture based		qPCR-based		
	Total cells	Viable cells	Filter plate culture	Liquid culture	Online qPCR	Laboratory semi-automated qPCR	Laboratory qPCR
5×10^4	*	*	73.4	12.6	62.7	38.1	36.5
5×10^5	9.5	20.7	72.5	14.5	57.6	38.8	49.7
5×10^6	10.6	15.26	83.2	14.5	79.3	48.2	54.3

* Not available, below limit of detection (LOD).

circuits was routinely monitored at the primary circulating pump by: (1) plate culture testing in a certified laboratory for monthly compliance and (2) bi-weekly direct plate culture and liquid culture performed in the facility laboratory. As laboratory qPCR presented similar results as semi-automated qPCR (Fig. 2, Table 2), the former was chosen to monitor throughout the system in addition to online qPCR at the pumps. For simplicity, qPCR will be used for short form of laboratory qPCR. There was close agreement between *Lp* levels detected by direct plate culture and liquid culture in most paired samples analysed by the facility laboratory and with certified external laboratory compliance samples, with slightly lower plate culture values (Fig. 3A). *Lp* culture results remained below or near the first surveillance threshold (10^4 CFU/L) prior to and a few days after the annual cleaning of the CT (June 21–23). Laboratory and online qPCR were within 1-log of each other and congruent with the culture results during this period (Fig. 3). For samples collected on June 28th, one week after the cleaning, the LOD for the certified laboratory plate culture analysis shifted from 5×10^3 CFU/L to 10^5 CFU/L due to an increase of interfering flora. In mid-July, the online qPCR detected a 100-fold increase in *Lp*, increasing from 10^3 GU/L to 10^5–10^6 GU/L, while culture results remained below or at action thresholds (Fig. 3C). As a result, a shock dosage of disinfectant and biodispersant were applied in the CT system on July 21. Despite the disinfection treatment, *Lp* DNA concentrations remained between 10^4 and 10^5 GU/L with online qPCR, and culturable cell concentrations measured by both the compliance lab and in-facility testing increased in the following weeks suggesting an actively growing source of *Lp* (Fig. 3A, B). Therefore, wo more disinfection treatments were applied on August 27 and September 7, but no reduction of culturable *Lp* levels were observed (Fig. 2A). Repeating disinfection two days later (September 9) finally decreased the in-facility and compliance testing *Lp* levels below the LOD. However, two weeks later (September 29) the in-facility plate culture results rose again to ~10^4 CFU/L, and liquid culture exceeded 10^4 MPN/L. These observations question the ability of shock disinfections alone to reduce *Lp* level in culture for more than a few days, suggesting that disinfectants are probably not reaching, or have inadequate contact time with, some *Lp* hot spots in the system. While closely repeating shock disinfection appears more effective, other operational changes, such as recirculation times and rotation of CT used, are likely needed to sustain repression of *Lp*.

The direct plate cultures consistently reported lower values than liquid culture in facility testing of paired samples at the primary pump (7/14), but values were within the same order of magnitude (Fig. 3A).

