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# Impact of recommissioning flushing on *Legionella pneumophila* in a large building during the COVID-19 pandemic

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COVID-19 shutdowns drastically increased the frequency and duration of water stagnation events in building plumbing systems, urging local authorities to issue guidance for the safe reopening of buildings mostly by recommissioning flushing. The objectives of this study were to document the dynamic changes of bacterial indicators [adenosine triphosphate (ATP), total and intact cell counts (TCC, ICC)] and the prevalence of *Legionella pneumophila* (*Lp*) in 20–21 showerheads in a large building before (16-week building closure) and then shortly (24 h) and monthly (4-week of distal water stagnation) after targeted recommissioning flushing. Following the 16-week shutdown, the highest mean of ATP (10 pg ATP/mL), TCC ( $1.7 \times 10^6$  count/mL) and ICC ( $5.2 \times 10^5$  count/mL) were measured in first draw samples. This bacterial amplification was mostly attributable to detachment from biofilm present in the distal devices and immediate connecting piping. Culture-based (mean of 4 487 MPN/L) and quantitative polymerase chain reaction (qPCR; mean of 63 822 gu/L) concentrations of *Lp* were respectively measured in 81 and 90% of first draw samples. Individual flushing of showerheads for 5 min resulted in 1.2–278-fold decreases in ATP, whereas TCC and ICC were lowered by 1.1- and 0.7-log on average. A one-log reduction in culture-based and qPCR *Lp* was only achieved in 63 and 29% of paired water samples, resulting in less than one-log reduction in mean risk values per exposure, thus demonstrating the limited effects of fixture-flushing for risk reduction. Clear short-term (24 h) benefits of device recommissioning flushing included lowered values of all bacterial indicators and *Lp* levels systematically under the common alert threshold of 1 000 MPN/L in first draws. However, after a period of 1 month without water use, these benefits were mostly lost with considerable rebounds of concentrations to similar levels than those measured following the 16-week building closure. Results highlight the temporary benefits of device recommissioning flushing for the control of *Lp* in shower systems, especially in buildings colonized by *Legionella*.

## KEYWORDS

legionella, flushing, recommissioning, stagnation, building plumbing, COVID-19

## Introduction

In early 2020, multiple sanitary measures were put in place to limit the spread of the COVID-19 virus, including the closure of non-essential venues and services. Consequently, numerous buildings were left vacant or partially occupied, thus increasing the frequency and duration of water stagnation events in building plumbing systems or at specific outlets.

Among concerns about water quality deterioration over the course of stagnation, microbial growth is well documented. Short water stagnation times (< 24 h) typically result in large increases in ATP concentrations, total and intact cell counts, or heterotrophic plate counts (HPC; Lautenschlager et al., 2010; Zlatanović et al., 2017; Bédard et al., 2018; Peng et al., 2020; Rahmatika et al., 2022). This suggests that short stagnation events may favor culturability and that microorganisms tend to remain metabolically active. However, longer periods of water stagnation (up to 2 weeks) commonly show plateaued bacterial concentrations (Lautenschlager et al., 2010; Manuel et al., 2010; Zlatanović et al., 2017; Bédard et al., 2018; Peng et al., 2020). Indeed, prolonged stagnation is likely to result in depleted-nutrient environments that may limit long-term bacterial growth.

Considering that many waterborne pathogens can survive in oligotrophic environments characterized by low levels of nutrients (e.g., stagnant drinking water), health concerns were raised after the extended (in terms of months) closure of buildings during the COVID-19 pandemic. Due to increasing incidence rates of Legionellosis cases in the United States and elsewhere (NASEM, 2019), most recommendations recently issued to address the safe reopening of buildings after the COVID-19 pandemic shutdowns were focused on *Legionella* bacteria (AWWA, 2020; CSA, 2020; Government of Quebec, 2020; Proctor et al., 2020; CDC, 2021). More specifically, *Legionella pneumophila* is responsible for most Legionellosis cases (Fields et al., 2002), for which the risk of infection is most important when susceptible populations are exposed to high concentrations and the production of water aerosols (NASEM, 2019) in the respirable-size range of 1–10 µm in diameter (Allegra et al., 2016).

Routine flushing of taps to replace stagnant water with fresh water carrying an oxidant residual (cold water) or a high temperature (hot water) are identified as key control measures in several *Legionella* control regulations and guidelines, which are most often based on risk and type of population present in the building (Australian Government, 2015; NHS England, 2016; ESCMID, 2017; NASEM, 2019; ASHRAE, 2020; AWWA, 2020; VHA, 2021). Preventative flushing is also a common practice for the commissioning of new buildings and after the completion of construction work or in response to delayed occupancy. Flushing and disinfection are recommended for the commissioning of whole buildings or sectors of a new

building after a period of more than 3 weeks (ASHRAE, 2018). ASHRAE Standard 188 also calls for preventative flushing if the occupancy of a new building sector is delayed after commissioning for a period of more than 2 weeks, but less than 4 weeks. Additionally, several guidance and standards warrant for the preventative flushing of low flow and stagnant areas in existing buildings, some setting a limit of 2 weeks for flushing after which disinfection must be (re)conducted (ASHRAE, 2018; CSA, 2020; Proctor et al., 2020).

Because of the widespread extreme water stagnation in buildings with no or low occupancy, a flurry of recommendations for the safe reopening of buildings was issued during the COVID-19 pandemic, with flushing being the most recommended corrective action (Proctor et al., 2020). The need to carry out recommissioning flushing was justified by water quality concerns (e.g., lead, copper, and *Legionella*), the duration of building shutdown, or the integrity of the entire building plumbing system (AWWA, 2020; CSA, 2020; Government of Quebec, 2020; Proctor et al., 2020; CDC, 2021). In all cases, buildings with vulnerable populations, such as healthcare facilities, and those with numerous aerosol producing devices (e.g., showers) were identified as high-risk priorities to investigate for the presence of *Legionella pneumophila*.

Evidence to support the definition of efficient remedial flushing (e.g., during recommissioning) practices is however limited, despite quite frequent prolonged shutdowns in buildings depending on their use (e.g., schools, seasonal venues). Individual flushing of cold-water taps for 3–6 min proved to be beneficial for reducing bacterial-ATP (cell-bound) concentrations by 6-fold on average (Lautenschlager et al., 2010), intact and total cell counts by more than 1-log (Bédard et al., 2018), and qPCR *Legionella* spp. by up to 2-log (Wang et al., 2012; Hozalski et al., 2020). Yet, there is a scarcity of data regarding the short- and long-term benefits of flushing toward *Legionella pneumophila* in building plumbing systems. Moreover, there is very scarce information on the water quality before the stagnation events, therefore limiting the extrapolation of findings after flushing.

The objectives of this study were to: (1) assess water quality of showers in a large sports complex following an extended period of stagnation during the COVID-19 pandemic shutdown (16-week), and (2) evaluate the short- (24 h) and long-term (4-week) effectiveness of targeted recommissioning flushing on general bacterial indicators and the occurrence of *Legionella pneumophila*. This study provides insights into the efficacy of flushing procedures which have been widely recommended as the first level of remedial action in most recommissioning flushing guidance recommendations following shutdown due to COVID-19 pandemic restrictions.

## Materials and methods

### Characteristics of the building and showers

A five-story sports complex built in 1976 (Canada) was selected for this study given its large number of showers ( $n = 114$ ). The building plumbing system was supplied at one point of entry by municipal chlorinated water (0.41–0.64 mg  $\text{Cl}_2/\text{L}$ ). The hot water system included four hot water tanks (30–58°C) in series delivering hot water through a single central recirculating line. The prevalence of *Legionella pneumophila* in the municipally distributed water was previously found to be very low with rare positives detectable only in concentrated large volumes (Prévost et al., 2019).

The building was closed to the public from March 13 to July 6, 2020, due to COVID-19 pandemic restrictions orders. After this, normal summer activities (day camps) resumed on a daily basis, although the access to showers remained closed. Most showers sampled were part of a large grouped shower system in which one single bimetallic strip thermostatic mixing valve (TMV) supplies mitigated water to 20–22 showerheads (Supplementary Figures S1A,B). These showerheads were manually activated by a pressure-button with each activation resulting in a flow lasting 10 s–1 min. A few other showerheads sampled were either manually activated by a pressure-button or with a user-adjusted TMV and received mitigated water by a single TMV supplying less than four showerheads at once (Supplementary Figures S1C–E).

None of the pre-set TMV could be bypassed nor set differently during the study period. Pipe materials were not systematically identified as building plumbing was not easily accessible, except for large grouped shower systems in which copper was found from the TMV to each timer valves, and flexible plastic hoses from the latter to each showerhead.

