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POLYTECHNIQUE MONTRÉAL

affiliée à l'Université de Montréal

Occurrence and mitigation of opportunistic drinking water pathogens in large building water systems

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Thèse présentée en vue de l'obtention du diplôme de *Philosophiæ Doctor*

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Occurrence and mitigation of opportunistic drinking water pathogens in large building water systems

présentée par **Marianne GRIMARD-CONEA**

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a été dûment acceptée par le jury d'examen constitué de :

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RÉSUMÉ

L'eau potable est reconnue comme étant une source importante d'infections hydriques en raison de la présence de bactéries pathogènes opportunistes, telles que *Legionella pneumophila* (*Lp*), *Pseudomonas aeruginosa* (*Pa*) et les mycobactéries non tuberculeuses (MNTs). Ces bactéries représentent des enjeux majeurs de santé publique lorsqu'ils sont amenés à proliférer dans les systèmes d'eau, en particulier au sein des biofilms sur les surfaces internes des tuyaux de plomberie et des appareils de robinetterie. Contrairement aux pathogènes francs, ces bactéries opportunistes ne provoquent pas de maladies graves chez les individus en bonne santé, mais elles peuvent engendrer des infections sévères chez les personnes immunodéprimées, les personnes âgées et celles atteintes de maladies chroniques.

- ***Legionella pneumophila*** est responsable de la majorité des cas de maladie du Légionnaire en Europe/Amérique du Nord, une forme grave de pneumonie transmise par l'inhalation d'aérosols contaminés. Bien que la majorité des cas soient d'origine communautaire ou liés aux voyages, les infections nosocomiales entraînent un fardeau sanitaire disproportionné, en raison de leur gravité et de la présence de populations vulnérables.
- ***Pseudomonas aeruginosa*** est responsable de nombreuses infections acquises en milieu hospitalier affectant plus particulièrement les victimes de brûlures, et les patients atteints de mucoviscidose. Une étude européenne récente a révélé que 31% des souches étaient résistantes à au moins un groupe d'antibiotique, compliquant les efforts de traitement.
- **Les mycobactéries non tuberculeuses**, un groupe varié causant des maladies pulmonaires chroniques et des infections cutanées, voient leur incidence augmenter mondialement. Elles sont également associées au plus grand nombre d'enquêtes sur les infections nosocomiales reliées à l'eau aux dans les établissements de soins de santé aux États-Unis.

Face à ces tendances préoccupantes, la gestion des systèmes d'eau dans les grands bâtiments est devenue un défi complexe, mais crucial. Ces systèmes d'eau présentent des conditions hétérogènes d'opération, de conception et d'utilisation créant un environnement favorable à la croissance et à la dispersion de microorganismes. Malgré ces défis, les réglementations et pratiques spécifiques à la gestion des systèmes d'eau dans les bâtiments demeurent limitées ou incohérentes.

L'objectif principal de ce projet de recherche est de fournir des recommandations fondées sur des données probantes afin d'améliorer la surveillance, l'exploitation et la conception des systèmes de

plomberie dans les grands bâtiments. En combinant des outils novateurs, cette recherche vise à étudier l'occurrence de bactéries pathogènes opportunistes (*Lp*, *Pa*, MNTs) et évaluer l'efficacité de mesures préventives et correctives destinées à réduire le risque d'exposition des utilisateurs. Ce projet cherche aussi à informer les gestionnaires de bâtiments de leur devoir de diligence en proposant des solutions pratiques pour une gestion proactive des systèmes d'eau de leurs bâtiments.

La première partie du projet examine les défis liés à la désinfection secondaire dans les grands bâtiments. Assemblant 1 737 échantillons provenant de neuf grands bâtiments institutionnels (hôpitaux, universités, écoles, centre sportif), une méta-analyse a été réalisée afin de déterminer si les gestionnaires de bâtiments peuvent activement compter sur les résiduels en chlore libre des réseaux d'aqueduc municipaux pour prévenir la prolifération microbienne. Les résultats ont montré que les concentrations de chlore libre dans les échantillons au premier jet atteignaient le niveau guide de 0,2 mg/L dans 26%, 6% et 2% des échantillons d'eau froide, tiède, et chaude, respectivement. Un rinçage pendant deux à 60 minutes augmentait significativement ce ratio uniquement dans l'eau froide (83%), sans toutefois atteindre les niveaux mesurés à l'entrée d'eau des bâtiments investigués. Le chlore libre était faiblement corrélé ($R < 0,2$) à l'adénosine triphosphate (ATP), aux bactéries hétérotrophes aérobies ou anaérobies facultatives (BHAA), ainsi qu'aux comptes totaux et viables par cytométrie de flux, démontrant que le résiduel contribue tout de même à réduire la biomasse bactérienne viable et cultivable. La détection de *Lp* cultivable, s'étendant sur quatre ordres de grandeur logarithmique, n'a eu lieu que lorsque le chlore libre était inférieur à 0,2 mg/L, mais aucune tendance claire n'a pu être distinguée pour *Pa* cultivable. Des températures d'eau inférieures à 20 ˚C et supérieures à 60 ˚C ont complètement inhibé la détection de *Lp* par culture. En général, la majorité des paramètres mesurés montraient des concentrations microbiennes élevées dans les points d'usage distaux ainsi que dans l'eau tiède et l'eau chaude, où le chlore libre est rapidement dissipé par la stagnation et l'augmentation de la température.

Dans la seconde partie de cette thèse, l'efficacité du rinçage de remise en service, soit une mesure largement suggérée pour la réouverture sécuritaire des bâtiments vacants durant la pandémie de COVID-19, a été évaluée à 23 pommeaux de douche d'un grand centre sportif. Après une fermeture de 16 semaines, les concentrations moyennes les plus élevées des indicateurs microbiens généraux (ATP, comptes totaux/viables) ont été mesurées dans les échantillons au premier jet alors que la bactérie *Lp* a été détectée dans respectivement 81% et 90% des échantillons par culture et par PCR quantitative (qPCR). Le rinçage de remise en service s'est avéré bénéfique à court terme (24

heures) pour réduire les risques associés à *Lp* en abaissant les concentrations en dessous du seuil d'alerte commun de 1 000 MPN/L dans les bâtiments publics. Cependant, suivant une période d'un mois sans utilisation de l'eau aux douches du centre sportif, des rebonds considérables des concentrations des indicateurs microbiens généraux et de *Lp* à des niveaux similaires que ceux mesurés après la fermeture prolongée ont été observés. Ces résultats mettent en évidence les bénéfices temporaires du rinçage de remise en service sur la prévalence de *Lp* à des dispositifs générant de grandes quantités d'aérosols.

La troisième partie du projet de recherche se concentre sur des mesures correctives couramment appliquées pour gérer la contamination par *Lp* dans les bâtiments, soient la chloration choc et le rinçage de (re)mise en service. Dans cette étude, l'impact à court terme (trois semaines) de la chloration choc (20 – 25 mg/L de chlore libre pendant 16 heures) et du rinçage (5-min à tous les pommeaux), tous deux combinés à différents régimes de rinçage préventif (quotidien, hebdomadaire, aucun), a été évalué en duplicata à des pommeaux de douche du même centre sportif. Les résultats ont démontré que lorsqu'aucun rinçage préventif (ou aucune utilisation de l'eau) ne suivait la chloration choc, une croissance microbienne importante était favorisée. En revanche, le rinçage suivi d'une période de stagnation distale a entraîné une recroissance complète, voire parfois même plus importante, de la cultivabilité et des concentrations par qPCR de *Lp*. Indépendamment de l'intervention appliquée, les pommeaux rincés quotidiennement présentaient des niveaux significativement plus faibles en ATP et compte totaux, ainsi que des concentrations réduites de Lp, que ceux rincés sur une base hebdomadaire. Enfin, invariablement du régime de rinçage implémenté, la chloration choc a permis de supprimer la cultivabilité de *Lp* (réduction de trois logs) pendant deux semaines avant que des faibles rebonds soient observés, alors que le rinçage n'a eu qu'un impact minimal sur la prévalence de Lp. Cette étude offre des perspectives sur les combinaisons optimales à court terme de stratégies correctives et préventives pouvant être envisagées en attendant la mise en place de contrôles opérationnels plus adaptés ou de traitements systémiques, à l'échelle du bâtiment.

Finalement, le dosage *in situ* de monochloramines dans le système d'eau chaude d'un grand hôpital a été implémentée comme alternative à l'échelle du bâtiment en réponse à la persistance de *Lp* dans le réseau d'eau chaude malgré la mise en place d'un régime thermique de contrôle (> 55 ˚C) et d'interventions répétées de désinfection choc. Plus spécifiquement, cette dernière étude examine les impacts longitudinaux et transversaux des monochloramines générées sur site pour atténuer la

prévalence de nombreux pathogènes et hôte. Au total, 544 échantillons ont été récoltés à partir de 22 points d'utilisation (postes de lavage des mains, robinets d'eau chaude, pommeaux de douche), sélectionnés en fonction des risques pour les patients, et de 10 points représentatifs du système de distribution d'eau chaude l'hôpital (retours d'eau chaude, alimentation d'eau chaude, points d'utilisation éloignés), et ce, avant et après l'introduction des monochloramines avec des concentrations cibles variant entre 1,5 et 3,5 mg/L. Les monochloramines ont rapidement éliminé le réservoir de Lp, avec des réductions allant jusqu'à trois logs en culture (< 24 heures) et deux logs par qPCR (< quatre semaines). Les concentrations de *Vermamoeba vermiformis* (*Vv*), un hôte prédominant pour les Légionelles et les mycobactéries non tuberculeuses, ont diminué de deux logs en 24 heures avant de se stabiliser. En revanche, les mycobactéries non tuberculeuses ont montré une nette persistance, avec des concentrations moyennes variant entre 10^4 et 10^6 gc/L, malgré une diminution d'environ un log dans les points distaux et de deux logs dans les points systèmes au bout de deux semaines. Les plus fortes réductions de ces microorganismes ont été observées pour des concentrations de monochloramine de 2 à 3 mg/L et des températures au-dessus de 55 ˚C. Cependant, l'interruption du dosage a entraîné des rebonds rapides des mycobactéries non tuberculeuses, des Légionelles et de *Vv* à des niveaux similaires à ceux mesurés avant l'introduction des monochloramines, démontrant ainsi leur persistance dans les biofilms. Par ailleurs, le traitement a induit des changements significatifs dans la composition des communautés bactériennes et eucaryotes, augmentant notamment la richesse des taxons, mais réduisant leur répartition en raison de l'émergence de taxons peu abondants. Les analyses de diversité ont aussi révélé des groupes distincts spécifiques à chaque phase de traitement, avec une hétérogénéité spatiale marquée entre les points d'utilisation et ceux sur le système d'eau chaude. De plus, la persistance de souches pathogènes (probables) appartenant aux genre *Legionella* et *Mycobacterium* souligne l'importance de réaliser des évaluations compréhensives des risques pour orienter les efforts de mitigation. Dans l'ensemble, cette étude fournit des données exploitables pour optimiser la mise en œuvre pratique des systèmes de génération de monochloramine sur site dans les bâtiments accueillant des populations vulnérables.

ABSTRACT

Drinking water is recognized as a significant source of waterborne infections when opportunistic pathogenic bacteria are present, such as *Legionella pneumophila* (*Lp*), *Pseudomonas aeruginosa* (*Pa*), and nontuberculous mycobacteria (NTMs). These bacteria pose major public health concerns when they proliferate in water systems, particularly within biofilms that adhere to the internal surfaces of plumbing pipes and fixtures. Unlike frank pathogens, these opportunistic bacteria do not cause severe diseases in healthy individuals, but they can lead to acute, sometimes fatal, infections in immunocompromised populations, the elderly, and those with chronic illnesses.

- ***Legionella pneumophila*** is responsible for the majority of Legionnaires' disease cases in Europe/North America, a severe form of pneumonia-like infection transmitted by inhaling contaminated aerosols. Although most cases are community-acquired or travel-related, nosocomial infections impose a disproportionate health burden due to their severity and the vulnerability of the populations involved.
- ***Pseudomonas aeruginosa*** causes numerous hospital-acquired infections, especially among burn victims and cystic fibrosis patients. A recent European study found that 31% of strains were resistant to at least one group of antibiotics, therefore complicating treatment efforts.
- **Nontuberculous mycobacteria** are a diverse group that can cause chronic pulmonary diseases, as well as skin and soft tissue infections, with an alarmingly increasing incidence rate globally. They are also linked to the highest number of investigations into water-related nosocomial infections in the United States.

Given these concerning trends, managing water systems in large buildings has become a complex, but crucial challenge. These systems feature heterogeneous conditions of operation, design, and usage, thus creating environments conducive to the growth and dispersal of microorganisms. Despite these challenges, regulations and practices specific to managing building water systems remain limited or inconsistent.

The primary goal of this research project is to provide evidence-based recommendations to improve the monitoring, operation, and design of plumbing systems in large buildings. By combining novel tools, this research aims to study the occurrence of opportunistic pathogens (OPs) (Lp, Pa, NTMs) and assess the effectiveness of preventative and corrective measures designed to reduce users'

exposure risks. This thesis also seeks to inform building managers of their duty of diligence by suggesting practical solutions for proactive management of their buildings' water systems.

The first part of this research project laid the groundwork for understanding challenges associated with secondary disinfection in large buildings. Gathering 1,737 samples from nine institutional buildings (hospitals, universities, schools, sports center), a meta-analysis was conducted to determine whether building managers can rely on municipal drinking water system's free chlorine residuals to prevent microbial proliferation. The results showed that free chlorine concentrations in first draws reached the guideline level of 0.2 mg/L in 26%, 6%, and 2% of cold, tepid, and hot water samples, respectively. Flushing for two to 60 minutes only significantly increased this ratio in cold water (83%), but did not systematically allow to reach levels measured at the building's point of entry. Free chlorine was weakly correlated ($R < 0.2$) with adenosine triphosphate (ATP), heterotrophic plate count (HPC), and total and viable flow cytometry counts, demonstrating that the residual nonetheless contributed to reduce viable and cultivable bacterial biomass. The detection of culturable *Lp*, spanning over four logs, only occurred when free chlorine was below 0.2 mg/L, yet no clear trend was distinguished for culturable *Pa*. Water temperatures below 20 ˚C and above 60 ˚C completely inhibited the presence of *Lp*. In general, the majority of parameters showed high microbial concentrations at distal points of use as well as in tepid and hot water, where free chlorine is quickly dissipated by stagnation and elevated temperatures.

In the second part of this thesis, the effectiveness of recommissioning flushing, a measure widely suggested for the safe reopening of vacant buildings during the COVID-19 pandemic, was evaluated at 23 showerheads in a large sports center. After a 16-week closure, the highest mean concentrations of general microbial indicators (ATP, total/intact cell counts) were measured in first draw samples, while *Lp* was detected at 81% and 90% of samples by culture and quantitative PCR (qPCR), respectively. Recommissioning flushing was beneficial short-term (24h) for reducing risks associated with *Lp* by lowering concentrations below the common alert threshold of 1,000 MPN/L in public settings. However, following a one-month period without shower use, significant rebounds of general microbial indicator concentrations and *Lp* levels similar to those measured after the prolonged closure were observed. These results highlight the temporary benefits of recommissioning flushing on the prevalence of *Lp* at devices generating large amounts of aerosols.

The third part of the research project focused on corrective measures commonly applied to manage *Lp* contamination in building plumbing systems, namely shock chlorination and recommissioning flushing. In this study, the short-term impact (three weeks) of shock chlorination (20 – 25 mg/L of free chlorine for 16h) and flushing (5-min flush at each showerhead), both combined with different regimes of preventative flushing (daily, weekly, none), was evaluated in duplicate of showerheads in the same previous sports center. The results demonstrated that when no preventative flushing (no water use) followed shock chlorination, significant microbial regrowth was favored. Conversely, recommissioning flushing followed by a period of distal stagnation also led to complete regrowth, and even, at times, higher levels of culturable and qPCR *Lp*. Regardless of the intervention applied, showerheads flushed on a daily basis exhibited significantly lower concentrations of ATP and total/intact cell counts, as well as *Lp*, than those flushed each week. Invariably of the flushing regime implemented, shock chlorination suppressed *Lp* cultivability (3-log reduction) for two weeks before small rebounds were observed, while recommissioning flushing only had a minimal impact on the abundance of *Lp*. This study offers insights into most optimal short-term combinations of corrective and preventative strategies that could be considered pending the implementation of more appropriate operational controls or building-wide treatments.

Finally, the *in situ* dosing of monochloramines into the hot water system of a large hospital was implemented as a building-wide alternative to control *Lp* in response to the persistence of *Lp* in the hot water system despite the establishment of a thermal control regime (> 55 ˚C) and repeated heat shock interventions. More specifically, this latest study examined the longitudinal and transversal impacts of *in situ* monochloramine on the mitigation of numerous pathogens and hosts. A total of 544 water samples were collected from 22 distal points of use (handwashing stations, hot water taps, showerheads), selected based on patient risks, and from 10 representative points in the hospital's hot water distribution system (hot water return loops, hot water supply, remote points of use), both before and after the introduction of monochloramine with targeted concentrations ranging 1.5 to 3.5 mg/L. Monochloramine rapidly eliminated the reservoir of *Lp*, with reductions of up to 3-log in culture (< 24h) and 2-log by qPCR (< four weeks). Concentrations of *Vermamoeba vermiformis* (*Vv*), a predominant host for *Legionella* species and NTMs, decreased by 2-log within 24 hours before stabilizing. In contrast, NTMs showed a clear persistence, with average concentrations ranging between 10^4 and 10^6 gc/L, despite an initial reduction of about 1-log and 2-log at distal and system sites in the first two weeks, respectively. The greatest reductions of these

microorganisms were observed for monochloramine concentrations of 2 to 3 mg/L and when temperatures were above 55 ˚C. However, the interruption of dosage led to rapid rebounds of *Legionella* species, NTMs and *Vv*, thus demonstrating their persistence in biofilms. Moreover, the treatment induced significant changes in the composition of bacterial and eukaryotic communities, notably increasing taxa richness, but reducing their evenness, likely due to the emergence of less abundant taxa. Diversity analyses also revealed distinct groups specific to each treatment phase, with marked spatial heterogeneity between distal points of use, as well as those collected across the hot water distribution system. Additionally, the persistence of probable clinically relevant strains belonging to the genera *Legionella* and *Mycobacterium* underscores the importance of conducting comprehensive risk assessments to guide mitigation efforts. Overall, this study provides actionable data to optimize the practical implementation of onsite monochloramine generation systems in buildings hosting vulnerable populations.

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LIST OF SYMBOLS AND ABBREVIATIONS

List of acronyms and abbreviations presents, in alphabetical order, the acronyms and abbreviations used in the thesis and their meaning.

˚C	Celsius degree
ASV	Amplicon sequence variants
ATP	Adenosine triphosphate (English), Adénosine triphosphate (Français)
BHAA	Bactéries hétérotrophes aérobies et anaérobies facultatives
BLAST	Basic local alignment search tool
CEAEQ	Centre d'expertise en analyse environnementale du Québec
CFU	Colony-forming unit
CIEP	Chaire industrielle en eau potable de Polytechnique Montréal
Cl_2	Free chlorine
CREDEAU	Centre for research, development and validation of water treatment technologies and processes
Cu	Copper
DALY	Disability-adjusted life year
DBPs	Disinfectant by-products
DNA	Deoxyribonucleic acid
DWDSs	Drinking water distribution systems
DWPIs	Drinking water-associated pathogens causing opportunist infections
EPANET-MSX	Environmental protection agency network evaluation tool multi-species extension
eq.	Equivalent
FC	Flow cytometry
Fe	Iron

gc	Gene copy
gu	Genomic unit
h	Hour
HCFs	Healthcare facilities
HDPE	High-density polyethylene
HPC	Heterotrophic bacteria count
ICC	Intact cell counts
ICU	Intensive care unit
IPC	Infection and prevention control
L	Liter
LD	Legionnaires' disease
LoD	Limit of detection
log	Logarithmic
LoQ	Limit of quantification
Lp	*Legionella pneumophila*
*L*spp	*Legionella* species
MAB	*Mycobacterium abscessus* complex
MAC	*Mycobacterium avium* complex
mg	Milligram
min	Minute
mL	Milliliter
Mn	Manganese
MNT	Mycobactéries non tuberculeuses
MO	Month

MPN	Most probable number
n	Number of samples/buildings/or else
NCBI	National center for biotechnology information
NH_2Cl	Monochloramine
NICU	Neonatal intensive care unit
NSERC	Natural sciences and engineering research council of Canada
NTMs	Nontuberculous mycobacteria
OPs	Opportunistic pathogens
OTU	Operational taxonomic unit
p	p-value or significance level
Pb	Lead
PCR	Polymerase chain reaction
PES	Polyethersulfone
PoE	Building point of entry
PoU	Building point of use
Pa	*Pseudomonas aeruginosa*
PCoA	Principal coordinates analysis
pg	picogram
PR2	Protist ribosomal reference database
QMRA	Quantitative microbial risk assessment
qPCR	Quantitative polymerase chain reaction
R	Correlation coefficient
RF	Regrowth factor
RNA	Ribonucleic acid

rRNA	Ribosomal ribonucleic acid
s	Second
SBT	Sequence-based typing
sg	Serogroup
SILVA	rRNA database derived from the Latin word forest
spp.	Species
S/V	Surface-to-volume ratio
T	Temperature
TCC	Total cell counts
TMV	Thermostatic mixing valve
TOC	Total organic carbon
µg	Microgram
µL	Microliter
µS/cm	MicroSiemens per centimeter
VBNC	Viable but not culturable
vPCR	Viability PCR
Vv	*Vermamoeba vermiformis*
WE	Week
WSP	Water safety plan

LIST OF APPENDICES

CHAPTER 1 INTRODUCTION

1.1 Background

Water is recognized as an important source of infections since the British physician John Snow demonstrated that a cholera outbreak in London (1854), caused by the bacterium *Vibrio cholerae*, was traced back to a contaminated water pump (Paneth et al., 1998). Historically, public health efforts in water safety focused on controlling bacterial pathogens like cholera and typhoid, which were ultimately eliminated with improved water treatment and sanitation practices, at least in high-income countries (Ashbolt, 2015). Nowadays, controlling enteric protozoa such as *Giardia* and *Cryptosporidium* remains a critical component of modern water treatment systems, but their reduced incidence rates have shifted the focus to a newer public health concern with very unique challenges: OPs associated with drinking water biofilms (Collier et al., 2021). Unlike frank pathogens, opportunistic waterborne microorganisms are not generally harmful to healthy individuals, but can cause severe infections in people with weakened immune systems, the elderly, and those affected with chronic illnesses. Ensuring the safe distribution of drinking water has currently become a pivotal public health priority for several key reasons, including protecting the most vulnerable populations and reducing the economic burden of healthcare costs arising from waterborne infections (Collier et al., 2021). Most importantly, safe drinking water is a fundamental human right, aligning with the global Goal six of the United Nations sustainable development goals (2015 – 2030), which aims to achieve universal access to safe drinking water, among other water-related objectives (UN, 2015). Several OPs, including *Legionella pneumophila*, NTMs, and *Pseudomonas aeruginosa*, have become noteworthy public health hazards due to their ability to thrive in building plumbing systems, and most particularly in biofilms adhering to the inner surfaces of pipes and fixtures. They each present singular health risks and are associated to most hospitalizations and deaths from drinking water exposure (Gerdes et al., 2023).

Legionella pneumophila is responsible for most cases of Legionnaires' disease (LD) worldwide, an acute form of pneumonia, and Pontiac fever, a less severe flu-like illness. It predominantly affects older adults, smokers, and individuals with chronic lung diseases or impaired immune systems through the inhalation of contaminated water aerosols (< 10 μm dia.) (Fields et al., 2002). Between 2008 and 2017, nearly 8% of reported cases of LD in Europe were attributable to hospital-acquired infections (nosocomial), the vast majority (98.1%) of which were caused by the specie

Legionella pneumophila. Although community-acquired cases (71%) and travel-associated cases (20%) accounted for most LD cases in Europe, the case-fatality ratio was significantly higher in healthcare facilities (29%) than in the community (9%) (ECDC, 2023). In a CDC report from the United States for the year of 2015, 20% of LD cases confirmed by culture were imputable to nosocomial cases, with mortality rates similar to European data, amounting to 25% for definitive cases and 10% for probable cases (Soda et al., 2017). Furthermore, a fivefold increase in the incidence rate of LD was observed in the population from 1992 – 2002 (0.48 cases/100,000) to 2018 (2.71 cases/100,000) (Barskey et al., 2022).

Pseudomonas aeruginosa causes a variety of infections, including skin, urinary tract, blood, and respiratory infections depending on the exposure route. It is particularly notorious for causing chronic lung infections in cystic fibrosis patients and severe infections in burn victims (Gellatly et Hancock, 2013). *Pseudomonas aeruginosa* mostly affects individuals with compromised immune systems, such as cancer patients, transplant recipients, those with chronic illnesses or invasive devices, and neonates (Bédard et al., 2016a). In the United States, *Pseudomonas aeruginosa* is the sixth most common cause of nosocomial infections, representing about 7% of all hospital-acquired infections (not only waterborne) (Magill et al., 2014). The incidence of *Pseudomonas aeruginosa* infections remains high, partly because of its resistance to multiple antibiotic thus complicating treatment efforts. Indeed, a European study on the prevalence of infections among intensive care patients in 2017 demonstrated that nearly 16% of patients had developed an infection to the bacteria, and 3% of them were infected with a strain resistant to beta-lactams (Vincent et al., 2020). Correspondingly, data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) showed that 31% of isolates reported by EU and EEA countries were resistant to at least one monitored antibiotic group (ECDC and WHO, 2023).

NTMs are a diverse group of environmental mycobacterial species that can cause pulmonary diseases, as well as skin and soft tissue infections. Similarly to the other two OPs, NTMs take advantage of the vulnerability of someone's immune system to cause an infection (Dowdell et al., 2019). The annual incidence rate of NTMs is progressively increasing worldwide (Dahl et al., 2022). For example, from 2008 to 2015 in the United States, a large healthcare program revealed that the prevalence of pulmonary infections by NTMs had jumped from 6.78 to 11.6 cases per 100,000 persons per year (Winthrop et al., 2020). According to a recent CDC's report on consultations related to nosocomial waterborne infections, investigations involving NTMs

accounted for the largest number of investigations between 2014 and 2017 (about 30%) (Perkins et al., 2019). Generally, data on hospital-acquired infections by this group of bacteria remain limited (non-notifiable in the United States and in Europe), but outbreaks and pseudo-outbreaks (apparent increase in cases) are not uncommon (Decker and Palmore, 2013).

Given the increasing disease burdens associated with *Legionella pneumophila*, *Pseudomonas aeruginosa*, and NTMs, the challenges, complexity, and scale of managing large building plumbing systems become even more critical to address. Large buildings have extensive plumbing networks with numerous pipes, storage tanks, water heaters, and fixtures. This overall complexity increases the difficulty of maintaining consistent water quality throughout the system up to consumer's point of use, in addition to ensuring adequate thermal regime and disinfectant residuals far and wide. These systems have large surface area for biofilms to grow, variable flow patterns and water temperatures, and a broad diversity of plumbing materials, creating various intricate environments where different microorganisms can both selectively grow and disperse (Cullom et al., 2020). Such heterogeneous conditions and levels of intricacy make it challenging to prevent and/or mitigate the colonization of OPs with a one-size-fits-all solution.

1.2 Structure of the dissertation

This thesis is structured into ten chapters, each focusing on crucial aspects of the occurrence and mitigation of OPs in large buildings.

Chapter 1: Introduction. This chapter sets the foundation for the thesis by discussing the importance of opportunistic drinking water-associated pathogens like *Legionella pneumophila*, *Pseudomonas aeruginosa*, and NTMs. The chapter also reviews the increasing incidence rates of these pathogens in the population and highlights the public health risks they pose.

Chapter 2: Literature review. This chapter presents a comprehensive review of existing research on the occurrence of these pathogens in large building plumbing systems. It covers monitoring strategies and factors that affect their growth. The chapter further discusses various mitigation strategies, including preventive interventions, corrective actions, and emergency measures, as well as challenges introduced by the prolonged building closures related to the COVID-19 pandemic lockdowns. A section about the context in the province of Québec explores the prevalence of waterborne infections in Québec's water systems, as well as the existing regulatory framework

aimed at managing the risks associated with the targeted opportunistic drinking water-associated pathogens. Finally, a critical analysis of the literature review is presented.

Chapter 3: Objectives and methodology. This chapter details the main objective, the specific objectives, and the research hypotheses of this thesis. It gives an overview of the physico-chemical and microbiological parameters used throughout this project, while the full methodologies of each study are provided in the subsequent chapters presenting the individual research articles.

Chapters 4 to 8: Peer-reviewed and submitted articles. These chapters feature the five scientific papers written during the course of this thesis. Chapter 4 presents the first research article, which investigates the effectiveness of maintaining free chlorine residuals in preventing microbial growth in nine large building plumbing systems (published in Science of the Total Environment). The second article is presented in Chapter 5 and focuses on the impact of recommissioning flushing as the first level of action to address stagnation issues that arose due to reduced building occupancy during the COVID-19 pandemic (published in Frontiers in Water). Chapter 6 presents the third article, which evaluates the effectiveness of combining remedial interventions, such as shock chlorination and recommissioning flushing, with different preventive flushing regimes (published in Microorganisms). The fourth article explores the use of onsite monochloramine disinfection as a strategy to control OPs in a hospital hot water system and is presented in Chapter 7 (submitted to Water Research). Finally, Chapter 8 presents the final research article of this dissertation, which investigates microbial community changes associated with the *in situ* dosing of monochloramine in the hospital hot water system (submitted to Science of the Total Environment).

Chapter 9: General discussion. In this chapter, the overall findings from the research articles are synthetized and discussed. The general discussion addresses the broader implications of the studies in the context of water safety planning. It also reflects on the limitations of the current research and provides critical analysis linking the findings to existing knowledge.

Chapter 10: Recommendations and conclusions. The final chapter draws key conclusions regarding the findings on the occurrence and mitigation of these OPs in different large buildings. The chapter also provides recommendations to improve water management practices in large buildings and suggests areas for future research.

CHAPTER 2 LITERATURE REVIEW

Although each published or submitted paper within this thesis (Chapters 4 to 8) presents a literature review tailored specifically to its content, a broader range of references are provided in Chapter 2 to enrich understanding and shape the research hypotheses and objectives presented further on.

2.1 Opportunistic drinking water-associated pathogens

Despite the multi-barrier approach designed to ensure the safe distribution of drinking water from its source intake through its transportation across distribution systems and consumers taps, many opportunistic drinking water-associated pathogens have adapted to withstand current treatments and persist within engineering water systems, such as building plumbing. Their ability to capitalize on weakened immune systems makes them particularly harmful to at-risk (vulnerable) populations, including the elderly, infants, or any individuals with compromised immunity and chronic illnesses (Falkinham III, 2015).

2.1.1 *Legionella*

Description. *Legionella* is a genus of Gram-negative, aerobic bacteria comprising over 61 species among which half have been associated with human infections. The predominant *Legionella* species responsible for LD exhibit clear geographic and habitat variations. *Legionella pneumophila* is most frequently isolated in patients exposed to contaminated water systems in North America and Europe, whereas *Legionella* longbeachae, typically found in potting soils, is more commonly associated to community-acquired LD cases in Oceania and some parts of Asia (NASEM, 2019). Other *Legionella* species known to cause infections in susceptible individuals essentially include *Legionella anisa*, *Legionella micdadei*, *Legionella bozemanii*, *Legionella dumoffi*, *Legionella feeleii*, and suchlike (Muder and Yu, 2002). *Legionella* bacteria utilize an aerobic metabolic pathway, requiring oxygen for energy production, along with specific nutrients such as ferric iron and amino acids, which serve as their primary sources of carbon and energy. Notably, most species rely on L-cysteine (amino acid) for growth, unless they acquire it from host organisms during intracellular replication (Ewann and Hoffman, 2006). In regard to *Legionella pneumophila*, these specific nutritional requirements are closely linked to its parasitic lifestyle, as the bacterium primarily replicates within protozoan cells. Indeed, during its life cycle, *Legionella pneumophila* transitions from a transmissive form (virulent) to infect host cells to a replicative one (metabolically

active) once internalized to exploit the nutrient-rich environment within the eukaryotic cell. After replication, the bacterium transitions back to its transmissive form to evade the host cell and disseminate into the surrounding environment (Oliva et al., 2018). This intricate process of infection, internalization, replication, and egress from the host cell is strongly related to *Legionella pneumophila*'s pathogenicity, which is modulated by a vast array of effector proteins enhancing its ability to infect, manipulate host cell processes, and multiply upon invasion (Barbosa et al., 2024).

Ecology. The ecology of *Legionella* is intricately tied to protozoa, whose habitat is the biofilm. Most *Legionella* species are mesophilic, thus thriving at temperatures between 25 and 45 ˚C, but survival is possible outside of this range (NASEM, 2019). In fact, *Legionella pneumophila* has been repeatedly detected from cold water taps (Donohue et al., 2014; Donohue et al., 2018; Li et al., 2018a; Buse et al., 2020) and hot water distribution systems and points of use (Borella et al., 2004; Leoni et al., 2005; Bukh et Roslev, 2014; De Filippis et al., 2018; Hayes-Phillips et al., 2019) in building water systems (offices, commercial buildings, hospitals, hotels, retirement homes, day care centres, houses). A literature review conducted by van Heijnsbergen and colleagues (2015) identified several water systems as confirmed reservoirs of *Legionella*, with the highest level of evidence based on matching clinical and environmental *Legionella pneumophila* strains. These environmental sources that caused LD cases included baths, fountains, wastewater treatment plants, room humidifiers, ice machines, mist machines, thermal springs, natural soils, milling machine, ship water pump, and foot bath. Although showers and taps were excluded from this review, these reservoirs were identified as sources of definite community-acquired LD cases in a more recent review by Orkis and colleagues (2018).

2.1.2 *Pseudomonas aeruginosa*

Description. *Pseudomonas aeruginosa* is a Gram-negative bacterium possessing a single flagellum, which provides motility. It has a flexible glycolytic pathway for glucose degradation, utilizing either oxygen or nitrogen as the final electron acceptor under aerobic or anaerobic conditions, respectively (de Sousa et al., 2021). The organism can feed on a variety of organic compounds as sources of carbon and energy, including sugars, fatty acids, and amino acids, and it can also survive in oligotrophic (i.e., low-nutrient) environments (Bédard et al., 2016a). The pathogenicity of *Pseudomonas aeruginosa* bacteria stems from a wide array of virulence factors associated to both cellular (e.g., flagellum, biofilm formation, lipopolysaccharides, pili) and extracellular processes

(e.g., production of exotoxins, cytotoxins, proteases, hemolysins, etc.). Most of these extracellular virulence factors, as well as its ability to produce extracellular polymeric substances required for biofilm formation, are governed by quorum sensing, a cell-to-cell communication mechanism in which bacteria exchange information about population density for survival purposes or metabolic resources management (Strateva et Mitov, 2011).

Ecology. *Pseudomonas aeruginosa* thrives best around 37 ˚C, although it can survive in water temperatures ranging from 4 to 42 ˚C. However, some of its virulence pathways are affected when temperatures fall below 30 ˚C (LaBauve et Wargo, 2015). Biofilm formation is a fundamental ecological strategy for this OP, as it enhances its propensity to persist and proliferate in both engineered water systems and the human body. Indeed, building plumbing and fixtures are notable reservoirs for *Pseudomonas aeruginosa* as they provide extensive surface areas and diverse materials supporting biofilm colonization and formation by the bacterium. Occurrence of *Pseudomonas aeruginosa* has been confirmed in a myriad of hospital-associated components, such as faucets, showers, baths, sinks, drains, medical equipment (e.g., endoscopes, respiratory devices), humidifiers, and hydrotherapy pools (Bédard et al., 2016a). The microorganism is also frequently isolated from recreational settings like hot tubs, Jacuzzis, and swimming pools (Caskey et al., 2018), which typically operate within the bacterium's preferred temperature for growth.

2.1.3 Nontuberculous mycobacteria

Description. There are over 190 species NTMs, which are environmental mycobacteria that do not cause tuberculosis or leprosy. Most clinically relevant species include the *Mycobacterium avium* complex (MAC), the *Mycobacterium abscessus* complex (MAB), the Mycobacterium fortuitum complex, the *Mycobacterium mucogenicum* complex, *Mycobacterium xenopi*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium chelonae*, among others with fewer cases (CDC, 2024). NTMs are acid-fast bacteria, which refers to the high proportion of mycolic acids (long chain fatty acid) in their cell walls. This key morphological characteristic renders them resistant to antimicrobial agents, difficult to identify through the Gram staining method, and highly hydrophobic, as the rich lipid layer reduces cell permeability (Pereira et al., 2020). These aerobic, sometimes microaerophilic (i.e., requiring low oxygen levels) bacteria are typically classified into two groups depending on their culturable growth rate: slow-growing organisms taking weeks to grow into colonies (e.g., MAC) and fast-growing species (e.g., MAB) forming colonies within days

(Gupta et al., 2018). NTMs are considered oligotrophic microorganisms, capable of growing in environments with low carbon levels. Similar to *Legionella*, certain NTMs exhibit resistance to ameba-mediated phagocytosis, allowing them to persist and potentially replicate intracellularly within protozoan hosts (Greub et Raoult, 2004).

Ecology. This group of environmental mycobacterial species are widely distributed across different ecological niches, particularly in natural aquatic environments, engineered water systems and soils (Falkinham III, 2009). They are remarkably adaptable to a broad range of temperatures, which support their ubiquitous presence in diverse ecosystems and settings. While individual species have specific temperatures that they thrive in, NTMs generally grow optimally at warm (25 to 45 ˚C) temperatures (Pereira et al., 2020). For example, species like *Mycobacterium fortuitum*, *Mycobacterium gordonae*, and *Mycobacterium mucogenicum* were identified in pseudo-outbreaks associated with ice machines (cold water) (Millar and Moore, 2020), whereas species like *Mycobacterium avium* are frequently isolated from hot water systems (hot water) (Li et al., 2017). The environmental sources in which these organisms are found determines the route of exposure, which ultimately affects the type of infection. NTMs causing pulmonary infections are commonly linked to contaminated water aerosols from faucets and showerheads, while those responsible for hypersensitivity pneumonitis (i.e., a type of allergic reaction leading to lung inflammation) are frequently traced to exposure in recreational settings, including hot tubs, spa baths, and swimming pools (Honda et al., 2018). Skin infections have been associated to community exposures, such as whirlpool baths in nail salons and contaminated water in tattoo ink (Honda et al., 2018). In contrast, soft tissues and post-surgical sites infections are mostly acquired in hospitals, typically resulting from inadequate sterilization or cleaning of medical equipment and tubing (Li et al., 2017). Finally, bloodstream infections due to NTMs have been associated to dialysis machines, heater-cooler devices in cardiac surgery, and tap water from faucets and showers in hematology-oncology units (Li et al., 2017). The prevalence of these bacteria in building plumbing systems and their components is largely attributed to their ability to assemble into biofilms and adhere to various moist surfaces, a result of their hydrophobic nature, which serves as a major ecological strategy for their enhanced persistence (Falkinham III, 2009).

2.1.4 OPs monitoring strategies

Monitoring OPs in building plumbing systems is crucial for maintaining water safety, protecting public health, preventing outbreaks, and evaluate the effectiveness of corrective measures.

Sampling methodologies. Assessing OPs involves careful considerations regarding sample collection methods and site selection. In the context of routine monitoring, sites representative of different parts of the building plumbing system (cold and hot water systems) are chosen, most often focusing on areas at increased risk of OPs growth (e.g., low-use, dead-ends, warmer temperatures, etc.), exposure (e.g., high potential for generating water aerosols), or based on the presence of vulnerable people or previous positive contamination issue (Wang et al., 2017).

Furthermore, OPs can be present in the bulk water and biofilm phases, as well as in particular niches at specific points of use (distal contamination), or throughout the building plumbing system (systemic contamination). Water and biofilm samples capture microbial communities which are not consistently representative of one another, sharing only a variable portion of the same core taxa as reported in studies conducted in large-scale drinking water distribution systems (Liu, 2014; Krishna et al., 2021; Ren et al., 2024), buildings (Huang et al., 2021; Cavallaro et al., 2024), or experimental setups (Douterelo et al., 2013; Ji et al., 2017). In fact, biofilm samples provide a direct view of microorganisms adhering to inner pipes and fixtures, but due to the heterogeneity of biofilms (Wimpenny et al., 2000), they exclusively picture the microbial diversity within the swabbed surface. Contrastingly, water samples collect free-floating (planktonic) microorganisms and those that have detached from biofilms with flow induced during sample collection, thereby portraying solely a snapshot of microbial communities.

Finally, considering the complex architecture and varying flow patterns found in buildings (Proctor et al., 2020), sampling timing and volume will capture how water quality varies across a plumbing system. First draw samples (i.e., samples collected immediately after a tap is opened), also referred to as distal samples, correspond to what the user is exposed and are typically associated to increased likelihood of OPs detection. Flushed or semi-flushed samples (i.e., samples collected after running water) are indicative of the upstream system water quality (Wang et al., 2017; Bédard et al., 2018). Both type of samples yield complementary insights into water quality, allowing for a more comprehensive assessment of the occurrence of OPs, but remain contingent to monitoring purposes. First draw samples are collected from distal sites (i.e., distal sections of building

plumbing, hence points of use) whereas flushed samples can act as control points and are typically collected from sites across the cold and hot water distribution systems (i.e., point of entry, risers, hot water recirculation lines, or after a certain flush at points of use) (Wang et al., 2017).

Detection methods. Screening for the occurrence of OPs requires reliable and accurate analytical methods. Methods vary in terms of sensitivity, specificity, and speed of execution, and are selected based on the monitoring objective (e.g., outbreak investigation, routine surveillance, mitigation assessment, research) and available technical, economical and human resources.

Culture-based methods are traditionally regarded as the gold standard for identifying, quantifying, and typing OPs. They produce culturable, viable counts that are used to set thresholds for regulatory compliance. However, most bacteria can transition to a viable, but non culturable state (VBNC) under stressful conditions, making them undetectable by culture (Li et al., 2014). Although requiring long incubation times delaying response during outbreak investigations or interventions, culture methods allow for identifying and typing strains, which is essential for matching clinical and environmental isolates and confirming the outbreak source (Wang et al., 2017). Molecular methods like qPCR are widely used for the rapid detection and quantification of OPs. These methods target specific DNA sequences unique to the pathogen of interest, allowing for highly sensitive and specific detection, even at low concentrations (Girones et al., 2010). PCR-based methods are high-throughput and fast, thus relevant for quickly identifying anomalies or sites to focus culturing as a broad outbreak response (Lu et al., 2016). However, traditional qPCR does not differentiate between DNA from live and dead cells, which can lead to overestimations of risks (Ditommaso et al., 2015). Therefore, viability PCR (vPCR) addresses this limitations by selectively suppressing the amplification of DNA from dead cells, ensuring that only DNA from viable or VBNC cells is further amplified (Delgado-Viscogliosi et al., 2009).