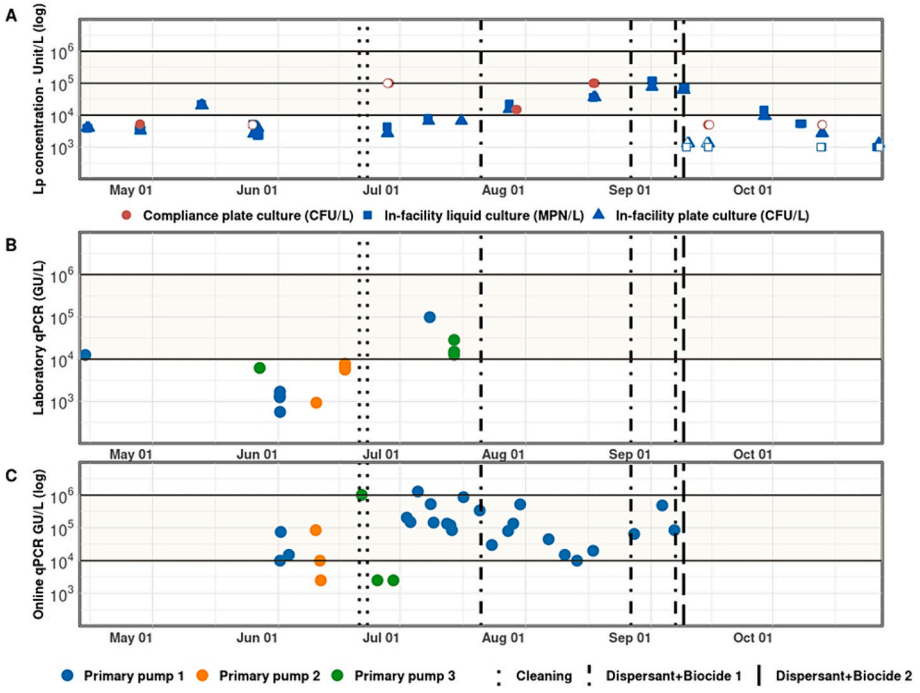

Fig. 3. Comparing A) culture-based, B) laboratory qPCR, and C) online qPCR methods for monitoring *Lp* at the primary circulating pump of the CT system. Empty symbols represent values under the LOD. Vertical dotted lines indicate full cleaning on June 21–23 and shock dosage of disinfectants on later dates. Black horizontal lines represent threshold limits in Quebec, 10^4 CFU/L is the enhanced surveillance threshold, and 10^6 CFU/L is the health risk threshold requiring immediate interventions (Quebec Government). The 10^5 CFU/L intervention threshold used in other regulations is also represented (Public Works and Government Services Canada (PWGSC), 2013). These limits (shaded yellow) only apply to plate culture in regulations but are indicated in qPCR as a visual reference.

This closer agreement relative to laboratory comparison likely stems from the omission of filtration in field plate culture, improving recovery but increasing the LOD. Overall, the similar performance of both culture methods further support liquid culture as an equivalent to plate culture for *Lp* quantification in the field. The high LOD for culture after CT cleaning measured by the certified laboratory can be attributed to the drastic increase in interfering flora. The LOD for qPCR and online qPCR were as not impacted by the high bacterial count environment since the rate of inhibition remained low (<5 %). However, when PCR inhibitors were present, a dilution was required which raised the LOD. Generally due to its lower detection limit, qPCR enabled the observation of fluctuations and trends even when the concentration was below 10^3 GU/L, as opposed to liquid culture which has a limit of detection 10-fold higher than qPCR. Overall, liquid culture and plate culture methods used for monitoring of a complex CT system provided comparable results and trends, especially to track impact of disinfection. Online qPCR ascertained the increase in *Lp* two to three weeks prior to culture.

3.2.2. *Monitoring 1 - online qCPR at primary and secondary pumps*

The online qPCR system was used to investigate *Lp* concentrations at the pumps from May 27 to September 13, 2021. Samples were collected every two or three days at the primary and the secondary pumps, resulting in 36 sampling days and 70 measurements in total (Fig. 4). At any given day, one of the three pumps was turned off and was neither primary nor secondary. During this period, pump 1 was used 75 % of the time as the primary circulating pump and was turned off only 19.4 % of the time. Pump 2 and pump 3 were mainly used as secondary pumps, only acting as primary for 8.3 % and 16 % of the sampling days respectively.

Online *Lp* monitoring levels varied greatly from day to day, both at the primary and at the secondary pump. Considering all detections shown on Fig. 4, the 10^5 GU/L threshold was more frequently exceeded at the secondary designated pump (24/41–59 %) than at the primary pump (12/29–41 %). Considering only the 28 paired measurements obtained on the same day at the primary and the secondary pumps, the mean levels at the secondary pumps were generally higher, by 0.74 log, than those measured at the primary pump (17/28) (Fig. 4). These results suggest that secondary pumps that are turned on periodically could be more favorable to the growth or detachment of *Legionella* within the circuit.

3.2.3. *Monitoring 2 - liquid culture and online qPCR at primary pump*

Thirty-two water samples from the primary pump of the cooling tower circuit were analysed by liquid culture and online qPCR (Fig. 5). *Lp* concentrations measured by the liquid culture and online qPCR methods were within 1-log of each other and generally follow the same trend. Of the 32 samples analysed, 28 had a concentration greater than or equal to 10,000 GU/L and 4 were under this concentration. Three of these 4 samples gave results under LOD for liquid culture. Further investigation for concentrations <10,000 GU/L is desirable, but results are promising for continuous monitoring technology of *Lp* in CT.