### Sampling and targeted recommissioning flushing timelines

Sampling was first performed on July 2, 2020, to assess the water quality following a 16-week period of low building occupancy (< 1% of occupancy) at 21 selected showerheads distributed over seven locker rooms across the building (Supplementary Figure S1). On July 14, 2020, all showerheads ( $n = 114$ ) connected to the building plumbing system, including the ones investigated in this study, were successively flushed for 5 min each with mitigated water (25–39°C) as part of the recommissioning flushing program. No systemic flushing of the cold and hot water systems by opening all water points of use in the building was conducted although

recommended by several recent guidance documents (AWWA, 2020; CSA, 2020; Government of Quebec, 2020; Proctor et al., 2020; CDC, 2021). Therefore, the recommissioning program conducted should be viewed as partial device recommissioning targeted at high-risk areas only (showers). It is believed that few building managers have thoroughly conducted complete flushing of buildings after extended closures, partially because of the important resources to conduct full flushing, the lack of available information on the water systems or other considerations such as water conservation. Sampling was conducted again on the next day to evaluate the water quality within a short (24 h) period of time after targeted recommissioning flushing. Showers then remained unused despite the reopening of the building for day camps purposes. A third sampling event was carried out on August 19, 2020, to assess the long-term (4 weeks) impacts of targeted recommissioning flushing with no regular water use at showers. One showerhead from locker room E (Supplementary Figure S1C) could not be accessed for the last sampling event. All showers investigated remained untouched throughout the whole study period, with the exception of sampling days, hence the term distal water stagnation used in this present study.

In a subset of two showerheads, profile sampling was conducted over the course of the following year from the third recommissioning sampling event (August 19, 2020), without shower usage in the meantime, to measure total and intact bacterial cell counts after respective periods of distal water stagnation of 2 and 5 months. Profile flushing duration was based on the mitigated water volume between the outlet and the TMV (6 L) in the small grouped shower system (Supplementary Figure S1E) and on the volume between the outlet and the hot water recirculation line (250 L) in the large grouped shower system (Supplementary Figure S1A). Profile sampling was repeated after a successive 1 h distal water stagnation.

### Sampling methodology

For each large grouped shower system, sampling was performed by starting with the showerhead closest to the TMV and moving to the farthest. First draw samples (1 L) and 5 min flushed samples (1 L) were collected using sterile plastic bags attached to each showerhead so that exposure and generation of aerosols were reduced during sampling. A volume averaging 250 mL was drawn after each sample collection into a plastic beaker for onsite physico-chemical measurements (data not shown). Whenever free chlorine concentrations exceeded 0.05 mg/L, one mL of sodium thiosulfate (10% v: v) was added to the sample.

## Microbiological measurements processing

### Culture-based enzymatic *L. pneumophila* detection

In the field, an aliquot of 100 mL was transferred from each well-mixed one-liter sample to a sterile polypropylene container and kept at ambient temperature until analyzed in the laboratory. Within 12 h of sampling, aliquots were analyzed for the detection and quantification of *L. pneumophila* by culture-based enzymatic method with the 100 mL potable water Legiolert/Quanti-Tray kit (IDEXX Laboratories Canada Corp., Markham, Ontario, Canada) and incubated at  $39 \pm 0.5^\circ\text{C}$  for 7 days. Results were latterly determined by counting positive wells and expressed as most probable number (MPN) per liter concentrations (10–22 726 MPN/L).

### Bacterial-ATP through bioluminescence assay

Additional 50 mL aliquots were transferred from each well-mixed one-liter sample to distinct sterile polypropylene containers and kept on ice until transported to the laboratory. The protocol followed and the materials used were those provided by the manufacturer of the Dendridiag<sup>®</sup> SW kit (GL-Biocontrol, Cal Alpha, France), which has been specifically designed for bacterial-ATP quantification in drinking water. Water samples were filtered on  $0.45\ \mu\text{m}$  ( $\varnothing\ 33\ \text{mm}$ ) PES sterile membranes (CLEARLine<sup>®</sup> Biosigma S.p.A, via Valletta, Italy) to eliminate free ATP or other inhibitors that could interfere with bacterial-ATP detection (Hammes et al., 2008; Arroyo et al., 2017; Van Nevel et al., 2017). ATP was then extracted with a solution for cell lysis and immediately quantified through bioluminescence assay with the Kikkoman PD-30 Lumitester<sup>TM</sup> apparatus (Kikkoman Corp., Noda, Japan). The kit had a detection limit of 0.1 pg ATP/mL, and samples were processed within 12 h of sampling.

### Total and intact cell counts by flow cytometry

Within 24 h of sampling, aliquots of approximately 5 mL per sample were set aside in sterile tubes to assessed total and intact bacterial cell counts through flow cytometry on the BD Accuri<sup>TM</sup> C6 Plus Flow cytometer, along with the automatic sampling BD CSampler<sup>TM</sup> arm (BD-Biosciences, Mississauga, ON, Canada). Flow cytometry assays were performed according to Cazals et al. (2022). A volume ratio of water sample to reagent of 300:3  $\mu\text{L}$  was used for co-staining samples firstly with the SYBR<sup>®</sup> Green I fluorochrome to discriminate all bacterial cells, then with propidium iodide (PI) which stains only dead cells. The FL1 (533 nm) green fluorescence and FL3 ( $> 670\ \text{nm}$ ) red fluorescence density plots were selected for the enumeration of total and intact cell counts. The EAWAG water quality software analysis template for damaged and intact bacteria gating in

drinking water samples was adopted for all water samples to ensure reproducibility and comparison among results (Gatza et al., 2013). Viability of bacterial cells was therefore based on the integrity of cell membranes.

### DNA extraction and qPCR *L. pneumophila*

Remaining contents (600–800 mL) of bulk water samples were filtered on sterile  $0.2\ \mu\text{m}$  ( $\varnothing\ 47\ \text{mm}$ ) Supor<sup>®</sup> PES membranes (PALL Corp., Mississauga, ON, Canada) upon reception. Membranes were gently folded upon themselves and placed into sterile tubes for conservation at  $-80^\circ\text{C}$ . DNA was then extracted using the bead beating and centrifugation method adapted from Yu and Mohn (1999). For each sample, 1 mL of an extraction buffer (50 mM Tris-HCl, 5 mM EDTA, 3% SDS, and RNase  $20\ \mu\text{g}/\text{mL}$ ) was added to a FastPrep<sup>TM</sup> Lysing Matrix A tube containing the thawed membrane and agitated (6 m/s, 40 s) in the FastPrep-24 bead beater (MP Biomedicals, Solon, OH, USA) over two successive cycles, after which tubes were centrifuged for 5 min at 13 200 rpm. DNA purification was performed through impurities precipitation with ammonium acetate (100–300  $\mu\text{L}$ ) and DNA precipitation with isopropanol (100–750  $\mu\text{L}$ ) and glycogen (5  $\mu\text{L}$ , 0.02% v: v), followed by serial ethanol washes (1 mL 70% ethanol). DNA was finally resuspended in 100  $\mu\text{L}$  sterile qPCR grade water (Fisher Bioreagents, Waltham, MA, USA).

Quantification of *L. pneumophila* by real-time qPCR was performed in duplicates using the iQ-Check<sup>®</sup> Quanti *L. pneumophila* Real-Time PCR kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Inhibition testing and standard curve efficiency were assessed according to the manufacturer's recommendations. Fluorescence signals were recovered on the Rotor-Gene Q instrument (QIAGEN Sciences Inc., Germantown, MD, USA), and expressed into gene units (gu) per liter. No inhibition was detected, and qPCR efficiencies and correlation coefficients ( $R^2$ ) respectively ranged 86–121% and 0.99003–0.99818.

## Risk analysis

Risk characterization of both Legionellosis health outcomes (Pontiac fever – Sub-clinical infection and Legionnaires' disease – Clinical severity infection) was performed through quantitative microbial risk assessment (QMRA) using mean culture-based *L. pneumophila* concentrations as point estimates for first draw and flushed samples. Exposure assessment to *L. pneumophila* was adapted from Hamilton et al. (2019) to the specifics of the investigated building. A breathing rate associated to a moderate activity (USEPA, 2011) was considered since users typically take a shower after practicing physical activities, as well as a shower duration of 6.8 min represented by a right-skewed lognormal distribution (Wilkes et al., 2005)



and a value of one shower per day were used. Dose-response models for conventional ( $> 13$  lpm) and low-flow ( $< 7$  lpm) showerheads developed by [Hamilton et al. \(2019\)](#) were used accordingly to the R script code published by [Hozalski et al. \(2020\)](#). Finally, a disability-adjusted-life year (DALY) burden per Legionnaires' disease infection of 0.97 (0.90–1.05, uniform distribution; [Van Lier et al., 2016](#)) was also selected for the dose-response model.