Other rapid and cost-effective methods to assess the potential presence of OPs in building plumbing systems include the use of fast and/or cost-effective general microbial parameters (e.g., flow cytometry counts, ATP, HPC). These parameters provide insights into overall water quality, microbial activity, and water systems conditions that may favor OPs growth, making them suitable for routine surveillance and early warning systems. Flow cytometry is an optical-based technique that involves labelling cells with fluorescent markers that bind to specific cell components, such as nucleic acids, allowing quantification and differentiation between live and damaged cells (Safford

et Bischel, 2019). ATP is indicative of microbial activity as it measures this molecule which is only present in living cells (Prest et al., 2016), and has been demonstrated to correlate well with viable cells measured through flow cytometry assays (Hammes et al., 2010; Nescerecka et al., 2014). Then, HPC is a culture-based technique that quantifies the number of heterotrophic bacteria colonies in water, yet has a limited scope of detection since it captures only the cultivable fraction (< 1% of the total biomass) (Van Nevel et al., 2017). However, these surrogate parameters are most often lacking good correlation with OPs abundance (Duda et al., 2015; Arroyo et al., 2017).

2.1.5 Factors influencing the occurrence of OPs

Factors influencing the occurrence of OPs in large buildings are multifaceted, involving physical, chemical, and biological aspects. Unlike traditional water quality management that focuses primarily on the treatment at the plant, controlling OPs requires a detailed understanding of how internal system dynamics affect pathogen behavior. To effectively manage risks, it is necessary to address the underlying factors that control pathogen persistence and decay, as well as overall microbial growth. This section briefly explores each of these factors – water temperature (Section 2.2.2), plumbing design and materials, building age, hydraulic regime, disinfectant residuals (Section 2.2.2), incoming water chemistry, in-building water treatment (Section 2.2.3), water usage patterns, biofilms – to elucidate their roles in influencing pathogen dynamics (Figure 2.1).

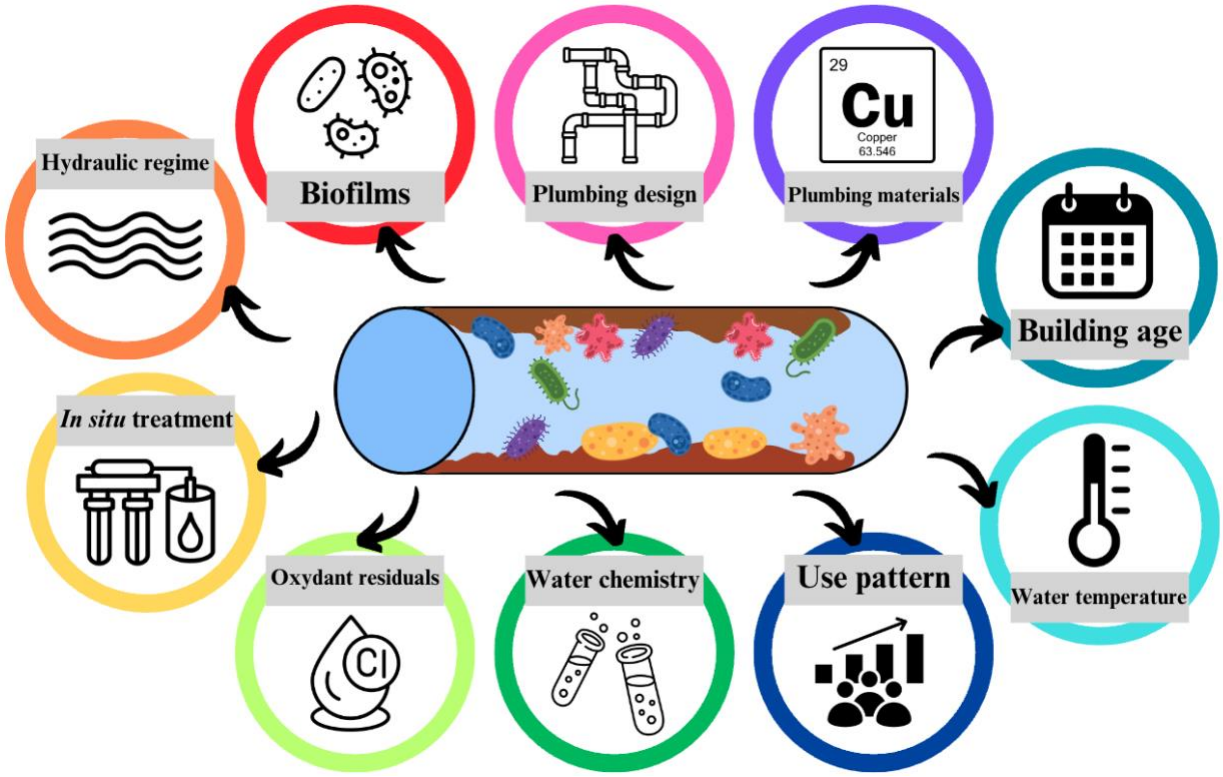

Figure 2.1 Overview of factors influencing the dynamics of OPs in buildings.

Plumbing design and materials. Specific plumbing design aspects, including the surface area to volume ratio of pipes, the complexity of legacy building plumbing, and other design features, create conditions that favor biofilm formation and OPs colonization. Large buildings are built as tree-like structures in which increasing surface to volume ratios (S/V) are found as the pipe diameter narrows to points of use. Distal sections with larger S/V ratios tend to support greater culturable cell counts due to enhanced biofilm development (Bédard et al., 2018), release more assimilable organic carbon from plastic materials (Bucheli-Witschel et al., 2012), and cause more pronounced changes in disinfectant profiles due to wall decay over increasing stagnation times (Ling et al., 2018). Sections of hot water pipes that experience cooling, or conversely sections of cold water pipes that experience heating, can provide ideal temperatures for pathogens to grow, particularly in systems not properly balanced. The presence of water storage tanks (Li et al., 2018) and electric water heaters (Cazals et al., 2022) create zones of thermal stratification leading to gradients in temperatures that may favor OPs growth, especially in oversized or poorly maintained systems.

Moreover, the diversity of points of use – such as faucets, showerheads, decorative fountains, ice machines, humidifiers, among many others – varies in terms of S/V ratios, materials, potential of

aerosolization, and flow characteristics, which are all particularities influencing pathogen fate or the risk of exposure through inhalation. Fixtures have complex inner geometries and plumbing materials supporting biofilm development. For example, data-based evidence demonstrated that higher prevalence of *Legionella* spp. (Sydnor et al., 2015) and *Pseudomonas aeruginosa* (Charron et al., 2012) was measured in electronically activated faucets compared to conventional ones, due to the complexity of their internal architecture and multiplicity of materials. In the context of indoor water use, risk-based critical culturable concentrations of *Legionella pneumophila* were two orders of magnitude lower for showerheads than for faucets, as showerheads generate a greater quantity of water droplets (Hamilton et al., 2019).

Piping and plumbing component materials significantly affect the composition of microbial communities (Ji et al., 2015; Proctor et al., 2016; Waines et al., 2011; Yu et al., 2010), as well as the structure and formation rat of adhered biofilms (Biedroń et al., 2017). These materials can also influence the decay rates of disinfectant residuals (Zhang et Edwards, 2009). Additionally, different materials exhibit varying leaching potentials, which can impact microbial growth (Proctor et al., 2016), and create diverse nutrient profiles that drive unique microbial catabolism (Biedroń et al., 2017). However, the impact of pipe materials if often modulated by water temperature and water chemistry, which both govern nutrient migration and other reactions (Proctor et al., 2017).

Building age. Older buildings often have aging, complex plumbing systems, and outdated plumbing materials. Large legacy buildings often feature highly intricate plumbing networks with extensive pipe runs, dead legs (i.e., sections of piping connected to points of use that are rarely used), and blind ends (i.e., sections of piping closed off at their rear end, from prior plumbing modifications or repurposing). These design particularities produce areas of low flow or stagnation, which provide ideal conditions for pathogens to settle and biofilm to develop, as these zones are infrequently disturbed by water movement, and impair the effectiveness of disinfection strategies. Renovations in these buildings can unintentionally introduce new complexities, such as additional dead legs and blind ends, thus disrupting established water flow patterns and creating new conditions even more conducive to biofilm growth (Scanlon et al., 2020). Older buildings have been associated to a greater probability of detecting a positive sample to *Legionella* in healthcare facilities (Gamage et al., 2022) and higher prevalence and abundance of culturable *Legionella* species (Barna et al., 2016). Considering offices and residences, Donohue and colleagues (2018) found no clear relationship between the age of the building and the occurrence of molecular

Legionella pneumophila and *Mycobacterium avium*, although there was a tendency for older buildings to support more persistent colonisation of these OPs. On the opposite, newer constructions and green-certified buildings may incorporate low-flow fixtures aimed at achieving water conservation goals, thus inadvertently contributing to microbial growth (Rhoads et al., 2015a). These low-flow fixtures are increasingly seen as problematic from a water quality perspective (Julien et al., 2020; Singh et al., 2020).

Hydraulic regime. The hydraulic regime in building plumbing systems is defined by flow velocity, turbulence, pressure changes, and water use patterns. It shapes biofilm structure by influencing shear forces that can compact or disrupt biofilm layers. Biofilms formed under turbulent or highly variable flow conditions exhibited increased formation rates (Liu et al., 2006; Simões et al., 2006) and enhanced microbial diversity, at least in municipal distribution systems (Douterelo et al., 2013; Fish et al., 2017), likely due to the continuous renewal of nutrients and microorganisms in the flowing water. Although these biofilms often have greater biomass, their more compact structure is associated with increased resistance to shear stress and detachment (Vieira et Melo, 1999). In contrast, biofilms grown under laminar flow tend to be rougher and characterized by interstitial voids between surface asperities (Stoodley et al., 1999), thereby creating ecological niches that may protect pathogens from detachment. Additionally, low hydraulic regimes (< 2h per month) in different hospital water systems were associated with significantly higher concentrations of qPCR *Legionella pneumophila* (Nisar et al., 2023) and culturable *Legionella* spp. (Totaro et al., 2018), the latter when combined with supplemental chlorine dioxide.

Incoming water chemistry. The incoming water chemistry is shaped by the water source and treatment processes at the drinking water treatment plant. Factors such as nutrient availability and pH, among other chemical properties, can directly affect the microbial ecology within building plumbing. Growth-promoting nutrients readily available for microorganisms and present in the incoming water from municipal distribution systems include assimilable organic carbon and biodegradable dissolved organic carbon (Park et al., 2021). High levels of organic carbon can fuel biofilm development by serving as primary energy source for heterotrophic bacteria, including OPs, and can also react with disinfectant residuals, thus promoting oxidant decay (Prest et al., 2016). Additionally, pH can influence the stability and efficacy of disinfectants (Li et al., 2019), which in turn impacts microbial composition. In a study simulating building plumbing systems at a pilot scale, Ji and colleagues (2017) determined that the pH of incoming utility water was one of

the key chemical parameters contributing to most variations in microbial community composition. The switch of source water in Flint, Michigan, from a non-corrosive to a corrosive supply was further hypothesized as a probable factor that contributed to a large LD outbreak (Rhoads et al., 2017), stressing the importance of carefully managing water quality from source to points of use.

Water usage patterns. Water usage patterns directly influence inter-use stagnation events occurring in distal parts of building plumbing systems, as the frequency, timing, and intensity of water use determine how often water is refreshed within the pipes. Short- and long-term periods of stagnation are not uncommon in large buildings, ranging from intermittent stagnation due to diversified daily water usage patterns, to weekend stagnation, and holidays, or prolonged closure of buildings or sections of a facility. Stagnation events allow disinfectant residuals to dissipate and temperature to stabilize, all of which promoting microbial growth (Proctor et al., 2020). During stagnation, metals such as lead and copper leaching from metallic pipes can pose health risks (Zlatanović et al., 2017; Doré et al., 2019), while plastic pipes can also leach organic compounds that increase nutrient availability (Kelley et al., 2014). At the residential level, shorter stagnation times (< 24h) have been shown to increase ATP concentrations by 4- to 18-fold, intact cell counts by 1.5- to 3.2-fold, and HPC by 4- to 580-fold (Lautenschlager et al., 2010; Zlatanović et al., 2017). Similar trends have been demonstrated in larger buildings (Bédard et al., 2018). These findings suggest that short-term stagnation promotes microbial culturability and maintains microbial metabolic activity. In contrast, longer stagnation periods (three days to two weeks) often result in plateaued intact cell counts, likely due to scarce nutrient conditions arising from the lack of drinking water renewal from water use. Stagnant conditions also lead to substantial shifts in both bulk water bacterial (Lautenschlager et al., 2010; Ling et al., 2018) and eukaryotic communities (Wang et al., 2014). Generally, reductions in microbial richness and evenness were observed after overnight (Lautenschlager et al., 2010) and weekly stagnation in bulk water (Ling et al., 2018), possibly indicating that some microbial populations become more dominant during stagnation.

Biofilms. Biofilms play a crucial role in the occurrence and persistence of OPs. In engineered plumbing systems, including distribution networks and buildings, over 95% of the total biomass is estimated to reside within the biofilm (Flemming et al., 2002; Liu et al., 2014). Biofilms harbor complex, diverse microbial communities, each contributing to the biofilm's structure, function, and resilience, and interacting synergistically together. The biofilm matrix offers several advantages to embedded microorganisms compared to planktonic ones: (1) it sequesters nutrients due to their

sorption properties (Flemming et al., 2007), (2) it acts as a protective barrier against antimicrobial agents such as disinfectants (Bridier et al., 2011), (3) it facilitates quorum-sensing communication through inter-cell molecular signaling (Li et Tian, 2012), and (4) it supports protozoan grazing (Huws et al., 2004), which, in turn, enables the intracellular replication of *Legionella pneumophila* (Shaheen et al., 2019), and potentially of some species of NTMs (Thomas et McDonnell, 2007).

Legionella pneumophila exhibits a unique and highly adaptive relationship with protozoan hosts, such as amoeba belonging to the genera *Acanthamoeba*, *Vermamoeba*, and *Naegleria* among others (Wadowsky et al., 1991; Cavallaro et al., 2022), and ciliates (Paranjape et al., 2020). These protozoa act as environmental reservoirs and natural hosts for *Legionella pneumophila*, allowing the pathogen to replicate intracellularly in a protected environment that shields it from external harmful conditions while providing nutrients. After being phagocytosed and entering the host cell, *Legionella pneumophila* manipulates the protozoan's cellular machinery to create a specialized compartment, the *Legionella*-containing vacuole, where it can replicate and grow to high densities (Oliva et al., 2018). Similarly, NTMs are known to interact and survive within free-living amoeba (Thomas et McDonnell, 2007), although their relationship is less well understood and documented compared to *Legionella* bacteria. For example, *Mycobacterium avium* was observed to withstand phagocytosis by *Acanthamoeba castellanii*, and replicate intracellularly while gaining virulence (Cirillo et al., 1997). Finally, the propensity to detect *Pseudomonas aeruginosa* in building plumbing is closely related to its ability to form or join biofilms (Bédard et al., 2016a).

2.2 Mitigation of opportunistic drinking water-associated pathogens

Understanding the occurrence and ecological habitats of OPs is essential to mitigate their growth and preventing their resurgence in buildings. Mitigation efforts are broadly categorized into preventive, corrective, and emergency measures. Preventive strategies aim to address potential risks before proliferation occurs, often through optimized building plumbing system design, routine maintenance, and operational controls. In contrast, corrective actions are implemented when OPs contamination is detected or after outbreak investigations, involving more long-term interventions. Emergency measures are typically quickly conducted as a one-time aggressive response to control contamination or when disease cases are reported, especially in healthcare settings.

2.2.1 Water safety planning

Waterborne infections can be prevented or greatly limited by implementing specific strategies that minimize the occurrence of OPs and prevent exposure risks to susceptible individuals. Notably, this approach is articulated in water safety plans (WSPs), which include comprehensive measures aimed at preventing and managing water-related risks. In addition to helping limit waterborne infections, the implementation of a WSP is necessary to ensure compliance with various standards and recommendations, as well as to respond promptly to high-risk events (e.g., outbreaks, system (re)commissioning). For building managers, developing a WSP provides evidence of due diligence.

The multi-barrier approach of WSPs is based on risk control through (WHO, 2023):

- The identification of priority risks.
- The implementation of risk control and prevention measures.
- The monitoring of control measures and water quality.
- The execution of emergency measures (corrective actions) if thresholds are exceeded.

After assembling a multidisciplinary team with diverse skills and responsibilities, the building's water systems are described in terms of design and water usage patterns in order to identify at-risk areas. The majority of guidelines available in the literature to define a WSP focus solely on control strategies targeting the prevention of *Legionella* (*pneumophila*), generally assuming that the practices are equally effective for other OPs (Australian Government, 2015; ESCMID, 2017; TPSGC, 2016; ASHRAE, 2020; CDC, 2021a; VHA, 2021, INSPQ, 2023a, HSE, 2024). However, recent guidance documents for implementing WSPs in healthcare facilities are now specific to the control of *Pseudomonas aeruginosa* (NHS, 2016) and NTMs (NHS, 2024), thereby expanding the focus on a wider range of microbial risks in building water systems.

Drawing from the guidelines just previously cited, key elements of a WSP apply universally among these guidance documents unless specified otherwise, and include the following:

- Definition of control targets (e.g., microbial, chemical), based on (1) intervention priorities of the infection prevention and control team (i.e., if in healthcare settings), (2) the history of previously confirmed or suspected cases and/or outbreak sources, (3) previous sampling results confirming the presence of specific OPs, (4) vulnerability of the building's patients

or occupants, (5) existing standards and regulations on water quality, and (6) available capacities and resources to implement control measures and routine monitoring.

- Identification of control measures, which predominantly target thermal regime and the maintenance of a certain disinfectant residual, their control limits and corrective actions when the latter are exceeded.
    - For cold water systems, most guidelines suggest maintaining temperatures below 20 – 25 ˚C from the building's point of entry to all points of use. In contrast, recommended temperatures for hot water systems vary by component. Water heaters generally need to maintain temperatures at or above 55 to 60 ˚C, while distribution lines are kept at 51 to 55 ˚C. At distal points, the recommendations range from a minimum of 49 ˚C (ASHRAE, 2020) to 55 ˚C or more to ensure effective control of *Legionella* bacteria, but some guidelines intended for healthcare settings allow temperatures as low as 43 ˚C to prevent burn injuries (CDC, 2019; TPSGC, 2016; VHA, 2021).
    - Maintaining a certain biocide concentration within buildings is the second key control strategy identified in WSPs guides, aimed at limiting microbial regrowth. Suggested limits include maintaining a free chlorine residual concentration above 0.2 mg/L in both cold and hot water systems (Australian Government, 2015; ESCMID, 2017; HSE, 2024), with higher concentrations (0.5 – 1.0 mg/L as Cl_2) where supplemental disinfection is applied.
- Validation of the WSP through water quality monitoring at specific sites, with specific alert and action threshold for each targeted environmental parameters. According to Gamage and colleagues (2021), the WSP can be validated through two distinct environmental water quality monitoring approaches. The first involves assessing general physico-chemical parameters (e.g., temperature, residual disinfectant, pH) alongside rapid microbiological indicators such as HPC and ATP. The second focuses on detecting and quantifying the specific OPs targeted by the WSP.

2.2.2 Preventive strategies

To prevent the growth of OPs, health regulators worldwide recommend several building-level preventive strategies, including temperature control, maintaining residual disinfectant, and flushing

stagnant water volumes. Control limits associated to the preventive strategies discussed below, which are derived from directives related to WSPs, primarily targeting the control of *Legionella* bacteria. However, it is generally assumed that these measures are also effective against other OPs, despite sharing somewhat different ecological niches.

Temperature control. In large building plumbing systems, effective temperature control requires setting water heaters at elevated temperatures and adequately insulating pipes to ensure consistent hot water temperatures throughout the system and reduce heat losses between the water heater and the point of use. According to most regulatory guidelines and documents on water safety planning, cold water systems should be kept below 25 ˚C, and ideally under 20 ˚C, whereas hot water systems should be maintained at a minimum of 60 ˚C in the water heaters, and preferably above 55 ˚C in hot water recirculation lines (Castex et Houssin, 2005; HPSC, 2009; Australian Government, 2015; PWGSC, 2016; UK Department of Health, 2016; ESCMID, 2017; OFSP et OSAV, 2018; CDC, 2019; ASHRAE, 2020; VHA, 2021; HSE, 2024). These temperature recommendations are set outside of the optimal growth zones of *Legionella*, *Pseudomonas aeruginosa*, and NTMs, thus creating unfavorable conditions to their growth. In fact, Bédard and colleagues (2016b) observed a gradual reduction in both culturable and qPCR concentrations of *Legionella pneumophila* when the hospital's water heater setting was increased from 55 ˚C to 60 ˚C, alongside other control measures, in the months following a LD outbreak. At the pilot-scale level, Rhoads and colleagues (2016a) reported significantly lower populations of *Legionella pneumophila* at 58 ˚C compared to 40 ˚C, along with non-detectable levels of *Vermamoeba vermiformis*, likely due to the encystment of this protozoan host at more elevated temperatures. Additionally, Falkinham III (2015) found significantly lower proportions of NTMs in water heaters set above 55 ˚C, further supporting the benefits of high temperatures in reducing these OPs. Increasing water temperatures from 20 to 37 ˚C was also found to decrease monospecies *Pseudomonas aeruginosa* biofilms in flow cells, by affecting the regulation of genes responsible for biofilm formation (Kim et al., 2020). Similarly, hot water temperatures affected negatively the abundance of culturable *Pseudomonas aeruginosa* in water samples collected from hotels comparatively to cold water (Chochlakis et al., 2023).

Disinfectant residuals. Maintaining disinfectant residuals (e.g., chlorine, monochloramine) is the second major preventive measure widely suggested to limit the growth of OPs, providing a continuous chemical barrier that reduces bacterial proliferation and helps in controlling biofilms. Limit values often range from 0.1 to 0.5 mg/L for free chlorine residuals, which is the most

commonly used biocide as a secondary disinfectant (Castex et Houssin, 2005; HPSC, 2009; Australian Government, 2015; ESCMID, 2017; VHA, 2021). However, the effectiveness of disinfectant residuals is often limited, as their concentrations tend to decrease in low-flow areas, in the presence of excessive organic (e.g., biofilm, bulk organic carbon) (Absalan et al., 2024) and inorganic matter (e.g., sediments, accumulated pipe scales) (Al-Jasser, 2007; Fisher et al., 2017), and with increasing water temperatures and water age (Bédard et al., 2018; Julien et al., 2022). Therefore, while residuals from the utility provide some baseline protection against microbial growth, notably by diminishing HPC concentrations in distribution networks (Nescerecka et al., 2014) or in buildings (Nguyen et al., 2012), additional in-building control measures are often necessary to enhance OPs control.

The type of disinfectant also impacts microbial communities and occurrence of OPs, as each disinfectant exerts distinct selective pressures (Bautista-de los Santos et al., 2016). For example, *Legionella pneumophila* appears to be less prevalent in monochloraminated distribution systems (LeChevallier, 2019) or in buildings receiving this disinfectant (Donohue et al., 2019; Dowdell et al., 2023) compared to systems supplied with free chlorine. Additionally, *Mycobacterium* species seems to be more prevalent in monochloraminated systems (Pryor et al., 2004; Gomez-Alvarez et al., 2012; Donohue et al., 2015), while disinfectant-free systems support more diverse and even microbial communities due to the absence of selective pressures (Bautista-de los Santos et al., 2016; Proctor et al., 2018; Dai et al., 2020). These systems foster greater competition among a broader range of species, which likely provides a protective effect against growth of OPs that are typically more tolerant to certain disinfectants (Roeselers et al., 2015).

Preventive flushing. Flushing of water outlets that are used irregularly is another common strategy recommended among guidance documents, as it reduces water age in distal parts of large building plumbing systems and restores, to a certain extent, control limits associated to water temperatures and biocide concentrations (Proctor et al., 2020). Some guidelines recommend weekly flushing (Australian Government, 2015; UK Department of Health, 2016; ESCMID, 2017; HSE, 2024) or twice-weekly flushing of distal taps (VHA, 2021). Individual flushing of taps has been shown to significantly reduce mean HPC (2- to 5-log) and ATP densities (5-fold), as well as intact and total cell counts in both cold and hot water (Lautenschlager et al., 2010; Bédard et al., 2018). These reductions are largely attributable to the lower cultivability and viability of upstream building water which has more elevated (or colder) temperatures and more adequate biocide levels (at least in cold

water). In a study surveying the occurrence of OPs in private households, Wang and colleagues (2012) observed significant decreases in qPCR concentrations of *Legionella* and *Mycobacterium* species, and the host *Vermamoeba vermiformis*, after a 3-min flush of distal outlets, thus resulting in 6- to 45-fold decreases compared to first draw samples.

Rhoads and colleagues (2016a) examined the influence of outlets usage frequency (three times/day, three times/week, one time/week) on the occurrence of *Legionella pneumophila* and *Vermamoeba vermiformis* in a controlled large-scale pilot rig. The study demonstrated that gene copies of *Legionella pneumophila* generally increased with higher flushing frequency for each water heater configuration (40 or 58 ˚C). Oppositely, low-use distal taps (one time/week) supplied by water at 58 ˚C exhibited the highest concentrations of *Vermamoeba vermiformis*. In one large green-certified building, implementing automatic flushing of taps using only 1% of the daily water demand was shown to effectively increase monochloramine residuals and reduce HPC levels by over 1-log, along with resolving previous aesthetic issues related to taste and odor (Nguyen et al., 2012). Similarly, the installation of timed flow taps which flushed 192 liters of water on a daily basis near dead-end branches in an Italian hospital significantly reduced culturable concentrations of *Legionella* species by more than 3-log (Totaro et al., 2018). These automated flushing systems helped maintain continuous *in situ* chlorine dioxide disinfection throughout the plumbing network without the labor-intensive efforts required by regular manual flushing of taps.

However, flushing alone is unlikely to completely remove the entire biofilm adhered to inner pipes as microorganisms can only partially dislodge during flushing (Fish et al., 2017). This is because the shear stress generated by flow during use primarily affects the interface between the bulk water and the biofilm. Flushing can further appear incompatible with water conservation goals, especially in green-certified buildings, but research indicated that only a small volume of flushed water is actually needed to address water quality issues associated with low water demands (Nguyen et al., 2012) and sectorial dead legs of building plumbing systems (Totaro et al., 2018).

2.2.3 Corrective actions

Corrective actions are generally implemented on a long-term continuous basis to provide ongoing and targeted protection against OPs. They are crucial components of OPs control strategies in large buildings, and most particularly in healthcare settings where vulnerable occupants are present. In general, the purpose of these interventions is to suppress or limit microbial growth and act as a

persistent barrier against recolonization of OPs. Therefore, they offer long-term control by addressing the root causes of contamination and enhancing overall water safety.

Chlorine-based interventions. Chlorine-based corrective actions, including the use of free chlorine, monochloramine, and chlorine dioxide, are widely implemented for controlling *Legionella* in building plumbing systems (U.S. EPA, 2016). Supplemental free chlorine levels of 2 mg/L injected into a hospital hot water system led to a significant reduction in the proportion of sites positive for culturable *Legionella pneumophila*, from 37% to 7% within six weeks, and no new cases of LD were reported over the subsequent two years (Snyder et al., 1990). Similarly, Orsi and colleagues (2014) observed that continuous chlorination at lower concentrations of 0.5 to 1.0 mg/L of free chlorine reduced the occurrence of *Legionella* spp. within 30 days, although the treatment did not completely eliminate the contamination issue.

Like free chlorine, chlorine dioxide is a powerful oxidizing agent used in water treatment and building water systems. In one hospital setting, the installation of time flow taps flushing water for one minute every two hours near all sectorial dead legs to bring chlorine dioxide (0.2 – 0.4 mg/L) significantly reduced the abundance of culturable *Legionella* spp. (Totaro et al., 2018). Only one site remained positive to *Legionella pneumophila* more than a year after their installation. Similarly, when comparing emergency measures such as heat shocks and hyperchlorination (Section 2.2.3) with the introduction of continuous low levels of chlorine dioxide (0.2 – 0.4 mg/L) in a large residential building, the latter proved more effective in reducing culturable concentrations of *Legionella pneumophila* ten weeks post-treatment (Lee-Masi et al., 2024).

Finally, monochloramine treatment is an increasingly adopted disinfection strategy by both utilities and building managers, specifically targeting *Legionella pneumophila* within water systems. Indeed, previous studies have shown that community-wide conversion to monochloramine reduced the prevalence of *Legionella* in buildings (Moore et al., 2006) and across different distribution network locations (Pryor et al., 2004), including all serogroups of *Legionella pneumophila* (Flannery et al., 2006). In-building monochloramine injected in the hot water system of several hospital settings (1 – 6 mg/L) has also generally led to a reduction, and sometimes even complete eradication, of culture-positive sites for *Legionella* species or *Legionella pneumophila* (Marchesi et al., 2012; Casini et al., 2014; Duda et al., 2014; Mancini et al., 2015; Coniglio et al., 2018; Lytle et al., 2021). However, concerns that monochloramine may lead to microbial shifts favoring a

higher prevalence of NTMs have been raised, although outcomes have been variable in previous studies (Casini et al., 2014; Duda et al., 2014; Lytle et al., 2021).

Copper silver ionization. Such treatments are particularly popular in hospitals as part of a water management strategy aimed at reducing *Legionella pneumophila* risks (LeChevallier, 2023). However, this corrective measure has consistently failed to control the growth of the pathogen, as nosocomial LD outbreaks continue to occur despite its implementation (Blanc et al., 2005; Demirjian et al., 2015; Bédard et al., 2016b). Moreover, there is evidence that *Legionella pneumophila* can develop tolerance or resistance to copper over time, thus altering the effectiveness of such treatment (Bédard et al., 2021).

2.2.4 Emergency measures

Emergency measures such as hyperchlorination and heat shocks are commonly recommended in *Legionella* control guidelines as an immediate action required to reduce *Legionella* contamination in WSPs. Both methods are designed as rapid, short-term responses that complement broader, ongoing preventive and corrective measures within water safety planning frameworks. These methods are typically reactive rather than preventive, thus addressing only the sudden issue without resolving other underlying concerns that allowed *Legionella* to proliferate in the first place.

Hyperchlorination. Hyperchlorination of water systems involves temporarily injecting 20 to 50 mg/L of free chlorine for shorter contact times (< 24h) (Campos et al., 2003; U.S. EPA, 2016). Such treatment applied to a hospital plumbing system resulted in significantly lower *Legionella* spp. occurrence rates, from 21.1% to 2.5% in the following 30 days (Orsi et al., 2014). Due to severe inorganic and organic deposits on inner pipes of this antiquated healthcare facility, concentrations of trihalomethanes, a potential carcinogen chlorination by-product, significantly increased post-treatment, highlighting the need to balance OPs control with chemical safety. Conversely, another study reported the persistence of a single strain of *Legionella pneumophila* serogroup 1 in a domestic shower in spite of frequent cycles of hyperchlorination conducted with 50 mg/L of free chlorine over contact time periods of one hour (Cooper et al., 2008). Persistence of *Legionella pneumophila* was attributed to increased resistance in some strains to chlorine-based disinfectants caused by this repeated exposure to elevated dose of free chlorine, thereby diminishing the overall disinfection impact over time. Moreover, if not carried out adequately, high chlorine concentrations cannot reach all areas of a building water system, particularly dead-ends

and low-flow sections, potentially leaving niches where *Legionella* can resurge. Protozoan hosts can further shield *Legionella* under stressing conditions (Kilvington et Price, 1990; Dupuy et al., 2011).

Heat shocks. Heat shock treatments involve raising water temperature generally above 60 ˚C, thus exceeding the near lethal threshold for *Legionella* bacteria (Rhoads et al., 2015b), and flushing outlets to ensure that hot water reaches all parts of the building plumbing system. In general, the ineffectiveness of heat shocks in sustainably reducing the prevalence of *Legionella pneumophila* in large buildings has been demonstrated in the literature (Chen et al., 2005; Bédard et al., 2016b; Lee-Masi et al., 2024). This can be explained by the tolerance of certain *Legionella pneumophila* lineages to withstand high temperatures (Allegra et al., 2008; Liang et al., 2023), the protection of the pathogen by biofilm-associated microorganisms, or the inability of the hot water plumbing system to maintain superheating temperatures at all distal points of the building.

2.2.5 COVID-19 pandemic context

During the COVID-19 pandemic, most large institutional and public buildings were closed or experienced significantly reduced occupancy due to lockdowns, remote working policies, or other restrictions. This led to prolonged periods of water stagnation, bringing to light the vulnerabilities of building water systems during extended closures. Primary concerns explicitly raised by worldwide stakeholders, which participated in the development of recommissioning guidance, were (1) growth of *Legionella* bacteria and (2) exposure to increased concentrations of lead and copper (Proctor et al., 2020). Conditions like decreased (or complete loss of) disinfectant residuals (Nguyen et al., 2012; Rhoads et al., 2016b), general microbial growth (Lautenschlager et al., 2010; Bédard et al., 2018), and leaching of plumbing metals (Zlatanović et al., 2017; Doré et al., 2019) have been previously associated to shorter stagnation periods or high water age in buildings prior to the pandemic. However, it was unclear how these reactions might be affected by prolonged stagnation lasting several weeks, even months. Other health-related issues, including disinfection by-products formation, taste and odor concerns, increased occurrence of NTMs and *Pseudomonas aeruginosa*, were also reported in guidance documents, although less frequently.

To mitigate risks associated with reopening buildings after extended closures, two main strategies were widely recommended: (1) routine/recommissioning flushing and (2) shock chlorination (Proctor et al., 2020). Flushing involves the systematic opening of all points of use (e.g., faucets,

showerheads, toilets, etc.) to ensure the removal of stagnant water. This process aims to replenish the building plumbing water system with “fresh” water coming from the municipal supply, thus restoring disinfectant residuals (if present in cold water system) and removing accumulated harmful biofilm-associated bacteria and plumbing metals. In general, guidelines recommend sequential flushing, starting with the outlets closest to the building point of entry and moving towards the farthest points, with both cold and hot water systems being targeted (AWWA, 2020; CSA, 2020; Government of Québec, 2020). Routine flushing during periods of low occupancy helps maintain water quality by regularly circulating fresh water and preventing stagnation at specific points of use, whereas recommissioning flushing is more intensive and conducted right before reoccupation to thoroughly remove stagnant water.

Shock chlorination has been suggested or discussed in a handful of guidance documents, most especially if (1) microbial contamination is suspected or confirmed, (2) vulnerable occupants are coming back, (3) depressurization occurred during the closure, or (4) the hot water system has been shut down (AWWA, 2020; Proctor et al., 2020). This remedial intervention involves injecting free chlorine (10 – 50 mg/L) into the water system, and allowing it to sit for a set duration (2 – 24 hours) to achieve effective disinfection (ESCMID, 2017; VHA, 2021). After the contact time period, the system is rigorously flushed to remove high chlorine levels, detached biofilm pieces and plumbing scales breakdown.

In addition to these mitigation procedures, a water system integrity check is a critical preparatory step that enhances the success of subsequent mitigation measures, safeguarding both the plumbing system and public health upon reoccupation (Proctor et al., 2020). These steps include actions such as (1) confirming backflow prevention and water treatment devices are intact, (2) assessing any structural weaknesses (e.g., pipe leaks, damages), (3) ensuring water heaters function properly and at adequate temperatures, and (4) reviewing the overall current state of the plumbing layout to optimize flushing or shock chlorination strategies. Finally, post-mitigation water quality testing is further recommended to confirm the effectiveness of the interventions and ensure building water system are safe for reopening. Testing include relevant water quality parameters that directly align with the targeted health concerns, warranting that the tests performed specifically address the contaminants and water conditions most likely to impact building occupant health.

2.3 Context in the province of Québec

Understanding the regional context of the occurrence and regulatory policies of these OPs is essential to validate current guidelines and proactively develop new strategies to protect public health. The condition of large buildings in Québec plays a significant role in the prevalence of waterborne pathogens. Many older buildings lack proper plumbing layout plans and the capacity to implement and monitor effective control measures, making them increasingly vulnerable to microbial contamination. Even newer facilities often fail to consider water quality and associated risks during both the design and commissioning phases, thus resulting in unforeseen health hazards. Routine monitoring of water quality is rare, except in certain cases (e.g., at the building's point of entry, lead in schools), and clinical surveillance of waterborne disease outbreaks is limited. Moreover, aggressive initiatives about water and energy savings in buildings have complicated the issue. Without appropriate plumbing materials and carefully designed hydraulics, significant cuts in water and energy demands contribute to elevated water age and reduced hot water temperatures, respectively, both of which promote microbial growth. The risks associated with these conditions remain poorly documented, with interventions often occurring sporadically and only in response to specific cases or outbreaks. Consequently, these challenges underscore the need for a more proactive, comprehensive strategy to safeguard building managers from liability risks in Québec and prevent waterborne infections putative to major OPs, especially where vulnerable occupants are present.

2.3.1 Occurrence of OPs

According to the Bulletin d'information en santé environnementale from the Institut National de Santé Publique du Québec (INSPQ), waterborne outbreaks remained relatively occasional from 2005 to 2018. The number of outbreaks ranged from 4 to 40 per year, involving a varying number of individuals depending on the context and extent of the outbreaks. During this period, the largest proportions of outbreaks were attributed to drinking water (41.2%) and recreational waters (38.2%), with lesser contributions from cooling tower installations (3.5%) and other unknown sources (17.1%). In healthcare facilities, 5% of outbreaks were linked to drinking water, 14.3% to cooling towers, and 6% to unidentified sources. Among the suspected infectious microorganisms, *Legionella pneumophila* (n = 15), *Giardia* spp. (n = 14), cercariae (n = 11), and *Pseudomonas*

aeruginosa (n = 10) were notably implicated in the highest number of outbreaks, regardless of the water source (Dubé et Lebel, 2022).

Legionella. Analyzing various *Legionella pneumophila* strains reported to the Laboratoire de Santé Publique du Québec (LSPQ), Lévesque and colleagues (2016) observed an increase in the incidence rate of LD in Québec between the period from 1990 to 2005 (average of 0.27 cases per 100,000 individuals) and the period from 2006 to 2015 (average of 1.91 cases per 100,000 individuals). Although LD has been a notifiable disease since 1987 in the province of Québec, the health network has raised growing concerns about this waterborne disease only more recently, following the massive outbreak in the city of Québec in 2012, where a cooling tower was identified as the outbreak source (182 people affected and 13 deaths) (Lévesque et al., 2014). To prevent Legionnaires' disease, conduct epidemiological surveillance, and track trends over time at the provincial level, the LSPQ implemented a *Legionella* strain detection and genotyping service for both clinical and environmental specimens (INSPQ, 2015). Government authorities have also established a provincial register of cooling towers and legal obligations for installations owners to perform monthly samplings and inspections, in addition to maintaining an adequate maintenance and intervention plan (Gazette officielle du Québec, 2014).

LSPQ activity reports have shown an increase in the number of strains and specimens analyzed or genotyped for *Legionella* species and *Legionella pneumophila* over the past two decades. These analysis have also confirmed the prevalence of different serotypes of *Legionella pneumophila*, as well as a diversity of species within the *Legionella* genus associated to infections, including *Legionella bozemanii*, *Legionella longbeachae*, *Legionella quinlivanii*, *Legionella wadsworthii*, and *Legionella micdadei* (INSPQ, 2010, 2011, 2012, 2013, 2014, 2015, 2016). Several studies conducted by the Industrial Chair in Drinking Water (CIEP) at Polytechnique Montréal and other researchers have further revealed the occurrence of *Legionella pneumophila* in Québec and across different types of buildings, such as hospitals, nursing homes and long-term care homes, a sports center, and educational facilities. Indeed, *Legionella pneumophila* was measured in environmental water samples from distinct water points in large buildings, including hot water production systems (e.g., water heaters, hot water recirculation loops, plate heat exchangers), faucets, and showers (Bédard et al., 2016b; Bédard et al., 2016c; Boppe et al., 2016; Bédard et al., 2021; Cadieux et al., 2019; Grimard-Conea et al., 2022; Cazals et al., 2023; Grimard-Conea and Prévost, 2023; Dowdell et al., 2023; Grimard-Conea et al., 2024). Identical *Legionella pneumophila* strains have been

detected in different buildings, suggesting the presence of a common strain originating from the municipal distribution network, although the probability of detecting the bacteria in treated water is typically very low (Dias et al., 2019) compared to that in large, old, and complex buildings.

Pseudomonas. The LSPQ performs molecular typing of *Pseudomonas* spp. from clinical specimens obtained from sputum and throat samples of patients with cystic fibrosis or other non-strerile sites, according to available activity reports (INSPQ, 2010, 2011, 2012, 2013, 2014, 2015, 2016). However, the number of specimens analyzed is lower than those for *Legionella pneumophila* or *Legionella* species. Despite pneumonia or septicemia diseases caused by *Pseudomonas* species not being notifiable in Québec, *Pseudomonas* infections are associated with nearly 5% of primary and secondary nosocomial bacteremia reported between 2020 and 2023 by healthcare facilities across Québec participating in the pan-hospital bacteremia surveillance program (SPIN). Isolates of *Pseudomonas* species associated with an "extreme" antibiotic resistance profile (i.e., resistant to five classes of antibiotics) remain relatively rare among participating facilities. Nonetheless, an important proportion of nosocomial isolates showed resistance to at least one class of antibiotics (3 to 12% of isolates, depending on the antibiotic class) (INSPQ, 2023b). Similarly to *Legionella pneumophila* bacteria, sampling campaigns organized by the CIEP have demonstrated the occurrence of *Pseudomonas aeruginosa* at various water points of use, including electronic and manual faucets, drains, and ice machines (Charron et al., 2014; Bédard et al., 2015; Cazals et al., 2023; Grimard-Conea et al., 2024). Finally, a study conducted by the INSPQ revealed that *Pseudomonas aeruginosa* was detected in nearly 41% of public spas (n = 95) investigated in three regions of the province of Québec (INSPQ, 2009).