3.2.4. *Investigating correlation of qPCR and liquid culture results throughout CT circuits*

Detailed investigative monitoring of *Lp* was performed to quantify the variability of *Lp* across various locations within the complex CT circuits from April 7th to July 14th, 2021. Samples were collected on eight dates, at up to 13 locations per date, and grouped into 4 categories: basins, chillers, tanks and pumps (Fig. 1). More samples were positive by qPCR (108/111) than by enzymatic liquid culture (31/111) and qPCR consistently resulted in higher counts.

Paired samples analysed by qPCR and liquid culture (Fig. 6) show spatial variability in levels of *Lp* by location type. This variation was greater than what was observed in the laboratory comparison, strongly suggesting a real difference in the level of *Lp* across the system. The highest values for both qPCR and liquid culture were found in the chiller and pumps, pointing to them as high-risk areas that better suited as control monitoring sampling points. The correlation between liquid culture and qPCR was evaluated with all paired samples using Pearsons' product-moment correlation coefficient after non-detects were removed (Fig. 7). Although moderate, a significant correlation ($r = 0.66$, p-value <0.01) was found for positive samples by both methods (Fig. 7), which agrees with previously published results (Monteiro et al., 2021). This limited correlation is not surprising considering that qPCR detects DNA from all cells whereas liquid culture detects only culturable cells.

3.3. *Monitoring and regulatory implications*

Cooling tower monitoring regulations in Canada often require that one sample be taken once a month from a single location in the system and analysed for *Legionella* by plate culture (PWGSC, 2013; GNB, 2024;

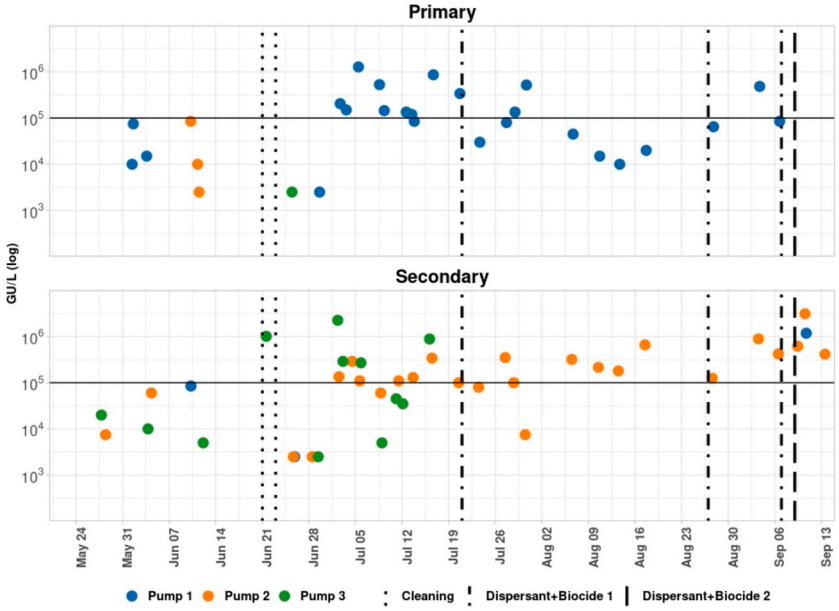

Fig. 4. *Lp* quantification by online qPCR in primary and secondary pumps. Dotted lines indicate cleaning (June 21–23), or shock dosage of disinfectants. Black horizontal line represents a site-specific action level (10^5 GU/L), which is suggested by Young et al. (2021) to be more appropriate for management purposes.