## Data analysis

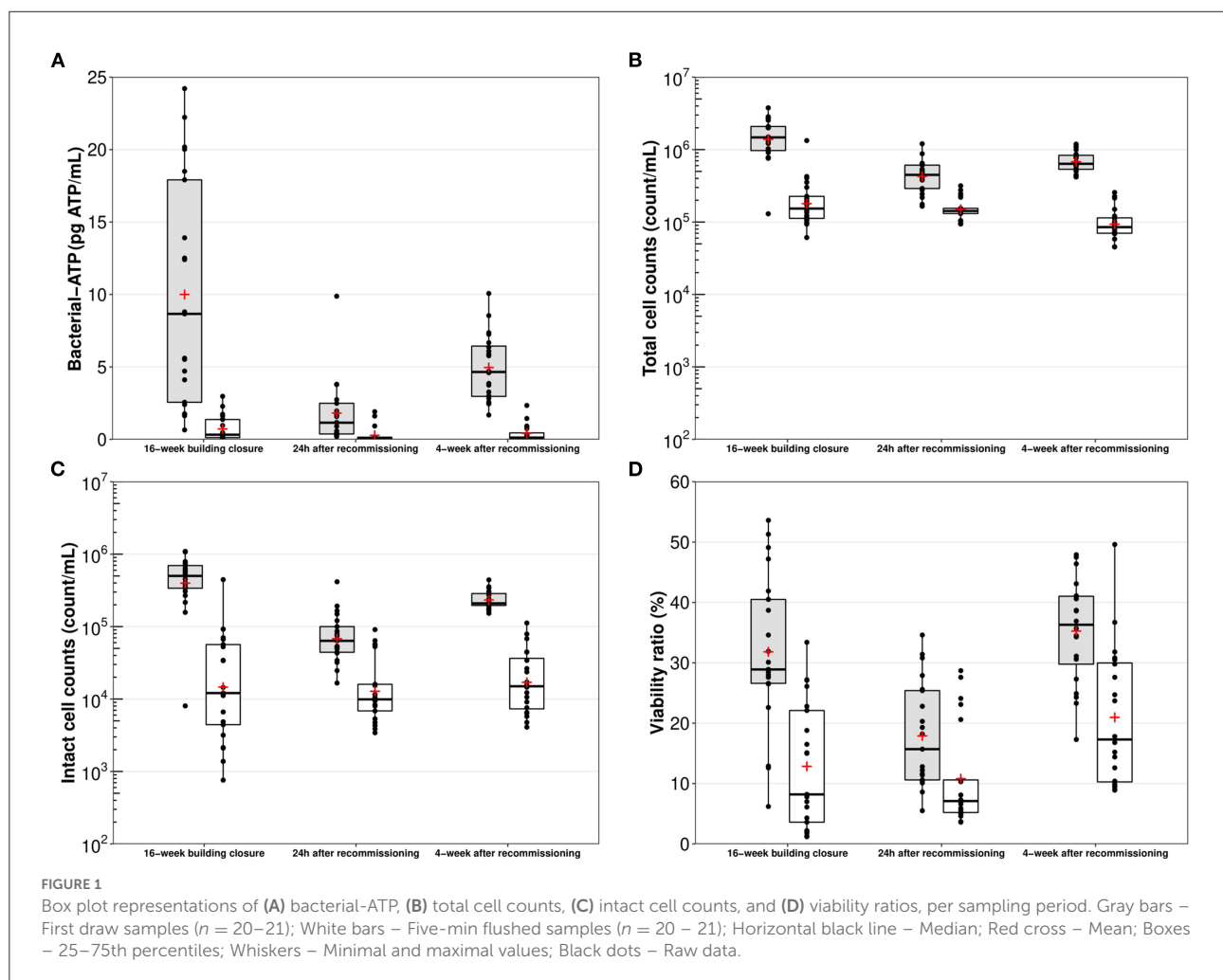
Normality of distributions of microbiological water quality indicators was first assessed generating histograms. Given that variables were all non-parametric, the Wilcoxon test was used to assess statistical differences between paired first draw and flushed samples, as well as between sampling events. For statistical purposes, any sample with a culture-based or a qPCR *L. pneumophila* concentration outside the detection limits of each method was respectively set at 5 or

30 000 MPN/L, or at 10 gu/L. Correlation among variables was evaluated through Spearman's rank correlation test by considering separately first draws and flushed samples due to the flushing intervention.

Significance level was set at a  $p$ -value of 0.05 unless stated otherwise and all analysis and graphs (except for line graphs which were produced on Microsoft Excel version 16.59) were performed on RStudio version 4.1.1 (2021.09.0).

## Results

Overall, 124 water samples from showerheads located in a large sports complex were analyzed for general bacterial indicators and *L. pneumophila*. First draw and 5 min flush samples were collected after a 16-week building shutdown, shortly (24 h) following targeted recommissioning flushing, and 4-week after the former intervention without water use in between (distal water stagnation). Summary statistics are available as [Supplementary Table S1](#).



## Trends in bacterial indicators

Measured bacterial indicators followed similar trends during the study. The highest mean bacterial-ATP (10.0 pg ATP/mL; Figure 1A), total cell counts ( $1.7 \times 10^6$  count/mL; Figure 1B) and intact cell counts ( $5.2 \times 10^5$  count/mL; Figure 1C) were measured in first draws ( $n = 21$ ) after the prolonged 16-week building closure (Figure 1). These indicators respectively reached values as high as 24.2 pg ATP/mL,  $3.8 \times 10^6$  count/mL, and  $1.1 \times 10^6$  count/mL (Supplementary Table S1). Targeted recommissioning flushing resulted in significant ( $p < 0.001$ ) reductions in these microbiological concentrations in first draw samples collected 24 h after flushing, and maximum values thereby measured remained below previously recorded mean. However, concentrations generally increased back during the monthly distal water stagnation period, indicating that detachment or regrowth of bacteria likely occurred near the points of use. Viability percentages in first draw samples ranged from 5 to 54% (Figure 1D).

Throughout the study, flushed samples ( $n = 62$ ) showed significantly lower ( $p < 0.01$ ) bacterial indicators concentrations than their first draws counterparts, although differences were slightly less marked 24 h after the recommissioning intervention. Globally, bacterial-ATP were reduced by 1.2–278-fold in flushed samples, whereas viability ratios were reduced by 0.5–48%. In terms of total and intact bacterial cell numbers, flushed samples were on average (per sampling event) 0.5–0.9-log and 0.7–1.4-log lower than first draws.

## Profile sampling of mitigated water in two selected showerheads

In order to investigate the relative contribution of growth during extended stagnation and detachment from biofilm, two showerheads from one small grouped shower system (Supplementary Figure S1E) and one large grouped shower system (Supplementary Figure S1A) were sampled again after respective periods of distal water stagnation of 2–5 months. Profile sampling of mitigated water collected from both showerheads after prolonged distal water stagnation (Figures 2A,B) showed that total and intact bacterial cell counts were of the same orders of magnitude ( $10^5$ – $10^6$  count/mL) in the first liter of collected water than what was measured after the 16-week building closure (Figures 1B,C). Flushing the mitigated water volume of 6 liters between the showerhead and the TMV in the small grouped shower system resulted in total and intact cell numbers reduced by 0.7- and 0.8-log (Figure 2A). In the large grouped shower system, total and intact cell numbers were more markedly reduced, respectively by 1.7- and 2.7-log (Figure 2B), as water was flushed until reaching the hot water recirculation line (250 liters). Following a 1 h period of distal water stagnation, both total and intact cell counts measured in the first few liters of water from each

showerhead remained at similar levels than those found in the last flushed sample collected 1 h before. However, as sample collection proceeded, a gradual increase in total and intact cell counts was observed in the showerhead from the small grouped shower system (Figure 2A), as well as incidental increases in the showerhead from the large grouped shower system (Figure 2B). This was likely due to higher levels of bacterial indicators found in the upstream plumbing system (i.e., upstream the TMV) or repeated shear forces induced by sample collection.

## *L. pneumophila* occurrence in showerheads

Throughout the study period, highest mean culture-based (Figure 3A) and qPCR *L. pneumophila* concentrations (Figure 3B) were measured in both first draw (Legiolert culture: 4 487 MPN/L; qPCR: 63 822 gu/L) and flushed samples (Legiolert culture: 1 622 MPN/L; qPCR: 3 461 gu/L) following the prolonged 16-week building closure. Culture-based *L. pneumophila* concentrations spanned over the complete range of the culture-based enzymatic method used ( $< 10$  MPN/L to more than 22 726 MPN/L), whereas qPCR concentrations spiked up to more than  $10^6$  gu/L. Conversely, lowest mean values were detected 24 h after targeted recommissioning flushing in first draw samples (Legiolert culture: 100 MPN/L; qPCR: 6 839 gu/L) and flushed samples (Legiolert culture: 23 MPN/L; qPCR: 687 gu/L). After the monthly distal water stagnation, mean concentrations bounced back to comparable levels than those found after the 16-week shutdown in both first draw samples (Legiolert culture: 2 368 MPN/L; qPCR: 47 007 gu/L) and flushed samples (Legiolert culture: 450 MPN/L; qPCR: 3 158 gu/L).

Five water samples (out of 124) had a higher culture-based concentration of *L. pneumophila* than its qPCR counterpart, as likewise reported by Dowdell et al. (2022; manuscript submitted to Environmental Science and Technology). This was likely due to DNA losses during the extraction phase or poorly mixed split water volumes. Nonetheless, specifically for first draw and flushed samples, qPCR concentrations were on average (per sampling event) 0.2–4.0-log and 0.4–3.4-log higher than culture-based results. However, mean log differences between the two *L. pneumophila* quantification methods used were generally larger in flushed samples, probably due to a greater proportion of dead or viable-but-non-cultivable *L. pneumophila* cells upstream in the hot water system. Linear regression analysis with first draw samples ( $n = 62$ ) further yielded a stronger correlation coefficient ( $R^2 = 0.64$ ) than for flushed samples ( $n = 62$ ;  $R^2 = 0.51$ ; Figure 4).