Nontuberculous mycobacteria. In Québec, three species belonging to the *Mycobacterium avium* complex are predominant: *Mycobacterium intracellulare*, *Mycobacterium chimaera*, and the four subspecies of *Mycobacterium avium* (*avium*, *hominissuis*, *silvaticum*, *paratuberculosis*) among clinical samples (all exposure pathways combined) (Akochy, Pierre-Marie [INSPQ], 2022, personal communications). Infection cases by the slow-growing species *Mycobacterium chimaera* have been associated with a particular model of heater-cooler units used during cardiac surgery in Canada and worldwide (Ogunremi et al., 2017). Among patients with cystic fibrosis, the *Mycobacterium abscessus* complex is primarily dominant. This clade of NTMs poses considerable treatment challenges because most cases of *Mycobacterium abscessus* involve strains that are multidrug-resistant. Other NTMs also causing atypical infections in the most vulnerable

populations in Québec include *Mycobacterium kansasii*, *Mycobacterium xenopi*, *Mycobacterium fortuitum*, *Mycobacterium chelonae*, and *Mycobacterium gordonae* (Akochy, Pierre-Marie [INSPQ], 2022, personal communications). Data on infections by NTMs remain scarce in Québec, as only leprosy and tuberculosis, caused by *Mycobacterium leprae* and the *Mycobacterium tuberculosis* complex, respectively, are notifiable diseases and require mandatory treatment.

2.3.2 Regulatory guidelines

In the province of Québec, water quality standards are defined in the Règlement sur la qualité de l'eau potable ("Regulation respecting the quality of drinking water") (Environment Quality Act, chapter Q-2, r. 40). Water quality measurements require that samples, collected at the water treatment plant outlet and throughout the distribution networks, contain no more than 10 total coliforms per 100 mL of water, must be free of fecal indicators such as Escherichia coli and enterococci, and should maintain a minimum residual disinfectant concentration of 0.3 mg Cl2/L and 1 mg Cl2/L in chlorinated and chloraminated systems, respectively. Routine sampling of buildings is uncommon and, when conducted, typically occurs at the building's water entry, except for lead and copper, which are sampled directly from consumers' taps.

Apart from the requirements specified in the Code de Sécurité of Québec ("Safety Code") (Building Act, chapter B-1.1, r. 3) for *Legionella* testing in cooling towers every 30 days after winterization, routine surveillance sampling for OPs in public building plumbing (institutional, commercial) is rarely carried out. It is even less common at the residential level due to private ownership constraints. Currently, there is no definitive stance from the Ministry of Health and Social Services of Québec on the obligation of developing WSPs where vulnerable occupants are found, thus leaving a gap in standardized guidance for managing water-related health risks. Nonetheless, some preventive measures against *Legionella* growth are mentioned in the Code de la Construction of Québec ("Construction Code") (Building Act, chapter B-1.1, r. 2), including maintaining a temperature of 60 ˚C at the water heater outlet and limiting the use of thermostatic mixing valves to within the bathroom perimeter. To avoid scalding issues, showerheads and bathtub taps should be set at a maximum temperature of 43 ˚C in healthcare settings and long-term care homes (Building Act, chapter B-1.1, r. 3). The INSPQ also recommends considering the implementation of control measures (e.g., thermal regime, supplemental disinfection) to reduce *Legionella* risks in

hospitals for future updates relative to the Loi sur les services de santé et les services sociaux of Québec (“Act respecting health services and social services) (chapter S-4.2) (INSPQ, 2016).

2.3.3 Recommendations for reopening buildings

Although initially developed at the onset of the COVID-19 pandemic to ensure the safe reopening of facilities and address water stagnation, Québec’s recommendations for restoring water service are applicable to any facility that has been closed or partially occupied (Government of Québec, 2020). This flushing procedure is recommended whenever a building has had less than 25% of its normal occupancy for over a month, which is the double threshold that triggers action.

Depending on the size of the facility, the (re)commissioning procedure varies slightly. For smaller properties (without hot water recirculation, three stories or fewer, and covering 600 m2 or less), the first step is to verify the integrity of the hot water production system. This includes ensuring that heaters operate at a minimum of 60 ˚C and that distal sections and points of use reach at least 55 ˚C. If the water heater was shut down during the closure, it has to be restarted and run properly for 24 hours before proceeding with the flushing protocol. Flushing begins with the cold and hot water points of use located farthest from the building point of entry and the water heater, respectively, and lasts for 30 minutes. Then, all points of use, both cold and hot water, are flushed for two minutes each, moving progressively from the farthest outlet toward the entry point and the heater. For larger buildings (with a hot water recirculation system, three stories or more, and covering over 600 m2), once the integrity of the hot water production system is ascertained, flushing begins at the building point of entry for at least 30 minutes, if no plumbing plans are available. The process continues floor by floor, flushing all points of use for five minutes with cold water and two minutes with hot water. If plumbing plans are available and are up to date, the flushing protocol starts by flushing each main cold and hot water riser for 15 minutes, then proceeding floor by floor.

2.4 Critical review of the literature

In large building plumbing systems, the occurrence of OPs, such as biofilm-associated *Legionella pneumophila*, *Pseudomonas aeruginosa*, and NTMs can pose significant public health risks, highlighting the need for a comprehensive review of current knowledge on factors influencing their proliferation and the effectiveness of various mitigation strategies. In Québec, the absence of a

clear regulatory framework and guidance on best management tools for the control of these OPs presents considerable challenges to ensure water safety. This regulatory gap is further widened by the absence of practical data that bridge research and real-world applications. To effectively carry out preventive and corrective actions, there is a need for well-defined standards, guidelines, and codes that outline procedures and operational methods for building water systems. However, once water enters the portion of the distribution system that lies beyond a property line and within the building itself, the responsibility for ensuring water quality shifts to the building owner or manager. Despite these key responsibilities, there are limited guidelines and regulatory requirements specifically tailored for buildings, which are most often overlooked by water utilities (Logan-Jackson et al., 2023). Detailed protocols that have been validated for operating adequately large building plumbing systems and monitoring specific risks associated with the presence of opportunistic pathogens are lacking, thus leading to inconsistent practices. Furthermore, OPs monitoring is essential to verify that WSPs are functioning as intended, but the absence of such overall framework complicates consistent application and enforcement across different settings.

Control guidelines for *Legionella* worldwide recommend maintaining free chlorine residuals as a primary preventive strategy. Specifically, these guidelines commonly suggest maintaining free chlorine concentrations above 0.2 mg/L, sometimes even more than 0.5 mg/L, to inhibit the growth of *Legionella pneumophila*. However, despite widespread adoption, the effectiveness of recommendations are not supported by evidence. Studies that systematically evaluated the effectiveness of chlorine levels in real-world buildings are scarce, and existing research often fails to account for the variability in terms of design and operation of water systems across different facilities. Guidelines often recommend identical residuals concentrations for both cold and hot water systems, as well as for both control and distal sites of building plumbing. This generalized approach and oversight disregard free chlorine decay reactions occurring in these distinct systems, thus leading to unrealistic expectations for building managers to ensure consistent water quality. The lack of evidence-based differentiation between residual targets at control versus distal sites, and cold versus hot water points, underscores the need for more nuanced recommendations that reflect chlorine dissipation occurring across large building plumbing networks.

The COVID-19 pandemic led to unprecedented prolonged building closures and low occupancy levels, which raised urgent concerns about their impact on water quality and how buildings should be safely reopen to public. The growth of *Legionella pneumophila* was identified as a main risk in

most recommissioning guidance documents, prompting the rapid development of various flushing protocols and disinfection procedures aimed at ensuring water safety upon building reoccupation. However, these recommendations were largely reactive, based on the limited empirical data available in the literature, and frequently lacked specificity regarding the efficacy and duration of the suggested interventions. Prior to the pandemic, studies that conducted some flushing at points of use showed short-term benefits on the reduction of general microbial indicators (HPC, ATP, cell counts) and *Legionella* concentrations, yet their long-term effectiveness remains unclear. The need for robust recommissioning guidance extends beyond the pandemic, as prolonged closures are not uncommon in various settings, including schools during summer breaks, seasonal buildings in northern countries (e.g., hotels, camping), or closed wings of larger facilities during construction activities. This highlights the need for comprehensive studies that provide clear and actionable evidence-based strategies for building managers regularly facing long periods of closures or low occupancy.

The frequency of flushing of irregularly used outlets and the optimal combination of preventive and corrective actions for controlling *Legionella pneumophila* in large building water systems is poorly defined in the current literature and control guidelines. This lack of definition creates considerable challenges for building managers in implementing these strategies. Indeed, the literature lacks consensus on the most effective flushing regime, (e.g., daily, weekly, or any other time interval) to sustain reductions in *Legionella pneumophila* concentrations, and most especially during periods of low occupancy or following recommissioning efforts after prolonged closures. Moreover, the literature reports variable results regarding hyperchlorination efficacy, with studies showing only temporary reductions in *Legionella* levels and rapid recolonization, questioning its long-term benefits. Nevertheless, it is still recommended widely regardless of potential secondary effects on plumbing systems. Its limited effectiveness underscores the need for additional continuous preventive actions, such as regular flushing, to ensure long-term control. Furthermore, the limited data on the combined efficacy of independently moderately successful strategies complicates decision-making for building managers, especially in situations that require immediate responses to contamination issues while awaiting the implementation of more sustainable, long-term solutions. Providing clearer guidance would enable more proactive and effective risk management strategies.

The use of monochloramine as a continuous disinfection strategy in hospital hot water systems has been fairly studied. However, significant gaps remain in the comprehensiveness and applicability of these studies. Many of the existing investigations primarily focus on OPs control, often targeting *Legionella pneumophila* as the sole indicator of water safety. While these studies have demonstrated monochloramine's potential to selectively reduce *Legionella pneumophila* levels, they often neglect other clinically relevant pathogens. This narrow scope limits the generalizability of the findings, as it fails to account for the diverse microbial communities present in complex plumbing systems, particularly in healthcare settings where patient safety is paramount. Another critical shortcoming is the poor resolution of sampling intervals, site selection and environmental conditions reported within these studies. Key factors such as water temperature, water demand, disinfectant concentration, and the diversity of sampling sites are underreported or insufficiently assessed, creating a disconnect between study conditions and the operational realities of large hospital water distribution networks. This absence of context-specific information reduces the replicability of findings in different buildings. Additionally, the resolution of sampling in both spatial and temporal terms is often too low, resulting in data that do not adequately capture the dynamic nature of microbial communities over time, particularly in response to disinfection. Actionable data, characterized by comprehensive, high-resolution insights into OPs dynamics and environmental conditions, are crucial for the formulation and implementation of robust water safety plans, which rely on detailed, context-specific data to identify critical control points, assess risks, and establish monitoring protocols that are both effective and feasible.

Finally, efficient OPs control would benefit from the use of surrogate parameters (e.g., HPC, ATP, etc.) that can provide rapid, practical, and real-time assessments of the probability of presence of OPs. These parameters offer a dynamic assessment of the overall microbial water quality within building plumbing systems, and could serve as early warning indicators of potential risks before OPs reach levels of concern. However, there is a need to understand if they can reliably predict OPs in large and complex buildings, especially in response to operational changes (e.g., temperature improvements, disinfection strategy, etc.). Incorporating these parameters into water safety plans would provide a continuous feedback loop that supports proactive risk management and timely intervention, thereby contributing to the comprehensive effectiveness of OPs monitoring and control strategies in large building water systems. One critical challenge moving forward lies in establishing threshold values relevant to OPs risk, and validating their use.

CHAPTER 3 OBJECTIVES AND METHODOLOGY

3.1 Research objectives and hypotheses

The primary objective of this doctoral project is to provide evidence-based recommendations to improve the monitoring, operation, and design of plumbing systems in large buildings. Combining novel tools, this research aims to investigate the occurrence of OPs and evaluate the effectiveness of preventive and corrective measures intended to lower the user's exposure risk. Additionally, this project seeks to safeguard building managers from potential liability risks by offering actionable guidance into proactive management of building water systems.

Therefore, this doctoral project focuses on the following specific research objectives:

- Assess the impact of maintaining free chlorine residuals across nine large building systems on the occurrence of *Legionella pneumophila* and *Pseudomonas aeruginosa*.
- Examine the short-(24h) and long-term (4-week, low water demand) effectiveness of device recommissioning flushing after a 4-month inoccupation on the occurrence of *Legionella pneumophila* at 23 showerheads of a large sports complex.
- Determine the most appropriate combination of preventive flushing regimen (daily/weekly flushes, or no water demand) and remedial intervention (device recommissioning flushing or shock chlorination) on the occurrence of *Legionella pneumophila* at 12 showerheads.
- Evaluate the longitudinal (> 1-year) impact of *in situ* monochloramine disinfection of a large hospital hot water system on microbial diversity and the abundance of *Legionella pneumophila*, *Legionella* species, NTMs and *Vermamoeba vermiformis*.

The following research hypotheses are suggested to address the previous specific objectives:

1) Water temperature is a major predictor of the maintenance of free chlorine residuals in large building plumbing systems.

Temperature plays a fundamental role in the stability of free chlorine residuals, which decay faster at elevated water temperatures. Despite this, *Legionella* control guidelines most often suggest maintaining the same residual concentration in cold, tepid, and hot water systems, an approach that fails to consider the accelerated decay of free chlorine at with increasing water temperatures. New manufacturers entering the market promote the installation of *in situ* supplemental disinfection systems in shower and hot water systems, which again overlooks the basic relationship between

temperatures and free chlorine levels. Therefore, data-based evidence challenging these conventional guidelines and demonstrating the need for temperature-specific disinfection strategies are needed.

2) Free chlorine concentrations above 0.2 mg/L, a common guide level, can significantly reduce the occurrence of *Legionella pneumophila* and *Pseudomonas aeruginosa*, and the overall microbial biomass culturability and viability in large building plumbing systems.

Investigating the impact of maintaining free chlorine concentrations over 0.2 mg/L – a commonly recommended guide level – on the reduction of *Legionella pneumophila* and *Pseudomonas aeruginosa*, and overall microbial biomass is crucial for validating existing control guidelines. Yet, this specific concentration threshold hypothesized to effectively suppress pathogenic bacteria and microbial load is based on limited data and does not consider heterogeneity of environmental conditions across different sampling sites (e.g., cold versus hot water, control versus distal sites, stagnation).

3) Device recommissioning flushing conducted after a prolonged period of low occupancy can significantly lower the exposure risk to *Legionella pneumophila* at showerheads, but only short-term (24h).

Determining the impact of recommissioning flushing is critical for understanding the limitations and effectiveness of this widely recommended intervention to address the safe reopening of building water systems during the COVID-19 pandemic. Fixture-flushing is commonly used as a preventive measure to remove stagnant water and reduce microbial loads, yet the duration of its effectiveness remains uncertain. Demonstrating that flushing significantly lower *Legionella pneumophila* exposure risk only for a short duration before reoccupation would emphasize the need for more sustained mitigation strategies.

4) The combination of daily flushing and shock chlorination is the most optimal strategy to reduce significantly the abundance of *Legionella pneumophila* at showerheads.

Daily flushing and shock chlorination are preventive and corrective measures commonly employed to mitigate *Legionella pneumophila* contamination, but their effectiveness as individual strategies can be limited by rapid recolonization. The combination of these two strategies aims to understand their synergistic effects and provide critical evidence for implementation in water safety plans in response to contamination issue or disease cases.

5) *In situ* dosing of monochloramine in a hospital's hot water system can selectively eliminate *Legionella pneumophila* long-term (> 1-year), but is less effective on the abundance of NTMs, *Legionella* species and *Vermamoeba vermiformis*.

Monochloramine is increasingly recognized as a disinfection strategy in healthcare settings due to its selective effectiveness against *Legionella pneumophila*. However, the differential efficacy of such disinfectant on a broader spectrum of pathogens, including nontuberculous mycobacteria, other *Legionella* species, or host organisms like *Vermamoeba vermiformis*, remains poorly understood. The importance of this research lies in its potential to provide actionable and data-driven information, highlighting strengths and limitations, while evaluating its long-term impact over extended periods and over a wide range of conditions. Findings could support the development of more effective, tailored protocols that balance the need for pathogen control with the practicalities of system operation in complex and large building water systems like healthcare facilities.

6) The introduction of monochloramine results in speciation of *Legionella* and NTMs due to changes in microbial communities over time.

Investigating the impact of monochloramine on microbial communities is critical for understanding its broader ecological effects and the selective pressures it imposes, which may drive shifts in species composition and promote the emergence of other clinically relevant pathogens. Evaluating the long-term implications of monochloramine use and the dynamic changes in microbial populations induced by disinfection highlights will highlight if potential adaptation and survival of resistant pathogenic strains could pose additional health risks to vulnerable occupants.

7) Surrogate parameters, such as ATP, HPC, and flow cytometry cell counts, can reliably predict the presence and the abundance of *Legionella pneumophila*, *Pseudomonas aeruginosa* and nontuberculous mycobacteria.

Surrogate parameters like HPC, ATP, and flow cytometry cell counts offer rapid, sensitive, and comprehensive assessments of overall microbial water quality, providing early warning signals that can help predict potential OPs risks. Ultimately, if found reliable, integrating surrogate parameters into water management and water safety planning could significantly improve monitoring efforts.

3.2 Experimental approaches

Detailed methodologies relative to this research project are extensively described in the subsequent chapters (Chapters 4 to 8) corresponding to each research article. To comprehensively assess the factors influencing OPs occurrence and control, a series of physico-chemical (Table 3.1) and microbiological measurements (Table 3.2) were performed throughout the research project, providing essential data on both general and specific water quality dynamics and treatment effectiveness. The specific parameters assessed for each part of the project are described in each respective research paper they appear. Temperature, pH, conductivity, dissolved oxygen, and both free and total chlorine were measured in the field, typically using a well-mixed water sample volume of 150 to 250 mL. In contrast, total organic carbon, plumbing metals, nitrites, nitrates, and ammonium concentrations were conveyed to the laboratory for analysis.

Table 3.1 Summary of physico-chemical parameters and their corresponding analysis method.

Physico-chemical parameter	**Method**
Temperature	Digital thermometer
pH, Conductivity, Dissolved oxygen	Multiparameter probe HQ40d™ HACH
Free and total chlorine	Spectrophotometer DR 2800TM HACH, methods HACH DPD Powder Pillows 8021 (free chlorine) and 8167 (total chlorine)
Total organic carbon (TOC)	TOC analyzer Sievers M5310C Veolia
Plumbing metals (0.15% v: v acidified)	Inductively coupled plasma mass spectrometry (ICP-MS) (external lab)
Nitrites, nitrates	Ion chromatography using the analysis method MA. 300 – Ions 1.3 (external lab) (CEAEQ, 2020)
Ammonium	Colorimetry with sodium salicylate using the analysis method MA. 300 – N 2.0 (external lab) (CEAEQ, 2014)

Microbiological parameters, including ATP, intact and total cell counts, and culturable *Legionella pneumophila*, were systematically measured in the CIEP laboratory within 24h of sampling. The parameters listed in Table 3.2 do not include additional parameters that have been included in the first part of this research project (meta-analysis) as they were measured part of previously published papers from colleagues. For molecular analysis, water samples were always vacuum filtered on 0.2 µm (Ø 47 mm) Supor® PES membranes upon reception in the lab. The membranes were then aseptically folded and stored at -80 ˚C until DNA extraction. Two DNA extraction methods were employed in this project. The first one, detailed in Chapter 5 of the thesis, involved a bead beating and ammonium acetate precipitation method adapted from Yu and Mohn (1999). For the remaining studies of this thesis (project Parts 3 and 4), the FastDNA Spin kit was used for DNA extraction. Post-extraction, DNA templates were stored at -20 ˚C until further molecular analyses, such as qPCR and Illumina sequencing.

Table 3.2 Summary of microbiological parameters and their corresponding analysis method.

Microbiological parameter	**Method**
Intracellular-ATP	Dendridiag® SW kit (Project Part 2) LuminUltra Quench-Gone™ Aqueous kit
Intact and total cell counts	BD Accuri™ C6 Plus flow cytometer
Culturable *Legionella pneumophila*	100 mL potable water Legiolert/Quanti-Tray IDEXX kit
qPCR *Legionella pneumophila*	iQ-Check® Quanti *L. pneumophila* Real-Time PCR Bio-Rad kit
qPCR triplex *Legionella* (*Legionella* species, *Legionella pneumophila*, and *Legionella pneumophila* serogroup 1)	Microproof® *Legionella* quantification triplex Bio-Rad kit
qPCR *Mycobacterium* species	Adapted protocol from Haig and colleagues (2018)
qPCR *Vermamoeba vermiformis*	Protocol from Kuiper and colleagues (2006)
16S rRNA gene amplicon sequencing	Illumina MiSeq v2 250 reads (F515/R806) targeting the V4 region of the 16S rRNA gene (external lab)
18S rRNA gene amplicon sequencing	Illumina MiSeq v2 250 reads (F1391/EuKBR) targeting the V9 region of the 18S rRNA gene (external lab)

CHAPTER 4 ARTICLE 1: CAN FREE CHLORINE RESIDUALS ENTERING BUILDING PLUMBING SYSTEMS REALLY BE MAINTAINED TO PREVENT MICROBIAL GROWTH?

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Regulatory requirements for maintaining free chlorine residuals are commonly suggested in drinking water distribution systems to prevent microbial regrowth and address potential system breaches. However, there are no specific guidelines for building managers unless supplemental disinfection is implemented. While maintaining free chlorine residuals in distribution system is already challenging due to chlorine decay, building plumbing systems face additional difficulties, such as increased water residence times and higher temperatures, which accelerate chlorine depletion.

This study analyzed 1,737 samples collected by the CIEP research group between 2012 and 2022 from nine large institutional buildings (hospitals, sports complex, and schools) in Canada. The objective was to assess the reliability of maintaining free chlorine residuals in building plumbing systems to prevent microbial growth and the presence of specific opportunistic pathogens, including *Legionella pneumophila* and *Pseudomonas aeruginosa*. Findings provide evidence-based support for the development of guidelines on maintaining free chlorine residuals in building plumbing systems for risk assessment monitoring. Finally, this study highlights the challenges building managers face when relying solely on residuals from municipal service lines to mitigate microbial risks in their facilities.

Can free chlorine residuals entering building plumbing systems really be maintained to prevent microbial growth?

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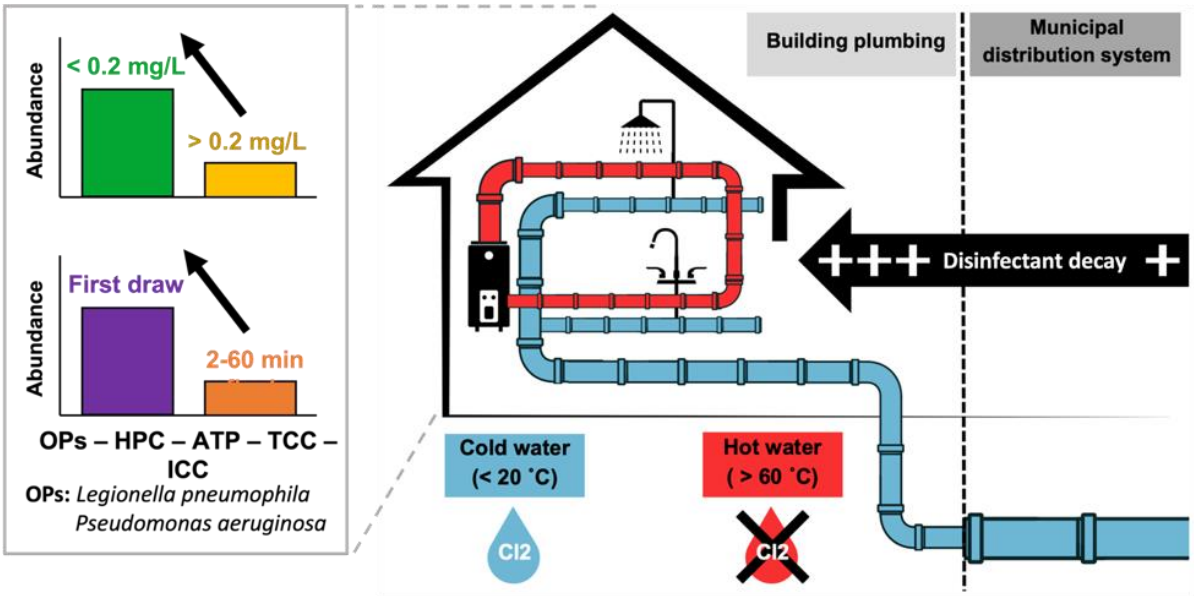

Figure 4.1 Graphical abstract

Highlights

- Free chlorine is absent in hot water and in first draws due to fast depletion
- Flushing increases residuals but mostly in cold water and is site-specific
- Free chlorine is correlated to decreased bulk microbial abundance and viability
- Residuals cannot predict robustly OPs presence in building plumbing
- Water temperatures outside 20 – 60 ˚C prevented *L. pneumophila* culture detection

Abstract

Secondary disinfection aims to prevent microbial regrowth during distribution by maintaining disinfectant residuals in water systems. However, multi-factorial interactions contribute to free chlorine decay in distribution systems, and even more so in building plumbing. Assembling 1,737 samples from nine large institutional buildings, a meta-analysis was conducted to determine

whether building managers can actively rely on incoming free chlorine residuals to prevent in-building microbial amplification. Findings showed that free chlorine concentrations in first draws met the 0.2 mg/L common guide level in respectively 26%, 6% and 2% of cold, tepid and hot water samples, whereas flushing for 2-60-min only significantly increased this ratio in cold water (83%), without reaching background levels found in service lines. Free chlorine was significantly but weakly ($R \leq 0.2$) correlated to adenosine triphosphate, heterotrophic plate count and total and intact cell counts, thus evidencing that residuals contributed to decreased culturable and viable biomass. Detection of culturable *Legionella pneumophila* spanning over a 4-log distribution solely occurred when free chlorine levels were below 0.2 mg/L, but no such trend could be distinguished clearly for culturable *Pseudomonas aeruginosa*. Water temperatures below 20˚C and greater than 60˚C also completely prevented *L. pneumophila* detection. Overall, the majority of elevated microbial counts were measured in distal sites and in tepid and hot water, where free chlorine is less likely to be present due to stagnation and increased temperature. Therefore, building managers cannot solely rely on this chemical barrier to mitigate bacterial growth in bulk water.

Keywords: Chlorine; Building plumbing; *Legionella*; *Pseudomonas*; Flushing.

4.1 Introduction

Drinking water supply systems are designed to deliver water that meets stringent water quality standards and objectives, addressing both health and aesthetic concerns. One crucial step in the treatment process involves the establishment of a secondary disinfection barrier, with free chlorine being the most widely employed disinfectant (van der Kooij et al., 1999). The main objective of primary disinfection is to inactivate viruses and harmful microorganisms present in the source water. Subsequently, secondary disinfection can be implemented to maintain a persistent disinfectant residual from the point of delivery across the drinking water distribution system (DWDS) up to points of use in buildings and residences. This residual serves multiple purposes, including limiting microbial regrowth and mitigating microbial risks arising from intrusion during events such as main breaks, cross-connections, or pressure losses. Additionally, it acts as a swift and cost-effective means of monitoring potential intrusion in DWDSs when the system's integrity is compromised (Haas, 1999; LeChevallier, 1999; Hatam et al., 2020).

Regulatory requirements for the maintenance of free chlorine residuals in DWDSs vary across countries, and occasionally, between jurisdictions, provinces or states within the same country. In

North America, typical operational range for residuals in DWDSs are between a measurable level (generally 0.2 mg/L) and a maximum of 4.0 mg/L (U.S. EPA, 1989) or 5.0 mg/L (Health Canada, 2009) to prevent taste and odor concerns and comply with standards on disinfectant by-products (DBPs). Similarly, the World Health Organization (WHO) guidelines on drinking water quality suggest a minimal free chlorine concentration of 0.2 mg/L throughout DWDSs, which can thereupon be increased to at least 0.5 mg/L during waterborne outbreaks (WHO, 2022). Yet, there are no specific requirements for the maintenance of free chlorine residuals across building plumbing systems, unless supplemental onsite chlorine disinfection is implemented or in the event of established *Legionella* contamination for which cases higher concentrations are commonly suggested than those for DWDSs. In guidelines for the prevention and control of Legionella bacteria, minimum free chlorine concentrations of 0.5 mg/L (HPSC, 2009; ACHD et PRHI, 2014; HSE, 2014; VHA, 2022) or 1.0 mg/L (ESCMID, 2017) are typically recommended throughout the plumbing system and at points of use for in-building chlorine disinfection, regardless of the water system temperature (cold or hot).

Maintaining free chlorine throughout DWDSs presents a considerable challenge due to various factors contributing to chlorine decay. As water travels through DWDSs, chlorine can be depleted by bulk demand from inorganic and organic matter, and wall decay especially in the presence of metallic pipes with accumulated scales (Al-Jasser, 2007; Fisher et al., 2017). Higher residence times combined with greater surface to volume ratios in smaller diameter pipes lead to increased disinfectant decay and microbial growth in the extremities of DWDSs (Prévost et al., 1998; Dias et al., 2019). Seasonal increases in chlorine decay also result from elevated water temperatures, particularly during summer in northern regions (Hua et al., 1999), and the interplay between temperature and dissolved organic carbon (Absalan et al., 2024). All these factors are exacerbated in building plumbing systems with extended residence times, intermittent periods of water stagnation, elevated water temperatures (> 55 ˚C) and larger surface-to-volume pipe ratios (Bédard et al., 2018; Julien et al., 2022). The installation of building point of entry treatments (e.g., water softeners, carbon filters, ultra-violet disinfection) can also lower or remove incoming residuals (ASHRAE, 2020).

While primary disinfection has considerably reduced waterborne outbreaks related to fecal contamination of source water, the use of secondary disinfection is questioned by a growing body of evidence showing the adaptation of opportunistic pathogens (OPs) to disinfectant, primarily due

to their persistence in biofilms (Falkinham III et al., 2015; Leslie et al., 2021). *Legionella*, *Pseudomonas* and nontuberculous mycobacteria are recognized biofilm-associated OPs, contributing significantly to hospitalizations and deaths in the United States, as indicated by surveillance data (Collier et al., 2021). To mitigate the growth of OPs, water utilities could increase free chlorine residuals, but this results in an increased formation of DBPs which have been associated with adverse health outcomes (Hrudey, 2009). In fact, DBPs formation and OPs control in DWDSs have distinct and even opposite responses to increased residuals, and associated health risks should be considered carefully (Dion-Fortier et al., 2009; Zhang et Lu, 2021).

In several European countries, including the Netherlands, Denmark, Switzerland and parts of Germany and Austria, secondary disinfection is currently avoided to balance OPs- and DBPs-risk (van der Kooij et al., 1999; Bertelli et al., 2018). The more diverse microbial communities measured in disinfectant-free DWDSs (Bautista-de los Santos et al., 2016; Dai et al., 2020) is hypothesized to safeguard from OPs growth due to a more stable and competitive microbiome (Roeselers et al., 2015). Such risk mitigation solution yet involves additional protective barriers throughout the supply chain, thereby requiring specific high-standard practices regarding the quality of the source, treatment, distribution and monitoring of drinking water (Hydes, 1999; van der Kooij et al., 1999; Hambsch et al., 2007; Smeets et al., 2009). Considering aging and increasingly susceptible water infrastructures in North America, alongside poor surveillance of DWDSs, some argue that the maintenance of a certain disinfectant residual is a compelling benefit in terms of microbial prevention (Haas, 1999; LeChevallier, 1999).

To use the maintenance of disinfectant residuals as a control strategy, building managers need to be aware of residuals levels that can actually be maintained in cold, hot and tepid water systems. Therefore, published and novel results from multiple sampling campaigns (2012 to 2022) in nine large Canadian buildings were gathered to quantify the potential for maintaining free chlorine residuals and its impact on bacterial densities and the prevalence of selected OPs. The objectives of this study were to determine whether free chlorine residuals can be maintained in large building plumbing systems and to assess if free chlorine concentrations are predictive of the presence of culturable *Legionella pneumophila* and *Pseudomonas aeruginosa*, and other general microbial density indicators. To this end, it was hypothesized that water temperature was a major predictor of the presence of free chlorine residuals in buildings and that free chlorine levels were associated with decreased culturable and viable biomass.

4.2 Material and methods

4.2.1 Sampling data collection

Samples were collected from nine large buildings between 2012 and 2022, including hospitals (n = 4), sports complex (n = 1), elementary schools (n = 2) and university buildings (n = 2), located in four municipal chlorinated DWDSs in Québec, Canada. Some of the results were published as aggregated data (Bédard et al., 2016; 2018; Charron et al. 2014; Grimard-Conea et al., 2022; 2023), while additional data sets were obtained and new data measured. Among the studied buildings, two were LEED-certified and one followed LEED guidelines for water conservation purposes. Only one hospital setting had supplemental onsite disinfection with copper-silver ionization. Overall, 1,737 data points were incorporated into the present meta-analysis, and physico-chemical and microbiological measurements performed specifically during each study are summarized in Table 4.1.

Table 4.1 Overview of the number of data points and water quality analysis performed in each investigated building. Legend: Temp. – Temperature, HPC – Heterotrophic plate count, ATP – Adenosine triphosphate, ICC – Intact cell counts, TCC – Total cell counts, *Lp* – *Legionella pneumophila*, and *Pa* – *Pseudomonas aeruginosa*.

Building	Date	# of samples[1]	Physico-chemical (section 2.2) and microbiological (section 2.3) analysis performed						
			Chlorine residuals	Temp. (°C)	HPC	ATP	ICC and TCC	*Lp*	*Pa*
Hospital A	2016	6	Full	Full	Full	None	None	None	None
Hospital B	2013[2]	316	Full	Full	None	None	None	None	None
Hospital C	2012, 2015, 2016[3], 2017	681	Full	Full	Subset (178/681)	None	Subset (172/681)	Subset (183/681)	Subset (136/681)
Hospital D	2021, 2022	163	Full	Full	None	Subset (95/163)	Subset (95/163)	Subset (162/163)	None
Sports complex	2020[4,5], 2021	287	Full	Full	None	Subset (230/287)	Subset (230/287)	Full	None
Elementary school A	2018	6	Full	Full	Full	None	Full	None	None
Elementary school B	2018	13	Full	Full	Full	None	Full	None	None
University building A	2020	126	Full	Full	Subset (75/126)	Subset (75/126)	Subset (29/126)	Subset (83/126)	Subset (75/126)
University building B	2020	139	Full	Full	Subset (93/139)	Subset (75/139)	Subset (29/139)	Subset (94/139)	Subset (75/139)
Total of samples		1,737	1,737	1,737	375	475	574	809	286

[1] The number of samples is based on the number of available data on free chlorine concentrations.

[2] Data set from Charron et al. (2014)

[3] Data set from Bédard et al. (2016)

[4] Data set from Grimard-Conea et al. (2022)

[5] Data set from Grimard-Conea et al. (2023)

Water samples (0.25 to 2.5 liters) were collected from cold (building points of entry, risers, drinking water fountains, washing basis hoses, manual faucets), tepid (hands free faucets [electronic, foot pedal-activated, knee pedal-activated], showerheads, bath faucets), and hot water points (manual faucets, hot water return loops, risers). In this meta-analysis, first draws represented the first few milliliters (mL) to liters of water collected from the sampling points, whereas post draws were water samples collected right after first draws collection. Water samples collected after flushing devices for 2 – 60 minutes were also included in the data set to assess water quality from the upstream building plumbing. All samples were collected into sterile polypropylene bottles.

4.2.2 Onsite physico-chemical measurements

Temperature was measured using digital thermometers that were directly inserted into beakers containing 100 – 250 mL of the sampled water or straight from the water coming out of the point of use. Free (Hach Method 8021) and total (Hach Method 8167) chlorine were measured either on the portable Hach DR 2800TM spectrophotometer (Hach, London, ON, Canada) or portable colorimeters (Hach DR300 or Pocket Colorimeter II) (Hach, Loveland, CO, USA) using 10 mL aliquots of the sampled water according to the instructions of the manufacturer. Water samples with free chlorine concentrations over 0.05 mg/L were neutralized by the addition of one mL of sterile sodium thiosulfate (10% v:v).

4.2.3 Microbiological measurements processing

All samples were processed within 24 hours from their respective sampling time. The total number of culturable heterotrophic bacteria (HPCs) was assessed in compliance with the standard method 9215D (APHA et al., 2005). Briefly, several dilutions of 1 – 250 mL of sampled water were vacuum filtered on 0.45 µm pore size MF-Millipore™ membranes (ø 47 mm gridded) (Sigma-Aldrich, Oakville, ON, Canada) and aseptically transferred on R_2A agar petri dishes (18.1 g/L) for subsequent incubation at room temperature in the dark for a period of seven days.

The enumeration of intact (ICC) and total bacterial cell counts (TCC) was performed by different staining methods relying on the integrity of bacterial cell membranes as the viability criterion to distinguish dead cells (compromised cell membrane) from their live counterparts (intact cell membrane). In studies carried out before 2018, ICC and TCC were quantified by fluorescence microscopy with the LIVE/DEAD® BacLight™ Bacterial Viability kit (Molecular Probes,

Eugene, OR, USA) according to Bédard and colleagues (2014), whereas in recent studies (since 2018), flow cytometry was used on either the BD Accuri™ C6 flow cytometer or the BD Accuri™ C6 Plus flow cytometer according to the procedure detailed in Grimard-Conea and colleagues (2023). In the fluorescence microscopy assay, all observations of "intermediate" states, referring to the extent of damage of the outer membrane of bacteria (Berney et al., 2007), were considered as injured cells, and therefore enumerated as dead microorganisms. To reduce bias, all interpretations were made by the same analyst when using the LIVE/DEAD® BacLight™ Bacterial Viability kit (SYTO9 and propidium iodide).

Intracellular adenosine triphosphate (ATP) from live microorganisms was measured using approximately 50 – 60 mL of water. For samples collected before 2022, ATP measurements were done with the Dendridiag® SW kit (GL-Biocontrol, Clapiers, France), as specified in Grimard-Conea and colleagues (2022, 2023), whilst measurements made since 2022 were done with the LuminUltra Quench-Gone™ Aqueous test kit (LuminUltra Technologies Ltd., Fredericton, NB, Canada). For the latter, a calibration was made using the UltraCheck™ 1 standard provided in the manufacturer's kit as well as a verification of the contamination level of the Luminase™ enzyme reagent over each sampling event. Then, water samples were aseptically filtered on Quench-Gone™ membrane filters to remove extracellular-ATP and other dissolved inhibitors or impurities. Intracellular-ATP was subsequently extracted from filters using the UltraLyse™ lysis solution supplied in the kit and further diluted in UltraLute™ tubes containing a buffer solution. At last, ATP concentrations were estimated in picograms (pg) of ATP per mL using the relative light units (RLUs) measured by the PhotonMaster™ using the calibration curve.

Culturable concentrations of *Legionella pneumophila* were determined by the AFNOR (Association Française of Normalisation: the French Standards Association) standard NF-T90-431 culture method on glycine, vancomycin, polymyxin and cycloheximide (GVPC) selective agar, as described in Bédard and colleagues (2016), for 45 out of the 809 water samples included in this study. The liquid culture-based enzymatic method with the 100 mL potable water Legiolert™/96-well Quanti-Tray kit (IDEXX Laboratories Canada Corp., Markham, ON, Canada), as detailed in Grimard-Conea and colleagues (2022, 2023), was used for the remaining set of samples. Concentrations of culturable *Pseudomonas aeruginosa* were quantified using the 100 mL potable water Pseudalert™/51-well Quanti-Tray kit (IDEXX Laboratories Canada Corp., Markham, ON, Canada) in accordance with manufacturer's instructions. Sealed Quanti-Tray plates were incubated

at 39 ± 0.5 ˚C for seven days and 38 ± 0.5 ˚C for 24 hours for *Legionella pneumophila* and *Pseudomonas aeruginosa*, respectively.

4.2.4 Data analysis

Data analysis and graphic viewing were conducted on both Microsoft Excel version 16.59 and RStudio version 4.1.1. Statistical correlations among parameters were evaluated with the Spearman's rank correlation coefficient test given that distributions of data for most variables did not follow a bivariate normal pattern. Significance level was set at a p-value of 0.05. For statistical and graphical viewing purposes, samples with culturable *Legionella pneumophila* or *Pseudomonas aeruginosa* concentrations lower than the detection limits of each method were set to 5 most probable number per liter (MPN/L) (or 5 CFU/L for samples analyzed by the GVPC method), whereas results over the detection limits were set to 30,000 MPN/L (i.e., appropriate dilutions for the GVPC method ensured no upper detection limit exceedance).

Samples from both *Legionella pneumophila* culture methods were grouped since recent studies reported MPN and CFU units results to be comparable in potable water samples (Sartory et al., 2017; Scaturro et al., 2020; Boczek et al. 2021). TCC and ICC concentrations measured by both viability assays were combined to increase the data set size. Data from both ATP bioluminescence assays were also pooled for analysis.

4.3 Results

4.3.1 Free chlorine residuals in cold, tepid and hot water systems

Free chlorine concentrations (n = 1,737) measured in cold (7 – 35 ˚C), tepid (10 – 52 ˚C) and hot water (10 – 63 ˚C) from nine large buildings are presented in Figure 4.2. Free chlorine was most likely detectable in cold water at first draw (mean of 0.14 mg/L, n = 103), and even more so after flushing for 2 – 60 minutes (mean of 0.40 mg/L, n = 296). In tepid and hot water, mean free chlorine results were systematically lower than 0.2 mg/L, the minimum concentration most frequently recommended in north American DWDSs (Health Canada, 2009; ASDWA, 2020), regardless of the sample type. Indeed, the proportion of first draws above the 0.2 mg/L threshold was 26%, 6% and 2% in cold, tepid and hot water, respectively. Flushing increased this ratio significantly ($p < 0.05$) in cold water only (83%), comparatively to tepid (15%) and hot water (1%). Similarly, the 0.5 mg/L threshold recommended in many *Legionella* prevention and control guidelines for

supplemental onsite disinfection (HPSC, 2009; ACHD et PRHI, 2014; HSE, 2014; VHA, 2022) was met in a lower proportion and mainly in cold water samples (first draws: 9%, flushed samples: 36%). These results are in accordance with previous studies in which disinfectant residuals were for the most part measurable when water temperatures were cold (Donohue et al., 2019; Salehi et al., 2020) or when devices were more frequently used (Lipphaus et al., 2014) or daily flushed (Grimard-Conea et al., 2023).