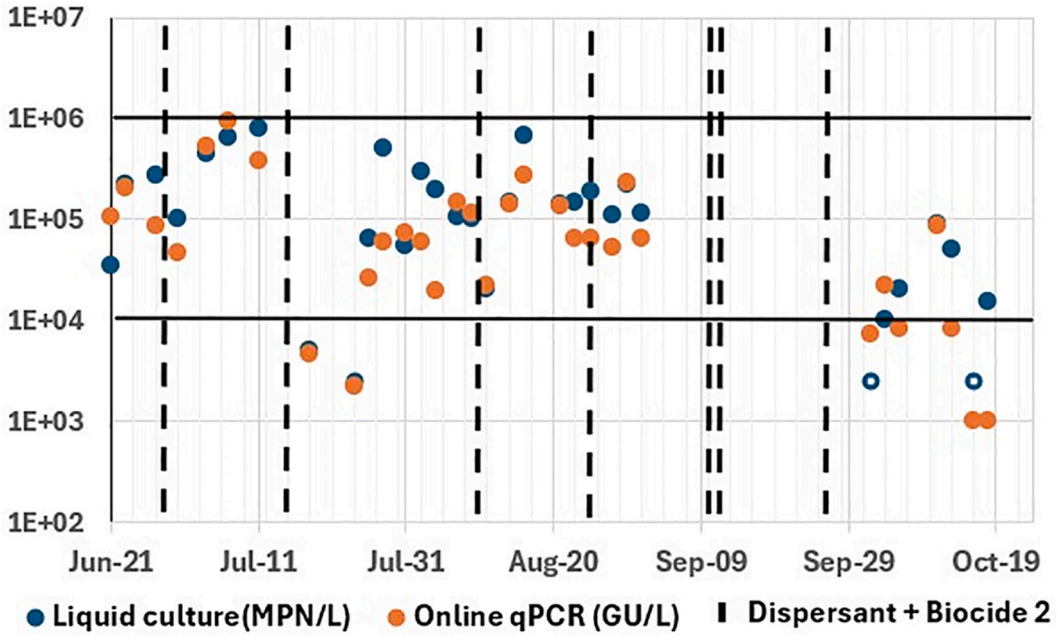

Fig. 5. Comparing liquid culture and online qPCR for monitoring *Lp* at the primary pump of the CT system. Empty symbols represent values under the LOD. Black horizontal lines represent threshold limits in Quebec.

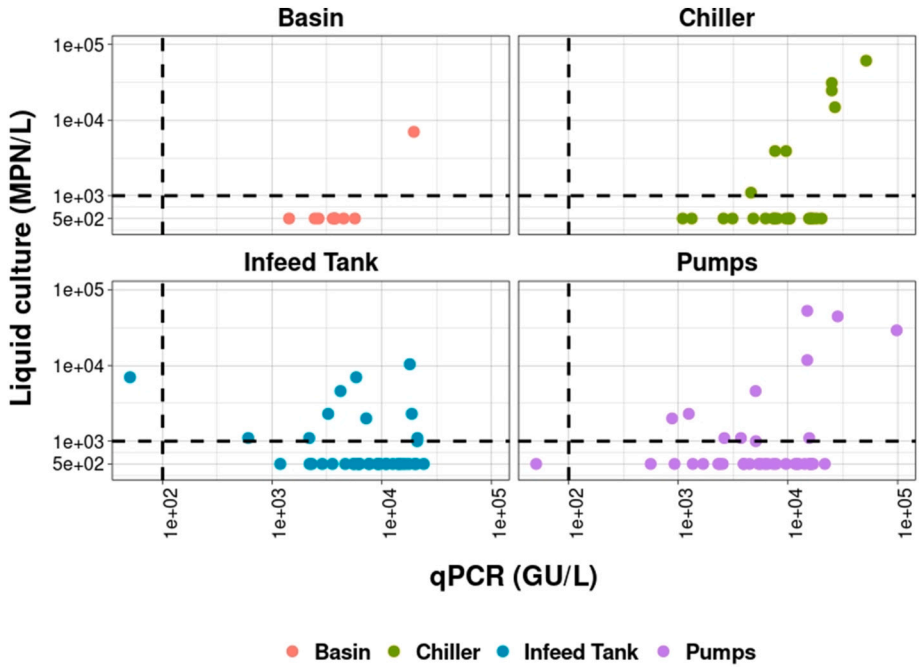

Fig. 6. Comparing liquid culture and qPCR results across the CT system. Dashed lines indicate the limit of detection (LOD) of each method, with those falling below marked as 50 % of the LOD.

RBQ, 2014; Radziminski and White, 2023). However, based on results from this study, the detection method,sampling frequency and the selection of the sampling location should be reconsidered for this CT system to improve monitoring.

3.3.1. *Selection of methods for improved compliance and timely response*

CT managers and regulators should move towards more rapid, precise and accurate detection methods for improved compliance and risk management. The two-weeks wait time associated with plate culture methods have resulted in major delays before establishing whether cleaning and biocide treatments were effective or were needed to reduce the level of *Lp*. During that period, CT systems continue to operate and can represent an important health risk if the intervention was not effective (Fig. 3).