Overall, individual flushing of showers for 5 min with mitigated water resulted in average log reductions (per sampling event) of 0.4–1.1 and 0.4–0.6 respectively for culture-based (Figure 3A) and qPCR *L. pneumophila* concentrations

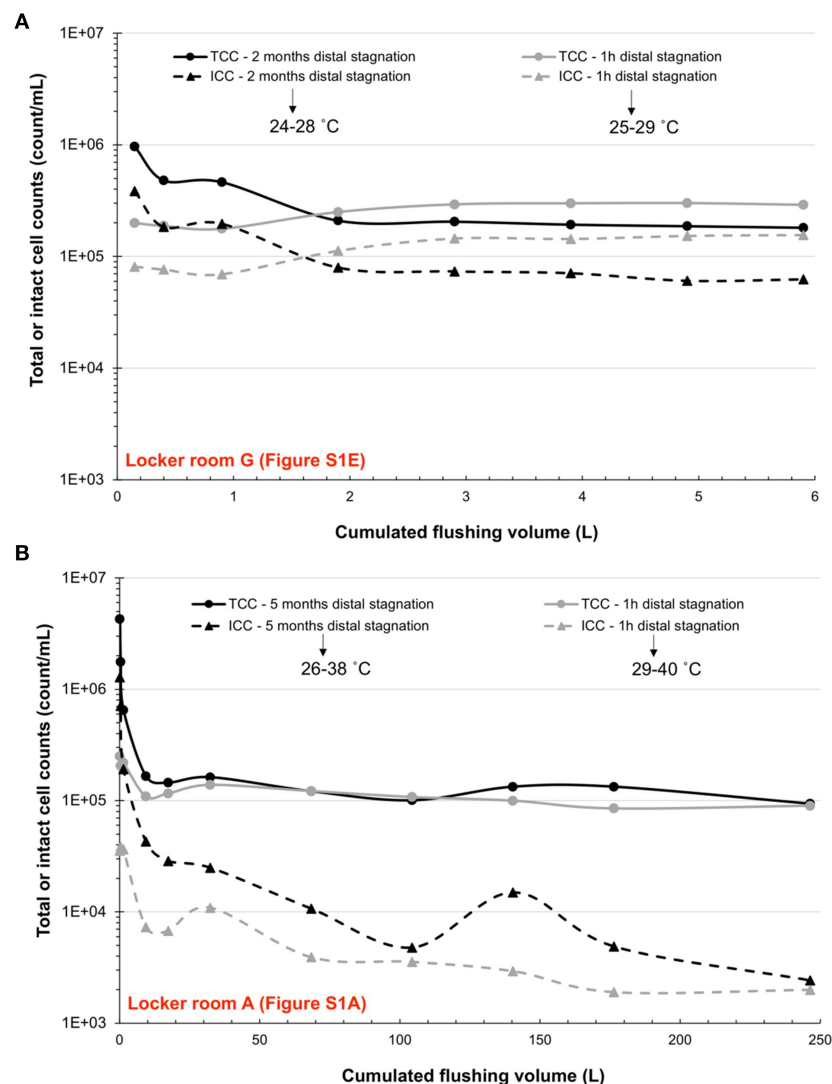


FIGURE 2

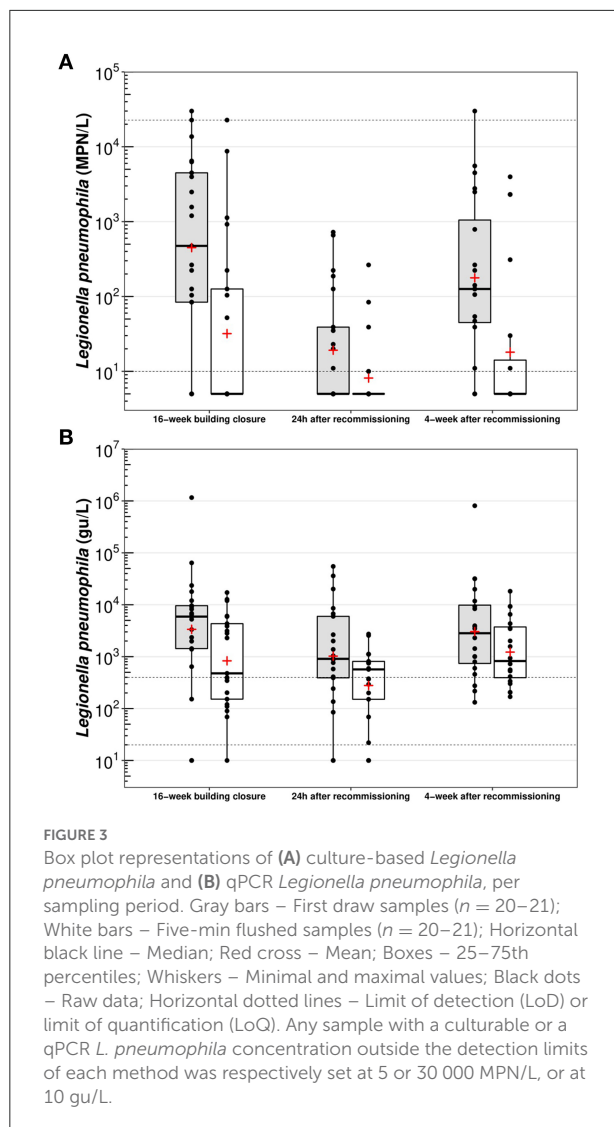
Total (TCC) and intact cell counts (ICC) profiles over cumulated flushing water volumes in two showerheads from a (A) small grouped shower system and a (B) large grouped shower system.

(Figure 3B). Considering paired samples, mean reductions in the concentration between first draw and flushed samples were always significant ( $p < 0.05$ ) over the study period, with the exception of qPCR results measured during the last sampling event ( $p = 0.07$ ). Contrary to expectations, increases ranging 0.1–1.8-log in culture-based *L. pneumophila* concentrations were observed in a subset of paired water samples ( $n = 7$  out of 62) in different showerheads. In four of these, a comparable increase ranging 0.3–1.4-log in qPCR *L. pneumophila* concentrations was seen, whereas decreasing qPCR concentrations were measured for the others.

Following the 16-week building closure, 81% (17/21) of showerheads were positive ( $> 10$  MPN/L) for culture-based

*L. pneumophila* in first draw samples (Table 1). Comparably, 90% (19/21) of showerheads had detectable *L. pneumophila* DNA (qPCR) in first draw samples. This proportion slightly increased to 95% (20/21) in flushed samples. The targeted recommissioning flushing intervention then lowered the proportion of positive culture-based *L. pneumophila* samples to 48% (10/21) in first draw samples, and to an even lower proportion (24%, 5/21) after a brief 5 min flush. Culture-based concentrations of all water samples also remained under 1 000 MPN/L. However, 90% (19/21) and 86% (18/21) of first draw and flushed samples still yielded detectable *L. pneumophila* DNA through qPCR. On the last sampling event, after a period of distal water stagnation of 4-week following the targeted

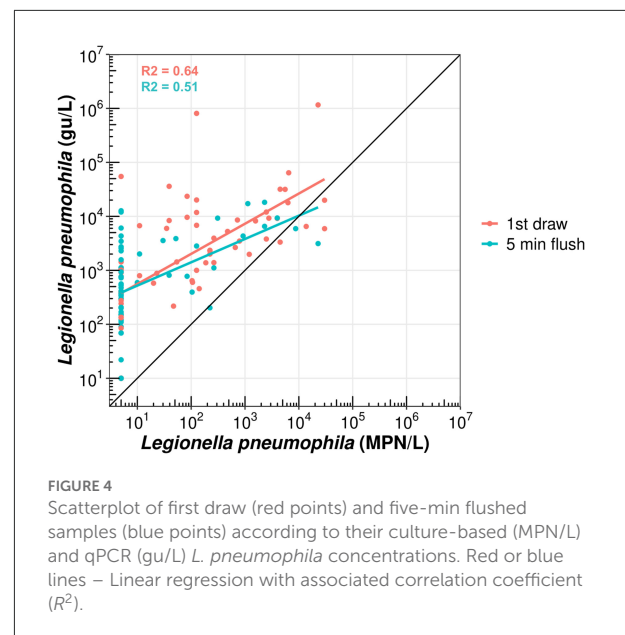




recommissioning flushing, *L. pneumophila* DNA through qPCR was detectable in all water samples. The proportion of positive samples to culture-based *L. pneumophila* increased accordingly to 85%, although the fraction of first draw samples over 1 000 MPN/L (25%) observed was less than after the initial 16-week building closure (48%; Table 1).