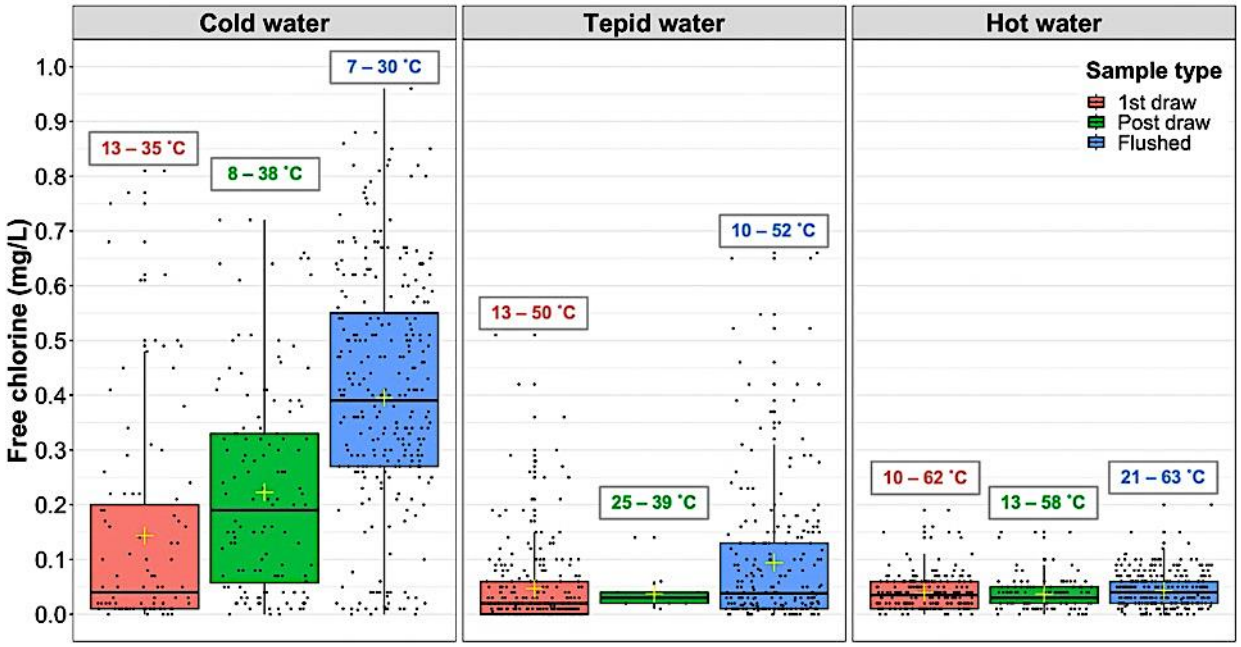

Figure 4.2 Free chlorine concentrations (n = 1,737) in first draws, post draws, and 2-60-min flushed samples from cold, tepid, and hot water points. Legend: Yellow cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Whiskers – 1.5 times the interquartile range, Black dots – Raw data.

Free chlorine concentration profiles in cold water for six points of use located in three of the nine buildings showed that even after a 30-min flush, chlorine values equivalent to the point of entry of each building were rarely reached (Figure 4.3). Indeed, free chlorine residuals remained 0.01 – 0.5 mg/L below what was measured at building inlets. Based their respective flowrates, some taps reached steady free chlorine concentrations within the first few liters of cold water being flushed (1 – 5 liters), whereas others necessitated several minutes of flushing before increases were observed. One point of use located far from its connecting cold water riser had a delay of about 15 min, corresponding to 200 liters of water, before a gradual increase in free chlorine was even measured. Furthermore, these profiles were obtained during normal occupation (Hospital C) or

minimum occupation (< 5%) in new (University building A) and old (University Building B) buildings. Flushing times required to reach conditions at the point(s) of entry were site-specific, thus contingent to the flowrate at which the point of use was being flushed and the pipe length between the building cold water riser and the device. Comparably to prior research also taking place in northern DWDSs (Ley et al., 2020; Salehi et al., 2020), incoming free chlorine concentrations from DWDSs varied markedly across seasons, with mean levels of 0.5 mg/L and 0.72 mg/L in warmer (April to October) and colder months (November to March), respectively. Overall, a moderate inverse and significant correlation between free chlorine and water temperature was found ($R = -0.53$, $p < 0.001$) (Figure A.1). As water temperatures increased and residual decay occurred, especially in hot water, no such clear relationship was observed due to the large number of samples with low (< 0.2 mg/L) to absent residuals (Figure A.2a).

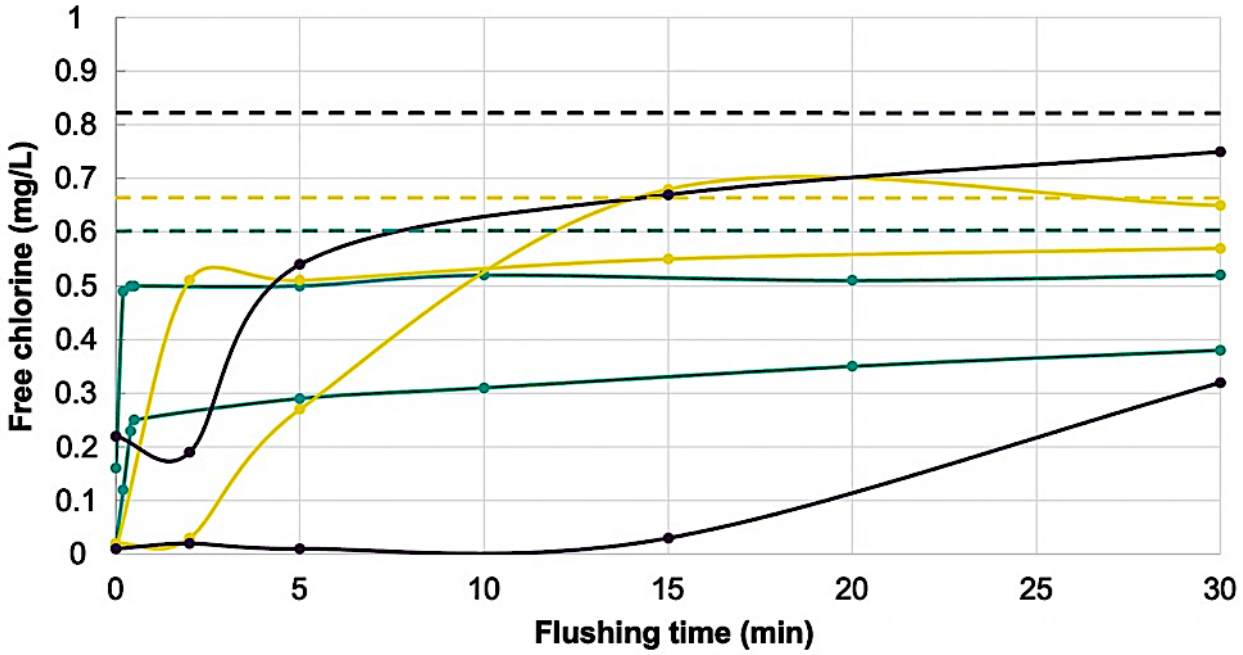

Figure 4.3 Free chlorine concentrations profiles in cold water (9 – 30 ˚C) over flushing time for six points of use located in Hospital C (blue-green lines), University Building A (yellow lines), and University Building B (purple lines). Note: dotted lines represent free chlorine concentrations at the point of entry of each building at the time of sampling.

4.3.2 Correlations between free chlorine and microbial densities

ATP and free chlorine concentrations showed a moderate and significant negative correlation in both first draws ($R = -0.38$, $p < 0.001$) and flushed samples ($R = -0.51$, $p < 0.001$) ($n = 475$) (Figure

4.4a). There was a clear trend of water samples with free chlorine residuals being lower than 0.2 mg/L harboring much higher ATP concentrations spanning up to 28 pg ATP/mL. Contrastingly, flushed water with a measurable free chlorine concentration (0.2 – 0.82 mg/L) was characterized by lower ATP concentrations (< 5 pg ATP/mL). Overall, ATP values in 92% of samples (437/475) were below 10 pg ATP/mL, a risk threshold suggested by manufactured ATP kits for drinking water applications. Similarly, concentrations of HPC and free chlorine showed a weak and inverse relationship in first draws ($R = -0.17$, $p = 0.02$), although it was only significant in flushed samples ($R = -0.33$, $p < 0.001$) ($n = 375$) (Figure 4.4b). The majority of samples with a free chlorine concentration higher or equivalent to 0.2 mg/L had HPC counts below 500 CFU/mL. Correlation results for both ATP and HPC were generally stronger in cold or tepid flushed samples, due to the higher free chlorine residuals measured compared to first draws and hot water samples (Figure A.2b and Figure A.2c).

When using 0.2 mg/L of free chlorine as a discriminatory concentration, significant ($p < 0.01$) differences in cell counts were observed. When free chlorine was below 0.2 mg/L, mean TCC and ICC concentrations were of 3.7E+05 cell/mL and 1.1E+05 cell/mL, respectively. Mean TCC and ICC concentrations were approximatively 1- and 2-logs lower when free chlorine was greater than 0.2 mg/L, regardless of the sample type. TCC and ICC negatively correlated to free chlorine residuals in both first draws (TCC: $R = -0.49$, $p < 0.001$; ICC: $R = -0.54$, $p < 0.001$) and flushed samples (TCC: $R = -0.63$, $p < 0.001$; ICC: $R = -0.57$, $p < 0.001$) (Figure 4.4c and Figure 4.4d). This was mainly driven by cold and tepid water samples (Figure A.2d and Figure A.2e). Similar correlation trends were reported in previous studies, both with data collected from DWDSs (Gillespie et al., 2014; Li et al., 2018b; Dias et al., 2019; Kennedy et al., 2021) and buildings (Ley et al., 2020; Greenwald et al., 2022), thus showing the impact of free chlorine on reducing bulk biomass in water.

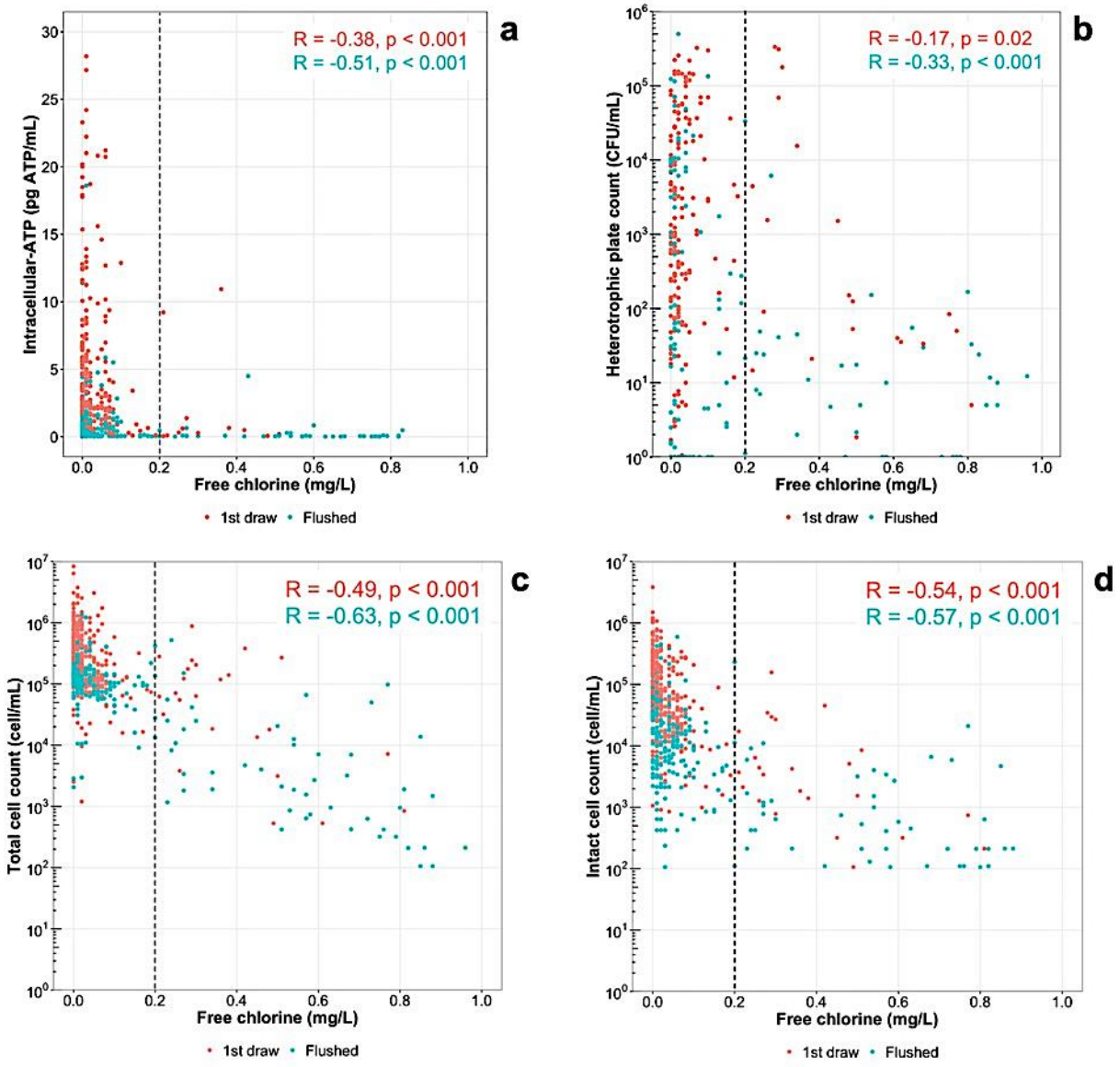

Figure 4.4 Scatter plots of free chlorine concentrations (x-axis) against (**a**) Intracellular-ATP (n = 475, 4 buildings), (**b**) Heterotrophic plate count (n = 375, 6 buildings), (**c**) Total cell counts (n = 574, 7 buildings), and (**d**) Intact cell counts (n = 563, 7 buildings) in first draws and flushed samples. Legend: Spearman rank correlation coefficient (R) and p-value (p) are given for first draws (red) and flushed samples (blue); black dotted line is the recommended free chlorine concentration in DWDSs.

4.3.3 Occurrence of OPs in bulk water

Detection of culturable *L. pneumophila* solely occurred when free chlorine residuals were below the 0.2 mg/L threshold (Figure 4.5a). Odds of detecting culturable *L. pneumophila* in first draws were 5.1 times (95% CI: 3.5 – 7.4) higher compared to flushed samples. Paired first draws and

flushed samples showed significantly ($p < 0.05$) higher concentrations in first draws, hence confirming the prevailing vulnerability of distal parts of building plumbing to *Legionella* growth. Concentrations spanned over a large range of values, from 10 to more than 10,000 MPN/L (or CFU/L), but all *L. pneumophila* positive samples were measured when water temperature was tepid or hot (Table 4.2). No samples with temperatures above 60˚C or below 20˚C were positive for culturable *L. pneumophila*, thus clearly demonstrating the benefits of maintaining an adequate thermal regime. Increasing temperature from the range 40 – 50 ˚C to 50 – 60 ˚C further reduced by almost half the ratio of positive samples. These results distinctively show zones of risk for *L. pneumophila* proliferation despite comparable mean and maximum concentrations among temperature ranges. Previous studies in residences and building (Ley et al., 2020; Julien et al., 2022), some with pre-flushing (Donohue, 2021), in DWDSs (LeChevallier, 2019) and in lab-scale glass water heaters (Martin et al., 2020) have failed to establish a satisfactory correlation between *L. pneumophila* (culture or molecular) and free chlorine. In this study, the small range of samples with a measurable free chlorine concentration as shown on Figure 4.5a limits the ability to establish such a correlation unless first draws and flushed samples are combined together. However, weak yet significant ($p < 0.001$) inverse correlations were observed in first draws ($R = -0.18$) and flushed samples ($R = -0.29$), separately (Figure 4.5a).

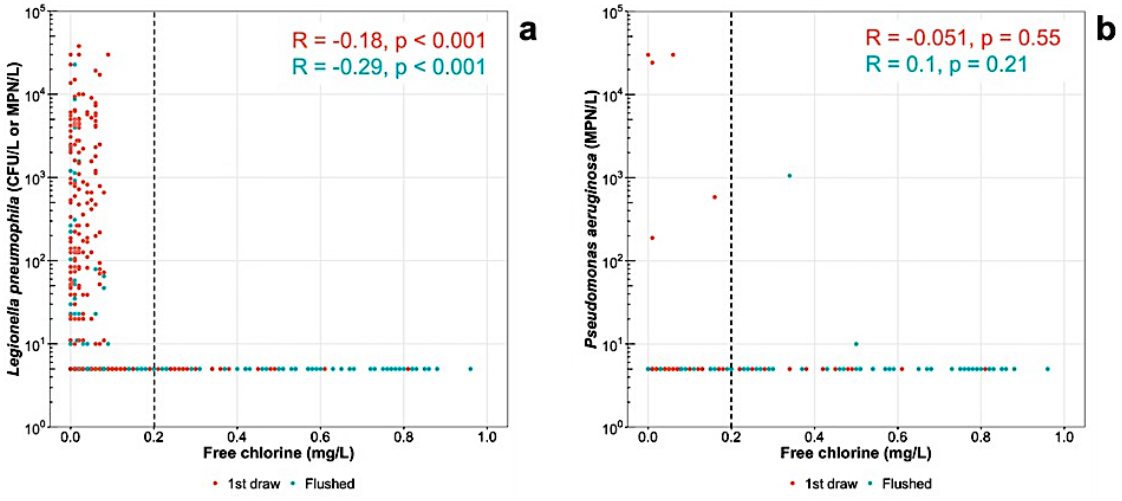

Figure 4.5 Scatter plots of free chlorine concentrations (x-axis) against (**a**) Culturable *Legionella pneumophila* (n = 809, 5 buildings) and (**b**) Culturable *Pseudomonas aeruginosa* (n = 286, 3 buildings) in first draws (red) and flushed samples (blue). Legend: black dotted line is the typical minimum desired free chlorine concentration in DWDSs.

No correlations with residual chlorine could be established for culturable *P. aeruginosa* in first draws and flushed samples, which is to be expected given the considerably lower subset of positive samples (7/286). Nonetheless, highest concentrations (over 10,000 MPN/L) were measured in water samples with barely detectable free chlorine residuals (0.01 – 0.06 mg/L) (Figure 4.5b), which were associated to tepid and hot water as well (Figure A.2g). Despite the presence of 0.34 and 0.50 mg/L of free chlorine in two cold water samples, culturable *P. aeruginosa* were still detected at 1,054 and 10 MPN/L, respectively. These observations suggest that positives resulted from the detachment of *Pseudomonas* spp. from the adjacent biofilm. Positivity in samples with higher disinfectant residuals is probably attributable to extracellular polymeric substances (EPS), which protect bacteria from environmental stressors such as chlorine (Bertelli et al., 2018; Leslie et al., 2021).

Table 4.2 Number and percentage of positive samples to culturable Legionella pneumophila (Lp) by temperature range in five building water systems (n = 809).

Culturable *Lp* (MPN/L or CFU/L)	Temperature range (°C)						
	0 – 10	10 – 20	20 – 30	30 – 40	40 – 50	50 – 60	60 – 70
Ratio of negative samples	100% (7/7)	100% (62/62)	71% (220/312)	78% (165/211)	52% (47/91)	74% (71/96)	100% (23/23)
Ratio of positive samples	0% (0/7)	0% (0/62)	29% (92/312)	22% (46/211)	48% (44/91)	26% (25/91)	0% (0/23)
Min culturable *Lp* value	N/A	N/A	1.00E+01	1.00E+01	1.00E+01	1.00E+01	N/A
Max culturable *Lp* value	N/A	N/A	3.80E+04	2.27E+04	1.92E+04	9.40E+03	N/A
Mean culturable *Lp* value	N/A	N/A	2.80E+03	2.81E+03	1.73E+03	2.38E+03	N/A

4.3.4 Statistical correlations among microbial parameters

Using all data points from the dataset, temperature was weakly but significantly ($p < 0.001$) correlated to ATP ($R = -0.16$), TCC ($R = -0.14$) and ICC ($R = -0.17$) (Figure A.1). Higher water temperatures were generally associated to lower microbial abundance, confirming the impact of thermal regime on the bulk biomass. ATP values were moderately and strongly correlated to ICC ($R = 0.65$) and TCC ($R = 0.56$) concentrations, respectively. However, HPC only correlated weakly to cell counts (ICC: $R = 0.16$; TCC = 0.26) despite a moderate relationship to ATP ($R = 0.43$). Both flow cytometry and ATP assays were thus more accurate and sensitive for the enumeration of microbial cells in drinking water than HPC which relies exclusively on cell culturability for

quantification (Siebel et al., 2008). Culturable *L. pneumophila* was significantly ($p < 0.01$) associated to ATP and ICC, although only to a very poor degree for both ($R = 0.12$) (Figure A.1). The significant association ($p < 0.001$) of culturable *P. aeruginosa* with ATP is driven by atypical high values of ATP (13 – 21 pg ATP/mL) in three positives samples, again suggesting detachment of biofilm.

4.4 Discussion

4.4.1 Challenges of maintaining free chlorine residuals in large building water systems

In this study, elevated temperatures (tepid or hot) and first draw samples (i.e., typically characterized by preceding short to long periods of distal stagnation depending on the usage pattern), were generally depicted by low (< 0.2 mg/L) or absent free chlorine residuals. These data suggest that minimum free chlorine levels recommended for the prevention of microbial regrowth in DWDSs (> 0.2 mg/L) cannot be reliably maintained in large buildings or for supplemental onsite disinfection in hot water systems. Consequently, building managers should not rely on incoming residuals nor on free chlorine generated *in situ* as a chemical barrier against microbial growth in their building water systems. The rapid decay of free chlorine in hot water and the frequent inter-use stagnation clearly limit the ability of chlorine residuals to persist as shown by the low residuals observed in hot water and in first draws (Figure 4.2). Although flushing water for 2 – 60 minutes increases free chlorine concentrations in cold water, conditions from DWDSs were rarely reached because of disinfectant decay occurring within the building plumbing. Profile samplings at a subset of cold water taps demonstrated that flushing times required to reach conditions nearing those from municipal service lines are site-specific and flushing can extend to more than 30 minutes (Figure 4.3). Moreover, residuals in the incoming water at the building entry point will likely show significant spatial and temporal variations as evidenced by several studies (Ley et al., 2020; Saetta et al., 2020; Salehi et al., 2020).

4.4.2 Free chlorine tends to decrease viable bulk biomass

The inverse relationships observed between free chlorine and general microbial parameters (ATP, HPC and flow cytometry cell counts) confirm that the presence of a chlorine residual contributes to decrease bulk viable biomass in building plumbing (Figure 4.4). Chlorination treatment typically

results in damaged cells, thus diminishing the fraction of culturable cells in the bulk water phase during distribution (Nescerecka et al., 2014), but has a minor impact on cells in plumbing biofilms (De Beer et al., 1994; Chen et Stewart, 1996). The consistent loss of residuals in distal ends and in tepid or hot water systems, where microbial amplification is more likely to occur (Lautenschlager et al., 2010; Lipphaus et al., 2014; Bédard et al., 2018), questions the value of solely relying on incoming residuals to prevent microbial regrowth in buildings. Chlorine concentration is a good predictor of microbial counts in DWDSs, thereby serving as a useful means of routine monitoring purposes (Prévost et al., 1998; Kennedy et al., 2021). In the case of building water systems such as those considered in this study, free chlorine concentrations were below the 0.2 mg/L threshold in the vast majority of samples. At these low chlorine concentrations, microbial concentrations varied across a very large range (ATP: 0 – 28 pg ATP/mL, HPC: 8-log span, flow cytometry counts: 5-log span). Therefore, the sole monitoring of residuals in buildings cannot be indicative of microbial risks, and should preferably be paired with other measurements for surveillance purposes or in water safety plans.

4.4.3 Implications for OPs prevention and control guidance in large buildings

In this study, free chlorine concentrations were generally negatively correlated to culturable *L. pneumophila* and *P. aeruginosa* (Figure 4.5). First draw samples and tepid to hot water temperatures (20 – 60 ˚C) were also associated to increased probability of detecting culturable *L. pneumophila* (Table 4.2). This suggests that cold water temperatures (< 20 ˚C) and the presence of free chlorine residuals (> 0.2 mg/L) prevented the growth of these OPs. The detection of OPs in the first draw of water despite their absence in municipal or cold water risers is indicative of operational and design plumbing concerns that are conducive to their proliferation as discussed previously (Charron et al., 2014; Bédard et al., 2016; Grimard-Conea et al., 2022; 2023). Considering that numerous factors can contribute to OPs presence in building plumbing and that free chlorine residuals are hardly maintained in tepid and hot water systems, free chlorine should not be reckoned as the sole parameter controlling their growth. Increasing free chlorine residuals from the utility is consequently not a suitable alternative for OPs mitigation in buildings, especially since it can increase markedly DBPs formation. The focus should rather be put towards other proven strategies, such as ensuring appropriate thermal control (Bédard et al., 2016; Ji et al., 2018; Cazals et al., 2022), balancing adequately hydraulics (Boppe et al., 2016), favoring taps with simple architecture and minimal plumbing material diversity (Charron et al., 2014), implementing *in situ*

monochloramine disinfection in buildings where vulnerable occupants are found (Duda et al., 2014; Baron et al., 2015; Mancini et al., 2015; Lytle et al., 2021), or remedial and preventative flushing (Hozalski et al., 2020; Grimard-Conea et al., 2022; 2023; Meegoda et al., 2023).

4.5 Conclusions and recommendations

Comprehensive review of 1,737 data points in nine large institutional buildings in Canada confirms the limited advantages of actively maintaining free chlorine residuals in building plumbing systems to limit microbial growth. While some benefits of ensuring sufficient residuals to inhibit *Legionella* growth were observed in cold water, this study highlights challenges that building managers face to maintain this residual throughout their tepid or hot water systems, up to water usage, to prevent general microbial proliferation and exposure to OPs.

- In hot water, free chlorine concentrations were mostly undetectable due to fast depletion with high temperature. Building managers cannot rely on the presence of incoming residuals for the prevention and control of microbial amplification in these systems. Proven alternatives for OPs mitigation are needed in hot water systems, such as ensuring an adequate and balanced thermal regime (> 55˚C) or implementing onsite supplemental disinfection with disinfectants that can actively persist in hot water, most especially where vulnerable occupants are present.
- In tepid water, free chlorine concentrations were hardly maintained in first draws (6% of samples were above 0.2 mg/L). Flushing increased this ratio to 15%, but it was largely dependent on the temperature setting of the point of use. Therefore, the focus should be put towards minimizing the surface area available for biofilm growth and the diversity of materials found in plumbing devices that provide tepid water, as well as the volume of tepid water between sites and cold and hot risers.
- In cold water, free chlorine residuals complied with the 0.2 mg/L and 0.5 mg/L thresholds in 26% and 9% of first draws, respectively, whereas flushed samples met these levels in 83% and 36% of data points. However, profile sampling of a subset of cold water taps located in three different buildings demonstrated that conditions from service lines were rarely reached and were site-specific. Frequent flushing of taps would be necessary to replenish stagnant water with depleted free chlorine residuals.

- Negative relationships between free chlorine and ATP, HPC, TCC and ICC were observed, thus suggesting that the presence of free chlorine residual was beneficial to suppress microbial abundance and viability in the bulk water. However, these correlations were mostly driven by cold flushed water samples, therefore highlighting the microbial amplification occurring most particularly at distal sites of the building plumbing and in taps with higher water temperatures. Although providing a good assessment of bulk water dynamics with free chlorine and temperature changes, these parameters could not predict with robustness the presence of OPs in building plumbing.
- Free chlorine concentrations over 0.2 mg/L generally resulted in undetectable culturable *L. pneumophila* and *P. aeruginosa*. Similarly, water temperatures outside the range 20 – 60 ˚C completely prevented the detection of *L. pneumophila*. Despite the absence of culturable OPs from service lines, findings from this study confirm that conditions within plumbing systems were conducive for OPs growth and temperature played a more significant role than free chlorine for their prevention. As free chlorine decays rapidly in buildings, water utilities only have a limited power of action to prevent OPs growth at the point of use, thus pinpointing the paramount importance of rather adequately operating and designing buildings.

Credit authorship contribution statement

Marianne Grimard-Conea: Conceptualization, Data collection, Formal analysis, Visualization, Writing-original draft, Writing-review & editing. Emilie Bédard: Conceptualization, Data collection, Writing-review & editing. Michèle Prévost: Conceptualization, Funding acquisition, Writing-review & editing.

Declaration of competing interest

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

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Appendix. Supplementary data

Additional figures presented in this article are available as Supplementary Materials in Appendix A.

CHAPTER 5 ARTICLE 2: IMPACT OF RECOMMISSIONING FLUSHING ON *LEGIONELLA PNEUMOPHILA* IN A LARGE BUILDING DURING THE COVID-19 PANDEMIC

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Recommissioning flushing has been widely recommended as the first level of action to prevent growth of *Legionella* in guidance documents developed for the safe reopening of building plumbing systems during the COVID-19 pandemic. However, the short- and long-term impacts of this intervention on the occurrence of *Legionella pneumophila* remain poorly investigated.

This study evaluated the dynamic changes in common bacterial indicators (ATP, total and intact cell counts) and *Legionella pneumophila* at 20 – 22 showerheads of a large sports complex. Sampling was conducted after a 16-week building closure, then shortly (24h) and four weeks following device recommissioning flushing of high-risk water devices, with no shower water usage between events. Findings highlight the limited effectiveness of device recommissioning flushing in reducing the occurrence and abundance of *Legionella pneumophila* over periods of prolonged distal stagnation.

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

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Abstract

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 (24h) 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 X 10^6 count/mL) and ICC (5.2 X 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 five minutes 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 (24h) 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 one 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.

5.1 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 (< 24h) 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 Québec, 2020; Proctor et al., 2020; CDC, 2021b). 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, 2021; 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 three 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 four 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 two weeks for flushing after which disinfection must be (re)conducted (ASHRAE, 2018; Proctor et al., 2020; CSA, 2021).

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 Québec, 2020; Proctor et al., 2020; CDC, 2021b). 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 to 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 towards *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- (24h) 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.

5.2 Materials and methods

5.2.1 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 Cl2/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 13th to July 6th, 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 (Figure B.1A, Figure B.1B). These showerheads were manually activated by a pressure-button with each activation resulting in a flow lasting 10 seconds to one minute. 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 (Figure B.1C, Figure B.1D, Figure B.1E).

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 TMW to each timer valves, and flexible plastic hoses from the latter to each showerhead.

5.2.2 Sampling and targeted recommissioning flushing timelines

Sampling was first performed on July 2nd, 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 (Figure B.1). On July 14th, 2020, all showerheads (n = 114) connected to the building plumbing system, including the ones investigated in this study, were successively flushed for five minutes 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 Québec, 2020; Proctor et al., 2020; CDC, 2021b). 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 (24h) 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 19th, 2020, to assess the long-term (four weeks) impacts of targeted recommissioning flushing with no regular water use at showers. One showerhead from locker room E (Figure B.1C) 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 19th, 2020), without shower usage in the meantime, to measure total and intact bacterial cell counts after respective periods of distal water stagnation of two and five months. Profile flushing duration was based on the mitigated water volume between the outlet and the TMV (six liters) in the small grouped shower system (Figure B.1E) and on the volume between the outlet and the hot water recirculation line (250 liters) in the large grouped shower system (Figure B.1A). Profile sampling was repeated after a successive one-hour distal water stagnation.

5.2.3 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 (one liter) and five-minute flushed samples (one liter) 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.

5.2.4 Microbiological measurements processing

5.2.4.1 Culture-based enzymatic *Legionella 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 hours 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 ± 0.5 ˚C for seven days. Results were latterly determined by counting positive wells and expressed as most probable number (MPN) per liter concentrations (10 to 22,726 MPN/L).

5.2.4.2 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® 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 µm (Ø 33 mm) PES sterile membranes (CLEARLine® 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™ apparatus (Kikkoman Corp., Noda, Japan). The kit had a detection limit of 0.1 pg ATP/mL, and samples were processed within 12 hours of sampling.

5.2.4.3 Total and intact cell counts by flow cytometry

Within 24 hours of sampling, aliquots of approximatively five mL per sample were set aside in sterile tubes to assessed total and intact bacterial cell counts through flow cytometry on the BD Accuri™ C6 Plus Flow cytometer, along with the automatic sampling BD CSampler™ arm (BD-Biosciences, Mississauga, ON, Canada). Flow cytometry assays were performed according to Cazals and colleagues (2022). A volume ratio of water sample to reagent of 300 µL: 3 µL was used for co-staining samples firstly with the SYBR® 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 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.

5.2.4.4 DNA extraction and qPCR *Legionella pneumophila*

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

5.2.5 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 and colleagues (2019) to the specifics of the investigated building. A breathing rate associated to a moderate activity (U.S. EPA, 2011) was considered since users typically take a shower after practicing physical activities, as well as a shower duration of 6.8 minutes represented by a right-skewed lognormal distribution (Wilkes, 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 and colleagues (2019) were used accordingly to the R script code published by Hozalski and colleagues (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.

5.2.6 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).

5.3 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 five-minute flush samples were collected after a 16-week building shutdown, shortly (24h) 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 material (Table B.1).

5.3.1 Trends in bacterial indicators

Measured bacterial indicators followed similar trends during the study. The highest mean bacterial-ATP (10.0 pg ATP/mL) (Figure 5.1A), total cell counts (1.7 X 10^6 count/mL) (Figure 5.1B) and intact cell counts (5.2 X 10^5 count/mL) (Figure 5.1C) were measured in first draws (n = 21) after the prolonged 16-week building closure (Figure 5.1). These indicators respectively reached values as high as 24.2 pg ATP/mL, 3.8 X 10^6 count/mL, and 1.1 X 10^6 count/mL (Table B.1). Targeted recommissioning flushing resulted in significant ($p < 0.001$) reductions in these microbiological concentrations in first draw samples collected 24 hours 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 – 54% (Figure 5.1D).

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 hours 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 to 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.

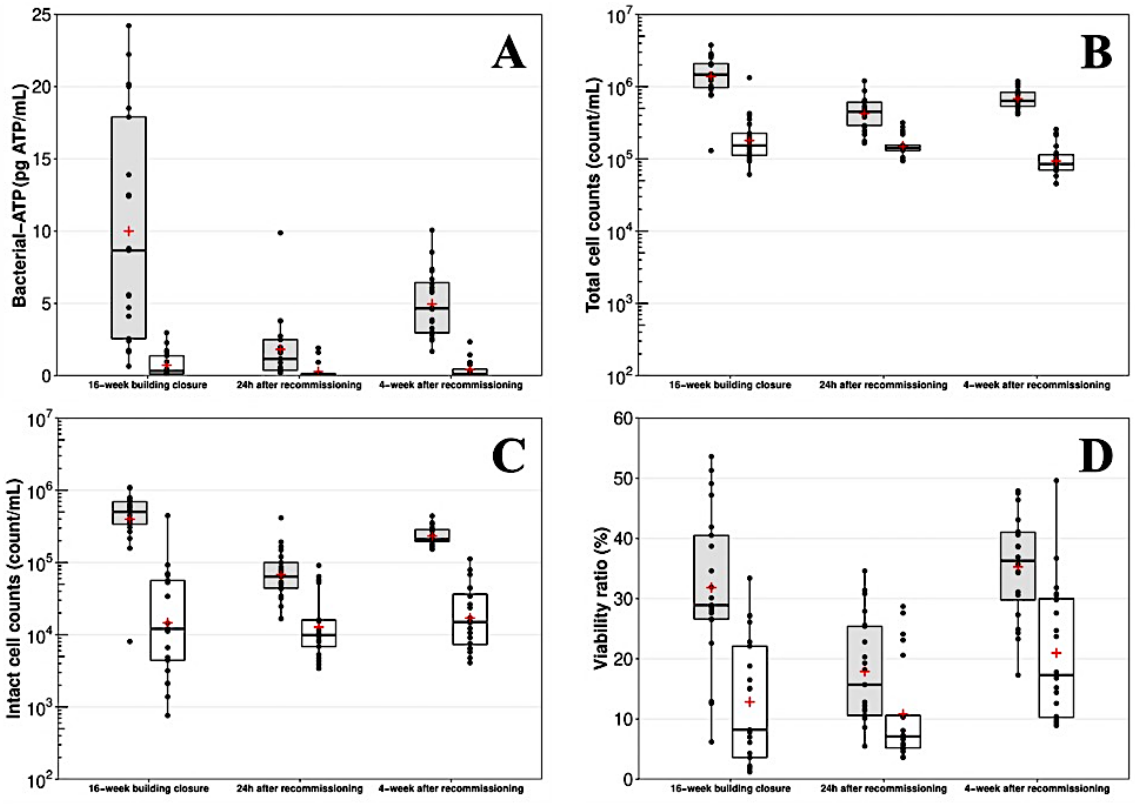

Figure 5.1 Box plot representations of (**A**) bacterial-ATP, (**B**) total cell counts, (**C**) intact cell counts, and (**D**) viability ratios, per sampling period. Gray bars – First draw samples (n = 20 – 21); White bars – Five-min flushed samples (n = 20 – 21); Horizontal black line – Median; Red cross – Mean; Boxes – 25 – 75th percentiles; Whiskers – Minimal and maximal values; Black dots – Raw data.

5.3.2 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 (Figure B.1E) and one large grouped shower system (Figure B.1A) were sampled again after respective periods of distal water stagnation of two and five months. Profile sampling of mitigated water collected from both showerheads after prolonged distal water stagnation (Figure 5.2A, Figure 5.2B) 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 (Figure 5.1B, Figure 5.1C). Flushing the mitigated water volume of six 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 5.2A). In the large grouped shower system, total and intact cell numbers were more markedly reduced, respectively by 1.7- and 2.7-log (Figure 5.2B), as water was flushed until reaching the hot water recirculation line (250 liters). Following a one-hour 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 one hour 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 5.2A), as well as incidental increases in the showerhead from the large grouped shower system (Figure 5.2B). 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.

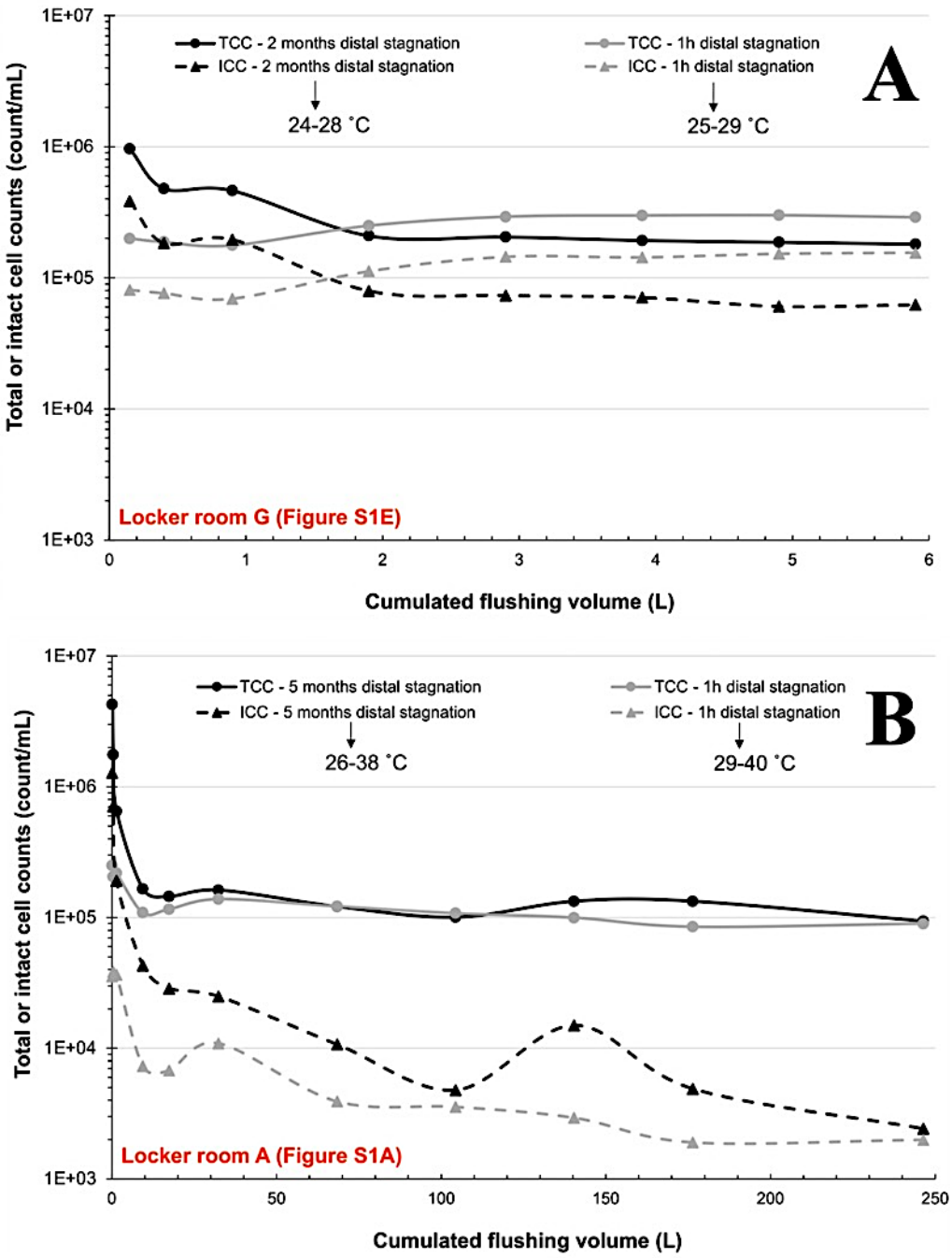

Figure 5.2 Total (TCC) and intact cell counts (ICC) profiles over cumulated flushing water volumes in two showerheads from a (**A**) small grouped shower system and a (**B**) large grouped shower system.

5.3.3 *Legionella pneumophila* occurrence in showerheads

Throughout the study period, highest mean culture-based (Figure 5.3A) and qPCR *L. pneumophila* concentrations (Figure 5.3B) 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 hours 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).