The use of liquid culture enables the direct application of existing alert levels based on culturability, since both CFU and MPN results are considered equivalent (AFNOR, 2019; ISO6107:2021, 2021; Sartory et al., 2017; Walker and McDermott, 2021). Liquid culture provides higher precision, isolate recovery capacity and improved response time (AFNOR, 2019; Bernadett et al., 2021; SCA, 2020, Matthews et al.,

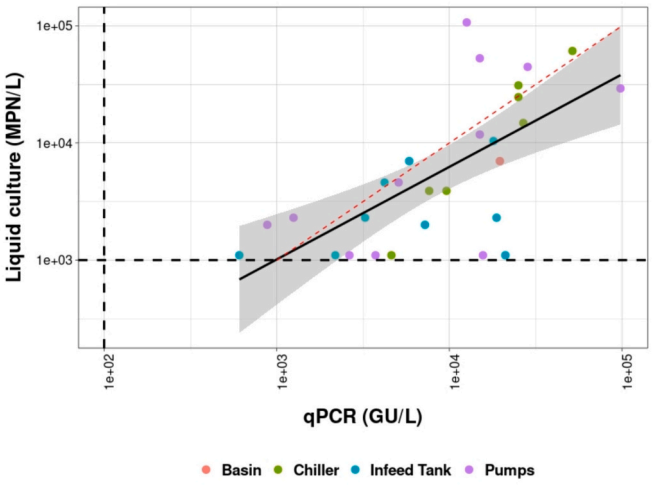

Fig. 7. Correlation between liquid culture and qPCR measurements across the CT system. Dashed black lines indicate the limit of detection (LOD) of each method and the red-dashed line is a theoretical correlation line if methods were perfectly equivalent. The grey zone represents the 95 % confidence interval. Non-detects are not shown ($N = 28$, r = 0.66, p-value <0.01).

2022), suggesting a more adequate choice of method for timely and responsive monitoring of CTs. In the context of an outbreak, a reduction of 3–7 days of exposure will likely reduce the number of infected people. qPCR-based methods shorten the time delay even further (Walker and McDermott, 2021), allowing prompt operator response in case of elevated *Lp* levels (6-72 h for qPCR vs. 7–14 days for culture). In this study, online qPCR established within a few days that the shock treatments had no or minimal impact on *Lp* levels measured at the primary pump (Fig. 3). The greater sensitivity and rapid turnaround time of qPCR methods enables early detection of increasing Lp levels, allowing timely intervention before reaching alert or regulatory thresholds.

3.3.2. *Benefits of increased number of sampling locations and frequency for improved compliance monitoring*

Typically, regulations in Europe and Canada prescribe monthly monitoring at a single designated location, most commonly the basin (GNB, 2024; PWGSC, 2013; RF, 2013). Complex CT systems, which consist of multiple towers and pumps, and CTs that have historically been challenging to control would benefit from optimizing monitoring requirements in terms of sampling locations and frequency. A meta-analysis of regulatory monthly compliance data in CTs (Sylvestre et al., 2024) revealed that a small number of CTs account for most non-compliance cases (60 CT/ 2 % of CTs) and had a very high risk with significant uncertainty. Often these high-risk towers had at least two incidences of high Lp (>10^5 CFU/mL) in a season. Therefore, monthly monitoring alone became insufficient to evaluate risk in these cases. Instead, a multipoint survey to identify hot spots within the system would be more useful. Cooling tower owners could use this information to adjust treatment, direct repairs, or modify systems accordingly. In this study, as shown on Fig. 6, chiller and pumps were found to be the site with the most positivity and highest concentration by both qPCR and liquid culture. Samples collected at chillers were approximatively 1 log higher in culture and qPCR than the corresponding measurements at primary pumps on June 1st and June 10th. These samples collected at the chiller allowed prompt response and comprehensive changes in CT operation, which involved not only another shock disinfection treatment but a change in the pump usage regime to reduce stagnation in the other sections of the circuit. However, collecting sampling at chiller is challenging and impractical, and so is unsustainable in the long-term here.