## Differences between types of shower systems

Two types of shower systems were investigated, including large grouped shower systems in which multiple ( $n > 20$ ) showerheads were supplied with mitigated water from a single TMV (Supplementary Figures S1A,B), and small grouped shower systems in which a small number of showerheads ( $n <$



4) were fed by a single TMV (Supplementary Figures S1C–E). These two shower systems differed for the most part by the total volume of mitigated water found between the TMV and each showerhead. This volume was approximatively of 300 liters of mitigated water in the large grouped shower systems, whereas it was less than 6 liters in the smaller ones.

Following the prolonged 16-week building closure, sampled showerheads associated with large grouped shower systems ( $n = 13$ ) showed similar mean culture-based *L. pneumophila* concentrations and total and intact cell numbers than showerheads associated with small grouped shower systems ( $n = 6$ ). However, significantly higher ( $p < 0.05$ ) bacterial-ATP and qPCR *L. pneumophila* concentrations in first draw samples were measured in showerheads from large grouped shower systems. Targeted recommissioning flushing appeared to bring more benefits in first draw samples collected from the large grouped shower systems as lower concentrations of all microbiological measurements were indeed measured, with the exception of *L. pneumophila* through qPCR. This may be attributable to the larger volume of water moved by the flushing of multiple showerheads at once, resulting in a greater turnover of water than in small grouped shower systems, which were oddly located further from the hot water recirculation line.

## Correlation analysis

Through Spearman's rank test, bacterial-ATP was found to have a strong and significant ( $p < 0.001$ ) positive relationship with intact cell counts in first draw samples ( $\rho = 0.8$ ,  $n = 62$ ; Supplementary Figure S2A), but a weak ( $\rho = 0.32$ ,

TABLE 1 Proportion of positive samples to culturable *Legionella pneumophila* (MPN/L) categorized in common sub-categories for drinking water.

Categories (MPN/L)	16-week building closure		24 h after recommissioning		4-week after recommissioning	
	First draw	5-min flush	First draw	5-min flush	First draw	5-min flush
Non-detect ( $< 10^1$ )	4/21 (19.1%)	13/21 (61.9%)	11/21 (52.4%)	16/21 (76.2%)	3/20 (15.0%)	14/20 (70.0%)
“Under control” ( $10^1 \leq Lp < 10^3$ )	7/21 (33.3%)	5/21 (23.8%)	10/21 (47.6%)	5/21 (23.8%)	12/20 (60.0%)	3/20 (15.0%)
Alert level ( $10^3 \leq Lp < 10^4$ )	7/21 (33.3%)	2/21 (9.5%)	0	0	4/20 (20.0%)	3/20 (15.0%)
Action level ( $\geq 10^4$ )	3/21 (14.3%)	1/21 (4.8%)	0	0	1/20 (5.0%)	0
Total positive ( $\geq 10^1$ )	17/21 (81.0%)	8/21 (39.0%)	10/21 (47.6%)	5/21 (23.8%)	17/20 (85.0%)	6/20 (30.0%)

$n = 62$ ), yet significant ( $p < 0.05$ ), correlation in flushed ones (Supplementary Figure S2B). This last observation could be attributed to the reduced bacterial-ATP sensitivity and higher variability commonly reported at lower cell concentrations (Hammes et al., 2010), given that flushed samples generally exhibited lower cell densities. Indeed, flushed samples generally showed low bacterial-ATP ( $< 1$  pg ATP/mL), but values spanned over 3 orders of magnitude in intact cell counts ( $10^3$ – $10^6$  count/mL). Hence, ATP-per-cell values, represented by the amount of bacterial-ATP over the number of cells with an intact membrane (Hammes et al., 2010; Lautenschlager et al., 2010), could be evaluated adequately at least in first draws. Despite higher bacterial-ATP and intact cell counts predominantly measured in first draw samples after the 16-week building closure and the monthly distal water stagnation period, ATP-per-cell ratios did not follow the same trends observed for the other bacterial indicators (Supplementary Table S1). Mean values in first draw samples did not change significantly ( $p = 0.34$ – $0.96$ ) across the sampling events. Nonetheless, values calculated for flushed samples were significantly ( $p < 0.05$ ) lower.

A significant ( $p < 0.05$ ) but weak correlation was observed between the proportion of intact cell counts (viability ratios) and culture-based concentrations of *L. pneumophila* in first draw samples ( $\rho = 0.25$ ,  $n = 62$ ; Supplementary Figure S2A) and in flushed samples ( $\rho = 0.31$ ,  $n = 62$ ; Supplementary Figure S2B). Therefore, culture-based concentrations were classified in dichotomous variables (below or higher than 1 000 MPN/L) to evaluate differences in viability ratios among sample types. Statistically different ( $p < 0.05$ ) mean of the fraction of intact cells between water samples below 1 000 MPN/L (first draw samples: 25%; flushed samples: 13%) or above 1 000 MPN/L (first draw samples: 38%; flushed samples: 32%) were found (Supplementary Figure S3). Finally, despite a weak ( $\rho = 0.34$ ) but significant ( $p < 0.01$ ) correlation between culture-based and qPCR *L. pneumophila* concentrations in first

draw samples (Supplementary Figure S2A), no such outcome was observed in the set of flushed samples.

## Quantitative microbial risk assessment for *L. pneumophila*

Mean of per exposure risk and DALY values calculated with culture-based concentrations of *L. pneumophila* through the QMRA approach are available in Supplementary Table S2. Overall, reported values displayed similar trends than *L. pneumophila* concentrations. Throughout the study, individual flushing of showerheads for 5 min only resulted in less than one log decreases in per exposure risk and DALY values for both Legionellosis health outcomes considered. For example, following the 16-week building shutdown, per exposure mean risk values for first draw samples considering a Pontiac fever endpoint were respectively of  $1.4 \times 10^{-2}$  and  $2.6 \times 10^{-3}$  for conventional ( $> 13$  lpm) and low-flow ( $< 7$  lpm) showerheads, whereas these values were reduced to  $4.1 \times 10^{-3}$  and  $7.8 \times 10^{-4}$  after a brief 5 min flush. Rather considering a Legionnaires' disease endpoint, per exposure mean risk values for first draw samples were of  $1.4 \times 10^{-5}$  and  $2.6 \times 10^{-6}$  for conventional and low-flow showerheads, and were subsequently lowered to  $4.7 \times 10^{-6}$  and  $8.8 \times 10^{-7}$  (Supplementary Table S2).

## Discussion

### Elevated bacterial indicators loads are observed after long periods of distal water stagnation

A prolonged building shutdown (16-week) resulted in elevated concentrations of microbial indicators in first draw and flushed samples, indicative of significant water quality losses

both in distal points of use (showerheads) and upstream in the building plumbing system.

Values of bacterial-ATP ranging from 0.7 to 24.2 pg ATP/mL (Figure 1A) were measured in first draw samples, exceeding previously reported values for potable water in the built environment in Europe (Lautenschlager et al., 2010; Duda et al., 2015; Zlatanović et al., 2017). The wide variations in measured values could further be evocative of the detection of different bacterial profiles, as ATP produced by cells vary across species, or conditions of growth (Zhang et al., 2019). Although ATP has not been correlated to increased health risks, nor it can predict accurately the presence of opportunistic pathogens (Duda et al., 2015), a cellular concentration over 10 pg ATP/mL is often suggested as a threshold for the implementation of corrective actions in some commercial kits. Nonetheless, the detection of high amounts of bacterial-ATP following the extended 16-week building shutdown reflects important bacterial activity or detachment from the biofilm, whereas the upsurge observed after the recommissioning intervention indicates possible bacterial regrowth.

Similarly, mean total and intact bacterial cell counts respectively reached  $1.7 \times 10^6$  count/mL (Figure 1B) and  $5.2 \times 10^5$  count/mL (Figure 1C) in first draw samples after the 16-week building closure and bounced back to  $7.2 \times 10^5$  count/mL and  $2.4 \times 10^5$  count/mL at the end of the monthly period of distal water stagnation which followed the targeted recommissioning flushing. Overall, these cell densities in first draw samples after long periods of no use in the distal parts of the building plumbing system largely exceed formerly published values after shorter stagnation times (Lipphaus et al., 2014; Zlatanović et al., 2017; Montagnino et al., 2022; Rahmatika et al., 2022). However, they are consistent with values measured in cold and hot water at taps after varying lengths of stagnation in buildings located in the same water distribution system than the sports complex sampled in the present study (Bédard et al., 2018; Dias et al., 2019). Observations from this study also appear consistent, yet with slightly higher values of total cell counts, than those measured after a 7-week shutdown in Swiss research buildings during the COVID-19 pandemic (Rhoads et al., 2022). Mean values for the proportion of intact cells (Figure 1D) were in agreement with prior observations of first draw samples in chlorinated water containing 15–41% of viable cells after 24 h to 10 days of distal water stagnation (Bédard et al., 2018).