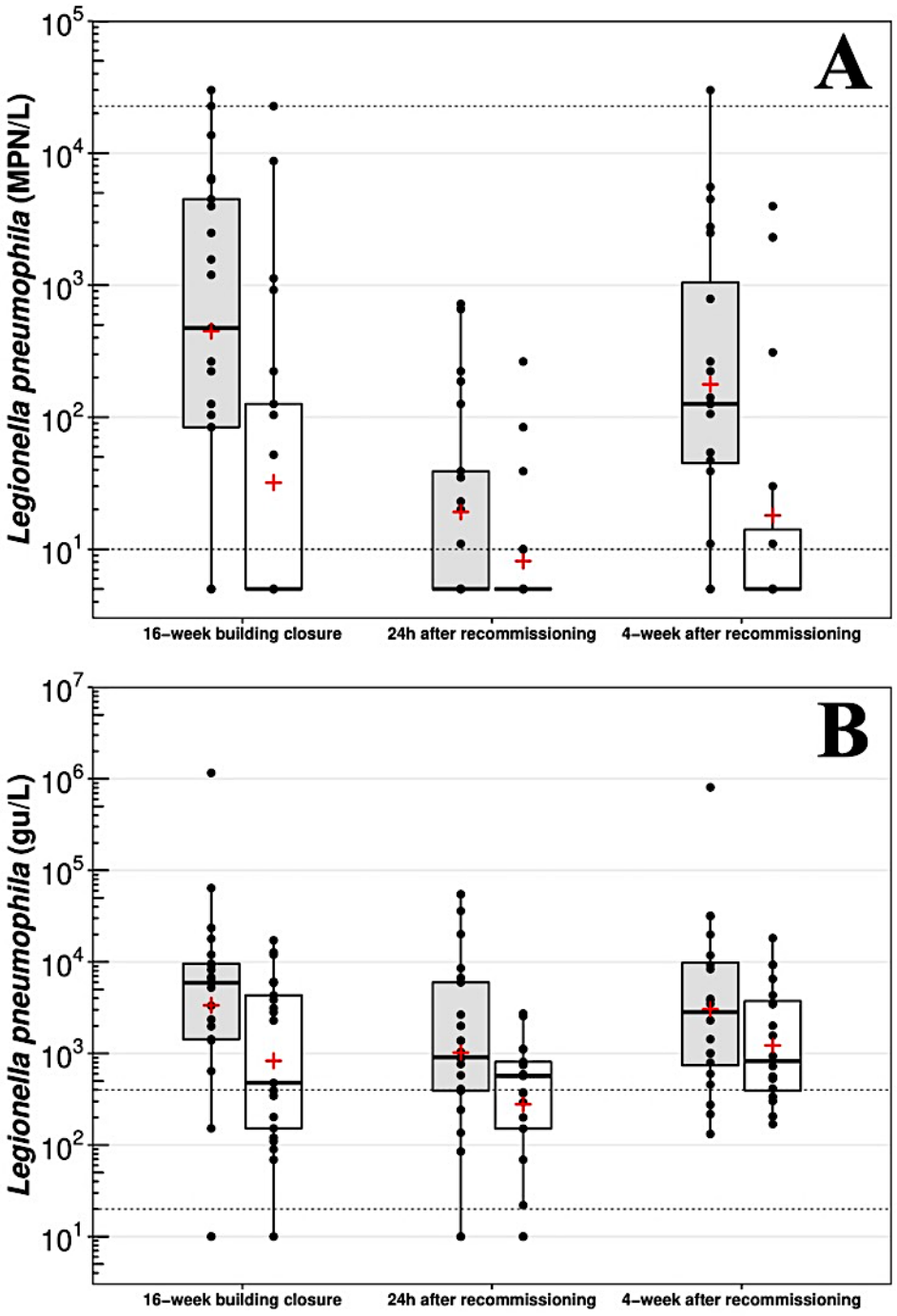

Figure 5.3 Box plot representations of (**A**) culture-based *Legionella pneumophila* and (**B**) qPCR *Legionella pneumophila*, per sampling period. Gray bars – First draw samples (n = 20 – 21); White bars – Five-min flushed samples (n = 20 – 21); Horizontal black line – Median; Red cross – Mean; Boxes – 25 – 75th percentiles; Whiskers – Minimal and maximal values; Black dots – Raw data; Horizontal dotted lines – Limit of detection (LoD) or limit of quantification (LoQ). Any sample

with a culturable 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.

Five water samples (out of 124) had a higher culture-based concentration of *L. pneumophila* than its qPCR counterpart, as likewise reported by Dowdell and colleagues (2022) (manuscript submitted to Environmental Science & 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- to 4.0-log and 0.4- to 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 5.4).

Overall, individual flushing of showers for five minutes 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 5.3A) and qPCR *L. pneumophila* concentrations (Figure 5.3B). 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 5.1). 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 five-minute 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 5.1).

Table 5.1 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	4/21	13/21	11/21	16/21	3/20	14/20
($< 10^1$)	(19.1%)	(61.9%)	(52.4%)	(76.2%)	(15.0%)	(70.0%)
"Under control"	7/21	5/21	10/21	5/21	12/20	3/20
($10^1 \leq Lp < 10^3$)	(33.3%)	(23.8%)	(47.6%)	(23.8%)	(60.0%)	(15.0%)
Alert level	7/21	2/21	0	0	4/20	3/20
($10^3 \leq Lp < 10^4$)	(33.3%)	(9.5%)			(20.0%)	(15.0%)
Action level	3/21	1/21	0	0	1/20	0
($\geq 10^4$)	(14.3%)	(4.8%)			(5.0%)	
Total positive	17/21	8/21	10/21	5/21	17/20	6/20
($\geq 10^1$)	(81.0%)	(39.0%)	(47.6%)	(23.8%)	(85.0%)	(30.0%)

5.3.4 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 (Figure B.1A, Figure B.1B), and small grouped shower systems in which a small number of showerheads (n < 4) were fed by a single TMV (Figure B.1C, Figure B.1D, Figure B.1E). 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 six 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.

5.3.5 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) (Figure B.2A), but a weak (rho = 0.32, n = 62), yet significant ($p < 0.05$), correlation in flushed ones (Figure B.2B). 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 (Table B.1). 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) (Figure B.2A) and in flushed samples (rho = 0.31, n = 62) (Figure B.2B). 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 (Figure B.3). 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 (Figure B.2A), no such outcome was observed in the set of flushed samples.

5.3.6 Quantitative microbial risk assessment for *Legionella pneumophila*

Mean of per exposure risk and DALY values calculated with culture-based concentrations of *L. pneumophila* through the QMRA approach are available in Table B.2. Overall, reported values displayed similar trends than *L. pneumophila* concentrations. Throughout the study, individual flushing of showerheads for five minutes 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 1.4×10^{-2} and 2.6×10^{-3} for conventional (> 13 lpm) and low-flow (< 7 Lpm) showerheads, whereas these values were reduced to 4.1×10^{-3} and 7.8×10^{-4} after a brief five-minute flush. Rather considering a Legionnaires' disease endpoint, per exposure mean risk values for first draw samples were of 1.4×10^{-5} and 2.6×10^{-6} for conventional and low-flow showerheads, and were subsequently lowered to 4.7×10^{-6} and 8.8×10^{-7} (Table B.2).

5.4 Discussion

5.4.1 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 5.1A) were measured in first draw samples, exceeding previously reported values for potable water in the built environment in Europe (Duda et al., 2015; Lautenschlager et al., 2010; 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 X 10^6 count/mL (Figure 5.1B) and 5.2 X 10^5 count/mL (Figure 5.1C) in first draw samples after the 16-week building closure and bounced back to 7.2 X 10^5 count/mL and 2.4 X 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; Montagnino et al., 2022; Rahmatika et al., 2022; Zlatanović et al., 2017). 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 5.1D) were in agreement with prior observations of first draw samples in chlorinated water containing 15 – 41% of viable cells after 24h to 10 days of distal water stagnation (Bédard et al., 2018).

Surprisingly, results of ATP-per-cell (Table B.1) 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) (Lautenschlager et al., 2010; Siebel et al., 2008). 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 versus 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 and colleagues (2018) suggest that extended stagnation from one-hour to four months can lead to very high levels of total and intact cell counts at the point of use and its immediate upflow plumbing.

5.4.2 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 hours) 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 hours). 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 and colleagues (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 one-hour (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 two and five months. Typically, meaningful bacterial growth is unlikely to be significant in less than one hour 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 (Figure 5.2A, Figure 5.2B). 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 one-hour periods of no use (Figure 5.2A, Figure 5.2B). 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 (Figure B.1A), was more beneficial at reducing total and intact cell counts (Figure 5.2B) than profiled flushing only until the TMV (six liters) (Figure 5.2A).

5.4.3 Widespread occurrence of *Legionella pneumophila* in showerheads

The high positivity rates of *L. pneumophila* by culture-based method and qPCR results (Table 5.1) 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 & 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) (Rhoads et al., 2022; De Giglio et al., 2020).

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 nine 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.

5.4.4 Interpretation of *Legionella 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 and colleagues (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 (Table B.2). However, flushing these showerheads for five minutes 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.

5.4.5 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 nine months.

To identify the cause of the high prevalence of *L. pneumophila* observed in this building, several investigations were conducted. As proposed by Bédard and colleagues (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 Québec (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 seconds, and preferably under 30 seconds (Singh et al., 2022). However, in the most distant large grouped shower system from the hot water recirculation line, it took 30 minutes 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 to 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 three liters or an elapsed time of 30 seconds 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.

5.4.6 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 (Figure B.2A, Figure B.2B), 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% versus 25%) and flushed samples (32% versus 13%) (Figure B.3). 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 modelled by a linear regression (Figure 5.4). 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- to 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 et Taylor, 2014; Dowdell et al., 2022, submitted to Environmental Science & Technology).

5.4.7 Implications for flushing guidance recommendations

5.4.7.1 Impact of distal flushing on microbiological measurements

In this study, flushing showerheads for five minutes with mitigated water produced clear and immediate benefits on a microbiological perspective. Overall, bacterial-ATP levels were lowered by 1.2 to 278-fold in flushed samples (Figure 5.1A), whereas total (Figure 5.1B) and intact (Figure 5.1C) bacterial cell counts were on average 1.1- and 0.7-log lower than in first draw samples. The proportion of intact cells was sightly lowered by about 14% (Figure 5.1D). 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 artefacts 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 (Bédard et al., 2018; Lautenschlager et al., 2010; Lipphaus et al., 2014), although values observed in this study remained slightly higher.

Consistent trends were also observed for culture-based (Figure 5.3A) and qPCR concentrations of *L. pneumophila* (Figure 5.3B) 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 (Table B.2). 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 five minutes after extended stagnation can be examined by excluding data collected 24 hours 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 five 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- to 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 (Hozalski et al., 2020; Richard et Boyer, 2021; Wang et al., 2012), 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).

5.4.7.2 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 5.1A), total (Figure 5.1B) and intact (Figure 5.1C) cell counts, viability ratios (Figure 5.1D), and culture-based *L. pneumophila* concentrations (Figure 5.3A) were observed in first draw samples collected shortly (24h) after the targeted recommissioning flushing intervention. More importantly, culture-based *L. pneumophila* concentrations were lowered below the 1,000 MPN/L alert threshold (Table 5.1) in all first draw samples. However, despite small decreases, qPCR concentrations (Figure 5.3B) 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 5.1).

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 5.1) and *L. pneumophila* loads (Figure 5.3) 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 – 24 days) thereafter flushing points of use for 45 minutes (Hozalski et al., 2020). These increases in distal parts of the building plumbing system could either reflect bacterial growth occurring under stagnant conditions (Bédard et al., 2018; Lautenschlager et al., 2010; Peng et al., 2020; Rahmatika et al., 2022; Zlatanović et al., 2017), 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 5.2). It is likely that the water turnover resulting from the recommissioning flushing intervention repleted 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; Buse et al., 2020; Donohue et al., 2018). Generally, taps which were persistently positive for the presence of *L. pneumophila* tended to harbor higher concentrations of the bacteria (Buse et al., 2020; Donohue et al., 2018), 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.

5.4.7.3 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; CDC, 2021b; CSA, 2020; Government of Québec, 2020; Proctor et al., 2020). 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 ˚C), then with a cold-water residual (> 0.5 mg Cl_2/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 one or two 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.

5.5 Conclusions

- The present study is the first to evidence the high prevalence and prolonged culturability (four to nine months) of *Legionella 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 two weeks). Mean values of *Legionella 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 nine 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 *Legionella 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 five minutes resulted in significant ($p < 0.05$) decreases in all measured bacterial indicators. Culture-based and qPCR concentrations of *Legionella pneumophila* were also reduced by respectively 0.8- and 0.5-log on average. Profile sampling showed that prolonging flushing from one minute to 35 minutes provided additional reductions of concentrations of total and intact cell counts.
- Targeted recommissioning flushing was beneficial at short-term (24h) to lower risks as all showerheads remained under the common alert threshold of 1,000 MPN/L for the monitoring of *Legionella 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 *Legionella 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 constructed as a potential conflict of interest.

Publisher's note

Supplementary material

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

CHAPTER 6 ARTICLE 3: CONTROLLING *LEGIONELLA PNEUMOPHILA* IN SHOWERHEADS: COMBINATION OF REMEDIAL INTERVENTION AND PREVENTIVE FLUSHING

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Shock chlorination and flushing are widely used to mitigate *Legionella pneumophila* contamination in building plumbing systems, including during system (re)commissioning. However, while the long-term persistence of *Legionella pneumophila* post-remediation has been documented, there is limited data on the short-term (less than one month) efficacy of these interventions, especially under varying water use patterns typical of building plumbing systems.

This study evaluated the short-term (3-week) weekly effectiveness of combining shock chlorination (20 – 25 mg/L of free chlorine for 16h) or device recommissioning flushing (five minutes at each showerhead) with different preventive flushing regimes (daily, weekly, stagnant). The assessment was conducted at duplicates of showerheads in two large grouped shower systems with a known *Legionella pneumophila* contamination issue within a large sports complex. Findings highlight the most effective combinations of corrective and preventive strategies to mitigate growth of *Legionella pneumophila* at distal sites, thus providing valuable insights for risk management pending the implementation of additional engineering controls or building-wide solutions.

Controlling *Legionella pneumophila* in showerheads: combination of remedial intervention and preventative flushing

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Abstract

Shock chlorination and remedial flushing are suggested to address *Legionella pneumophila* (*Lp*) contamination in buildings or during their (re)commissioning. However, data on general microbial measurements (adenosine tri-phosphate [ATP], total cell counts [TCC]), and the abundance of *Lp* are lacking to support their temporary implementation with variable water demands. In this study, the weekly short-term (3-week) impact of shock chlorination (20 – 25 mg/L free chlorine, 16h) or remedial flushing (5-min flush) combined with distinct flushing regimes (daily, weekly, stagnant) was investigated in duplicates of showerheads in two shower systems. Results showed that the combination of stagnation and shock chlorination prompted biomass regrowth, with ATP and TCC in the first draws reaching large regrowth factors of 4.31-7.07-fold and 3.51-5.68-fold, respectively, from baseline values. Contrastingly, remedial flushing followed by stagnation generally resulted in complete or larger regrowth in *Lp* culturability and gene copies (gc). Irrespective of the intervention, daily flushed showerheads resulted in significantly ($p < 0.05$) lower ATP and TCC, as well as lower *Lp* concentrations than weekly flushes, in general. Nonetheless, *Lp* persisted at concentrations ranging from 11 to 223 as the most probable number per liter (MPN/L) and in the same order of magnitude (10^3 – 10^4 gc/L) than baseline values after remedial flushing, despite daily/weekly flushing, unlike shock chlorination which suppressed *Lp* culturability (down 3-log) for two weeks and gene copies by 1-log. This study provides insights on the most optimal short-term combination of remedial and preventative strategies that can be considered pending the implementation of suitable engineering controls or building-wide treatment.

Keywords: *Legionella pneumophila*; building plumbing; flushing; chlorination; stagnation.

6.1 Introduction

Legionella pneumophila is an opportunistic pathogen that can be distributed in potable water systems and is transmitted through the inhalation of contaminated water aerosols, causing either Pontiac fever, a milder form of the infection, or Legionnaires' disease, a severe pneumonia-like infection in vulnerable or immunocompromised individuals (CDC, n.d.). In the last decades, *Legionella* infections, and more specifically Legionnaires' disease, were associated with an increasing health burden, reflected in a large number of hospitalizations and deaths (Collier et al., 2021).

Water safety plans (WSP) rely on a multi-barrier approach including the implementation of control strategies and environmental monitoring to manage risks associated with water in building plumbing (Gamage et al., 2021). Mostly in response to positive *Legionella* water samples or in the aftermath of nosocomial Legionnaires' disease cases, corrective actions are performed to suppress its growth. Among emergency measures, shock chlorination with free chlorine concentrations exceeding maximum allowable levels for drinking water (10 – 50 ppm) over a more or less prolonged contact time period (1 – 24h) is commonplace (PWGSC, 2016; U.K. Department of Health, 2016; ESCMID, 2017; CDC, 2019; VHA, 2022), and even more so when thermal shock conditions (65 – 75 °C) cannot be applied (Lin et al., 2011). In building plumbing, several studies have nevertheless reported the long-term persistence of *Legionella* bacteria despite the implementation of repeated shock chlorination as a remedial treatment (Cooper et al., 2008; García et al., 2008; Orsi et al., 2014). Recolonization or long-term recalcitrant *Legionella* positivity have been mostly attributed to (1) the limited penetration rate of chlorine into pipe bio-films (Chen et Stewart, 1996; De Beer et al., 1994), (2) the intracellular protection of *Legionella* within protozoan hosts (Kilvington et Price, 1990; Storey et al., 2004; García et al., 2007; Dupuy et al., 2011), (3) the intrinsic resistance of *Legionella* to chlorine (NASEM, 2019), and (4) the difficulty to reach tar-get-free chlorine concentrations at all distal outlets in large, legacy, and complex buildings (Muraca et al., 1990; Wiedenmann et al., 2001).

WSP and *Legionella* regulation guidelines further advocate for preventative (routine) flushing of water points that are irregularly used, although, if even mentioned, frequencies, duration, and flow conditions vary greatly. Some prescribe a weekly flush of taps (HPSC, 2009; Ministry of Health of New Zealand, 2012; Government of South Australia, 2013; HSE, 2014; Hong Kong

Government, 2016; ESCMID, 2017; Australian Government, 2015; OFSP et OSAV, 2018; ASHRAE, 2020), whereas others advise for more frequent flushes such as flushing on a daily basis (HPSC, 2009; Haut Conseil de la Santé Publique, 2013) or twice a week to prevent water stagnation (HSE, 2014; VHA, 2022). Nonetheless, there is yet to find a consensus on the most suitable flushing regime that prevents or controls the growth of *Legionella*. In one hospital building plumbing system, Gavaldà and colleagues demonstrated that the probability of recovering culturable *Legionella* in taps with occasional water use was multiplied by more than two-fold as compared to taps used on a daily basis (Gavaldà et al., 2019). However, the statistical analysis seemed rather based on qualitative observations, nor was there any indication on the amount of water necessary to account for daily or occasional water usage. In another hospital setting, Totaro and colleagues observed a dramatic reduction in *L. pneumophila* serogroups 2 – 14 in semi-flushed hot water samples after chlorine dioxide residuals were maintained (0 – 0.3 mg/L) by sectorial preventative flushing. A high flushing frequency of one minute every two hours at all five dead-end branches of the hospital water network was required to be effective, while flushing every six hours was not (Totaro et al., 2020). Similarly*, Legionella pneumophila* concentrations were found to be lowered by 6.3-fold in high water use taps (21 flushes/week) in a pilot-scale study as opposed to low water use taps (one flush/week) (Rhoads et al., 2015b). Such higher water use frequencies are, however, impractical in the context of routine manual flushing of taps, requiring instead the use of more expensive auto-flush devices.

Similar strategies were also put forward by local jurisdictions worldwide to reduce microbial risks associated with stagnant water during COVID-19 pandemic building shutdowns. Flushing to renew aged water with fresh water as a one-time event (re-commissioning) or with repeated routine flushing was frequently suggested in guidance documents (AWWA, 2020; CSA, 2020; Government of Québec, 2020; Proctor et al., 2020; CDC, n.d.), but showed only temporary benefits (Hozalski et al., 2020; Greenwald et al., 2022; Grimard-Conea et al., 2022; Rhoads et al., 2022). Moreover, shock chlorination was recommended if (1) occupants vulnerable to waterborne diseases were returning to the building (Connecticut Department of Public Health, 2020; CSA, 2020; Ohio Environmental Protection Agency, 2020; Washington State Department of Health, 2020; Government of Ontario, n.d.), (2) a depressurization incident occurred during the closure (Proctor et al., 2020), (3) the hot water system was turned off for energy conservation purposes during the closure (ESCMID, 2020), or (4) there was a strong suspicion or testing confirmation of

microbial contamination (e.g., to *Legionella*) (Connecticut Department of Public Health, 2020). The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) study group for *Legionella* infection further recommended shock chlorination with 50 ppm of free chlorine prior to building shutdown if water systems were to be drained, or if they had not recently been disinfected or experienced operational issues with temperature and disinfectant residuals (ESCMID, 2020).

Additionally, water flushing and shock chlorination of building plumbing are practices commonly recommended in the American Society of Heating, Refrigerating and Air-conditioning Engineers (ASHRAE) Standard 188 during the commissioning of new building plumbing (ASHRAE, 2018). If building occupancy is postponed between two and four weeks following shock chlorination, ASHRAE then requires a thorough flush of all water fixtures. If the delay exceeds four weeks or more, the need for shock chlorination, flushing, or both shock chlorination and flushing of unoccupied sectors should be re-evaluated. In the province of Québec (Canada), small seasonal building systems (e.g., managed by national parks) typically undergo flushing and shock chlorination before seasonal reopening (Deshommes et Sauvageau, 2021), as per required as well by the Revised Total Coliform Rule in the United States (U.S. EPA, 2015). However, it remains unclear how remedial or preventative flushing as well as shock disinfection procedures should be applied in the context of existing buildings undergoing construction, renovation, and demolition activities, which are fairly recurrent in large and older building plumbing. Although not every construction activity involves building plumbing, they can still lead to the closure of sectors of the building for a short to a prolonged period of time, followed by the gradual incoming of occupants. More specifically, Scanlon and colleagues highlighted the lack of prevention strategies including specific practices to tackle water management before, during, and after the completion of such activities to address the high prevalence of nosocomial waterborne infections associated to these activities for patients with overnight stays (Scanlon et al., 2020).

Studies with controlled variable water use patterns mimicking different water demand following either (re)commissioning flushing or shock chlorination are lacking to study their short-term efficacy towards the inactivation of *Legionella* and broader microbial indicators. Therefore, the main aim of this study was to assess the short-term (three weeks) effectiveness of the combination of remedial flushing or shock chlorination with preventative flushing (daily, weekly, left stagnant) on the occurrence of planktonic *L. pneumophila* and other microbial measurements in shower

systems. It was hypothesized that such combinations of remedial and preventative actions can only temporarily limit the regrowth of *L. pneumophila* and that daily flushes are more beneficial at maintaining lower microbial loads.

6.2 Materials and Methods

6.2.1 Shower System Description

Two large, grouped shower systems in which a single bimetallic strip thermostatic mixing valve (TMV) feeds 20 to 22 showerheads at once with mitigated water were selected. Shower systems and building plumbing were described in a previous study (Grimard-Conea et al., 2022). Both shower systems were left completely unused for 12 weeks (distal stagnation) prior to the start of this study despite the reopening of the building from July to September 2020. The building was shut down during the second COVID-19 pandemic lockdown (October 2020 to March 2021). As the study took place in November and December 2020, the building plumbing was under low water demand throughout the study (less than 5% of normal building occupancy).

All investigated showerheads were manually activated to ensure a flush of 5 min by activating a pressure button with resulting flow duration per activation ranging from 10s to more than one minute. Copper was identified as the piping material across the building plumbing including the main piping of the two large, grouped shower systems, except for the connecting pipe between each showerhead and timer valve, which was made of flexible polymer. The interior of each TMV casing, where cold and hot water are mixed, was made of several materials including brass, ethylene propylene diene monomer (EPDM), stainless steel, and other plastics (WATTS Eurotherm Ultramix®, North Andover, MA, USA).

6.2.2 Study Timeline and Water Sample Collection

The first sampling event took place on 16 November 2020, after a 12-week period without any shower usage, after which shock chlorination with free chlorine and remedial flushing were immediately carried out on the same day. Then, sampling events were conducted in the following three weeks from each mitigation intervention, thereby on November 23rd, November 30th, and December 7th, 2020 (on Mondays) (Figure C.1).

During each sampling event, the cold and hot water supplied to each TMV were first individually sampled (one liter) after a brief five-minute flush to assess the upstream water quality in the

building plumbing. Afterwards, the first draw (one liter) of water from the interior of each TMV casing and their immediate connecting pipe filled with mitigated water was sampled. Then, in a subset of six showerheads per shower system, first draws (one liter) were collected, followed by five-min flush samples (one liter). A sterile plastic bag was used to collect water from each showerhead in order to facilitate water collection and reduce exposure to water aerosols. All water samples were collected in sterile high-density polyethylene (HDPE) bottles. Finally, on every following weekday (Monday to Friday), showerheads that were flushed on a daily basis (Section 6.2.2.2) were also assessed for free and total chlorine by collecting first draws and five-min flush samples (Figure C.1). The incoming cold water from the municipal distribution system and the hot water return loop were sampled once (November 30th, 2020) after brief flushes of 5 – 15 min.

In mid-September 2021, building managers improved thermal control in the hot water distribution system by removing (1) the mixing point between a fraction of the hot water return loop coming back to the water heaters and the hot water delivered to the building, which increased temperature in both lines by up to 5 °C, as well as (2) the fraction of the hot water recirculation loop that was pre-heated in a small plate heat ex-changer before being supplied to the first heater of the series of four. These modifications were selected based on previous observations made during the characterization of the centralized hot water system (Grimard-Conea et al., 2022). Then, from mid-September 2021 to early January 2022, building managers implemented daily flushes (Mondays to Sundays) in selected shower systems of the building (seven shower systems covering about 90 showerheads out of 114) by (1) flushing for 15 – 30 min with mitigated water the rear-end showerhead of large grouped shower systems followed by a 30s flush of each upstream showerhead, and (2) flushing for five minutes with mitigated water all showerheads of small grouped shower systems or independent ones (single shower). One last sampling campaign was carried out on November 3rd, 2021, at a subset of 27 showerheads to sample only culturable L. pneumophila at first draw. The building was closed to the public in accordance with new COVID-19 lockdown orders from December 20th, 2021, to mid – March 2022, hence after the last sampling.

6.2.2.1 Remedial Interventions

Shock chlorination was performed with a diluted (50% v:v) solution of liquid sodium hypochlorite (commercial household bleach) which was injected into the shower system just above the TMV casing in the mitigated water pipe through an available sampling port. The solution was pumped at

approximately 200 – 250 mL/min with the use of a Masterflex® L/S® digital standard drive pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Starting with the showerhead closer to the TMV and moving towards the rear-end one, each showerhead was flushed (5 – 40 min) until target-free chlorine concentrations of 20 – 25 mg/L were reached. Overall, free chlorine concentrations ranging from 21.9 to 25.2 mg/L were measured at the start of the contact time period of 16h. At the end of the contact time period, free and total chlorine concentrations ranged from 0.31 to 1.68 mg/L and 0.67 to 2.15 mg/L, respectively, corresponding to minimum disinfection CTs of 298 – 1,613 mg•min/L (concentration of free chlorine X contact time). The resulting large free chlorine demand (93 – 99%) can be attributed to the extended contact time ap-plied, as well as the demand exerted from copper piping, deposits, and biofilm. Then, each showerhead was flushed for five minutes, corresponding overall to approximatively three water turnovers of the shower system plumbing, with mitigated water to restore chlorine levels back to normal values (free chlorine: 0.02 – 0.11 mg/L; total chlorine: 0.14 – 0.15 mg/L).

Remedial flushing was carried out as previously described (Grimard-Conea et al., 2022). In short, each showerhead was flushed for five minutes with mitigated water, starting with the showerhead closest to the TMV and moving towards the rear-end one. Altogether, roughly 1,000 L of mitigated water were flushed during remedial flushing, which was equivalent to more than three complete water turnovers of the shower system plumbing.

6.2.2.2 Controlled Preventative Flushing in Duplicates of Showerheads

Following both remedial interventions, a controlled preventative flushing strategy was implemented in both shower systems at a subset of six showerheads per shower system. Briefly, the two showerheads closest to each TMV were flushed on a daily basis from Monday to Friday for five minutes with mitigated water (25 – 40 °C). Then, the two showerheads located at the middle of each shower system were flushed weekly (on Mondays) with mitigated water (35 – 38 °C), whereas the last two rear-end showerheads were left stagnant for the remaining part of the study (Figure 6.1).

6.2.2.3 Temperature Monitoring in the Shower Systems

Water temperature was monitored at several locations throughout the study, including the hot water leaving the heaters and the hot water return loop, the cold and hot water supplied to each shower

system's TMV, as well as the mitigated water leaving each TMV. Temperature dataloggers (OM-CP-TC Temp X Series, 4 channels, Omega, Saint-Eustache, QC, Canada) were directly attached to pipe segments without thermal insulation. Temperature was also recorded with small thermocouple data loggers (OM-EL-USB-TC-LCD, Omega, Saint-Eustache, QC, Canada) that were installed at a subset of the investigated showerheads including one showerhead per duplicates of each flushing strategy (daily, weekly, left stagnant) in both shower systems (Figure 6.1). Temperature monitoring was put in place to examine thermal regimes and ensure that controlled flushing frequencies were applied accurately during the study.

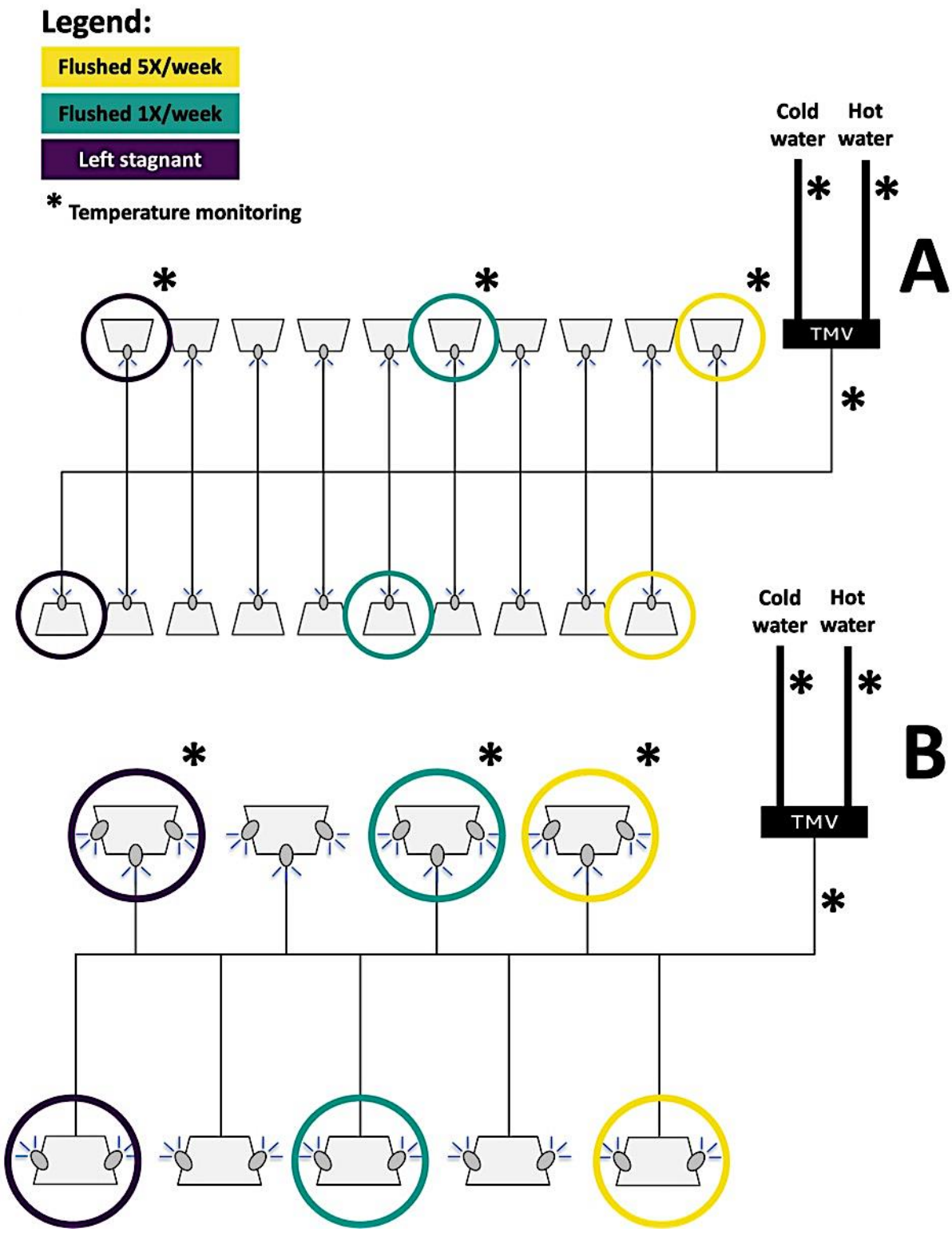

Figure 6.1 Schematic of the interventions and temperature monitoring performed in the shower systems in which (**A**) device recommissioning flushing and (**B**) shock chlorination were carried out, respectively.

6.2.3 Water Samples Processing

All water samples were immediately analyzed for onsite physico-chemical parameters, whereas laboratory measurements including intracellular adenosine tri-phosphate (ATP), flow cytometry, culturable *L. pneumophila* and water sample filtration, were processed within 12h of sampling.

6.2.3.1 Onsite Environmental Measurements

Temperature was measured using a digital thermometer (−50 – 300 °C), while pH (0 – 14 pH unit), conductivity (0.01 – 200 mS/cm), and dissolved oxygen (0 – 20 mg/L) were assessed with the HQ40d™ portable meter (HACH, London, ON, Canada) whose probes were inserted in a beaker containing approximatively 150 – 200 mL of the well-mixed water sample. Two successive aliquots of 10 mL were withdrawn from each well-mixed one-liter sample for assessment of free and total chlorine concentrations (0 to 2.00 mg/L), based on the HACH DPD Powder Pillows methods 8021 and 8167, respectively, with the portable DR 2800TM spectrophotometer (HACH, London, ON, Canada). During shock chlorination, dilutions (1:15) were made to measure free and total chlorine in the ap-propriate method range. Whenever free chlorine concentrations exceeded 0.05 mg/L, one mL of sterile sodium thiosulfate (10% v:v) was added. All probes and apparatus calibrations were performed before each sampling, according to the manufacturer's recommendations.

6.2.3.2 Intracellular-ATP and Flow Cytometry Assays

Fifty milliliters of water were used for intracellular-ATP quantification following the protocol specified by the manufacturer of the Dendridiag® SW kit (GL-Biocontrol, Clapiers, France). Briefly, samples were filtered on 0.45 µm (Ø 33 mm) polyether sulfone (PES) sterile membranes (CLEARLine® Biosigma S.p.A, Via Valletta, Italy) to eliminate free ATP and other inhibitors. Intracellular-ATP was then extracted with a solution for cell lysis provided in the kit and immediately quantified through bioluminescence assay with the Kikkoman PD-30 Luminester™ luminometer (Kikkoman Corp., Noda, Japan). At last, the measurements were validated with a standard also provided in the kit. Intracellular-ATP was expressed in picograms (pg) of ATP per millimeter and the kit had a detection limit of 0.1 pg ATP/mL.

Flow cytometry was conducted using a BD Accuri™ C6 Plus flow cytometer along with the automatic BD CSampler™ sampling arm (BD-Biosciences, Mississauga, ON, Canada) to

enumerate total (TCC) and intact cell counts (ICC) based on the integrity of cell membranes as the viability criteria. Quantification of cells was performed with four small aliquots of 300 µL (per water sample) to discriminate between dead and live cells in duplicates using (1) three µL of SYBR Green fluorochrome to stain all cells, and (2) three µL of a mix of SYBR Green and propidium iodide fluorochromes to stain damaged (dead) cells. Before the addition of dyes, aliquoted samples were incubated at 37 °C for three minutes, whereas samples with dyes were incubated once again at 37 °C for 10 min in the dark. Cells were enumerated using the FL3 (> 670 nm) and FL1 (530 – 533 nm) density plots, and bacteria gating was assessed according to the EAWAG water quality template previously developed to discriminate TCC and ICC (Gatza et al., 2013). Percentage of viable cells were calculated by dividing the number of ICC by the number of TCC.

6.2.3.3 Liquid Culture-Based Enzymatic *Legionella pneumophila*

The quantification of culturable *L. pneumophila* through the MPN method was per-formed using the 100 mL potable water Legiolert/Quanti-Tray kit (IDEXX Laboratories Canada Corp., Markham, ON, Canada) according to the manufacturer's instructions. In short, aliquots of 100 mL of water were transferred into sterile polypropylene vessels and first analyzed for water hardness using Aquadur® test strips (Macherey-Nagel, Düren, Germany). Due to overall low water hardness (zero to two positive pads on test strips), 0.33 mL of Legiolert Supplement were added to each vessel, as well as one Legiolert reagent blister pack. Finally, Legiolert water sample mixtures were transferred to 96-well plates and sealed with the IDEXX Quanti-Tray Sealer PLUS. Plates were then incubated at 39 ± 0.5 °C for seven days and results were read by counting any brown or turbid wells. The MPN method ranged from 10 to 22,726 MPN/L.

6.2.3.4 Water Samples Filtration and DNA Extraction

Approximatively 600 to 800 mL of the remaining water sample contents were vacuum filtered on sterile 0.2 µm (Ø 47 mm) Supor® PES membranes (PALL Corp., Mississauga, ON, Canada). Membranes were then gently folded and stored at −80 °C for prolonged conservation.

DNA extraction was carried out using an adapted protocol from the FastDNA Spin kit (MP Biomedicals, Solon, OH, USA). Membranes were first transferred to Lysing Matrix A tubes and fragmented with a flame-sterilized pair of scissors. A volume of 1.0 mL of a cell lysis solution for bacteria (CLS-TC) was added to each tube before homogenization in the MP Biomedicals

FastPrep-24™ bead beater for two successive cycles of 40 s at 6.0 m/s. Tubes were put on ice for two minutes in between bead beating cycles. Centrifugation was then performed at 14,000 g for 10 min at room temperature and the supernatant (700 – 750 µL) was collected into sterilized polypropylene 2.0 mL microcentrifuge tubes. An equal volume of Binding Matrix to that of the collected supernatant was added, and this mixture was gently agitated at 40 rpm and room temperature on a rotator for five minutes. From that point, the instructions specified in the FastDNA Spin kit manual were identically followed. DNA extracts (100 µL) were then stored at −20 °C.

6.2.3.5 Quantitative Polymerase Chain Reaction (qPCR) for *Legionella pneumophila*

The quantification of *L. pneumophila* DNA was conducted in triplicates by real-time qPCR according to the instructions of the iQ-Check® Quanti *L. pneumophila* Real-Time PCR kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Fluorescence curves were recovered on the Bio-Rad CFX Opus 96 Real-Time PCR Instrument and results were expressed in gene copies per liter. Inhibition was tested for each PCR plate in compliance with the instructions of the kit. For samples where inhibition was detected in only one of the triplicates, the inhibited triplicate was therefore not included in the analysis. Globally, amplification efficiencies and correlation coefficients (R2) of the qPCR standard curves ranged 94.7 – 96.4% and 0.992 – 0.996, respectively. The limit of detection was of 5 gc/reaction, whereas the mean lower and upper limits of quantification considering all PCR plates were of 19 gc/reaction and 30,285 gc/reaction.

6.2.4 Statistical Analysis and Graphic Viewing

Data exploration and statistical analysis were conducted on Microsoft Excel version 16.59 using the Formulas tab, and graphics were sketched on Rstudio version 2021.09.0, except for one line graph which was produced with Microsoft Excel. Due to small data sets, the Student's t test ("T.TEST()") was used to compare data sets means for different purposes. Statistical significance was set at a p-value of 0.05. For statistical and graphic viewing purposes, samples below the detection limit for culturable *L. pneumophila* were set at 1.5 MPN/L.

6.3 Results and Discussion

6.3.1 Elevated Baseline Microbial Contamination after the 12-Week Distal Stagnation Period

After the 12-week distal stagnation period, elevated concentrations of microbial measurements (ATP, TCC, and ICC) and *L. pneumophila* in first draw showerhead samples, the water in each TMV casing, as well as the incoming hot water feed to each TMV were observed. Results are presented in Table 6.1 and Table 6.2, respectively, for the shower systems that underwent remedial flushing and shock chlorination, and serve as baseline observations to evaluate the short-term impact of these remedial interventions. Overall, concentrations were comparable to those detected at these same shower systems in a previous study after prolonged (4- or 16-week) distal water stagnation (Grimard-Conea et al., 2022). Such significant water quality losses were attributed to biofilm growth during extended stagnation and biofilm detachment occurring near water collection points.

Table 6.1 Results of microbiological measurements in the shower system in which remedial flushing was carried out after the 12-week distal stagnation period (n = 1 for each TMV sample; n = 4 – 6 samples for distal and system samples). Legend: CW – Cold water, HW – Hot water, MW – Mitigated water, *Lp* – *Legionella pneumophila* concentrations, < LoD – Below detection limit.

Sampling Period	Water Sample	ATP (pg ATP/mL)	TCC (cell/mL)	Percent Viability (%)	Culturable *Lp* (MPN/L)	qPCR *Lp* (gc/L)
12-week distal stagnation	TMV—CW	0.84	7.10×10^3	8	<LoD	<LoD
	TMV—HW	0.80	1.45×10^5	22	<LoD	622
	TMV—MW	12.79	8.35×10^6	14	<LoD	427
	Distal—1st draw (mean)	8.26	1.07×10^6	27	340	8603
	System—5-min (mean)	0.36	1.40×10^5	5	<LoD	1087
1 week after remedial flushing	TMV—CW	0.08	1.87×10^3	53	<LoD	<LoD
	TMV—HW	1.28	2.30×10^5	24	23	415
	TMV—MW	4.21	3.69×10^5	38	<LoD	419
	Distal—1st draw (mean)	7.30	5.40×10^5	24	61	3657
	System—5-min (mean)	0.29	1.23×10^5	7	<LoD	248
2 weeks after remedial flushing	TMV—CW	0.04	1.82×10^3	43	<LoD	<LoD
	TMV—HW	1.88	3.04×10^5	24	35	394
	TMV—MW	1.78	2.80×10^5	36	<LoD	554
	Distal—1st draw (mean)	10.02	5.15×10^5	24	18	4460
	System—5-min (mean)	0.13	9.07×10^4	7	<LoD	189
3 weeks after remedial flushing	TMV—CW	0.08	3.39×10^3	35	<LoD	<LoD
	TMV—HW	1.10	1.50×10^5	20	<LoD	235
	TMV—MW	2.40	2.34×10^5	33	<LoD	149
	Distal—1st draw (mean)	6.33	6.22×10^5	30	126	11,118
	System—5-min (mean)	0.15	1.23×10^5	4	<LoD	531

Table 6.2 Results of microbiological measurements in the shower system in which shock chlorination was carried out after the 12-week distal stagnation period (n = 1 for each TMV sample; n = 4 – 6 samples for distal and system samples). Legend: CW – Cold water, HW – Hot water, MW – Mitigated water, *Lp* – *Legionella pneumophila* concentrations, < LoD – Below detection limit.