Online qPCR at the secondary pump revealed that *Lp* levels remained higher than in the primary pump even after disinfection interventions in 50 % (14/28) of paired measurements, with differences ranging between 0.1 and 1.43 log (an average of 0.74 log higher) (Fig. 4). Moreover, 59 % (24/41) of the secondary pump measurements had levels exceeding 10^5 GU/L compared to 41 % (12/29) of the primary pump measurements. The lower responsiveness of the secondary pump circuit to shock treatments was likely due to the low usage period restricted to a minimal daily recirculation time (1 h/day).

In light of the results, monitoring at the pumps is preferable for practical consideration and representative of water circulating in this type of system. Complex CT systems operating with multiple pumps are operated with different pumping strategies. When a pump is designated as primary while other remain in standby with minimum recirculation set points, regular monitoring should be conducted at the primary pump. In a CT system with alternating pumps, the active pump should be monitored. For due diligence, annual monitoring of high-risk sampling points, such as low use circuits, unused chillers, dead legs and stagnant zones, should be conducted to identify hot spots for *Legionella* growth, especially in complex systems. These results can be used to select the monitoring sampling points and to improve the operation to eliminate those hot spots. Ideally, this monitoring should be conducted during period of high thermal loads of the CT systems, as they represent high risk periods for the growth for *Legionella* and peak bioaerosol release.

Our results also highlighted that for complex systems, monthly monitoring is insufficient and misses exceedances of the intervention thresholds that occur between two compliance sampling dates (Fig. 3, in July) or in other parts of the system. New regulations could include more frequent sampling at one or multiple critical points in the system. qPCR-based techniques are best suited since results can be obtained within a day when shipped to a specialized laboratory (Walker and McDermott, 2021), or within few hours when online qPCR is used. While there are still questions about a defined conversion factor between GU/L and CFU/L (or MPN/L), a correlation with culturable *Lp* was observed (Fig. 5) and sudden changes in qPCR trends are a clear indication of important changes in a system. Although no definite action and alert levels can be set for qPCR, levels ranging from 5×10^3 to 10^5GU/L have been proposed, and the implementation of site-specific levels may be more appropriate for management (Young et al., 2021). When established qPCR thresholds are surpassed, the culture methods can be deployed, prior to disinfection, to fully determine the health risk posed by the system. Currently, culturable *Lp* are considered representative of infection risk and enable the isolation of strains which is required to confirm environmental sources of LD clusters and outbreaks. Although GU/L are yet to be implemented into guideline documents and standard methods (Ditommaso et al., 2015; Hamilton et al., 2019; Lee et al., 2011; Yaradou et al., 2007), qPCR can be valuable for trend analysis and a suitable complementary method to culture for rapid corrective adjustment in a complex industrial CT system.

4. Conclusions

Monitoring plans prescribed by guidance and regulations can be updated to mitigate their current limitations including the low frequency of compliance testing (monthly), the selection and use of single sampling locations for complex and/or high-risk systems, and the reliance on plate culture methods alone.

- Five methods (qPCR, semi-automated qPCR, online qPCR, liquid culture and filter plate culture) to detect *Legionella pneumophila* were compared in the laboratory, in reference to flow cytometry. Considering both accuracy and precision, qPCR and liquid culture were the most effective method in quantifying culturable *Lp* in controlled laboratory experiment.
- In field samples, qPCR correlated well with liquid culture measures. qPCR-based methods (laboratory and online) offer the significant advantage of rapid turn around time, enabling quicker detection and response to increasing trends, thus mitigating potential risk of

exposure to *Lp*. However, current regulations are based on the culturable *Lp* which are representative of infection risk and enable the isolation of strains.

- A combination of liquid culture for compliance and frequent qPCR for process control provides a more agile and robust monitoring scheme than plate culture alone. Online qPCR further increases sampling frequency capability and most importantly offers the possibility for online process adjustment, preventing noncompliance and shutdowns.
- This study suggests that the primary pump should be designated for sampling, as it is the most practical and representative of the health risk for this specific site.
- The most effective monitoring approach is system dependent; annual multi point monitoring can identify hot spots for *Legionella* growth and assist in determining the best locations for monitoring.

CRediT authorship contribution statement

Hana Trigui: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sara Matthews:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Emilie Bedard:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Dominique Charron:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Sakona Chea:** Formal analysis, Methodology, Validation, Writing – review & editing. **Carole Fleury:** Formal analysis, Methodology, Validation, Writing – review & editing. **Juan Francisco Guerra Maldonado:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Mélanie Rivard:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Sébastien P. Faucher:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Michele Prevost:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175136.

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