Surprisingly, results of ATP-per-cell (Supplementary Table S1) in first draws after long periods of distal water stagnation were in the same order of magnitude than those calculated in previous studies for shorter stagnation times ( $< 2$  days; Siebel et al., 2008; Lautenschlager et al., 2010). The use of such ratio is beneficial to account for the heterogeneity of ATP concentrations produced by different bacterial species, or in contrasting growth conditions (e.g., starvation vs. exponential growth phase; Hammes et al., 2010). Indeed, starved bacterial cells are likely to have ATP-per-cell

values considerably lower as a consequence of reduced metabolic activity (Hammes et al., 2010), which was not observed in this study. These differences could also reflect higher nutrient loadings in the water source feeding the studied building, although dissolved organic carbon (DOC) consumption across the distribution system rarely exceed 0.1 mg C/L (Dias et al., 2017).

A weakness of most, if not all studies conducted during the COVID-19 pandemic, is the lack of baseline data collected during normal building operations before extended stagnation occurred. Also, the information on how microbial quality changes progressed during the building shutdown period is limited. Although it cannot be inferred that increasing stagnation duration will systematically exacerbate distal bacterial amplification, results from this study and report from Bédard et al. (2018) suggest that extended stagnation from one-hour to 4 months can lead to very high levels of total and intact cell counts at the point of use and its immediate upflow plumbing.

## Detachment occurs after prolonged periods of distal water stagnation

The observed distal amplification of bacteria in first draw samples collected after the prolonged 16-week building closure, and to a lesser extent after the monthly period of non-water use, can simultaneously be the result of bacterial growth and detachment from the adjacent biofilm. When flow resumes after stagnant conditions, it exerts important shear forces at the biofilm-water interface (Paul et al., 2012). Consequently, weakly embedded bacteria or biofilm pieces can be dislodged and dispersed into the bulk water phase through a sloughing phenomenon (Stoodley et al., 2001). Peng et al. (2020) demonstrated that short stagnation times ( $< 6$  h) resulted in increases in free-floating bacteria (planktonic growth), whereas biofilm-attached bacteria contributed more to growth in stagnant water during longer stagnation times (12–24 h). Following long periods of distal water stagnation, detached bacteria will accumulate in the bulk water and be washed out, thus likely resulting in larger amounts of biomass in water samples. As shown by Bédard et al. (2018), an increase in total cell counts and culturable bacteria (HPC) after stagnation periods ranging 2–10 days was mostly associated with detachment, reflecting the high surface to volume ratio in the distal point of use and immediate connecting pipe. The interplay between growth and flow induced detachment is also shown by the smaller amplification in the first distal volume after a shorter stagnation of 1 h (Bédard et al., 2018). In a medium-sized building, a tap unused for several months showed total bacterial cell counts more than 1-log higher in the first liter of water than in taps more frequently used (Lipphaus et al., 2014).

To assess whether the elevated concentrations of bacterial indicators were mostly due to detachment from the biofilm,

two showerheads from this study were sampled (profile sampling) after periods of distal water stagnation of 2 and 5 months. Typically, meaningful bacterial growth is unlikely to be significant in less than 1 h of stagnation (Lautenschlager et al., 2010). This was demonstrated in both showerheads by similar intact cell numbers measured between the last collected flushed sample after extended periods of no use and the first sample collected in the subsequent short stagnation time (Figures 2A,B). In profiling results, the differences between bacterial cell counts in the first liter of water and the last sample collected can indicate if detachment occurred. Indeed, results showed much larger differences, mirrored by rapid decreases in total and intact cell counts, in the first liter of water collected following the two- and five-month distal water stagnation periods than after the 1 h periods of no use (Figures 2A,B). This suggests that long periods of stagnation can increase bacterial detachment from biofilms present in distal devices. Additionally, a greater turnover of water through flushing (250 liters), as performed in the showerhead from one large grouped shower system (Supplementary Figure S1A), was more beneficial at reducing total and intact cell counts (Figure 2B) than profiled flushing only until the TMV (6 liters; Figure 2A).

## Widespread occurrence of *L. pneumophila* in showerheads

The high positivity rates of *L. pneumophila* by culture-based method and qPCR results (Table 1) recovered in first draw and flushed samples indicate a broad and systemic contamination of the building hot and tempered water systems. Whereas some studies carried out during the pandemic reported either no detectable *L. pneumophila* (Hozalski et al., 2020) or low positivity and concentrations of *L. pneumophila* (Dowdell et al., 2022, submitted to Environmental Science and Technology), others measured quantifiable concentrations in similar ranges as those observed in this study for samples representative of points of use ( $10^1$ – $10^4$  MPN/L or CFU/L; Giglio et al., 2020; Rhoads et al., 2022).

Typically, starved monocultured *Legionella* bacteria show complete loss of culturability within a few weeks (Al-Bana et al., 2014; Schrammel et al., 2018). However, the detection of culture-based *L. pneumophila* in showerheads which had not been used for 9 months (3 out of 21; data not shown) at levels comparable to those in showerheads unused only during the 16-week building shutdown suggests that conditions remained favorable for growth despite the probable depletion of nutrients. The establishment of a suitable microcosm in which *L. pneumophila* can seek nutrients through protozoal infection (Fields et al., 2002) could have contributed to its extended culturability in spite of environmental stressors, such as stagnant conditions. These results bring evidence that *L. pneumophila* can remain

culturable for a prolonged period of time in stagnant water at low temperatures (23–25°C) with nutrient limitations.

## Interpretation of *L. pneumophila* results

Alert and action thresholds for the detection of *L. pneumophila* almost all rely on culture-based methods as an epidemiological link has been established between culture-based concentrations and infection and disease (NASEM, 2019). Thresholds proposed vary significantly across control regulations and guidelines, and many are set to protect vulnerable people in the building in addition to considering the potential for aerosolization exposure. For public buildings serving a general population, a threshold of 10 000 CFU/L is suggested by the European Technical Guidelines for the prevention, control and investigation of infections caused by *Legionella* species (ESCMID, 2017). In contrast, for healthcare-associated settings, a lower action threshold of 1 000 CFU/L is recommended by the Veterans Health Administration (VHA) in the United States. More recently, the National Academies of Sciences, Engineering, and Medicine (NASEM) reviewed existing evidence and supported the definition of concentrations corresponding to an acceptable risk of infection and disease.

Using a reverse QMRA approach, Hamilton et al. (2019) calculated a risk-based concentration of 14.4 CFU/L and 1 400 CFU/L for a shower exposition, respectively for per exposure risk of  $10^{-6}$  DALY and  $10^{-4}$  annual infection benchmarks. Typically, the acceptable burden for both Legionellosis outcomes (Pontiac fever or Legionnaires' disease) strongly depends on which risk benchmark is selected. For example, in this study, per exposure risk values calculated for first draw samples collected after either the 16-week building closure or the 4-week distal water stagnation were on average above the common risk target of  $10^{-4}$  for a Pontiac fever endpoint, and above the more stringent risk target of  $10^{-6}$  DALY for a Legionnaires' disease endpoint (Supplementary Table S2). However, flushing these showerheads for 5 min did not result in sufficient risk reductions as per exposure mean risk values remained above the previous benchmarks.

According to the employees of the sports complex sampled, almost half of their clientele is composed of elderly, thus susceptible people to *Legionella* infection. Although a gap remains between observed health outcomes and results from environmental monitoring of *L. pneumophila* (culture-based or qPCR-oriented), the action limit for such building, and more particularly for showers which result in greater aerosolization, should be reconsidered. Hence, considering that one CFU is the near equivalent of one MPN in potable water samples according to previous studies (Sartory et al., 2017; Spies et al., 2018; Boczek et al., 2021), the detection of more than 30% of first draws above culture-based *L. pneumophila* concentrations of 1 000 MPN/L fully justifies the flushing recommissioning intervention.



## High *L. pneumophila* prevalence linked to poor design considerations

The high prevalence in the showers sampled strongly suggests that *L. pneumophila* had already colonized the building plumbing system prior to the period of no water use, although the extent of the contamination cannot be established. Most of the showers had not been used for 16 weeks, but some of the showers sampled were only used seasonally and had not been used for at least 9 months.

To identify the cause of the high prevalence of *L. pneumophila* observed in this building, several investigations were conducted. As proposed by Bédard et al. (2015), this investigation should first establish whether the main hot water system composed of the water heaters, reservoirs and recirculation loops were adequately operated to control *L. pneumophila* during the pandemic period. If the main distribution system is not operated to control *L. pneumophila* reliably, it will seed the secondary piping and distal sites and lead to widespread occurrence of the pathogen in distal points of use such as showerheads. In this study, low levels of culture-based *L. pneumophila* (20–65 MPN/L) were detected in the hot recirculation line, confirming the presence of sub-optimal conditions to control the pathogen.