Sampling Period	Water Sample	ATP (pg ATP/mL)	TCC (cell/mL)	Percent Viability (%)	Culturable *Lp* (MPN/L)	qPCR *Lp* (gc/L)
12-week distal stagnation	TMV—CW	0.27	1.26×10^4	12	<LoD	<LoD
	TMV—HW	0.62	1.45×10^5	20	1198	541
	TMV—MW	2.21	2.57×10^6	42	3071	44,000
	Distal—1st draw (mean)	3.79	6.15×10^5	43	3017	13,900
	System—5-min (mean)	0.35	1.63×10^5	11	10	6405
1 week after shock chlorination	TMV—CW	0.29	8.60×10^2	15	<LoD	<LoD
	TMV—HW	1.03	1.41×10^5	10	11	428
	TMV—MW	0.28	6.36×10^4	1	<LoD	1480
	Distal—1st draw (mean)	2.74	2.01×10^5	27	<LoD	920
	System—5-min (mean)	0.27	9.64×10^4	3	<LoD	441
2 weeks after shock chlorination	TMV—CW	0.01	9.60×10^2	47	<LoD	<LoD
	TMV—HW	0.96	1.53×10^5	22	10	385
	TMV—MW	0.05	6.25×10^4	6	<LoD	860
	Distal—1st draw (mean)	7.96	6.48×10^5	50	<LoD	1026
	System—5-min (mean)	0.12	1.20×10^5	4	<LoD	398
3 weeks after shock chlorination	TMV—CW	0.03	1.56×10^3	26	<LoD	<LoD
	TMV—HW	0.83	1.73×10^5	16	<LoD	700
	TMV—MW	0.12	7.38×10^4	4	<LoD	2990
	Distal—1st draw (mean)	7.45	1.13×10^6	47	32	1783
	System—5-min (mean)	0.50	1.45×10^5	6	ND	858

Culturable *L. pneumophila* were detected in 83% (10/12) of first draw showerhead samples with concentrations ranging 35 – 6,081 MPN/L, whereas *L. pneumophila* gene copies were detected in all samples at concentrations ranging 173 – 37,700 gc/L. Compared to levels measured prior to the start of the present study (Grimard-Conea et al., 2022), a 2-log increase in culturable *L. pneumophila* was observed in one showerhead, thus showing its persistence and capacity to grow in stagnant conditions on the long-term run. Conversely, the other showerheads typically showed either a slight rebound in culturability of 0.1-0.8-log or decreases of up to 1.2-log. Reductions of 0.1-1.3-log were also measured in terms of *L. pneumophila* gene copies in most of these showerheads. Notably, one showerhead that tested negative for the presence of culturable *L. pneumophila* in the previous study remained negative at first draw in this study, despite the detection of *L. pneumophila* gene copies. These observations demonstrate that once *L. pneumophila* bacteria are established in the biofilm, it can persist over a prolonged period of time at varying concentrations during stagnation. Similarly, small increases of 1-1.6-fold in ATP and of 0.1-0.2-log in TCC and ICC were observed over the 12-week distal stagnation period. Larger increases in concentrations of ATP, TCC, ICC, and *L. pneumophila* were rapidly observed four weeks after recommissioning (remedial) flushing in the previous study (Grimard-Conea et al.,

2022), thus suggesting that long stagnation times (> 4-week) may not systematically result in continuous microbial growth because of limiting factors in distal parts of building plumbing such as nutrients availability (Rhoads et al., 2015b).

Although a low percentage of viable cells (14%) was measured in the water sampled from the interior of the TMV casing from one shower system, the corresponding elevated ATP and TCC concentrations of 12.79 pg ATP/mL and 8.35E+06 cell/mL were especially noteworthy (Table 6.1). In comparison, for the same order of magnitude of TCC in the TMV casing from the other shower system, a greater viability percentage (42%) but a lower ATP concentration (2.21 pg ATP/mL) were measured (Table 6.2). Such a difference could be attributable to the presence of intensive ATP intake microbial species contributing to a greater recovery of ATP molecules in the first TMV (Zhang et al., 2019) despite prolific conditions of growth characterized by a higher percentage of ICC in the second TMV.

6.3.2 Microbial Concentrations from the System Are Amplified in the Distal Sections

The building incoming cold water from the municipal distribution system was characterized by low microbial concentrations (0.05 pg ATP/mL and 6.30E+02 cell/mL as of TCC) and non-detectable culturable *L. pneumophila* and gene copies, and the presence of an elevated free chlorine residual (0.72 mg/L). In the hot water return loop, ATP and TCC greatly exceeded levels found in the incoming cold water, reaching, respectively, 5.51 pg ATP/mL and 4.09E+05 cell/mL, and L. pneumophila was detected at culturable and qPCR concentrations of 65 MPN/L and 233 gc/L.

The first step of microbial amplification occurred between the incoming municipal water and the hot and cold water piping feeding both TMV. Although free chlorine concentrations in cold water samples ranged from 0.27 to 0.63 mg/L throughout the study, ATP and TCC concentrations in these cold water feeds were higher by 2–17-fold and 0.3-1.3-log, respectively, in comparison to the incoming municipal cold water. Viability percentages showed wide variations (8–53%), and *L. pneumophila* was never detected in any cold water samples (Table 6.1 and Table 6.2), despite previous studies reporting frequent detection in flushed (10 – 15s) cold water taps using qPCR and larger water volumes (Donohue et al., 2014; Buse et al., 2020). In contrast, the microbial amplification from the incoming municipal water was more excessive in the hot water supplied to each TMV, thus highlighting the shift in terms of conditions of growth when water temperatures

increase and free chlorine residuals are depleted (0 – 0.08 mg/L). In fact, ATP and TCC concentrations in hot water feeds were overall higher by 12-110-fold and by 2.5-log when compared to the cold water entering the building. Culturable *L. pneumophila* was further detected at low concentrations (non-detectable to 35 MPN/L), and once at 1,198 MPN/L in the hot water that remained stagnant for 12 weeks. L. pneumophila gene copies were for the most part in similar ranges to those detected in the hot water return loop. Then, mitigated water collected from the interior of each TMV casing showed ATP, TCC, and *L. pneumophila* gene copies that were typically in between measurements from the cold and hot water feeds, with the exception of the 12-week distal stagnation period after which mitigated water had unusually elevated microbial measurements. Therefore, stagnant water in each TMV casing was found to be utterly conducive to microbial growth, although microbial loads were further reduced with water usage during the study.

The second step of microbial amplification occurred between the TMV and the showerheads. At the showerhead level, mean values in first draws were systematically 1.7-159-fold higher in ATP and up to 1.2-log higher in TCC than measurements from the mitigated water contained within the TMV casings despite the completion of remedial treatments and the implementation of different flushing regimes (Table 6.1 and Table 6.2). These increases in first draws showed important distal microbial amplification occurring within the showerheads and its immediate connecting pipes. Distal amplification is generally observed within the first few liters of water collected from taps (Lautenschlager et al., 2010; Lipphaus et al., 2014; Bédard et al., 2018; Grimard-Conea et al., 2022) due to the high surface-to-volume ratio, the presence of heterogeneous materials and architectures, variable stagnation times, and favorable temperatures, which are all factors favoring biofilm growth (Flemming et al., 2013). *L. pneumophila* gene copies were higher by one order of magnitude in distal parts of both shower systems than the concentrations measured in the cold and hot water TMV feeds, or the mitigated water found in the TMV casings. Although flushing remains a temporary beneficial mitigation strategy to reduce microbial risks for users (Hozalski et al., 2020; Grimard-Conea et al., 2022), flushing these showerheads for five minutes with mitigated water further reduced microbial concentrations to values generally lower than the hot water supplied to each TMV (Table 6.1 and Table 6.2).

During this study, microbial concentrations were amplified from the incoming water to the showerheads, and the elevated microbial concentrations in the recirculated hot water appeared to

be the main source of microbial cells and *L. pneumophila*. The amplification in the mitigated shower system downflow of both TMV was attributed to operational considerations, extended stagnation, and the presence of large (300 L) mitigated (22 – 38 °C) water networks previously identified in this building (Grimard-Conea et al., 2022). Unless efficient engineering controls are applied to the hot water distribution system, contaminated hot water can continue to seed distal points of use where favorable conditions for biofilm growth prevail. Distal amplification observed in first draws at showerheads confirms microbial regrowth, whereas flushed samples collected from these same showerheads were indicative of influent water quality. As suggested by Ji and colleagues, a deeper analysis of the building plumbing microbiome could help better understand to what extent the water eventually delivered to users is shaped by the upstream water quality and microbiome (Ji et al., 2015), so that effective controls and suitable design can thus be implemented.

6.3.3 Impact of the Combination of Remedial Interventions with Different Flushing Regimes

6.3.3.1 Stagnation Tends to Promote Microbial Regrowth after Remedial Intervention

Microbial regrowth factors (RFs) in the duplicates of showerheads that were left stagnant following remedial interventions are reported for first draws in Table 6.3 to assess whether a short stagnation period of three weeks was sufficient to promote complete regrowth from baseline data in distal sections (i.e., in that case, the factor would be of at least one).

Table 6.3 Microbial regrowth factors in duplicates of showerheads that were left stagnant after remedial interventions (RF = value at the third week after the intervention over baseline value).

Intervention	Shower ID	ATP (pg ATP/mL)	TCC (cell/mL)	ICC (cell/mL)	Culturable *Lp* (MPN/L)	qPCR *Lp* (gc/L)
Remedial flushing	Stagnant_1	1.48	0.86	0.74	0	5.49
	Stagnant_2	1.18	0.82	0.83	1.00	1.53
Shock chlorination	Stagnant_1	7.07	5.68	7.70	0.02	0.17
	Stagnant_2	4.31	3.51	4.74	0.01	0.21

In the shower system that underwent remedial flushing, a 3-week stagnation period resulted in increased ATP concentrations (RF > 1) (Figure 6.2B) in the duplicate of stagnant showerheads, but was not long enough to fully recover TCC (RF < 1) (Figure 6.2C). The percentage of viable cells remained steady at 23 – 29% throughout the stagnation period (Figure 6.2D). However, complete

regrowth of *L. pneumophila* culturability (RF = 1) (Figure 6.3A) and gene copies (RF = 1.53) (Figure 6.3B) was observed in one of the duplicates of showerheads, unlike the other in which only a large increase in gene copies (RF = 5.49) was measured despite culturable *L. pneumophila* cells being non-detectable. Since qPCR assays do not differentiate between culturable, viable-but-non-culturable (VBNC), and dead cells, this apparent increase of 5.49-fold (0.7-log) in *L. pneumophila* gene copies could be attributed to the detachment of a biofilm fragment or to the incoming flow of VBNC or dead cells from the upstream water when flushing was carried out. Considering that *L. pneumophila* gene copies were only detected at low concentrations (194 gc/L) in the five min flushed water supplied to that specific showerhead during remedial flushing, this last hypothesis could not support the 0.7-log increase. This example illustrates how two neighboring showerheads part of one grouped shower system can result in variable outcomes despite receiving the same water. Therefore, the selection of water sampling points during routine monitoring or in the aftermath of Legionnaires' diseases cases can greatly influence environmental investigation results.

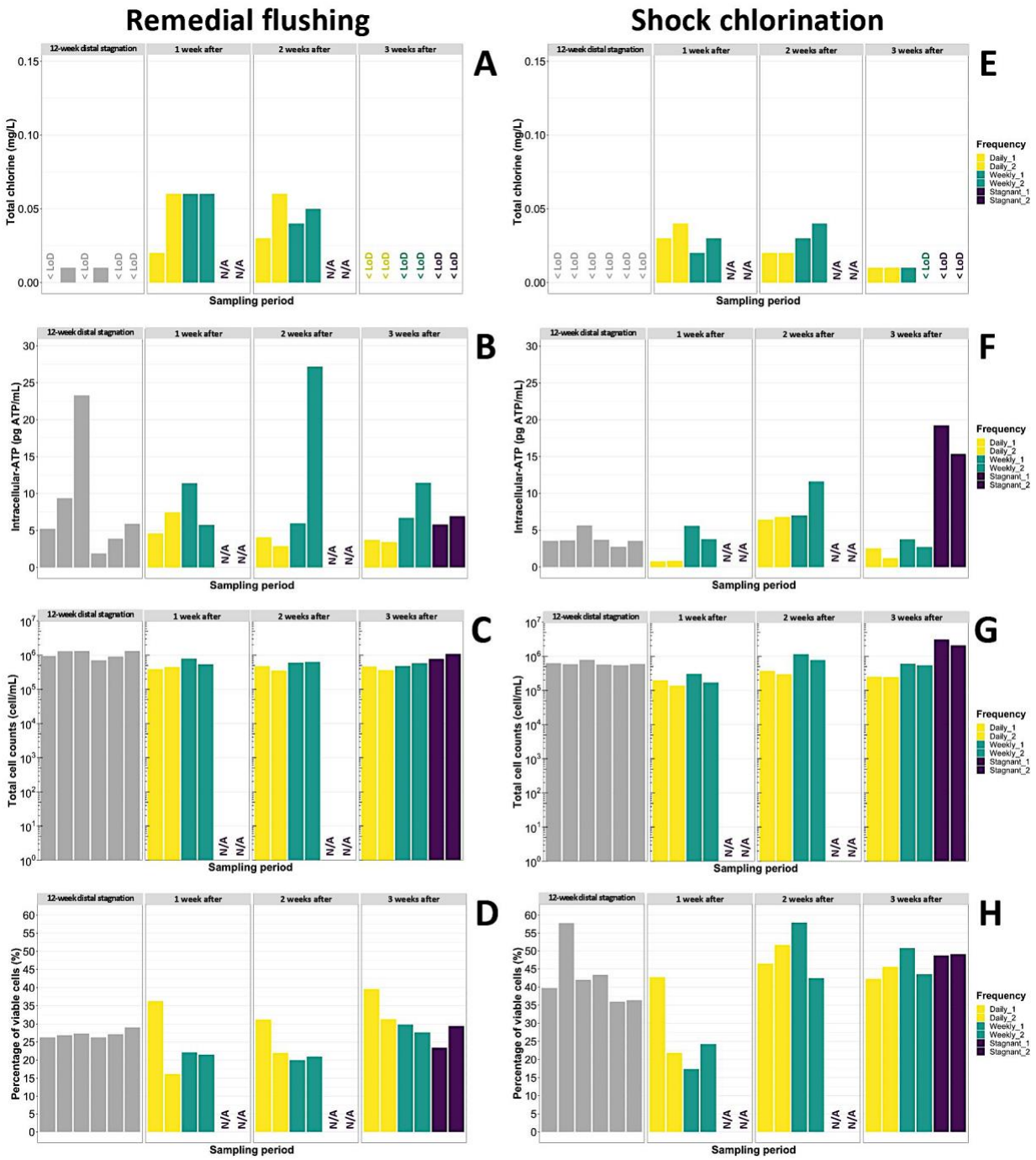

Figure 6.2 Weekly bar plot representations of (**A**) Total chlorine, (**B**) Intracellular-ATP, (**C**) Total cell counts, and (**D**) Percentage of viable cells after remedial flushing was carried out in one shower system, and weekly bar plot representations of (**E**) Total chlorine, (**F**) Intracellular-ATP, (**G**) Total cell counts, and (**H**) Percentage of viable cells after shock chlorination was carried out in the second

shower system. Legend: Grey bars – Baseline (12-week distal stagnation) values; N/A – Not evaluated; < LoD – Below the detection limit.

Shock chlorination followed by a 3-week distal stagnation period resulted in more important water quality losses than what was observed for the other duplicate of showerheads that underwent remedial flushing. Indeed, higher RFs were calculated for ATP (4.31 and 7.07), TCC (5.68 and 3.51), and ICC (7.70 and 4.74) in this shower system despite fairly similar baseline results (Table 6.3). Such a noteworthy difference among duplicates of showerheads is likely not to be the sole effect of biofilm detachment occurring after longer periods of stagnation (Grimard-Conea et al., 2022), but also the result of microbial regrowth. Free chlorine typically disrupts cell membranes of microorganisms, causing leakage of macromolecules (e.g., carbon, nitrates, phosphates) and resulting in the sudden bioavailability of nutrients essential for microbial growth. Previous studies have demonstrated that some microorganisms including *L. pneumophila* (Temmerman et al., 2006) and mixed drinking water bacterial communities (Chatzigiannidou et al., 2018) can sustain necrotrophic growth. Therefore, shock chlorination combined with a 3-week distal stagnation period prompted biomass regrowth as reflected by important increases in ATP (Figure 6.2F), TCC (Figure 6.2G), and percentage of intact cells (Figure 6.2H) in showerheads. In contrast, this combination was not sufficient to renew *L. pneumophila* culturability and gene copies within three weeks, as RFs remained low in the duplicate of showerheads (culturable: RF = 0.02 and 0.01; qPCR: RF = 0.17 and 0.21) (Table 6.3). Nonetheless, stagnation did promote the resurgence of culturability (20 – 60 MPN/L) (Figure 6.3C) in both showerheads that were left stagnant, although gene copies remained near 1-log lower than baseline values (Figure 6.3D). Shock chlorination combined with distal water stagnation further reduced to a greater extent the ratio of culturable cells to gene copies of *L. pneumophila* than remedial flushing and stagnation, thus demonstrating its benefits on all types of viable or VBNC cells. However, *L. pneumophila* growth is likely to recur in the long-term run as the persistence of *Legionella* in building water systems has been demonstrated in several studies despite chlorination treatments (Cooper et al., 2008; García et al., 2008; Orsi et al., 2014). Since ATP and TCC are general microbial measurements used to assess biostability (Prest et al., 2016), whereas qPCR and culturable *L. pneumophila* monitoring are pathogen-specific, it becomes essential to ensure that the microbiome did not shift towards an increased abundance of other opportunistic drinking water pathogens after such remedial treatment.

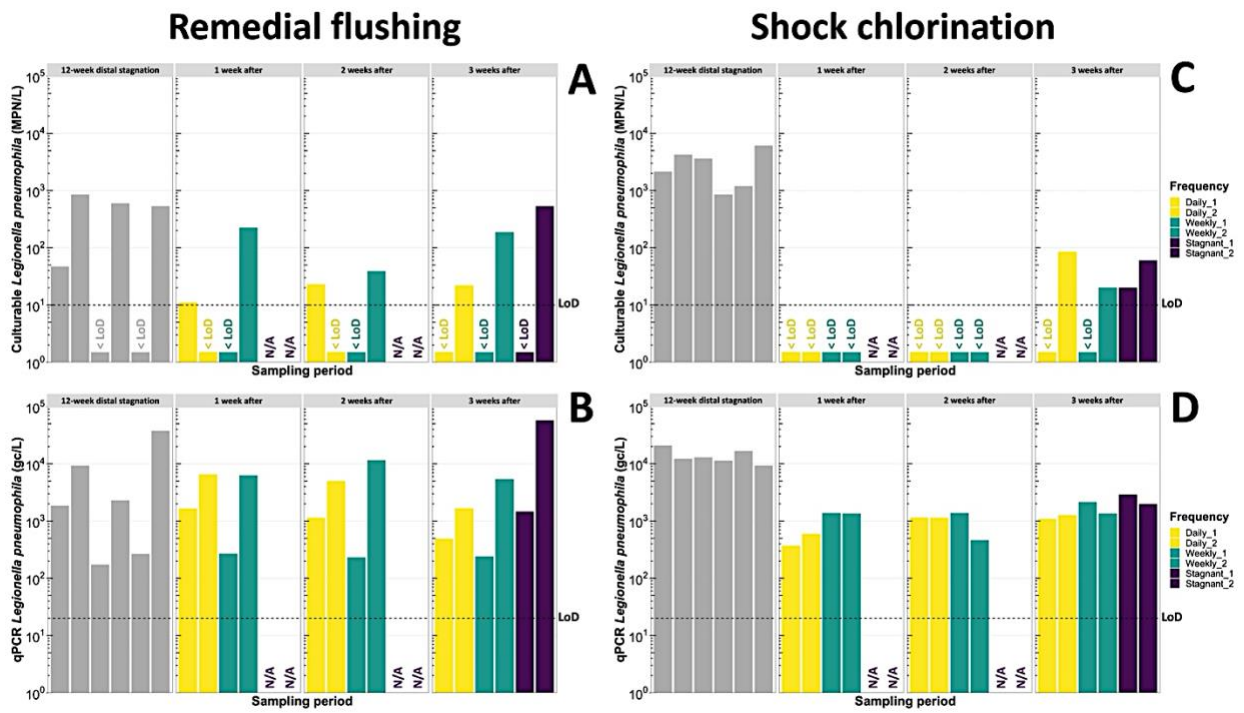

Figure 6.3 Weekly bar plot representations of (**A**) Culturable *L. pneumophila* and (**B**) qPCR *L. pneumophila* after remedial flushing was carried out in one shower system, and weekly bar plot representations of (**C**) Culturable *L. pneumophila* and (**D**) qPCR *L. pneumophila* after shock chlorination was carried out in the second shower system. Legend: Grey bars – Baseline (12-week distal stagnation) values; N/A – Not evaluated; < LoD – Below the detection limit.

Overall, different trends were observed when distinct remedial interventions were followed by short (3-week) distal water stagnation: complete distal regrowth of *L. pneumophila*, ATP, TCC, and ICC after remedial flushing because of the limited effect of flushing on biofilm removal (Meegoda et al., 2023), and important amplification of ATP, TCC and ICC after shock chlorination, likely due to the release of nutrients. Longer stagnation times may however alter these observations as *L. pneumophila* concentrations can gradually increase back after shock chlorination, whereas cell counts may reach plateaued concentrations over time (Meegoda et al., 2023) as nutrients are depleted. Nevertheless, distal water stagnation periods of three weeks promoted microbial regrowth in all showerheads regardless of the remedial intervention, thus highlighting the need for additional preventative measures (e.g., daily or weekly flushes) when plumbing systems are to be closed or left unused following corrective actions.

6.3.3.2 Daily Flushes Resulted in Significantly Lower ATP and TCC Concentrations Than Weekly Flushes

In this study, daily and weekly five min flushes of duplicates of showerheads were implemented after the completion of both remedial interventions. Such routine flushing protocols are typically recommended in several *Legionella* control guidance documents when taps are infrequently used (HPSC, 2009; Ministry of Health of New Zealand, 2012; Government of South Australia, 2013; Haut Conseil de la Santé Publique, 2013; HSE, 2014; Hong Kong Government, 2016; ESCMID, 2017; Australian Government, 2015; OFSP et OSAV, 2018; ASHRAE, 2020). Throughout the study, daily flushes resulted in significantly ($p < 0.05$) lower ATP (Figure 6.2B, Figure 6.2F) and TCC concentrations (Figure 6.2C, Figure 6.2G) than weekly flushes, regardless of the remedial intervention carried out. Nonetheless, the benefits of preventative flushing after shock chlorination were particularly meaningful as ATP and TCC remained lower by 5.1-16.4-fold and 0.2-0.8-log in the showerheads that were flushed either on a daily or weekly basis compared to those which were left stagnant. Therefore, periodic flushes of showerheads prevented biomass regrowth observed with the combination of shock chlorination and stagnation through the flushing of accumulated nutrients or dead microorganisms. This demonstrates the importance of maintaining a regular water flow in distal sections of plumbing systems following such remedial intervention. Whereas daily and weekly flushes slightly increased free and total chlorine residuals at first draw in showerheads (Figure 6.2A, Figure 6.2E), statistical differences between those two flushing regimes were not found to be significant ($p > 0.05$). As showerheads were flushed with mitigated water, chlorine concentrations additionally remained consistently low in five min flushed water samples (less than 0.13 mg/L as of free chlorine and 0.33 mg/L as of total chlorine) and even more so at first draw (less than 0.01 mg/L as of free chlorine and 0.05 mg/L as of total chlorine).

Frequent flushes can prevent bacterial accumulation resulting from biofilm detachment and suspended biomass growth, therefore resulting in lower microbial concentrations such as those observed in the duplicates of daily flushed showerheads in the present study. However, when water carries a certain load of nutrients, more frequent flushes can translate into higher nutrient delivery to distal parts and in a greater potential for microbial growth (Rhoads et al., 2015b; Rhoads et al., 2022), although it could be offset by the delivery of more frequent disinfectant residual if carried out with disinfected cold water. Ji and colleagues demonstrated that higher water usage frequencies

led to a lower proportion of shared operational taxonomic units (OTU) between water samples and their biofilm counterparts. This observation was attributed to the fact that microbial interactions among these two phases were less likely to occur during intermittent and short stagnation times (Ji et al., 2017). Consequently, when regular flushing is carried out under suitable preventative control regimes in terms of temperature or disinfectant residuals, the user is more likely to be exposed to upstream water that is typically characterized by lower microbial concentrations than the first few liters of water in distal sections, thus reducing microbial risks.

In this study, all water samples were collected on Mondays, thereupon after weekend stagnation to accommodate building staff availability. However, previous studies have shown that weekend-long stagnation periods can increase TCC in first draws by less than 1-log compared to measurements taken right before the start of the weekend (Lipphaus et al., 2014; Bédard et al., 2018; Montagnino et al., 2022). Indeed, the detachment in faucets was observed to occur mostly over the first 24h of stagnation (Bédard et al., 2018). Therefore, it is likely that statistical differences between daily and weekly flushes could be even greater if samples had not been collected after the weekend. Future research should aim to integrate daily flushes with cold, mitigated, or hot water in au-to-flush devices to prevent water stagnation and microbial growth.

6.3.3.3 The Combination of Preventative Flushing and Shock Chlorination Is the Most Effective to Reduce Temporarily *L. pneumophila*

Overall, larger decreases in both *L. pneumophila* culturability (Figure 6.3C) and gene copies (Figure 6.3D) were observed during the study after shock chlorination comparatively to remedial flushing (Figure 6.3A, Figure 6.3B). No culturable *L. pneumophila* cells were measured in first draws over the first and second week after shock chlorination, regardless of the flushing regime implemented and despite flushes supplying up to 834 gc/L (five min flushed samples) each time, whereas two showerheads persistently showed concentrations ranging 11 – 223 MPN/L after remedial flushing. *L. pneumophila* gene copies at first draw generally remained 1-log lower than baseline values in showerheads that underwent shock chlorination, whilst concentrations persisted in the same orders of magnitude (10^2 – 10^4 gc/L) than baseline measurements following remedial flushing. In general, the ratio of culturable to qPCR concentrations was diminished to a greater degree following shock chlorination than after remedial flushing, but there were no notable differences in these ratios when considering the flushing regime then implemented in each shower

system. As the discrepancy between culturable and qPCR *L. pneumophila* concentrations typically provides insights into the presence of VBNC and dead cells, the combination of preventative flushing and shock chlorination was more effective in reducing the proportion of VBNC and dead cells in this study.

Despite daily flushed showerheads being repeatedly exposed to temperatures favorable for *L. pneumophila* growth (32 – 40 °C) for five to 55 min after each flush (Figure 6.4), daily flushes did not yield statistically less *L. pneumophila* at first draw than weekly flushes ($p = 0.15 – 0.57$). The benefits of regularly washing cells away with the flow were therefore more important than the temporary establishment of conditions optimal for *L. pneumophila* growth in distal parts (Rhoads et al., 2016a). Nonetheless, the combination of remedial flushing or shock chlorination and daily flushes resulted in more important decreases than weekly flushes in terms of *L. pneumophila* culturability and gene copies over the first week after the intervention, as well as between baseline measurements and the third week of the study. By the end of the study, daily and weekly flushing of showerheads generally resulted in lower *L. pneumophila* culturable and qPCR concentrations than showerheads left stagnant, therefore demonstrating the beneficial effects of sustained (three weeks) preventative flushing when combined with remedial interventions.

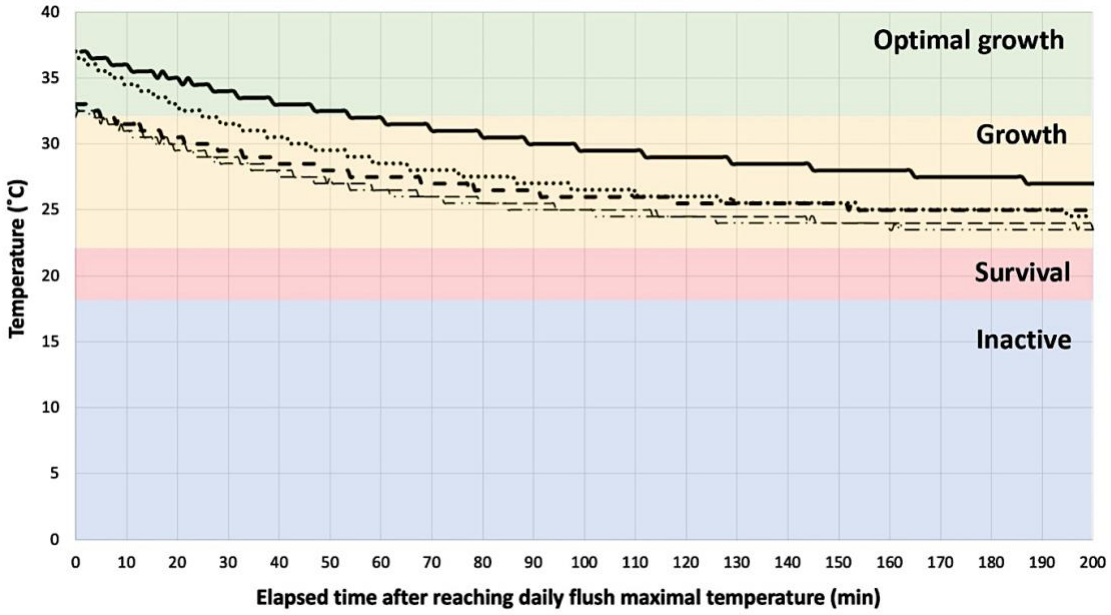

Figure 6.4 Temperature (y-axis) and elapsed time after reaching daily flush maximal temperature (x-axis) in showerheads flushed on a daily basis. Lines represent five different sets of temperature monitoring data over time. Colored temperature ranges – Typical conditions of growth of *Lp*.

Despite the clear short-term benefits (three weeks) of shock chlorination on *L. pneumophila*, such remedial intervention can accelerate plumbing corrosion and cause the formation of harmful disinfection by-products (Muraca et al., 1990). Additionally, it requires the assistance of professionals and prolonged time windows regarding the interruption of water usage. In this study, free chlorine residuals ranging from 21.9 to 25.2 mg/L were maintained at all showerheads for 16 h. Figure 6.3 shows culturable *L. pneumophila* regrowth observed by the end of the 3-week study period in some showerheads, which was likely due to (1) recolonization of *L. pneumophila* through flushing and seeding of planktonic unculturable *L. pneumophila* that could regain culturability over time, (2) persistence of *L. pneumophila* in the distal biofilm, and (3) the protection of *L. pneumophila* cells in protozoan hosts for a short period of time (Kilvington et Price, 1990; Storey et al., 2004; García et al., 2007; Dupuy et al., 2011). The limited short-term impact of remedial flushing on *L. pneumophila* can be attributable to the mechanical action of flushing, which only con-tributed to the removal of superficial biofilm cells that were poorly attached and planktonic cells controlled by the flow (Stoodley et al., 2001). These general trends should also take into con-sideration that the baseline concentrations of culturable and qPCR *L. pneumophila* varied substantially between showerheads, even though they were engineered identically and positioned

next to each other within the same grouped shower system. Interestingly, incoming concentrations of culturable and qPCR *L. pneumophila* after flushing that renew cells to distal points remained quite stable over time, reflecting the steady operations of the upstream distribution system.

6.3.3.4 Daily Thorough Flushes of Showerheads for Months can Reduce the Occurrence of Culturable *L. pneumophila* in Distal Sections

Even if providing actionable information to restart a system after extended stagnation, reduced building occupancy, or contamination events, this study does not provide more long-term evidence to assess whether the trends observed after both remedial interventions persist over time as the plumbing microbiome matures after being temporarily shifted. Long-term data would be beneficial to estimate the time duration by which *L. pneumophila* concentrations are likely to return to baseline values or not. Nonetheless, results presented in this study represent valuable information for building managers who want to temporarily lower microbial risks while implementing additional engineering controls (e.g., temperature correction) or building-wide treatment (e.g., *in situ* chloramine system). In contrast to distal water stagnation, the benefits of preventative flushing, and more particularly daily flushes of showerheads, combined with remedial interventions, hereby remedial flushing, and shock chlorination, were well demonstrated on the concentrations of ATP, TCC, and the percentage of intact cells, and to a lesser extent on the abundance of *L. pneumophila*.

As the pandemic persisted, building managers faced the issue of temporary closings and underuse of the facilities when reopening. To mitigate the potential risk to users, building managers thus implemented manual daily flushes of showerheads in most shower systems from mid-September 2021 to early January 2022 by flushing the rear-end showerhead of large, grouped shower systems for 15 to 30 min, followed by a brief 30s flush of all upstream showerheads. In fact, this approach has been considered as a more time-efficient alternative in recommissioning guidance (Government of Québec, 2020) to minimize the duration of flushing operations and the wastage of water after prolonged building inoccupation. Two months after implementing this modified flushing regime, *L. pneumophila* culturability persisted at a positivity rate of 41% (11/27) in first draws despite prior daily flushes, with positive concentrations ranging from 10 to more than 22,726 MPN/L (mean of positive samples of 3,163 MPN/L). It is noteworthy that all (n = 4) rear-end showerheads in each selected large, grouped shower system remained below the detection limit of 10 MPN/L for culturable *L. pneumophila*, with the exception of one showerhead in which a low

concentration of 74 MPN/L was measured. These levels were more than 3-log lower than those measured before the implementation of daily 15 – 30 min flushes (mean of 3,422 MPN/L). This shows that in a large, grouped shower system, a brief daily flush of 30s as opposed to more thorough flushes of rear-end water points as a time-efficient flushing strategy was not sufficient to depress culturable *L. pneumophila* in distal sections. Nonetheless, long-term (2-month) daily flushing of showerheads resulted in reduced culturable *L. pneumophila* concentrations below desirable thresholds of 1,000 and 10,000 colony-forming unit (CFU)/L (i.e., near equivalent of MPN/L [Sartory et al., 2017; Spies et al., 2018; Boczek et al., 2021]) in, respectively, 24 and 26 out of the 27 investigated showerheads.

6.4 Conclusions and Recommendations

In this study, the weekly short-term (3-week) effectiveness of the combination of shock chlorination (20 – 25 mg/L, 16h) or remedial flushing (five minutes at each showerhead) with different flushing regimes (daily, weekly, left stagnant) were implemented in duplicates of showerheads. Overall, this work demonstrated the temporary benefits of carrying remedial interventions despite the application of preventative flushing regimes.

The following recommendations are proposed to support building managers and other relevant authorities in the development of effective water management plans and environmental monitoring strategies.

In general:

- Monitoring microbial concentrations in plumbing piping sections from the incoming water to the points of use can locate sites of contamination so that targeted engineering controls or *in situ* treatments can be effectively applied. In this study, ATP, TCC, and *L. pneumophila* concentrations increased from the building cold water entry to the hot water return loop, then from the cold and hot water supplied to each shower systems' TMV towards each showerhead. Amplification of ATP, TCC, ICC, and *L. pneumophila* occurred clearly at distal sites as opposed to the upstream distribution system.
- System-specific *L. pneumophila* alert and action thresholds should be set to ensure minimum amplification at distal sites using qPCR as the first-tier surveillance and culture as the confirmation. Throughout this study, qPCR was a more conservative mean to assess

the weekly effectiveness of remedial interventions as it provided fairly steady measurements comparatively to culturable measurements which varied more substantially.

- In grouped distal sites, more than two water points should be at least monitored during routine (baseline) or investigative *L. pneumophila* monitoring. Neighboring showerheads that were engineered identically and received the same building plumbing water showed fairly different *L. pneumophila* culturable and qPCR concentrations in baseline measurements and had variable response to remedial interventions carried out.

For buildings with established contamination at distal sites:

- A combination of shock chlorination/preventative flushing is more effective than remedial flushing/preventative flushing to control temporarily (3-week) the regrowth of *L. pneumophila* at distal sites. Throughout the study, such combination (shock chlorination/preventative flushing) led to a 2-week suppression of *L. pneumophila* culturability and to greater decreases in qPCR concentrations than remedial flushing/preventative flushing for which *L. pneumophila* persisted at 11 – 223 MPN/L and 10^2 – 10^4 gc/L.
- The combination of preventative flushing (daily or weekly) and shock chlorination should be considered as a temporary measure to limit growth of *L. pneumophila* as it provided protection for at least three weeks at distal sites before small rebounds in culturability (20 – 84 MPN/L) were observed. Alternative mitigation strategies and engineering controls should consequently be considered to limit its long-term regrowth.
- Shock chlorination should not be followed by long periods (more than three weeks) of water stagnation as stagnant conditions can stimulate biomass regrowth. Such remedial intervention should therefore not be systematically conducted prior to low building occupancy or complete long-term building shutdown unless preventative flushing is implemented to wash away dead cells and nutrients to prevent microbial growth and necrotrophic growth. Indeed, larger microbial regrowth factors (RFs) were measured in showerheads left stagnant for three weeks after shock chlorination than following remedial flushing for concentrations of ATP (shock chlorination: RFs = 4.31 and 7.07; remedial flushing: RFs = 1.48 and 1.18), TCC (shock chlorination: RFs = 5.68 and 3.51; remedial flushing: RFs = 0.86 and 0.82) and ICC (shock chlorination: RFs = 4.74 and 7.70; remedial flushing: RFs = 0.74 and 0.83).

For buildings subjected to periods of low water use due to reduced occupancy (e.g., seasonal venues, partial building shutdown, construction activities):

- Without further evidence, daily flushes of distal sites as a measure to prevent water stagnation hazards should be considered where occupant exposure (e.g., showerheads) and susceptibility (e.g., vulnerable or immunocompromised individuals) are more risk critical. Indeed, regardless of the remedial intervention carried out (remedial flushing or shock chlorination), daily flushes of showerheads resulted in significantly ($p < 0.05$) lower ATP and TCC concentrations, as well as in generally lower *L. pneumophila* levels in this study.

Supplementary Materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1. Figure C.1: Chronological steps of the sampling events (orange) and interventions (green) carried out in both shower systems.

Author Contributions

Conceptualization, M.P. and M.G.-C.; Methodology, M.P. and M.G.-C.; For-mal analysis, M.G.-C.; Investigation, M.G.-C.; Writing—original draft preparation, M.G.-C.; Writing—review and editing, M.P. and M.G.-C.; Visualization, M.G.-C.; Funding acquisition, M.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 7 ARTICLE 4: MITIGATION OF OPPORTUNISTIC DRINKING WATER PATHOGENS BY ONSITE MONOCHLORAMINE DISINFECTION IN A HOSPITAL WATER SYSTEM

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The introduction of monochloramine as a building-level strategy to mitigate the prevalence of *Legionella pneumophila* in hospital's hot water systems has risen in popularity. While such systems have been implemented in healthcare facilities and evaluated for their effectiveness in reducing *Legionella* abundances, findings regarding their impact on nontuberculous mycobacteria and protozoan hosts remain inconclusive. This study offers several novel contributions compared to prior research, including multi-pathogen monitoring and a broad range of physico-chemical parameters, while carefully differentiating between distal points of use and hot water distribution system sites, thus recognizing their distinct dynamics and implications for monitoring purposes. The study also incorporates the influence of water use patterns, monitored through flushing devices, and examined varied operational conditions across common points of use (showerheads, hand washing stations, faucets) operating at different water temperatures. Additionally, it uniquely

evaluates the impact of short- and long-term dosing interruption periods (5-day and 4-week) resulting from operational alerts.

To this end, a total of 544 water samples were collected from 22 points of use selected based on patient risk, and 10 sites representative of the hot water flowing system, both before and after introducing monochloramine at target values ranging 1.5 to 3.5 mg/L. Findings provide actionable data and insights for building managers to optimize the practical implementation of onsite monochloramine generation systems in facilities hosting vulnerable populations.

Mitigation of opportunistic drinking water pathogens by onsite monochloramine disinfection in a hospital water system

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Abstract

In acute care hospitals, susceptible patients and large, legacy water systems contribute to increased risk of nosocomial infections associated with drinking water pathogens. This study aimed to evaluate the long-term (> 1-year) impact of onsite monochloramine treatment on *Legionella pneumophila* (*Lp*), nontuberculous mycobacteria (NTMs), *Vermamoeba vermiformis* (*Vv*), and physico-chemical water quality in a hospital hot water system. Using an innovative sampling approach, the efficacy of treatment was assessed at 22 distal sites (faucets, showerheads, handwashing stations) and compared to 10 control points representing the main flowing distribution system (return loops, heaters, remote sites). Monochloramine nearly eliminated *Lp*, achieving up to 3-log reductions in culturability (< 24h) and gene copies (4-week). Mean *Vv* concentrations decreased by 2-log within 24h, with no evidence of a shift towards increased NTMs. Optimal reductions in all organisms were observed at monochloramine concentrations of 2 – 3

mg/L combined with temperatures exceeding 55 ˚C. However, these conditions were only consistently maintained at control points, where post-treatment mean concentrations were systematically 1-log lower than those at distal sites. The interruption of dosage (5-day and 4-week) also revealed significant and rapid rebounds of *Legionella* species, NTMs, and *Vv* (> 1-2-log), highlighting their persistence in biofilms. Short-term increases in metal release were observed, with mean copper and lead concentrations rising 1.8- and 4.6-fold, respectively. Overall, results confirmed the high and rapid efficacy of onsite monochloramine to control *Lp* and other organisms. Analysis of water quality, temperature distribution, and usage patterns emphasize the importance of maintaining optimized hydraulic and thermal regimes to ensure effective pathogen control at points of exposure. This study provides actionable insights and practical evidence to support healthcare facilities in implementing robust long-term monitoring and control strategies.

Keywords: Monochloramine; Hospital plumbing; *Legionella*; Nontuberculous mycobacteria.

7.1 Introduction

Drinking water-associated pathogens causing opportunist infections (DWPI) in vulnerable individuals, including *Legionella pneumophila* and nontuberculous mycobacteria (NTMs), represent a major public health concern (Collier et al., 2021) because of their propensity to grow and persist in building plumbing systems (Falkinham III et al., 2015). Nosocomial transmission of *Legionella* bacteria has been associated with elevated mortality rates compared to community-acquired infections, both in the United States (Soda et al., 2017) and Europe (Beauté et al., 2020), with *Legionella pneumophila* identified most frequently in reported Legionnaires' disease (LD) cases. Additionally, significant healthcare prevalence of NTMs investigations (Perkins et al., 2019) represents a growing concern, particularly given the wide diversity of infectious *Mycobacterium* species and infection pathways (Dowdell et al., 2019). As healthcare facilities (HCFs) treat patients with heightened susceptibility, implementing comprehensive monitoring and mitigation strategies to reduce exposure risks to DWPI is increasingly recommended by guidance and regulations (NASEM, 2019).

In response to more stringent regulations to minimize disinfection by-products, water utilities have converted to monochloramine as a secondary disinfectant in distribution systems due to its greater stability and lower reaction rate with natural organic matter (Bradley et al., 2020). Early findings demonstrated that community-wide conversion to monochloramine reduced the prevalence of

culturable *Legionella* species (spp.) in the distribution system (Pryor et al., 2004), and drastically reversed prevalence in buildings (Moore et al., 2006), acting on all *L. pneumophila* serogroups (Flannery et al., 2006). A significantly lower incidence rate of nosocomial LD has been reported in hospitals supplied with monochloramine-treated water compared to those using other disinfectants (Heffelfinger et al., 2003). Lower rates of positive samples for culturable or quantitative PCR (qPCR) *L. pneumophila* have also been observed in distribution systems using monochloramine for secondary disinfection (LeChevallier, 2019). This impact is also shown by two extensive studies demonstrating lower *L. pneumophila* occurrence and abundance in buildings with residual monochloramine rather than with residual free chlorine (Donohue et al., 2019; Dowdell et al., 2023).

Several factors underscore the need for alternative mitigation solutions for *Legionella* control. These include (1) the limited long-term benefits of hyperchlorination (Orsi et al., 2014; Grimard-Conea et Prévost, 2023) and heat shocks (Chen et al., 2005; Bédard et al., 2016), especially when elevated temperatures (> 70 ˚C) are not reached (Ji et al., 2018; Cazals et al., 2022), to limit *Legionella* regrowth in response to colonization or nosocomial infection, (2) the limitations of *in situ* copper-silver ionization (Loret et al., 2005; Bédard et al., 2016) or chlorine dioxide (Marchesi et al., 2020; Lee-Masi et al., 2023) to fully prevent *Legionella* positivity, (3) the cost- and time-consuming installation of points of use (PoU) filter in high-risk areas of HCFs (Casini et al., 2014), (4) the limited long-term impact of device (Hozalski et al., 2020; Grimard-Conea et al., 2022) or building-wide flushing (Rhoads et al., 2022; Angert et al., 2023) on reducing *Legionella* loads, and (5) the poor persistence of incoming free chlorine residuals with stagnation and increased water temperatures (Grimard-Conea et al., 2024).

The introduction of monochloramine in the hot water system (HWS) of several large HCFs generally led to sharp reductions, and in some cases complete eradication of *Legionella* culture-positive sites (Marchesi et al., 2012; Casini et al., 2014; Duda et al., 2014; Mancini et al., 2015; Coniglio et al., 2018; Lytle et al., 2021). It is regarded as a highly effective building-level mitigation strategy to significantly reduce the risk of *Legionella* exposure, especially in areas with vulnerable populations. A major concern regarding the increased use of monochloramine is the potential for microbial shifts, particularly an increased prevalence of NTMs. However, studies investigating this possibility have produced contradictory results. Simultaneous increases in the positivity and mean concentrations (< 2-log) of culturable NTMs were measured in one study

(Casini et al., 2014). In contrast, Duda and colleagues (2014) reported no significant changes in culture-positive occurrence rates of NTMs, whilst considerable decreases in both culturable (< 1-log) and qPCR (up to 2-log) *Mycobacterium* spp. were documented by Lytle and colleagues (2021). When reviewing these studies, important inconsistencies between experimental approaches become evident, including the use of culture and qPCR-based detection methods, disparate sampling timeframes, pooling of samples for analysis, collection of first-flush or semi-flushed samples, types of PoU tested, and the limited characterization of the HWS. Additionally, as *L. pneumophila* and some species of NTMs rely on intracellular replication in hosts, the efficacity of monochloramine to inactivate trophozoites and cysts in water and in the biofilm needs additional research (Loret et al., 2005; Xi et al., 2024).

This study aims to comprehensively evaluate the longitudinal (1-year) effectiveness of *in situ* disinfection of a large hospital's HWS with monochloramine on the abundance of DWPI (*Legionella*, NTMs) and *Vermamoeba vermiformis*, a thermotolerant amoeba species frequently isolated from hospital water systems (Delafont et al., 2018), while also evaluating significant changes in water quality resulting from this intervention. Sampling was conducted at 22 distal sites under semi-controlled stagnation periods and different water temperature operation, including faucets, showerheads, and hand washing stations, and 10 points representative of the main flowing HWS. The effect of two dosage interruptions – one prolonged (4-week) and another shorter one (5-day) – was further assessed.

7.2 Materials and Methods

7.2.1 Hospital setting and rationale

The facility is a 540-bed (10 floors) acute care academic hospital built in 1954 (Québec, Canada). Most rooms have en-suite bathrooms (sink faucet and toilet) and accommodate overnight patients. Some rooms include a shower whereas in other sectors of the hospital, a shared shower is available for all rooms in the same hallway. The hospital complex features an extensive plumbing system centered around a main cross-shape building, which houses nearly all inpatient rooms. Additional facilities, including laboratories, outpatient care, radio-oncology, offices, and utility areas, extend from this central structure (Figure D.1). The HWS consists of two centralized 1500-liter pre-heated electrical water heaters, supplying hot water to all connected facilities. In the main cross-shape building, one hot water riser serves two adjacent rooms, with a shared horizontal hot water return

loop located in each wing. Additionally, a small 500-liter electrical water heater provides reheated water for Facility E. The hospital receives chlorinated water (0.37 – 0.64 mg/L during summertime) from the municipal system, and no culturable *L. pneumophila* has ever been detected at the hospital's point of entry in prior testing.

Two nosocomial cases of LD caused by the same *L. pneumophila* strain (including one mixed infection) were confirmed at the hospital in the two years prior to this study (2020 – 2021). These spatio-temporally linked cases prompted an extensive sampling campaign of the hospital's cold and HWSs, revealing high positivity rates for culturable (29%) and qPCR (81%) *L. pneumophila* in hot water (data not shown). Typing results from these water samples matched the strain isolated from clinical samples (Najeeb et al., 2024, Submitted to Journal of Hospital Infection). In response, short-term preventative and corrective actions were rapidly implemented, including sterile water protocols, PoU filters in targeted high-risk units, restrictions on showers and baths use to reduce water aerosolization and exposure, and two serial superheat-and-flush procedures at 65 °C, spaced three weeks apart. However, the poor efficacy of these heat shocks, as indicated by minimal changes in the occurrence rates and concentrations of culturable *L. pneumophila*, led to the installation of a monochloramine generator in 2022 as a long-term mitigation measure.

7.2.2 Monochloramine generator system and study timeline

In mid-June 2022, the monochloramine generator system (Sanipur Sanikill, PA, USA) was integrated at the hot water outlet of the water heater. This onsite system combines two monochloramine precursors: a solution of sodium hypochlorite (HOCl, Enoxin) to a solution of ammonium salts (NH_3, Zebion), which ratio is modulated by two metered pumps as a function of the cold make-up water flowrate supplied to the water heater and the redox potential of the hot water return. Sampling events were conducted at three monthly time-point before the introduction of monochloramine into the hospital's HWS, shortly (24h) after, then on a weekly (for four weeks on), monthly and bimonthly basis in the following year for a total of 17 sampling events. Due to unresolved alerts, dosage was periodically interrupted throughout the study, including for short (5-day) and prolonged (4-week) periods after which sampling was also carried out.

7.2.3 Sample collection and site location

For each sampling campaign, water samples were collected from 32 sites across the hospital water system, including 22 first draws from 16 manual faucets, four showerheads, and two electronic hand washing stations, and 10 two-min flushed samples from sites across the main flowing HWS, including two manual faucets remotely located from the water heaters, all six individual hot water return loops from the cross-shape main building, and the inlet and outlet of the water heaters (Figure D.1). First draws are herein defined as distal sites and flushed samples are designated as system sites, which are representative of the flowing (hot water recirculation) system. Distal sites were selected based on the presence of high-risk patients (hematology-oncology, kidney transplant, pneumology, neonatology, intensive care and COVID-19 units) and previous confirmed nosocomial LD cases and positive *L. pneumophila* measurements. Hot water was solely collected from manual faucets, whereas tepid water was collected from showerheads and hand washing stations. For all 544 samples gathered in this study, two sequential one liter of water were drawn in autoclaved HDPE bottles.

7.2.4 Water use monitoring

After the onset of treatment, 16 flushing monitors with hand-held shower adaptors and connectors for under-sink pipe connections (TapSnap™, Trusted Water LLC, Fort Collins, CO, USA) were installed at distal sites. Sensing vibrations imputable to water use, monitors showed a green light if at least one 30-second or more of continuous use was detected within the last seven days or a red light, otherwise reflecting no recent water use.

7.2.5 Physico-chemical and microbiological measurements

Field measurements included water temperature, pH, conductivity, dissolved oxygen, free and total chlorine, using approximatively 150 mL of water for onsite analysis. An additional 75 mL was reserved for laboratory measurements of nitrite, nitrate, ammonium, total organic carbon (TOC) and metals (manganese, iron, copper, lead), before adding one mL of sterile sodium thiosulfate (10% v:v) in all samples with chlorine concentrations above 0.05 mg/L. The remaining volume of water (1500 – 2200 mL) was processed in the laboratory within 12h of sampling for culturable *L. pneumophila* (culture-based enzymatic test), and further vacuum-filtered on sterile 0.2 µm (Ø 47 mm) Supor® PES membranes (PALL Corp., Mississauga, ON, Canada) for a triplex qPCR assay

simultaneously targeting *Legionella* spp., *L. pneumophila*, and *L. pneumophila* serogroup 1. Additional qPCR assays individually targeting *Mycobacterium* spp. and *V. vermiformis* were also performed. All qPCR analysis were processed in triplicates and DNA was extracted from membranes using an adapted protocol from the FastDNA® SPIN kit (MP Biomedicals, Solon, OH, USA) and diluted in 150 µl of sterile PCR water, as described in Grimard-Conea and Prévost (2023). Filtered membranes and DNA extracts were kept at -80 ˚C and -25 ˚C, respectively. Detailed descriptions of physico-chemical and microbiological analysis are provided as supplementary materials (Sections D.2, D.3, and D.4).

7.2.6 Data analysis

Statistical analysis and graphic viewing were conducted on RStudio version 2024.04.2+764. Correlations among parameters were evaluated through the Spearman's rank test and statistical differences between two conditions with the Wilcoxon test, using significance levels set at a p-value of 0.05.

7.3 Results and discussion

7.3.1 Physico-chemical parameters monitoring

During the study, temperature remained generally stable across the HWS (Figure 7.1a). Samples collected from the water heater outlet had a mean temperature of 58 ˚C, while those taken from the pipe consolidating all hot water return lines averaged 54 ˚C. Surprisingly, the return line from facility E (Figure D.1), equipped with an additional water heater, regularly failed to comply with the minimum temperature requirement of 55 ˚C mandated by the Construction Code of Québec (Building Act, chapter B-1.1, r. 2), rather fluctuating between 47 and 52 ˚C. At the two faucets remotely located from the water heater, a 2-min flush was sufficient to reach temperatures exceeding 55 ˚C, indicating good hydraulic efficiency to inpatient rooms. Temperatures in first draws at distal sites (Figure 7.1a) reflected the impact of mitigators at hand washing stations and showers, as well as the inter-use stagnation leading to water cooling in non-recirculated sections at faucets. Mean temperatures of 45 ˚C, 28 ˚C, and 27 ˚C were measured in manual faucets, showerheads, and hand washing stations, respectively. Free chlorine concentrations were consistently below 0.1 mg/L before onset of treatment, as expected with rapid decay at elevated temperatures and with stagnation (Grimard-Conea et al., 2024). PoU supplying tepid water

(showers, hand washing stations) generally had concentrations below 0.2 mg/L, a threshold under which microbial growth is insufficiently controlled and greater occurrence of DWPIs is more likely (Donohue et al., 2019; Grimard-Conea et al., 2024).

In the first three weeks of treatment, monochloramine was targeted at 1.5 mg/L, increasing to 2.5 mg/L thereafter. At the heater outlet injection point, total chlorine averaged 1.3 and 2.2 mg/L in the weeks leading to the dosage increase and in the subsequent months (excluding interruption periods), respectively (Table D.2). Mean total chlorine concentrations across system sites were consistently lower than at the heater outlet, and even more so at distal sites (Figure 7.1b), reflecting chlorine demand influenced by plumbing materials and scales, stagnation, increasing surface-to-volume ratio as hot water moves away from the heater, microbial growth, and temperatures (Grimard-Conea et al., 2024). During interruption periods, total chlorine was barely detectable at distal sites. Additionally, lower total chlorine concentrations were measured in Facility E's return loop (Table D.2), likely due to a lower capacity pump given the reduced water demand in this building.

Monochloramine did not alter pH (Figure D.2a) and conductivity (Figure D.2b), while ammonium concentrations varied with monochloramine dosage. Nitrite levels remained below detection limits (< 0.02 mg/L), and variations in dissolved oxygen (Figure D.2c), nitrate and TOC reflected seasonal changes in water quality from raw water. Nitrification is a major concern associated with the use of excess ammonia, leading to undesired microbial growth and disinfectant decay (Bradley et al., 2020). In this study, no concurrent pH decreases, nitrate increases, or dissolved oxygen decreases, common indicators of nitrification (Hossain et al., 2022), were observed.

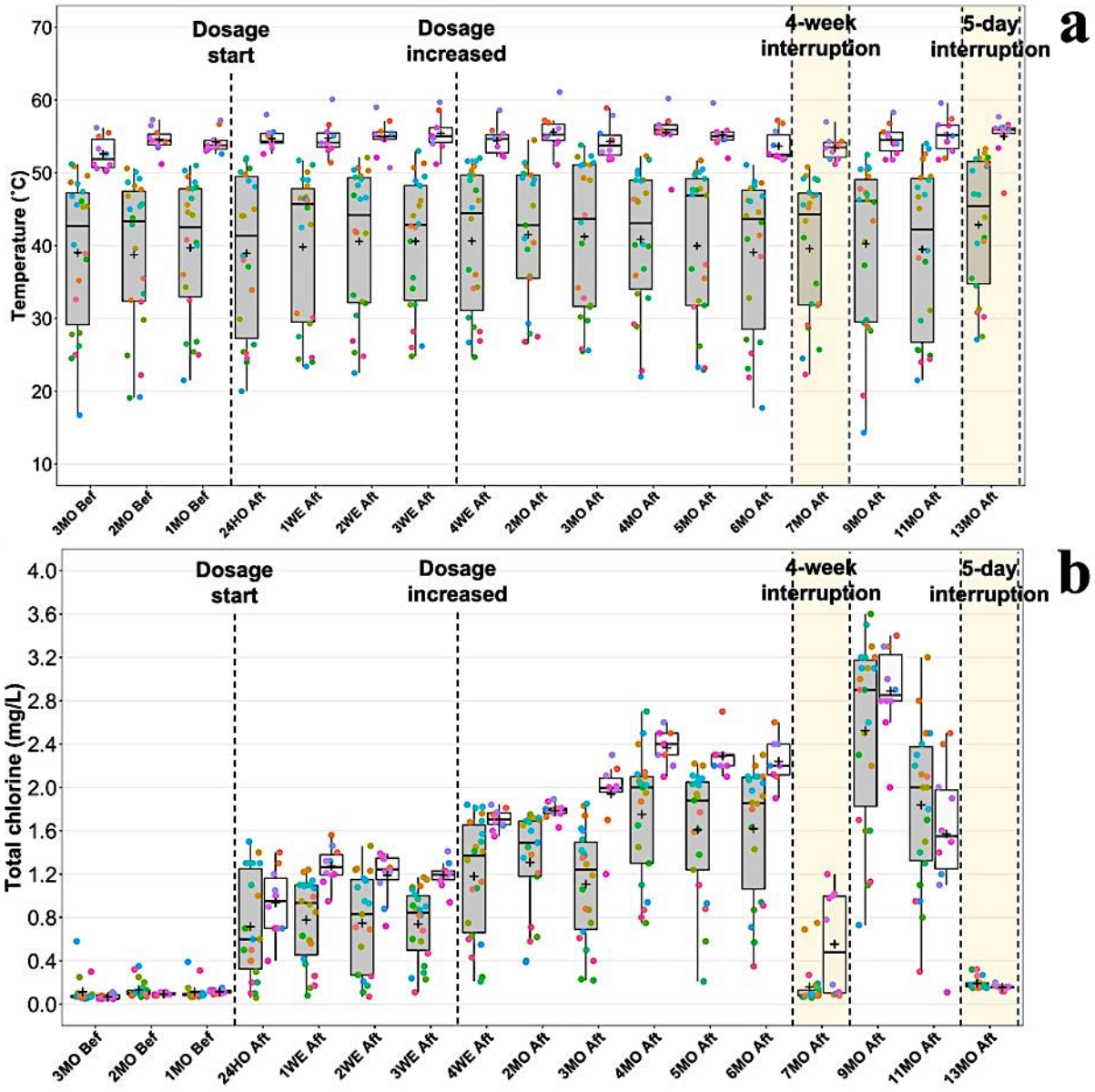

Figure 7.1 Box plots of (**a**) Temperature and (**b**) Total chlorine over time at distal (grey boxes, n = 22) and system sites (white boxes, n = 10). Legend: Black cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Colored dots – Raw data per sampling site, MO – Month, WE – Week, Bef – Before, Aft – After.

7.3.2 Plumbing metals

Monochloramine treatment notably increased metal concentrations, with mean levels of copper, lead, iron, and manganese rising from 393 µg/L, 4 µg/L, 38 µg/L, and 2 µg/L, to 633 µg/L, 5 µg/L, 78 µg/L, and 4 µg/L (Figure D.3a-d). Particulate and dissolved metals visibly accumulated on

membranes, highlighting the clear disruption of plumbing scales post-treatment (Figure D.4). Non-compliance rates to Canadian water quality guidelines – a maximum acceptable concentration (MAC) of 5 µg/L for lead and an aesthetic objective of 1,000 µg/L for copper – also increased from 19% to 29% for lead and from 0% to 9% for copper.

The hospital's aging unlined cast iron risers, secondary copper piping, and leaded brass fixtures and fittings most likely contributed to these increases, corroborating previous findings on the impact of disinfectant shift on copper, lead, and iron release from disrupted and corroded oxide layers (Edwards et Dudi, 2004; Edwards et al., 2011). As expected, monochloramine had a greater impact on metal concentrations at distal sites compared to those located within the HWS, which showed less variability and minor increases. At some distal sites, concentrations oftentimes spiked largely over MAC or aesthetic standards, reflecting more extended stagnation periods (Zlatanović et al., 2017), larger surface-to-volume ratios (Ling et al., 2018), and the accumulation of particulates which can exacerbate the release of metals.

7.3.3 Impact of monochloramine on microbial loads

7.3.3.1 *Legionella*

Before treatment, 61% (40/66) of distal sites were culture-positive for *L. pneumophila*, with concentrations ranging 10 to 19,226 MPN/L (Figure 7.2a). Nearly half of these (47%) exceeded the 100 MPN/L alert threshold recommended in *Legionella* guidelines for HCFs, and roughly a quarter exceeded 1,000 MPN/L, a value requiring immediate action to control growth (VHA, 2021; HSE, 2024). Elevated temperatures across the HWS likely inhibited culturability, as only four samples out of 30 samples were positive to *L. pneumophila* and all system sites were below 100 MPN/L. Nevertheless, *L. pneumophila* gene copies were detected at 98% (52 – 21,000 gc/L) and 100% (300 – 2,170 gc/L) (Figure 7.2b) of distal and system sites, respectively. At distal sites, *Legionella* spp. concentrations varied widely (10^2 – 10^6 gc/L), whereas system sites exhibited more consistent concentrations (10^3 – 10^4 gc/L) (Figure 7.2c). On average, *L. pneumophila* accounted for 19% (distal sites) and 25% of all *Legionella* spp. (system sites) during baseline months. Serogroups 2 – 15 were overwhelmingly dominant, accounting for 98% of the pre-treatment environmental samples. Despite serogroup 1 being responsible for the majority of nosocomial LD cases in Europe (Beauté et al., 2020) and the US (Kunz et al., 2024), hospitals have also reported cases caused by other serogroups and species. In fact, the two nosocomial LD cases originating

from this hospital belonged to serogroup 10. Notably, the underperforming return line from Facility E frequently had the highest *L. pneumophila* concentrations (47 – 79 MPN/L) during baseline months, stressing the need to maintain hot water temperatures above 55 ˚C as a first-level control measure.

The introduction of monochloramine rapidly suppressed (24h) and nearly eliminated culturable *L. pneumophila* across all sites, regardless of residual concentrations (0.1 – 3.6 mg/L) (Figure 7.2a). A single site (hand washing station) remained culture-positive (11 – 22,726 MPN/L) in the following months, despite receiving similar residuals (0.1 – 1.7 mg/L) than other tepid PoUs. Within 24h of dosing, *L. pneumophila* became highly dominant (50 – 80%) at all system sites, suggesting initial biofilm disruption releasing *L. pneumophila* into bulk water. Silva et al. (2024) found that L. pneumophila typically occupies the bottom layers of a *Pseudomonas fluorescens* biofilm. In contrast, this study suggests that in more diverse microbial communities, *L. pneumophila* likely resides in the top layers, making it more susceptible to sloughing under monochloramine exposure, thus explaining the long-term elimination of its reservoir. By the third week, qPCR *L. pneumophila* concentrations dropped below the limit of quantification in most samples, a decrease of up to 3-log. As the disinfectant quickly affected cell culturability, this 3-week period likely reflects a transition of *L. pneumophila* to a viable-but-non-culturable state before inactivation under prolonged monochloramine exposure. By the fourth month of treatment, *L. pneumophila* gene copies were undetectable. Given the pathogen's ability to regain culturability under favorable conditions, the progressive erosion of the qPCR signal underscores the complementary value of qPCR in monitoring long-term *Legionella* risks.

A gradual mean 2-log decrease in *Legionella* spp. gene copies occurred during the first month, further stabilizing at 10^2 – 10^4 gc/L, thus highlighting monochloramine's broad-spectrum effectiveness. The efficacy of such disinfectant to mitigate *Legionella* agrees with previous studies in legacy and complex HCFs, where similar target monochloramine concentrations (1.5 – 3.5 mg/L) (Marchesi et al., 2012; Casini et al., 2014; Duda et al., 2014; Coniglio et al., 2018; Lytle et al., 2021) or higher concentrations were used (6 – 10 mg/L) (Mancini et al., 2015). Notably, on the first day of treatment, *L. pneumophila* serogroup 1 were measured (42 – 843 gc/L) at six distal sites where only serogroups 2 – 15 were previously detected. Concentrations then quickly fell below detection limits, suggesting temporary detachment of *L. pneumophila* serogroup 1 from adjacent biofilms triggered by monochloramine.

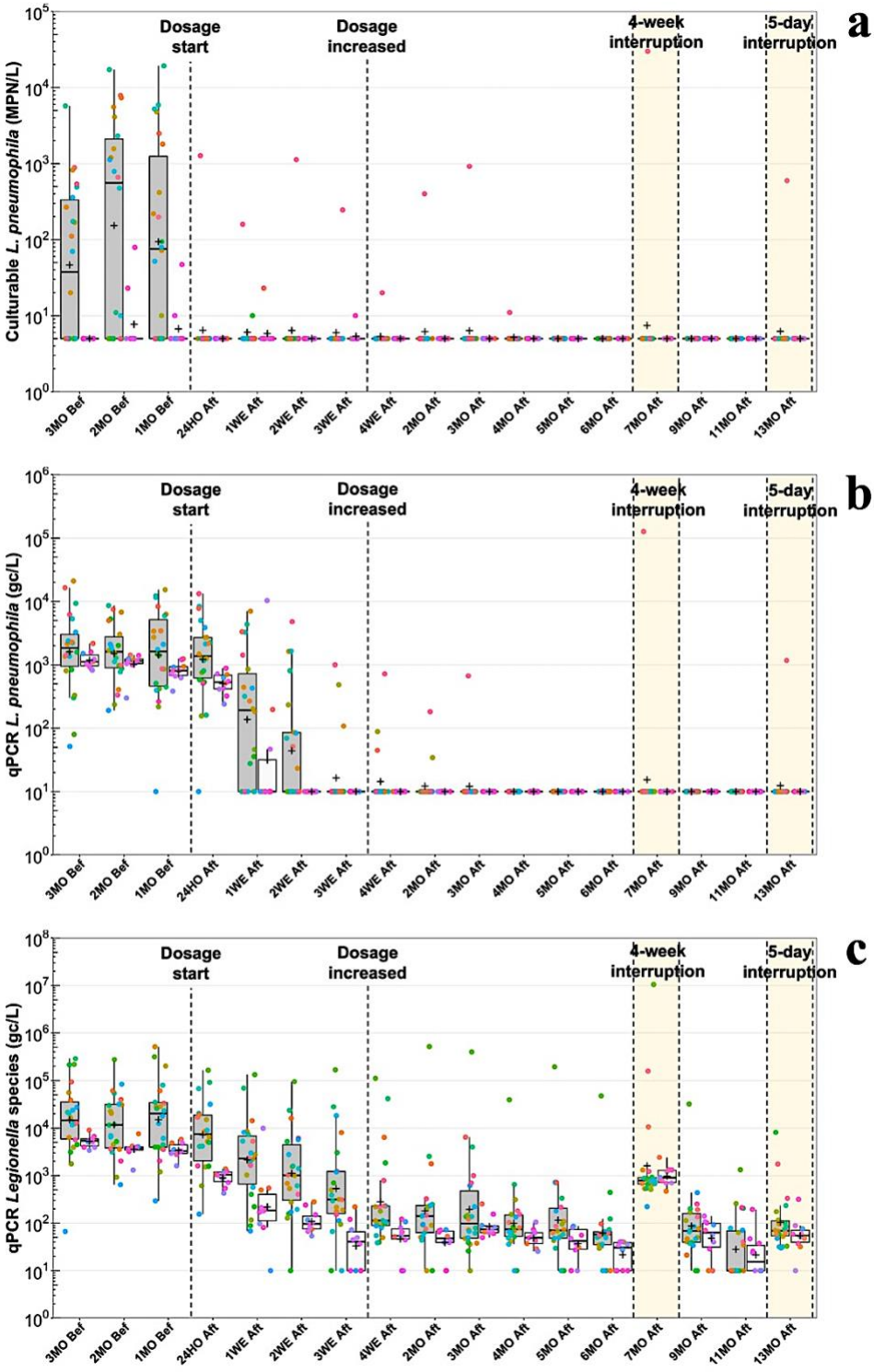

Figure 7.2 Box plots of (**a**) Culturable *L. pneumophila*, (**b**) qPCR *L. pneumophila*, and (**c**) qPCR *Legionella* species over time at distal sites (grey boxes, n = 22) and system sites (white boxes, n = 10). Legend: Black cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Colored dots – Raw data per sampling site, MO – Month, WE – Week, Bef – Before, Aft – After.

7.3.3.2 Nontuberculous mycobacteria

Prior to treatment, *Mycobacterium* spp. concentrations fluctuated between 10^5 and 10^7 gc/L, with comparable mean values at distal and system sites (Figure 7.3a). After the onset of treatment, mean concentrations decreased by 1-2-log and 1-3-log at distal and system sites, respectively. However, system sites, which experienced more stable flow, temperatures, and residuals, showed less variability, underscoring the differential impacts of monochloramine between distal points and the central HWS. Notably, distal sites exhibited a wide range of NTMs concentrations post-treatment, spanning over five logs. This greater variability observed for NTMs compared to *Legionella* spp. under the same site-specific conditions, including wide ranges of operating temperatures (14 – 55 ˚C), water demand patterns, and residual concentrations (0.1 – 3.6 mg/L) can be attributed to the higher monochloramine disinfection CT (product of the disinfectant concentration and the contact time) required for NTMs. For example, a 3-log inactivation (CT99.9%) at ambient temperatures using monochloramine is achieved with < 70 mg·min/L for different *Legionella* species, but requires 91–1,710 mg·min/L for *Mycobacterium avium* depending on strain susceptibility (Taylor et al., 2000).

NTMs persistence despite monochloramine exposure is also due to the hydrophobic and complex lipid composition of their cell walls, which enhance resistance to disinfectants (Loret et al., 2019). More particularly, their ubiquitous detection in monochloraminated systems is corroborated by studies showing greater planktonic abundance compared to systems with free chlorine (Donohue et al., 2019), or biofilm-associated cells compared to systems without residuals (Waak et al., 2019). Nevertheless, the reductions in NTMs observed in this study align with previous findings (Lytle et al., 2021). However, the present study reveals a more progressive and continuous reduction in qPCR over the 11 months of treatment, corresponding to an increase in combined residuals as shown on Figure 7.1b. Additionally, NTMs comprise a wide variety of species with varying disinfectant resistance, leading in distinct NTMs communities shaped by the type and disinfectant concentration (Yang et al., 2024), further supporting their survival.

7.3.3.3 *Vermamoeba vermiformis*

V. vermiformis ranged 10^2 – 10^5 cell equiv./L during baseline months (Figure 7.3b). With monochloramine dosing, rapid 2-log mean reductions were measured at system sites within 24h, with similar declines at most distal sites. This shows the disinfectant's effectiveness to reduce the

amoeba population during the initial phase of treatment. Then, concentrations at all sites fluctuated slightly in the subsequent months, but showed a progressive long-term reduction, with mean levels remaining below the detection limit (< 200 cell equiv./L).

L. pneumophila and certain NTMs are amoeba-resistant microorganisms that can evade phagocytosis, persisting intracellularly or replicating, especially under nutrient-limited conditions (Greub et Raoult, 2004). *V. vermiformis* is a well-documented host for *L. pneumophila* (Lau et Ashbolt, 2009) and a potential reservoir for NTMs (Delafont et al., 2014). Other pathogenic *Legionella* spp. co-occur with *Acanthamoeba* and *Naegleria fowleri* in engineered water systems, further supporting their reliance on these hosts for survival and replication (Logan-Jackson et Rose, 2021). Given the temperatures on Figure 7.1a, it is likely that *V. vermiformis* was predominantly in its cyst form at system sites, as Cazals and colleagues (2022) observed a dominance of cysts above 55˚C under similar conditions. However, at distal sites, temperatures would likely support the presence of trophozoites, as the shift to the cystic form occurs above 40˚C, thereby allowing replication of *L. pneumophila* at PoUs.

Culturable and molecular concentrations of *L. pneumophila* did not correlate ($R < 0.08$, $p > 0.05$) to concentrations of *V. vermiformis* in pre- and post-monochloramine samples, whereas *Mycobacterium* spp. significantly ($p < 0.05$), but only weakly correlated to *V. vermiformis* ($R = 0.34$). This reflects differences in host specificity, preferential detachment from biofilms, survival strategies, and the targeted eradication of the *L. pneumophila* reservoir. In fact, *L. pneumophila* is known to infect various protozoa, including *Acanthamoeba* and *Naegleria* species (Lau et Ashbolt, 2009), which may explain its weaker correlation with *V. vermiformis*, except at the hand washing station where *V. vermiformis* was abundant. Conversely, NTMs may not rely as heavily on hosts for replication, allowing them to survive even when *V. vermiformis* populations were lower (during treatment), while still benefiting from intracellular protection.

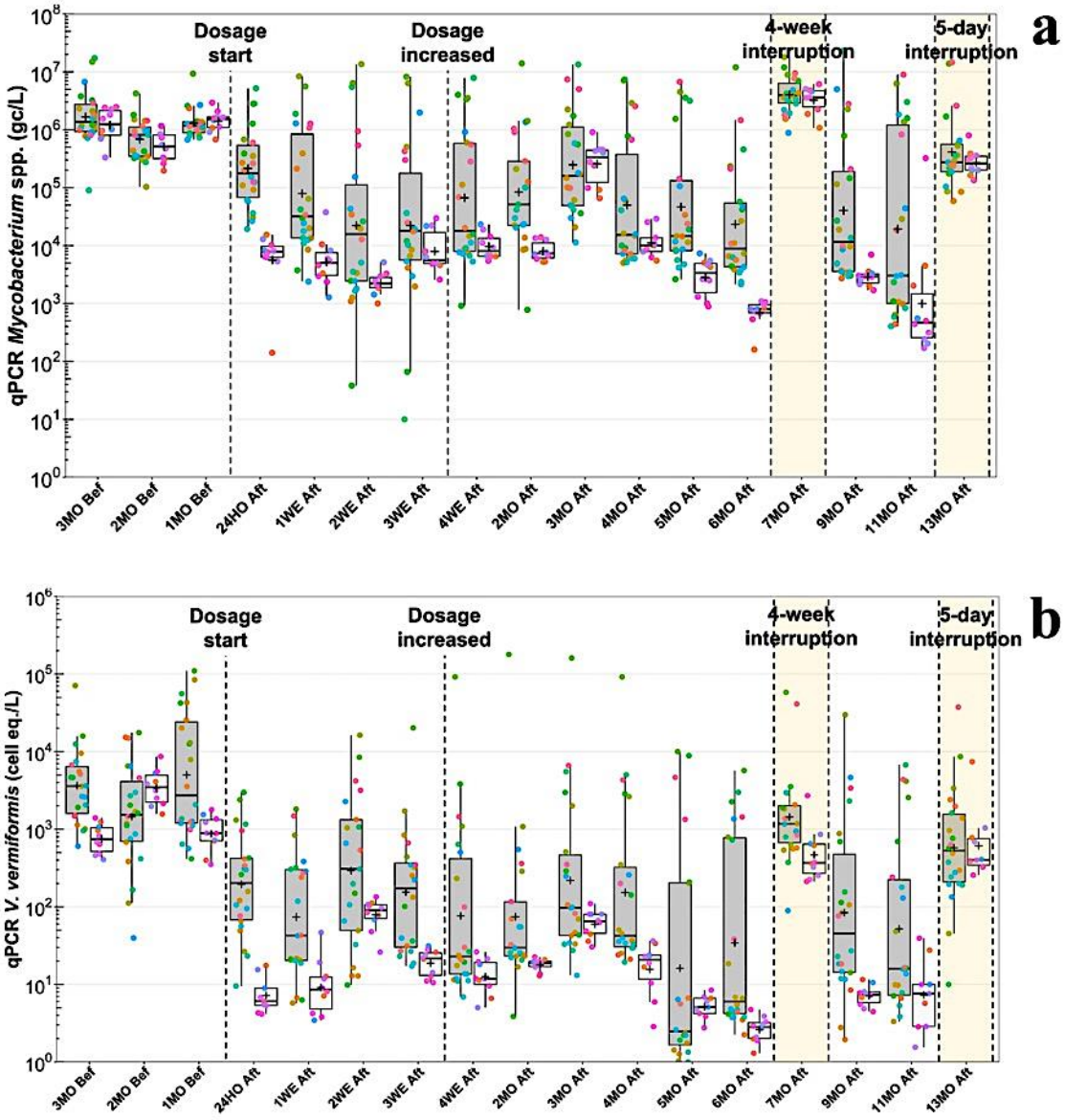

Figure 7.3 Box plots of (**a**) qPCR *Mycobacterium* species and (**b**) qPCR *Vermamoeba vermiformis* over time at distal sites (grey boxes, n = 22) and system sites (white boxes, n = 10). Legend: Black cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Colored dots – Raw data per sampling site, MO – Month, WE – Week, Bef – Before, Aft – After.

7.3.4 Impact of monochloramine dosing interruption

In this study, both short (5-day) and prolonged (4-week) interruptions of monochloramine dosing did not lead in *L. pneumophila* increases. The absence of *L. pneumophila* rebounds at the majority of sites during interruption of dosage further supports the idea that monochloramine effectively suppressed the reservoirs, thus preventing any host-prey relationships. This contrasts with

Legionella spp., which exhibited considerable increases during treatment interruptions, with gene copy concentrations increasing by more than one log at all sites during the longer dosage stop (Figure 7.2c). These rebounds suggest that non-*L. pneumophila* species may have species-specific survival and resistance mechanisms during monochloramine treatment, while *L. pneumophila* remained overall effectively controlled. Notably, some distal PoU with limited water usage and ineffective control, such as the hand washing station showing persistent contamination to *L. pneumophila* and showerheads with elevated *Legionella* spp. concentrations (> 10^4 gc/L), had the largest *Legionella* rebounds during interruptions. *Mycobacterium* spp. concentrations rebounded significantly (1-3-log), returning above or close to pre-treatment levels during the prolonged and shorter interruption periods, respectively (Figure 7.3a). These apparent increases are attributed to detachment from biofilms caused by changes in water chemistry rather than rapid growth, given the slow proliferation rates of many NTMs species (> 4 weeks) (Gupta et al., 2018). Additionally, one hypothesis for the striking efficacy of monochloramine in reducing *L. pneumophila* prevalence is its ability to induce amoebae to shift from their trophozoite active form to a cyst resistant form during selective pressures exerted by disinfectants, thereby preventing intracellular amplification of the pathogen (NASEM, 2019). In this study, large rebounds in *V. vermiformis* concentrations during discontinuation periods (Figure 7.3b) suggest that encystment occurred during treatment, followed by detachment of encysted and active *V. vermiformis* when conditions became more favorable.

7.3.5 Influence of abiotic factors

7.3.5.1 Temperature

Temperature is a critical factor in controlling DWPIs in buildings, with temperatures below 50 ˚C previously being associated to increased culturable *L. pneumophila* positivity (Grimard-Conea et al., 2024) in large buildings and qPCR NTMs in residential buildings (Falkinham III, 2011). Prior to monochloramine treatment, the highest concentrations and detection rates of culturable *L. pneumophila* were measured within the temperature range of 30 – 50 ˚C, consistent with its optimal growth conditions (Hochstrasser et Hilbi, 2022), whereas lower occurrences were measured below 30 ˚C and above 55 ˚C (< 8%) (Figure 7.4a). In contrast, high positivity was observed by qPCR for *L. pneumophila* (> 92%) (Figure 7.4b) and *Legionella* spp. (> 100%) (Figure 7.4c), regardless of the temperature. As monochloramine nearly eliminated *L. pneumophila*, no dependence to

temperature could be observed post-treatment, unlike *Legionella* spp. for which mean concentrations of data points above 55 ˚C were 2-log lower than that between 20 and 40 ˚C. *Mycobacterium* spp. (Figure 7.5a) and *V. vermiformis* (Figure 7.5b) were ubiquitous in all samples during baseline months. Notably, the combination of elevated temperatures (> 50 ˚C) and monochloramine treatment resulted in the largest reductions in mean concentrations of these two organisms (> 3-log), though this only significantly reduced positivity for *V. vermiformis*. These decreases align with the strong and significant negative correlations between temperature and qPCR concentrations of *Mycobacterium* spp. ($R = -0.56$, $p < 0.001$) and *V. vermiformis* ($R = -0.61$, $p < 0.001$) in monochloraminated samples, underscoring the added benefits of maintaining elevated temperatures alongside disinfection for their effective mitigation.

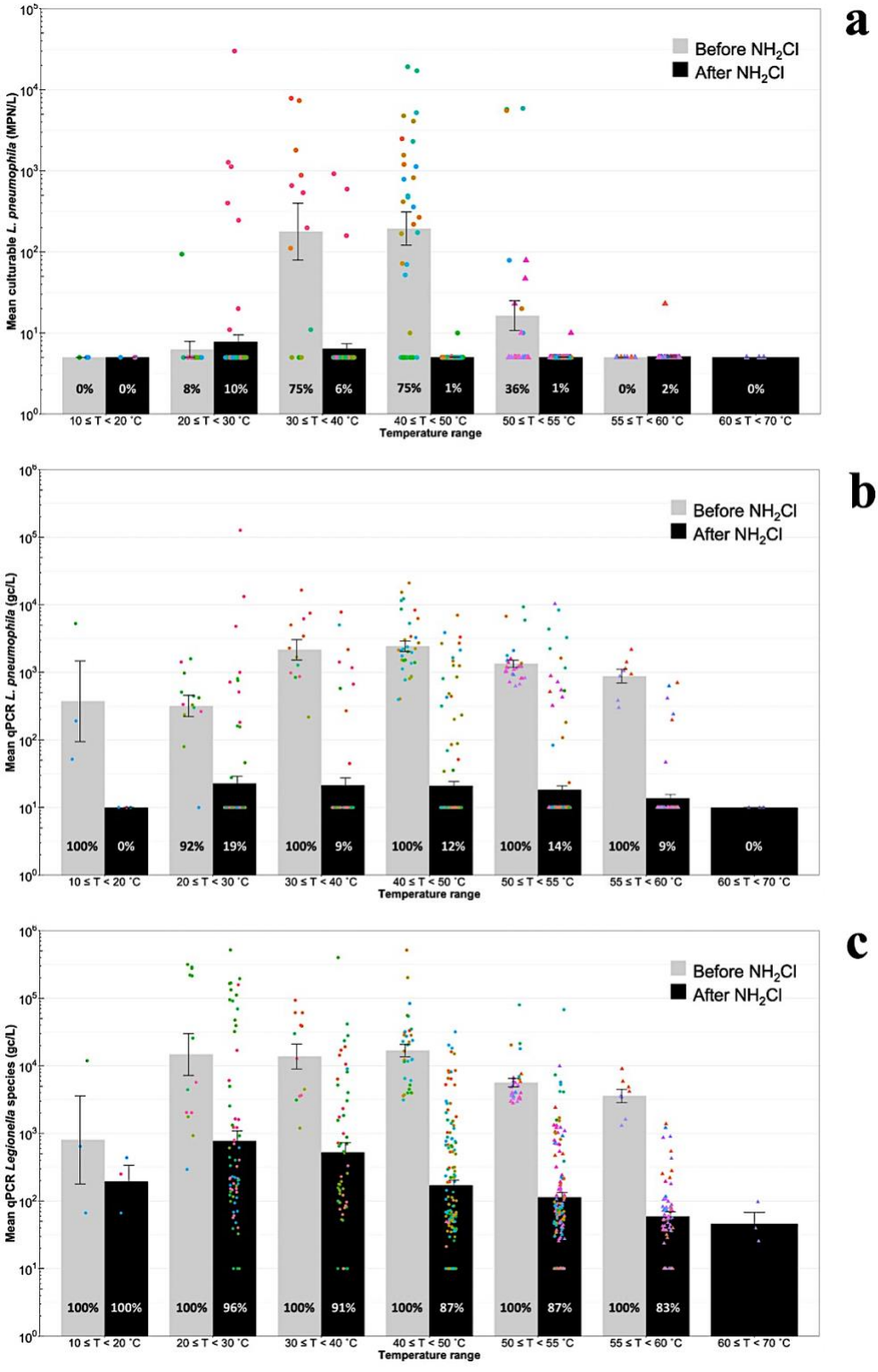

Figure 7.4 Bar plots of mean (**a**) Culturable *Legionella pneumophila*, (**b**) qPCR *Legionella pneumophila*, and (**c**) qPCR *Legionella* species per temperature range. Legend: Bar plot – Mean values, Bracket – Error bars, Circle points – Distal sites, Triangle-shaped points – System sites.