The characterization of the centralized hot water system through sampling and temperature monitoring revealed numerous design and operational issues that could contribute to the presence of *L. pneumophila* in the main hot water system. First, of the four water heaters in series, the penultimate from the hot water being supply to the building was turned off without first isolating it (e.g., through closure of isolation valves) from the others. The water heater was then operated at 30°C for months before, during, and after the study period, hence providing a water environment that could have acted as a *L. pneumophila* nursery, although not tested due to a lack of available sampling ports. Maintaining a temperature above 60°C, as recommended in the Construction Code of Quebec (Building Act, chapter B-1.1, r. 2) and elsewhere (NASEM, 2019), is a beneficial control measure to prevent the growth of *L. pneumophila* (Bédard et al., 2016a; Cazals et al., 2022). Secondly, the water heaters feed flow was modified recently to improve energy efficiency. To do so, part of the recirculated hot water returning to the first water heater of the series was rather blended with the hot water coming out from the last heater of the series. These modifications resulted in hot water supplied to the building at 53–55°C, despite the hot water leaving the last heater at a consistent 60°C. In turn, the temperature of the hot water returning to the series of heaters was lowered to 48–51°C, thus likely allowing the survival of *L. pneumophila*. Moreover, episodic drops in temperature down to 25°C were observed in a small plate heat exchanger which preheated a fraction of the hot water returning back to the first heater of the series. High surface area and favorable temperatures

measured in the plate heat exchanger may have further promoted the growth and dissemination of *L. pneumophila* (Bédard et al., 2016b). It is interesting to note that a similar design of mixing contaminated recirculation water with cold water inflow to a heat exchanger was previously shown to cause a Legionnaire's disease outbreak in a hospital (Bédard et al., 2016b).

Thirdly, the general configuration of the hot water system consisted of a central hot water recirculation loop feeding secondary piping that provided water to locker rooms with showers across the building. Because of this configuration, unrecirculated secondary connecting piping constituted extensive dead water volumes of hot water between the central hot water recirculation loop and the TMV of the shower systems. This dead volume was estimated to reach up to 300 liters in some locker rooms. Typically, the time required for a given point of use to reach the hot water temperature of the recirculation line should be less than 60 s, and preferably under 30 s (Singh et al., 2022). However, in the most distant large grouped shower system from the hot water recirculation line, it took 30 min for the hot water to get to the TMV when using only one showerhead at a time. Such prolonged elapsed time was likely due to imbalanced hydraulics, or the long pipe lengths and large diameter (2 ½ inches) of the hot water pipe supplying the TMV, which was designed for simultaneous use of showerheads in the large grouped shower systems.

Finally, considerable volumes averaging 200–300 liters of mitigated water (25–40°C), were measured in the large grouped shower systems between the TMV and its supplied distal showerheads, thus providing favorable conditions for *L. pneumophila* growth (Sharaby et al., 2017). According to several thermostatic mixing valve manufacturers recommendations, this volume should be minimized so that a maximum volume of 3 liters or an elapsed time of 30 s are found between the TMV and the farthest outlet. In addition, manufacturers also recommend monitoring and disinfecting such grouped shower systems with hot water (60–70°C) on a routine basis.

When considering the operational and design weaknesses documented, it was not surprising to find the high prevalence of *L. pneumophila* in the shower systems investigated. Apart from operational issues with the water heaters and recirculation water mixing, the building studied reflected an older design with little or no consideration to the limitation of volumes of unrecirculated hot water and tempered water. These conditions are likely to be present in many legacy buildings. Standards and plumbing codes related to grouped shower systems should be carefully reviewed in order to prevent these conditions highly favorable to *Legionella* growth. Additional research is needed regarding preventive control measures in large grouped shower systems, such as the installation of auto-flush valves situated at their rear-end to allow more frequent renewal of water.



## Lack of correlation between *L. pneumophila* presence and bacterial indicators

In the context of environmental routine monitoring, which is a central part of water safety management plans, the use of quick and accurate surrogate parameters to predict the presence of *L. pneumophila* in building plumbing systems is beneficial to identify more effectively at risk systems or conditions. Low cost indicators such as ATP have been used in building water quality monitoring to flag any changes in the water systems (Prest et al., 2016).

In this study, no strong correlations were observed between measured microbiological indicators and concentrations of *L. pneumophila*, even when considering first draw and flushed samples separately (Supplementary Figures S2A,B), in agreement with prior observations (Duda et al., 2015). However, comparatively to water samples with culture-based concentrations of *L. pneumophila* below 1 000 MPN/L, samples above 1 000 MPN/L tended to have significantly ( $p < 0.05$ ) higher proportions of intact cells in both first draw (38 vs. 25%) and flushed samples (32 vs. 13%; Supplementary Figure S3). This suggests that higher proportions of viable bacterial cells were favorable to the detection of culture-based *L. pneumophila*, possibly due to beneficial ecological interactions with other microorganisms, or due to detachment of biofilm, which is an ecosystem known to harbor most live cells.

Culture-based and qPCR concentrations of *L. pneumophila* also correlated strongly and moderately together when modeled by a linear regression (Figure 4). The proportion of dead or viable-but-non-culturable (VBNC) cells is likely to account for the large discrepancies between culture-based and qPCR concentrations (differences ranging 0.2–3.8-log), especially in flushed samples which are representative of more unfavorable environmental conditions (e.g., higher temperatures). These discrepancies are in agreement with previous studies (Lee et al., 2011; Whiley and Taylor, 2014; Dowdell et al., 2022, submitted to Environmental Science and Technology).

## Implications for flushing guidance recommendations

### Impact of distal flushing on microbiological measurements

In this study, flushing showerheads for 5 min with mitigated water produced clear and immediate benefits on a microbiological perspective. Overall, bacterial-ATP levels were lowered by 1.2–278-fold in flushed samples (Figure 1A), whereas total (Figure 1B) and intact (Figure 1C) bacterial cell counts were on average 1.1- and 0.7-log lower than in first draw samples. The proportion of intact cells was slightly lowered by about 14% (Figure 1D). The largest differences among paired samples were commonly observed in first draw samples with

especially elevated bacterial-ATP ( $> 10$  pg ATP/mL), total and intact cell counts over  $10^6$  and  $10^5$  count/mL, or percent of intact cells over 25%, which occurred mostly following the extended 16-week building closure. Reduced numbers and smaller reductions in measured bacterial indicators are artifacts of the upstream water quality, which is typically less vulnerable to microbial amplification due to the maintenance of a thermal barrier (in hot water) and measurable chlorine residuals (in cold water). Although culturability was evidently diminished with flushing, total cell counts almost 3-log higher than the incoming cold water entry (data not shown) suggest that a great deal of dead cells were nevertheless circulating in the building plumbing system. Observed decreases are comparable with previously reported observations in building plumbing systems and for similar flushing times in chlorinated and unchlorinated systems (Lautenschlager et al., 2010; Lipphaus et al., 2014; Bédard et al., 2018), although values observed in this study remained slightly higher.

Consistent trends were also observed for culture-based (Figure 3A) and qPCR concentrations of *L. pneumophila* (Figure 3B) as flushing lowered concentrations by 0.8- and 0.5-log on average, respectively. Although considerable reductions in culture-based *L. pneumophila* concentrations (over 1-log on average) were measured in flushed samples after the 16-week building shutdown and the monthly distal water stagnation period that followed the recommissioning flushing event, insufficient per exposure mean risk reductions (less than 1-log on average) were computed (Supplementary Table S2). This implies that a one log reduction in *L. pneumophila* culture-based concentrations might not be enough to reduce consequently the exposure risk to the pathogen in some instances. Moreover, considering each sampling event separately, the high proportions of flushed samples with detectable *L. pneumophila* by culture-based method (24–39%) or qPCR (86–100%) indicate that the contribution of biofilm during flow and upstream conditions allowed the persistence and the circulation of the bacteria across the building plumbing system, either in the VBNC form or dead.

The benefit of flushing some devices for 5 min after extended stagnation can be examined by excluding data collected 24 h after the whole recommissioning flushing conducted at every outlet. These data provide evidence of the benefits of partial distal flushing as a preventative measure. Flushing each outlet as recommended by recommissioning guidance is quite demanding and building managers may consider flushing selected points of use as an alternative method. Results show that 63 and 29% of paired samples ( $n = 41$ ) underwent a minimum of 1-log reduction in culture-based and qPCR *L. pneumophila* loads after 5 min of device flushing. Although there is limited guidance for the interpretation of qPCR results of *Legionella* spp. or *L. pneumophila*, some showerheads reaching the 1-log reduction after a brief five-min flush still had concentrations over  $10^3$  gu/L, yet undetectable culture-based *L. pneumophila*. Therefore,

longer flushing times may be required depending on the building plumbing system layouts to perform a water turnover equivalent to meeting upstream water quality.

It is noteworthy that flushing resulted in simultaneous increases of 0.1–1.8-log and 0.3- to 1.4-log in both culture-based and qPCR *L. pneumophila* concentrations in few showerheads. Such increases can be coincidental, due to odd biofilm detachment, or because of localized contamination. Although fixture-flushing is expected to reduce *Legionella* spp. levels (Wang et al., 2012; Hozalski et al., 2020; Richard and Boyer, 2021), it can lead to momentary detrimental effects. One study also demonstrated increases in *L. pneumophila* levels after flushing, which was associated to building-specific features and deleterious recommissioning flushing practices such as rapid depletion of boiler temperatures with water turnover in the hot distribution system (Rhoads et al., 2022).

### Short- and long-term effectiveness of targeted recommissioning flushing

Growth of *Legionella* bacteria and more particularly *L. pneumophila* were identified as a significant risk in most recommissioning flushing recommendations issued to address the safe reopening of building water systems during the COVID-19 pandemic. Yet, there is a scarcity of data regarding the short- and long-term benefits of such intervention because of the novelty of the prolonged building shutdowns that were observed during the COVID-19 pandemic.

Significant ( $p < 0.05$ ) reductions in bacterial-ATP (Figure 1A), total (Figure 1B) and intact (Figure 1C) cell counts, viability ratios (Figure 1D), and culture-based *L. pneumophila* concentrations (Figure 3A) were observed in first draw samples collected shortly (24 h) after the targeted recommissioning flushing intervention. More importantly, culture-based *L. pneumophila* concentrations were lowered below the 1 000 MPN/L alert threshold (Table 1) in all first draw samples. However, despite small decreases, qPCR concentrations (Figure 3B) did not significantly ( $p = 0.063$ ) change in first draw samples, likely due to its persistence in points of use and the great proportions of previously collected paired flushed samples that still had detectable *L. pneumophila* DNA through qPCR (Table 1).

Beneficial short-term impacts of targeted recommissioning flushing did not persist over the subsequent monthly distal water stagnation in shower systems. Indeed, bacterial indicators (Figure 1) and *L. pneumophila* loads (Figure 3) bounced back in first draws to more or less similar levels than those measured following the 16-week building closure. One study also demonstrated that *Legionella* species *ssrA* genes concentrations slightly rebounded in some taps during a follow-up period (2–4 days) thereafter flushing points of use for 45 min (Hozalski et al., 2020). These increases in distal parts of the building plumbing system could either reflect bacterial growth occurring under

stagnant conditions (Lautenschlager et al., 2010; Zlatanović et al., 2017; Bédard et al., 2018; Peng et al., 2020; Rahmatika et al., 2022), or result from higher detachment rates of bacteria from the biofilm during stagnation and when flow resumes after prolonged periods of no use (Stoodley et al., 2001; Figure 2). It is likely that the water turnover resulting from the recommissioning flushing intervention replenished nutrients near points of use, as well as dead biomass already circulating in the hot water distribution system, supporting regrowth of bacteria and biofilm grazing hosts of *Legionella*, amoeba (Rhoads et al., 2022). The implementation of routine flushing regimes at points of use should therefore be explored by building managers to prevent stagnant conditions.

Two showerheads that were negative for the presence of culture-based *L. pneumophila* at the beginning of the study finally became positive at concentrations of 141 and 264 MPN/L. The detection of the pathogen with qPCR concentrations ranging  $10^2$ – $10^3$  gu/L in their first draw samples after the 16-week building shutdown strongly suggests that the bacteria had already colonized the points of use but was mostly either in its VBNC form or dead. Aside from a single showerhead that only tested positive to culture-based *L. pneumophila* once, all others remained positive in both of sampling events characterized by prior distal water stagnation periods (first and last ones). This illustrates the persistence of the pathogen in distal areas, despite the targeted recommissioning flushing intervention. As flushing can only partially dislodge microorganisms found in the biofilm and stagnant water, its effect is likely to be short lived especially when followed by periods of no use.

Several authors have assessed the occurrence rate over time of the pathogen, and whereas one group found persistent contamination of *Legionella* species at all investigated taps throughout the year (Liu et al., 2018), others have mainly reported sporadic presence of *L. pneumophila* (Bédard et al., 2016a; Donohue et al., 2018; Buse et al., 2020). Generally, taps which were persistently positive for the presence of *L. pneumophila* tended to harbor higher concentrations of the bacteria (Donohue et al., 2018; Buse et al., 2020), similarly to what was observed in the present study. This suggests that when *L. pneumophila* is well established, its detection is likely to occur repeatedly regardless of the normal building water usage, although in dynamic concentration ranges.

### Incorporation of findings in sampling and flushing guidance for *Legionella* control

The observations from this study raise a few important issues on how and when preventative and curative flushing should be carried out, as well as how its efficacy should be assessed. The present study was conducted at a single site and would benefit from additional flushing efficacy studies on a wider set of building plumbing systems. In this specific sports complex, partial recommissioning flushing was mostly effective at short

term to lower planktonic *L. pneumophila* found in shower water, but building specifics at other sites could alter these trends.

### Complete building wide recommissioning flushing of hot and cold water systems should be prioritized

Flushing as performed in this study was conducted at the shower distal points, which are considered to be the most at risk devices for aerosol formation. A more extensive and systematic turnover of water through system-wide recommissioning flushing programs in which all water outlets are individually flushed with both cold and hot water is highly recommended (AWWA, 2020; CSA, 2020; Government of Quebec, 2020; Proctor et al., 2020; CDC, 2021). It is more likely to result in lower *Legionella* positivity on a short- and possibly long-term perspective. Indeed, flushing with a high hot water temperature ( $> 55^{\circ}\text{C}$ ), then with a cold-water residual ( $> 0.5\text{ mg Cl}_2/\text{L}$ ) is doubtlessly more effective to control the growth of a broad range of microorganisms, and should therefore be prioritized when possible. However, without other evidence, recommissioning flushing should preferentially be carried out within 1 or 2 days from the building reopening. As bacterial indicators concentrations and *Legionella* loads rebounded during the monthly distal water stagnation event, the 2-week (re)commissioning threshold proposed in the ASHRAE Standard 188 may not be sufficient to maintain low *Legionella* levels, especially in contaminated building plumbing systems.

### Levels of *Legionella* contamination vary widely between fixtures

This study shows the wide variations (up to 3-log at times) of concentrations of *L. pneumophila* found in neighboring showers. This important variation raises the issue of the effective selection of devices to be sampled to monitor the efficacy of flushing. Additionally, sampling the very first draw is necessary to assess distal contamination of showerheads, comparatively to what is suggested as monitoring strategies for microbiological sampling in some guidance document (ISO, 2006). The large differences between concentrations in first draw and flushed samples (up to 3-log) strongly support collecting a first draw sample as the best indicator of the maximum recovery level of contamination to which the user may be exposed (Wang et al., 2017; Hirsh et al., 2020).

Finally, additional research is needed to identify the most suited preventive and remedial flushing regimes to limit the growth of *Legionella* that building managers could easily implement in water safety plans, such as automatic flushing devices that are now commonly installed in healthcare facilities. Most urgent is the production of actionable data to determine the frequency at which preventative, recommissioning and curative flushing should be conducted.

## Conclusion

- The present study is the first to evidence the high prevalence and prolonged culturability (4–9 months) of *L. pneumophila* in distal parts of building plumbing systems.
- A prolonged building closure of 16-week revealed large amplification of bacterial-ATP (0.7–24 pg ATP/mL) and total and intact cell counts ( $10^4$ – $10^5$  count/mL) in first draw samples that exceeded previously published results for shorter stagnation times (less than 2 weeks). Mean values of *L. pneumophila* through culture-based and qPCR methods respectively reached 4 487 MPN/L and 63 822 gu/L, even in showerheads that were left unused for more than 9 months. These elevated loads were mostly attributable to detachment from the adjacent biofilm, which is thought to be more important after periods of prolonged stagnation.
- The widespread *L. pneumophila* contamination was presumably linked to poor design and operational considerations of the hot water system, including the use of TMV feeding a large ( $> 20$ ) number of devices, sub-optimal temperature conditions in the hot water recirculation line due to water heaters feed flow modifications, drastic drops in temperatures observed in a small plate heat exchanger, and overall large dead water volumes in secondary piping across the building.
- Individual flushing of showerheads for 5 min resulted in significant ( $p < 0.05$ ) decreases in all measured bacterial indicators. Culture-based and qPCR concentrations of *L. pneumophila* were also reduced by respectively 0.8- and 0.5-log on average. Profile sampling showed that prolonging flushing from 1 to 35 min provided additional reductions of concentrations of total and intact cell counts.
- Targeted recommissioning flushing was beneficial at short-term (24 h) to lower risks as all showerheads remained under the common alert threshold of 1 000 MPN/L for the monitoring of *L. pneumophila* in public buildings. However, recommissioning flushing was not sufficient to lower long-term (4-week) mean risks below benchmarks of  $10^{-4}$  infections per exposure for a Pontiac fever endpoint and below  $10^{-6}$  DALY per exposure for a Legionnaires' disease endpoint.
- The maximum stagnation period following commissioning should be reevaluated as significant positivity for *L. pneumophila* and rebound concentrations were observed during the monthly distal water stagnation after targeted recommissioning flushing.
- *Legionella pneumophila* positivity and concentrations by culture-based or qPCR methods could not be strongly predicted or correlated by any measured bacterial indicators in agreement with prior studies.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://doi.org/10.6084/m9.figshare.19807282.v3>.

## Author contributions

MP and MG-C conceptualized the study research methodology. MG-C conducted the investigations, analyzed the data, and wrote the original draft of the manuscript. EDe, EDo, and MP revised and edited the manuscript. MP acquired funding for this study. All authors have read and agreed to the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frwa.2022.959689/full#supplementary-material>

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