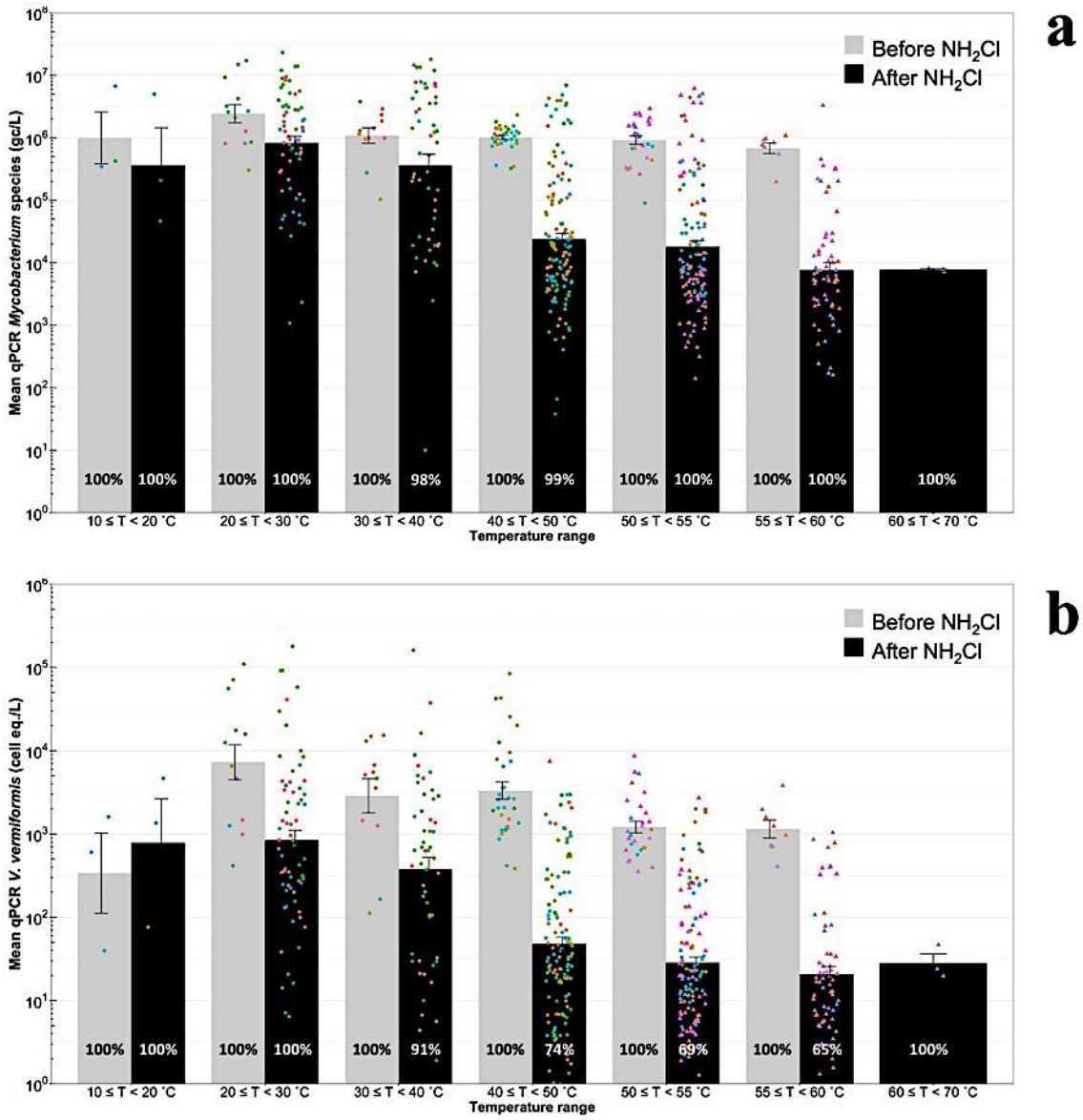

Figure 7.5 Bar plots of mean (**a**) qPCR *Mycobacterium* species, (**b**) qPCR *Vermamoeba vermiformis* per temperature range. Legend: Bar plot – Mean values, Bracket – Error bars, Circle points – Distal sites, Triangle-shaped points – System sites.

7.3.5.2 Disinfectant concentration

Mean concentrations of the targeted microorganisms for different total chlorine range during monochloramine dosing and interruption periods are presented in Table 7.1. Overall, both culturable and qPCR mean concentrations of *L. pneumophila* decreased as monochloramine levels increased, with qPCR signals becoming undetectable when total chlorine was maintained at 2 mg/L

or more. Compared to baseline months, *Legionella* spp. showed the most significant average decrease (2-log) when total chlorine levels were maintained at 2 – 3 mg/L, suggesting that further increasing the dosage did not result in a more considerable reduction. Likewise, results from Table 7.1 indicates that total chlorine above 2 mg/L was effective to erode the qPCR signal of *V. vermiformis*, but the data suggest that higher residuals (> 3 mg/L) would be required to achieve the most substantial reductions in *Mycobacterium* spp. concentrations. Due to chlorine demand during distribution throughout the hospital's HWS and dilution with cold water at tepid PoUs, concentrations of more than 2 mg/L are inconsistently reached at distal sites (Table D.2).

Table 7.1 Mean concentrations of targeted microorganisms for different total chlorine range. LoD: Limit of detection.

Targeted microorganism	Before	Stop	0 ≤ Cl < 0.5 mg/L	0.5 ≤ Cl < 1 mg/L	1 ≤ Cl < 2 mg/L	2 ≤ Cl < 3 mg/L	3 ≤ Cl < 4 mg/L
L. pneumophila (MPN/L)	1.1E+03	5.4E+02	7.7E+01	2.3E+01	< LoD	< LoD	< LoD
L. pneumophila (gc/L)	2.6E+03	2.3E+03	6.8E+02	3.5E+02	3.4E+02	< LoD	< LoD
Legionella spp. (gc/L)	3.5E+04	1.9E+02	2.3E+04	2.0E+04	1.6E+03	6.0E+01	4.3E+01
Mycobacterium spp. (gc/L)	1.7E+06	2.9E+06	2.1E+06	1.9E+06	4.8E+05	4.3E+04	5.1E+03
V. vermiformis (cell eq./L)	7.7E+03	3.2E+03	1.8E+03	8.6E+03	5.1E+02	4.2E+01	2.3E+01

7.3.5.3 Water use

The installation of flushing monitors at a subset of distal sites enabled the assessment of sites with very low water demand, defined as less than 30s of continuous use over the course of a week. These devices only allowed the identification of extreme low usage, as 30s of use per week remains very low. In this case, showerheads located in patient rooms within day cancer units, as well as the hand washing station at the entry of the neonatology ward, where hand sanitizer use is more prevalent, were rarely utilized. A modest increase of 0.2 mg/L in mean total chlorine (Figure D.5a) was measured in sites more regularly used. While no significant differences in *L. pneumophila* gene copies were observed between the different use regimes (Figure D.5b), a clear trend appeared with sites experiencing low water use showing significantly ($p < 0.05$) higher qPCR concentrations of *Legionella* spp. (Figure D.5c), *Mycobacterium* spp. (Figure D.5d), and *V. vermiformis* (Figure D.5e). These observations are consistent with results from another hospital, where significantly higher concentrations of qPCR *Legionella* spp. and *V. vermiformis* were also measured in

extremely low demand regime (< 2h per month) (Nisar et al., 2023). Similarly, automatic flushing taps near dead legs in a large hospital only reduced the prevalence of *L. pneumophila* when operated for one minute every two hours, compared to less frequent flushing (one minute every six hours) (Totaro et al., 2018). Indeed, resume of flow with flushing can cause detachment and even biofilm sloughing after longer periods of distal stagnation (Bédard et al., 2018; Grimard-Conea et al., 2022).

7.4 Implications for future guidance and conclusions

The recurring costs and inconveniences of PoU filters, along with labor- and logistics-intensive remedial actions like heat shocks and hyperchlorination underscore the need for effective, long-term solutions to control DWPIs in HCFs. In this study, despite prior optimization of thermal regimes across the hospital's HWS, *in situ* monochloramine application was needed to achieve prolonged mitigation control of *Legionella*, NTMs, and *V. vermiformis*. Notably, monochloramine strikingly eliminated *L. pneumophila* reservoirs, reducing rapidly culturable (< 24h) and progressively qPCR concentrations (< 4 weeks). Abundances of NTMs and *Legionella* spp., which comprise other pathogenic species, were further reduced and concentrations stabilized over the course of six months and four weeks, respectively. In this intervention study, total chlorine concentrations above 2 mg/L showed the greatest benefits on lowering abundances of *Legionella* spp., NTMs, and *V. vermiformis* at distal sites, which was only consistently reached when monochloramine was injected at 2.5 – 3.5 mg/L at the heater outlet. Additionally, a more aggressive dosage approach starting with the onset of treatment would limit the periods of persistence of *Legionella* spp. and NTMs. Overall, results emphasize the importance of multi-pathogen monitoring and risk assessments tailored to patient vulnerability throughout the duration of the intervention.

In large and complex HCFs, temperature and disinfectant act as combined selective pressures. In this study, the combination of monochloramine exposure and temperature had mixed results. Before onset of treatment, elevated temperatures (> 55 ˚C) were effective only at controlling culturable *L. pneumophila* occurrence, with no temperature dependency observed for the other targeted microorganisms. During monochloramine exposure, the largest reductions in *Legionella* spp., NTMs, and *V. vermiformis* were primarily observed at system points, exposed to higher residuals, thus underscoring the importance of maintaining consistent and adequate thermal control

throughout the HWS. Nevertheless, as exposure risk is primarily driven by concentrations, the significant reductions in their abundances despite their ubiquitous occurrence indicates that long-term risks associated with these organisms were still drastically reduced. The push to reduce energy consumption by lowering heater temperatures must be balanced against the need to maintain water safety, as failing to control the growth of less sensitive organisms can result in considerable public health risks in HCFs.

Rebounds in *Legionella* spp., *Mycobacterium* spp., and *V. vermiformis* concentrations during dosage interruptions demonstrate their persistence within biofilms, which serve as critical reservoirs allowing survival under adverse conditions and release into bulk water when treatment is disrupted. Even a brief 5-day stoppage contributed to elevate microbial concentrations to nearly pre-treatment levels, highlighting the need to rapidly respond to operational warnings from the monochloramine generator. Therefore, continuous monochloramine application and quick response protocols to dosage alerts should be initiated and clearly defined in water safety plans (WSPs) to prevent pathogen resurgence. Possible rebounds of clinically relevant species of *Mycobacterium* and *Legionella* other than *L. pneumophila* need further investigation to shed light on the benefits of multi-pathogen monitoring strategies and control measures. *V. vermiformis* was unlikely the primary host for *L. pneumophila* in this study, as no correlation was observed between their abundances. Nonetheless, understanding and managing the disruption of host-pathogen interactions remains crucial, especially in exploring the biotic factors contributing to the persistence and resurgence of these DWPIs.

In this study, monitoring both system and distal sites was essential for accurately assessing risk mitigation. Focusing solely on system sites would overlook the greater challenge of controlling DWPIs at distal points where exposure occurs. In general, results evidenced a higher DWPIs prevalence at distal sites, indicating that system sites, where water quality is typically monitored, were not predictive of distal sections. This reinforces the need for risk-based strategies in WSPs that extend beyond control points (system sites) to include comprehensive management of sentinel points (distal sites). Indeed, system sites alone do not provide a full picture of pathogen risks, particularly in buildings with diverse water use patterns and complex systems. Regular monitoring of DWPIs at sentinel sites is critical for early detection and timely intervention. Notably, after the onset of treatment, a greater variability in pathogen levels and plumbing metals was observed at distal sites, reflecting site-specific factors such as differences in water use patterns, disinfectant

residuals, and plumbing configurations and materials. Oppositely, system sites, which were exposed to more consistent temperatures and residuals, showed less variability. This variability calls for tailored control measures, including increased flushing, temperature control, and PoU filters in high-risk areas.

Furthermore, tepid and low-use sites exhibited higher microbial concentrations, showing the necessity for targeted control measures at these higher-risk PoUs. Discarding first draws of water, through regular flushing of taps is essential to minimize DWPIs exposure. This preventive measure can be carried out by maintenance staff during inpatient room turnover, or through the installation of auto-flush devices in critical areas or manual scheduled flushing protocols with the use of sensor-based monitoring systems implemented at strategic PoUs. Small amount of flushing (< 2 min) is typically required to reduce significantly microbial levels in first draws (Bédard et al., 2018; Grimard-Conea et al., 2022), although the frequency at which taps should be flushed remains site-specific (Grimard-Conea et Prévost, 2023). Tepid PoUs present additional challenges, as their operating temperatures consistently promote DWPIs growth. In the context of *in situ* monochloramine application, these sites are also compromised by diluted disinfectant concentrations resulting from the mixing of hot and cold water. While evidence of nitrification was not observed in this study based on variations of surrogate parameters (nitrogen species, dissolved oxygen, pH), tepid water temperatures may render these sites more vulnerable to growth of nitrifying bacteria. Therefore, regular flushing of tepid PoUs can prevent exposure to exacerbated pathogen growth, whereas in sections of the hospital housing very susceptible patients, installation of PoUs filters could provide a more robust layer of protection.

Finally, some transient release of metals (copper, lead, iron, manganese) was observed at both distal and system sites, as expected when shifting from free chlorine to monochloramine. However, with monochloramine infected in the HWS, the apparent increases in regulated metals are less concerning since hot water is not used for human consumption.

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Supplementary materials

Supplementary material is available with this article in Appendix D.

CHAPTER 8 ARTICLE 5: *IN SITU* DOSING OF MONOCHLORAMINE IN A HOSPITAL HOT WATER SYSTEM RESULTS IN DRASTIC MICROBIAL COMMUNITIES CHANGES

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This study represents the second phase of a broader investigation conducted at the hospital setting before and after the onset of monochloramine treatment, using a representative subset of samples from each intervention phase (before vs. during treatment, and over dosing interruption). Building on prior findings, this phase focuses on changes in bacterial and eukaryotic communities at distal and system sites based on raw sequencing reads processed though Illumina MiSeq. It addresses a critical gap in understanding the ecological impacts of introducing monochloramine on potential shifts in pathogen persistence or community turnovers, while investigating the diversity of ASVs of common opportunistic pathogens, including *Legionella* and nontuberculous mycobacteria.

Overall, this study provides insights into dominant lineages and the emergence of resistant strains during monochloramine disinfection and interruption of dosage. Additionally, it evaluates the use of ATP and flow cytometry cell counts as practical indicators of the presence of opportunistic drinking water pathogens, thus offering data-based evidence to support efficient monitoring of

microbiological content and the rapid identification of high-risk zones for proactive water safety planning and management.

In situ dosing of monochloramine in a hospital hot water system results in drastic microbial communities changes

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Abstract

Understanding changes in microbial composition under selective pressures is crucial to assess the emergence of resistant taxa and the survival of drinking water-associated pathogens. This study evaluated the impact of *in situ* monochloramine disinfection in a hospital hot water system on bacterial (16S rRNA gene amplicon sequencing) and eukaryotic communities (18S rRNA gene amplicon sequencing), and on general microbial measurements, including adenosine triphosphate (ATP) and flow cytometry counts. After the onset of treatment, ATP decreased by 1.2- and 3.5-fold, and total cell counts (TCC) dropped by 1- and 2-log at distal and system sites, respectively. During the dosage interruption (27-day), TCC rebounded to pre-treatment levels, but viability percentage decreased, indicating that cells were predominantly damaged. Low-use sites (e.g., showerheads) showed elevated ATP (> 15 pg/mL) and TCC (10^5 – 10^6 cells/L). Monochloramine

drastically altered bacterial and eukaryotic communities, with Alpha-diversity showing increased amplicon sequence variant richness, but reduced evenness during treatment due to these new, low-abundant taxa, and Beta-diversity revealed distinct shifts in community composition over time, with clusters corresponding to each treatment phase. Post-treatment, temporal and spatial heterogeneity was evident across distal sites, while elevated temperatures, consistent flow, and higher monochloramine concentrations in the hot water system resulted in more uniform communities at system sites. Additionally, the persistence of potential pathogenic strains belonging to *Legionella* and *Mycobacterium* genera highlights the value of comprehensive risk assessments. These findings emphasize the need to understand microbial shifts under disinfection stress and their public health implications, offering new insights into how treatment interventions shape microbial ecology and pathogen dynamics.

Keywords: Monochloramine; Hospital plumbing; *Legionella*; *Mycobacterium*; Diversity.

8.1 Introduction

Building plumbing water systems encompass a diverse mosaic of microbial niches shaped by a complex interplay of environmental and operational conditions such as temperature, disinfectant residuals, nutrient availability, water flow pattern, stagnation, and plumbing materials (Cullom et al., 2020). Biofilms formed on the inner surfaces of pipes and at point of use fixtures constitute major reservoirs for drinking-water associated pathogens involved in opportunist infections in susceptible populations (Falkinham III et al., 2015). These include many pathogenic species belonging to the genera *Legionella*, *Mycobacterium*, and *Pseudomonas*, which altogether pose significant public health burden in healthcare facilities (Collier et al., 2021) where a large concentration of immunocompromised individuals, elderly, and patients with chronic illnesses are accommodated or transiently present. Governed by miscellaneous ecological interactions, the diversity within these microbial communities can have contrasting deleterious or beneficial effects on the growth of opportunistic pathogens (OPs) or their attachment to or detachment from established biofilms.

Environmental stressors (e.g., temperatures, chemical disinfectants) and specific nutritional growth requirements warranting external sources not readily available in the environment induce *Legionella pneumophila* to transition from a metabolically active form to a virulent one, which facilitates its ability to infect host cells as a key aspect of its intracellular lifestyle (Oliva et al.,

2018). Some *Mycobacterium* species (Ben Salah et Drancourt, 2010) also adopt a parasitic lifestyle by replicating within protozoan hosts, including free-living amoebae and ciliates. Therefore, their ecology is strongly bound to that of protists, whose primary habitat is the biofilm. Contrastingly, *Pseudomonas aeruginosa* is frequently associated with biofilm formation given its ability to adhere and colonize various plumbing components by producing extracellular polymeric substances (Bédard et al., 2016). Investigating host-OPs relationships, or any other interactions with co-existing bacterial and eukaryotic populations, in engineered water systems is considered as one of the most suitable long-term approach to identify and interrupt OPs amplification niches (Cavallaro et al., 2022).

Installation of onsite monochloramine generator systems as an in situ disinfection method to control OPs and most especially *Legionella* bacteria is increasingly adopted by large hospital settings. Monochloramine introduced in hot water plumbing systems is generally associated with important and rapid reduction of *L. pneumophila*-positive sites in previous field studies (Marchesi et al., 2012; Casini et al., 2014; Duda et al., 2014; Mancini et al., 2015; Coniglio et al., 2018; Lytle et al., 2021; Grimard-Conea et al., 2025), although discrepant impacts on levels of nontuberculous mycobacteria (NTMs) and *Pseudomonas* are oppositely oftentimes observed. Given that sheltering of OPs within protists can enhance their persistence and survival during disinfectant exposure, it is essential to understand how supplemental disinfection can drive changes among microbial communities. This knowledge will help optimize monochloramine disinfection effectiveness as a holistic control strategy while minimizing selective pressures that are more likely to favor OPs presence. However, to date, only one prior hospital served as a testing ground to study bacterial ecology (Baron et al., 2014) and fungal diversity (Ma et al., 2015) using high-throughput sequencing methods in response to monochloramine treatment, thus highlighting a notable gap in the literature. It was demonstrated that monochloramine caused an immediate shift towards a significantly increased relative abundance of many genera containing potential pathogenic species, such as *Acinetobacter*, *Mycobacterium*, *Pseudomonas*, and *Sphingomonas*, yet without increasing nitrifying bacteria (Baron et al., 2014). The overall number of operational taxonomic units (OTUs), assessed by alpha-diversity of samples, was also statistically higher post-treatment, indicating competitive dynamics occurring once dominant bacterial groups were diminished upon monochloramination. Conversely, monochloramine did not result in significant changes in the fungal community, which was attributed to the greater disinfection resistance of fungi (Ma et al.,

2015). Moreover, the influence of design and operational factors on microbial populations after introducing monochloramine in full-scale water systems is not well studied in the context of disinfection. Nonetheless, a comprehensive investigation of these factors could provide valuable insights into components or situations supporting increased OPs risks, further aiding in regulatory compliance purposes, effective disinfection application, and enhanced system performance.

Moreover, additional studies should assess the impact of monochloramine on diversity of *Legionella* and *Mycobacterium* strains as many clinically relevant species are associated with waterborne infections in vulnerable individuals (Koh, 2016; Muder et Yu, 2022). One study reported a clear shift from *L. pneumophila* serogroup 1 as the dominant species (90%) isolated from fixtures in pre-monochloramine samples to *L. bozemanii* (70%) in post-monochloramine samples (Duda et al., 2014). Considering recurrent OPs contamination detected at distal sites despite the application of various disinfectants, examining OPs diversity can help track dominant lineages over time and space that may show new markers of resistance and adaptation. Data-based evidence to support the use of low-cost indicators for monitoring of microbiological content to quickly identify zones of risk during mitigation measures is also lacking.

The present study was conducted at a Canadian acute care academic hospital where two spatio-temporally linked nosocomial cases of Legionnaires' disease caused by the same strain of *L. pneumophila* triggered the in situ installation of a monochloramine generator in the hot water system following a series of heat shocks procedures failing to reduce *L. pneumophila* colonization (Grimard-Conea et al., 2025). The aims of this current study were to evaluate the impact of monochloramine introduction on the bacterial and eukaryotic planktonic communities, and on general microbial viability indicators, including adenosine triphosphate (ATP) and flow cytometry count cells. Diversity of major OPs was also assessed. Consequently, it was hypothesized that monochloramine disinfection drastically altered microbial community dynamics, while reducing overall general microbial loads.

8.2 Materials and Methods

8.2.1 Study ground

The study took place at a 540-bed (10 floor) acute care academic hospital in Québec (Canada) previously described by Grimard-Conea and colleagues (2025) (Submitted to Water Research). A monochloramine generator (Sanipur Sanikill, PA, USA) was installed at the water heater outlet to

control *Legionella* contamination, with dosage concentrations ranging from 1.1 to 3.3 mg/L as of total chlorine throughout the study timeline. Sampling campaigns were conducted on a monthly basis three times prior to the introduction of monochloramine into the hospital's hot water system. These were followed by sampling 24h post-dosage, then weekly for four weeks, and subsequently monthly and bimonthly over the following year, totaling 17 sampling events and 544 water samples. Several dosage interruptions occurred due to unresolved alerts during the study, including both short (5-day) and extended (4-week) periods. Of the collected water samples, 180 were processed for next-generation Illumina sequencing, while all 544 water samples were used for general microbial measurements. Sample collection and sampling site characteristics are fully detailed by Grimard-Conea and colleagues (2025).

8.2.2 General microbial measurements

Intracellular-ATP was measured through bioluminescence using the LuminUltra Quench-GoneTM Aqueous test kit (LuminUltra Technologies Ltd., Fredericton, NB, Canada), as previously described (Grimard-Conea et al., 2024). To enumerate total cell counts (TCC) and intact cell counts (ICC), flow cytometry using the BD Accuri^{TM} C6 Plus flow cytometer (BD-Biosciences, Mississauga, ON, Canada) was performed as outlined in Grimard-Conea et Prévost (2023).

8.2.3 Illumina MiSeq sequencing

Water samples and DNA extraction were processed as formerly described (Grimard-Conea et al., 2025), and 25 µL of extracted DNA per sample were sent on ice for Illumina MiSeq v2 250 reads sequencing at McGill Genome Center (McGill University, Montreal, QC, Canada). DNA products were cleaned up with AMPure XP Bead-Based Reagent (Beckman Coulter, Indianapolis, IN, USA) to improve DNA purification. Bacterial 16S rRNA gene amplicon sequencing was performed using a dual-index strategy with the forward primer sequence F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer sequence R806 (5'-GGACTACHVGGGTWTCTAAT-3') amplifying the V4 region of the 16S rRNA gene (Caporaso et al., 2011). The master mix composition for one reaction (20 µL) used to amplify the 16S rRNA gene contained 9.44 µL of nuclease-free water, 4 µL of 5X Platinum II PCR Buffer, 0.4 µL of 10 mM dNTP mix, 1 µL of 5 uM forward primer, 1 µL of 5 uM reverse primer, 4 µL of DNA template

diluted 1:10, and 0.16 µL of Platinum II Taq Hot-Start DNA polymerase. The cycling program included an initial denaturation step (94 ˚C, 2 min), followed by 30 cycles of denaturation (94 ˚C, 15 sec), annealing (56 ˚C, 15 sec), and elongation (68 ˚C, 15 sec).

Similarly, eukaryotic 18S rRNA gene amplicon sequencing was conducted in duplicates with the primer pair F1391 (5'-GTACACACCGCCCGT-3') and EuKBR (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Amaral-Zettler et al., 2009) which amplify the V9 region of the 18S rRNA gene. The amplification was done with a master mix (25 µL per reaction) composed of 2.5 µL of DNA template diluted 1:20, 2.5 µL of 2 uM forward primer, 2.5 µL of 2 uM reverse primer, 12.5 µL of 2X KAPA HiFi HotStart ReadyMix, and 5 µL of PCR-free water. Cycling conditions consisted of an initial denaturation step at 95 ˚C for three min, followed by 12 cycles of denaturation (95 ˚C, 30 sec), annealing (55 ˚C, 30 sec), and elongation (72 ˚C, 30 sec), and a final elongation phase at 72 ˚C for five min.

Due to insufficient DNA quantity (< 0.2 ng/µL) in some samples, amplification of the 16S rRNA and 18S rRNA gene was completed for 117 and 156 water samples, respectively, out of the 180 samples that were sent for sequencing.

8.2.4 Bioinformatics data processing

Illumina MiSeq raw sequencing reads were quality-filtered using the command-line tool fastp. Cleaned files were then processed using the Mothur pipeline (Kozich et al., 2013) based on previously published workflow (Paranjape et al., 2020). Initially, paired reads were joined into contigs. Assembled contigs containing ambiguous bases, sequences shorter than 8 bp, or exceeding 275 bp for the 16S rRNA gene sequencing and 250 bp for the 18S rRNA gene sequencing were removed. The remaining sequences were aligned to the SILVA Reference Database release 138.1 for bacterial sequences and the PR^2 v5 (Protist Ribosomal Reference) Database for eukaryotic sequences. Taxonomy comparison (results not shown) between the PR^2 and SILVA databases for eukaryotic sequences revealed that PR^2 provided more diverse eukaryotic lineages, thus enhancing retrieval of Amplicon Sequence Variants (ASVs). Ends and gaps of all sequences were then trimmed so that each sequence overlaps the same targeted region. Additionally, improved sequences were denoised using a pre-cluster algorithm command in Mothur which allowed for a maximum of two base differences between sequences. Chimeras were further culled from both the 16S rRNA and 18S rRNA sequencing results, as well as non-bacterial classified sequences (e.g.,

Eukaryotes, chloroplast, Mitochondria, and Archaea) from the 16S rRNA analysis and non-eukaryotic sequences from the 18S rRNA analysis. Finally, sequences were clustered into ASVs, a method that offers a more precise and accurate representation of the microbial communities by distinguishing sequence variants at a single nucleotide level. Samples were rarefied to 7,152 reads for the 16S rRNA gene analysis and 2,052 for the 18S rRNA gene analysis. However, due to low number of reads in many samples after rarefaction of the 18S rRNA sequencing data, 53 samples were filtered out.

For alpha-diversity analysis of the 16S rRNA and 18S rRNA genes, the Shannon index was calculated and plotted into groups referencing to the time of sampling and monochloramine dosage. Similarly, beta-diversity analysis was done using the Bray-Curtis index, and the ordination results were graphed in principal coordinates analysis (PCoA). Both diversity indexes were estimated by using Mothur. To assess differences among groups, the Kruskal-Wallis test was performed for alpha-diversity and the AMOVA and HOMOVA statistical tests for beta-diversity measurements. All ASV sequences belonging to the genus *Legionella* (n = 16), *Mycobacterium* (n = 8), and *Pseudomonas* (n = 5) were retrieved using the get.seqs command in Mothur, and subjected to NCBI BLAST Web search tool (BLAST+ 2.16.0) to identify strains using a per identity threshold over 99% (Table E.1, Table E.2). Since a very low number of reads (n = 62) were sequenced for *Pseudomonas* genus compared to *Legionella* (n = 494) and *Mycobacterium* (n = 6,270), ASV analysis of *Pseudomonas* was not considered. Graph visualization and statistics were conducted in RStudio version 4.1.1. using different R packages, including tidyverse, ggplot2, readxl, vegan, FSA, ggsignif, ggh4x, and data.table. The significance level was set at a p-value of 0.05.

8.3 Results and discussion

8.3.1 Monochloramine effectively reduces microbial loads and activity

8.3.1.1 Intracellular-ATP and flow cytometry

During the baseline months, the majority of samples remained below the 10 pg ATP/mL target recommended by ATP bioluminescence testing kits, with mean values of 4.2 pg ATP/mL (0.1 – 21.2 pg ATP/mL) and 1.5 pg ATP/mL (0.4 – 5.9 pg ATP/mL) at distal and system sites, respectively (Figure 8.1a). The consistently lower ATP measurements at system sites suggest that maintaining elevated temperatures (> 55˚C) had an additional inhibitory effect on microbial activity. Within the first three weeks of monochloramine dosage, concurrent decreases in ATP and

cell viability were measured (Figure 8.1b), thus demonstrating the immediate suppressive impact of the disinfectant on bacterial activity. The elevated concentrations of ATP (> 15 pg ATP/mL) observed at several distal sites following the dosage increase likely reflect the greater detachment of biofilm occurring after prolonged distal stagnation periods (Bédard et al., 2018; Grimard-Conea et al., 2022). These distal sites, primarily showers, were infrequently used between sampling events, which shifted to monthly intervals after the dosage increase, in contrast to the weekly sampling conducted at the start. Over both monochloramine dosing interruption periods, apparent rebounds in mean ATP values of 3- and 12-fold at distal and system sites were observed, respectively.

In pre-monochloramine samples, total cell counts showed minimal variation, with most measurements at both distal and system sites around 10^5 cell/mL (Figure 8.1c) and cell viability ratios averaging 20 – 25% (Figure 8.1b). After the onset of monochloramine treatment, total cell counts dropped consistently by 2-log at system sites within the first week, whereas distal sites showed smaller reductions of 1-1.5-log, but with high variability, spanning up to 3-log among different locations. Unlike total cell counts, which stabilized by the study's end, the fraction of viable cells continued to increase, displaying considerable variability over time. This initial rapid decrease in total and intact cell counts demonstrates the immediate impact of monochloramine in suppressing a large portion of the bacterial population. However, as the most susceptible cells were quickly inactivated, leading to a plateau in total cell counts, prolonged monochloramine exposure likely induced a shift within the microbial community. This shift, favoring more resistant strains, may have contributed to the fluctuations observed in cell viability as the population dynamics evolved. Similar to ATP levels, irregularly used showerheads had total cell counts persisting in the range 10^5 – 10^6 cell/mL, probably archetypal of biofilm detachment stressed by discontinued water flow. Despite significant increases of 1 – 2-log in total cell counts ($p < 0.05$) during dosage interruption periods, nearly reaching pre-treatment levels, the percentage of viable cells contrastingly decreased in comparison to the period of active dosing. This suggests that cells recovered when monochloramine was interrupted were apparently damaged or non-viable (dead), and predominantly detached once the biocidal pressure was removed. In previous studies, onsite monochloramine generation in hospital plumbing systems resulted in more than 2-log reductions in culturable heterotrophic bacteria (HPC) concentrations (Duda et al., 2014) and significant decreases in HPC levels by 55% (Mancini et al., 2015) and 83% (Lytle et al., 2021) at all distal

sites. However, HPC may not be the best surrogate parameter to assess changes in the microbiological water quality as it measures only bacteria growing under specific agar media and laboratory conditions compared to ATP or flow cytometry counts, thus leading to an underestimation of the total bacterial load (Allen et al., 2004).

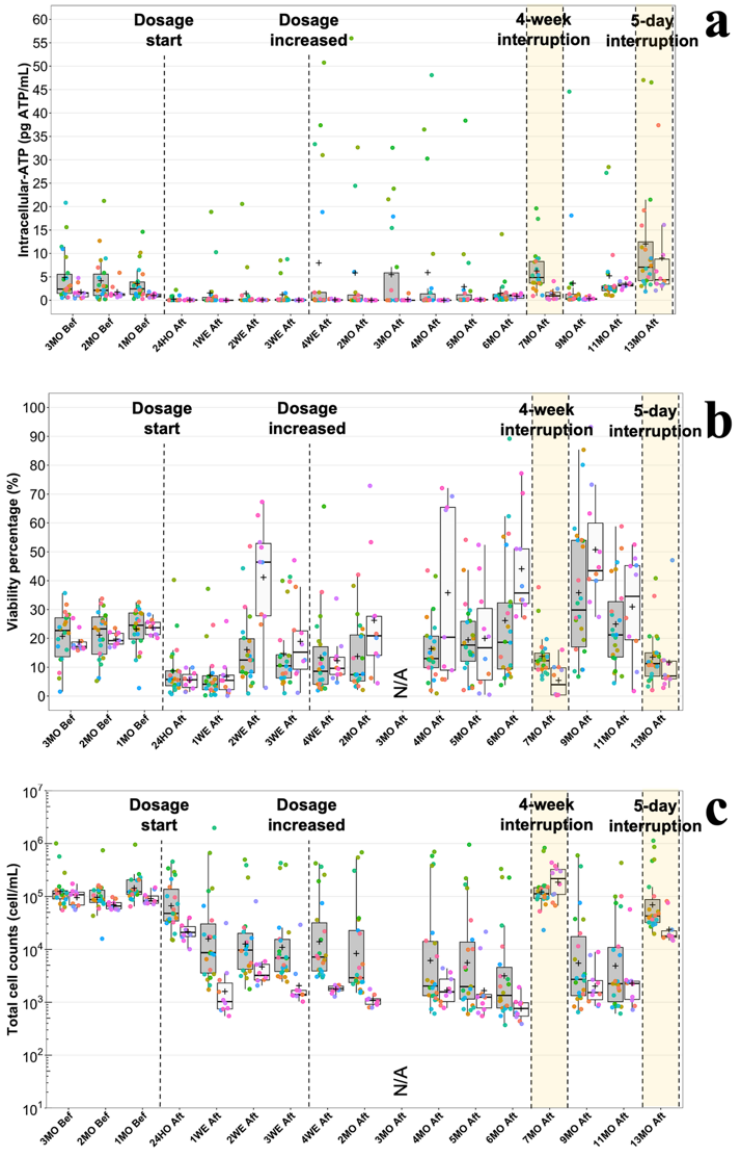

Figure 8.1 Box plots of sampling events (x-axis) against (**a**) ATP, (**b**) Viability percentage, and (**c**) Total cell counts at distal sites (grey boxes, n = 22) and system sites (white boxes, n = 10). Legend: Black cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Colored dots – Raw data per sampling site, Red/Blue solid lines – Mean heater outlet/return recorded by probes, MO – Month, WE – Week, Bef – Before, Aft – After, N/A – Not available.

8.3.1.2 Correlation analysis

ATP measurements correlated significantly ($p < 0.05$) and fairly strongly with both total cell counts ($R = 0.61$) and viable cell counts ($R = 0.65$), thus suggesting that ATP reflected adequately the metabolically active biomass and was a reliable monitoring indicator of disinfection effectiveness. ATP and flow cytometry cell counts showed negligible correlations ($R < 0.1$) with culturable and qPCR *L. pneumophila* (Table 8.1), likely because monochloramine suppressed this pathogen from the hospital water system (Grimard-Conea et al., 2025). The limited ability of ATP to specifically predict the abundance of *L. pneumophila* has been previously documented, even outside the context of disinfection (Duda et al., 2015; Arroyo et al., 2017; Grimard-Conea et al., 2022, 2024). The correlations between NTMs and ATP ($R = 0.48$), TCC ($R = 0.56$), and especially ICC ($R = 0.60$) were relatively strong, thus indicating that these microbial parameters are good surrogates for the presence of environmental NTMs and are closely associated with the overall microbial load and viable cell populations. For qPCR *Legionella* spp., correlations among parameters were generally weak ($R < 0.3$), thus limiting their potential use as predictors of *Legionella* burden in the context of disinfection. Notably, ICC were moderately good at predicting *V. vermiformis* trends ($R = 0.50$), comparatively to TCC measurements ($R = 0.38$), indicating a larger contribution of viable, intact cells to *V. vermiformis* amplification signals.

Table 8.1 Spearman rank correlation coefficients (R) between general microbial measurements and targeted microorganisms. Legend: Bold – significant correlation ($p < 0.05$).

Targeted microorganism	ATP	TCC	ICC
L. pneumophila (culture)	< 0.01	0.04	0.06
L. pneumophila (qPCR)	< 0.01	0.03	0.05
Legionella spp. (qPCR)	0.02	**0.20**	**0.25**
Mycobacterium spp. (qPCR)	**0.48**	**0.56**	**0.60**
V. vermiformis (qPCR)	**0.32**	**0.38**	**0.50**

8.3.2 Monochloramine alters bacterial and eukaryotic communities

8.3.2.1 Alpha- and beta-diversity indices

The Shannon alpha-diversity of the bacterial community in samples collected before introducing monochloramine into the hospital's hot water system was significantly ($p < 0.05$) different from

those collected during treatment, after the first interruption period, and once the dosage was restarted (Figure 8.2a). Upon initiating treatment, the number of ASVs increased from 849 to 1,361, indicating an important rise in community richness. This observation aligns with Baron and colleagues (2014), who also reported increased richness in monochloramine-treated samples compared to baseline months using OTU-based alpha-diversity. However, despite the increase in richness, the median Shannon index decreased during monochloramine treatment, suggesting that the additional ASVs detected were present at very low abundances, leading to reduced evenness in the bacterial communities. Monochloramine likely exerted a strong selective pressure on the communities by (1) creating new ecological niches where reduced bacterial competition allowed certain dominant strains to thrive, thereby decreasing evenness, and (2) disrupting biofilms, which led to the release of a broader array of bacterial strains into the water, thus increasing richness. Although fewer samples were sequenced during both treatment interruption and restart periods, there was a notable reduction in ASVs richness during these phases. Indeed, the bacterial community may not have fully recovered between treatment periods due to cumulative stress from the initial and subsequent round of monochloramine exposure. This likely resulted in a bottleneck effect, where only a small subset of highly tolerant ASVs survived, while many of the more sensitive strains that might have begun to recolonize during the dosage stop were ultimately eliminated. Similarly, alpha-diversity measurements revealed that the introduction of monochloramine had a profound impact on the eukaryotic community intra-samples, leading to a notable increase in detected ASVs from 157 to 974 (Figure 8.2b). However, the greater richness observed in samples during treatment may be partially attributed to the larger sample size in that second group, which naturally tends to capture more ASVs. Nonetheless, this increased richness may reflect how some eukaryotic species capitalize on the disturbance caused by monochloramine by feeding, among other things, on dead bacteria and metabolic by-products, thus favoring the survival of a wider spectrum of strains. As observed for bacterial communities, there is a trend for which richness decreases during the interruption of dosage, followed by a partial recovery in the number of ASVs once monochloramine treatment is restarted.

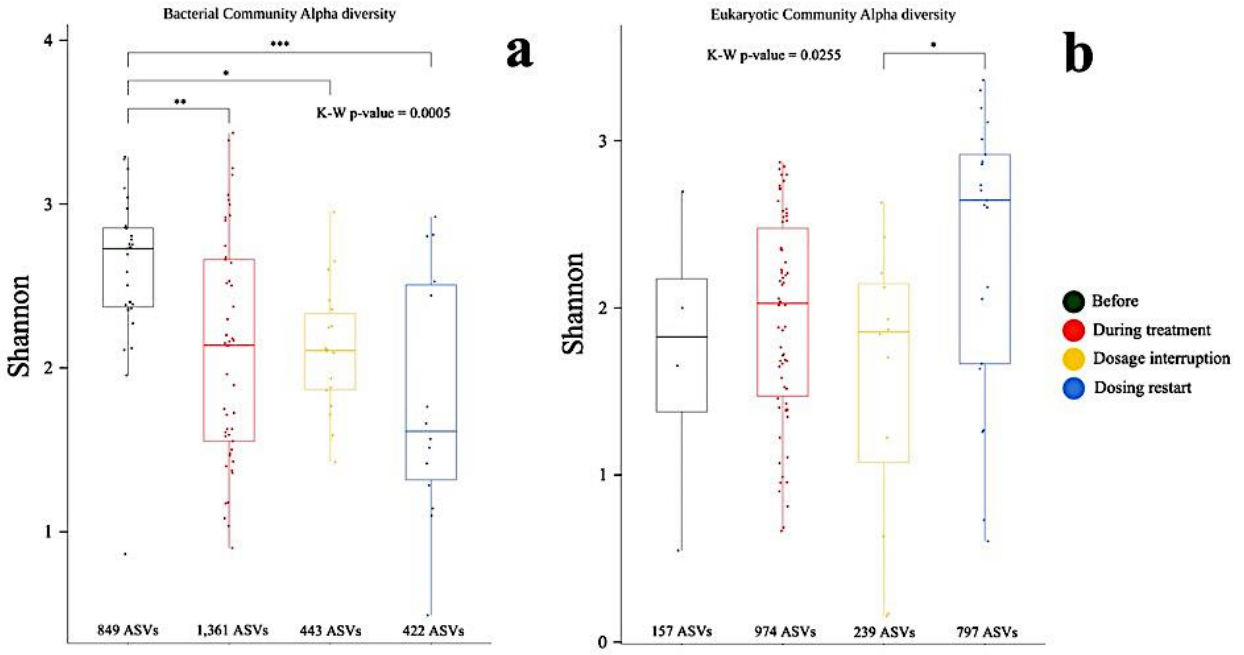

Figure 8.2 Shannon Alpha-diversity index for (**a**) Bacterial 16S community (n = 117 samples) and (**b**) Eukaryotic 18S community (n = 103 samples) across the four treatment phases. Legend: Horizontal line – Median, Boxes – 25th and 75th percentiles, Dots – Raw data.

The beta-diversity analysis of the bacterial communities, as shown in the Bray-Curtis dissimilarity plot (Figure 8.3a), highlights the significant and heterogenous impact of monochloramine on community composition. Prior to treatment, bacterial communities mostly clustered together, indicating a uniform and stable composition. Then, a distinct shift away from the pre-treatment cluster was observed, reflecting the rapid change in the bacterial community composition caused by monochloramine. Interestingly, data points from the dosage stop tend to form a separate group that partially shifted back toward the pre-treatment state, though with a unique composition likely influenced by the residual effects of monochloramine and the re-establishment of bacterial species. When treatment is restarted, the bacterial community underwent another shift, with the data points spreading widely once again. This dispersion mirrors the significant variability in community composition in response to monochloramine exposure across sampling sites. Similar results were observed in eukaryotic communities (Figure 8.3b), with each phase producing distinct shifts in composition. The introduction of monochloramine into the hospital's hot water system led to increased variability in the communities, with independent clusters corresponding to different sampling events and representing successional biofilm dynamics occurring after prolonged

exposure to the disinfectant. Similarly to bacterial communities, the interruption of treatment allowed for partial recovery of initial community composition, but restart of dosage triggered further changes. Overall, these findings indicate that monochloramine had a discerning and lasting impact on the composition of bacterial and eukaryotic communities, with apparent variabilities depending on treatment phase and sites.

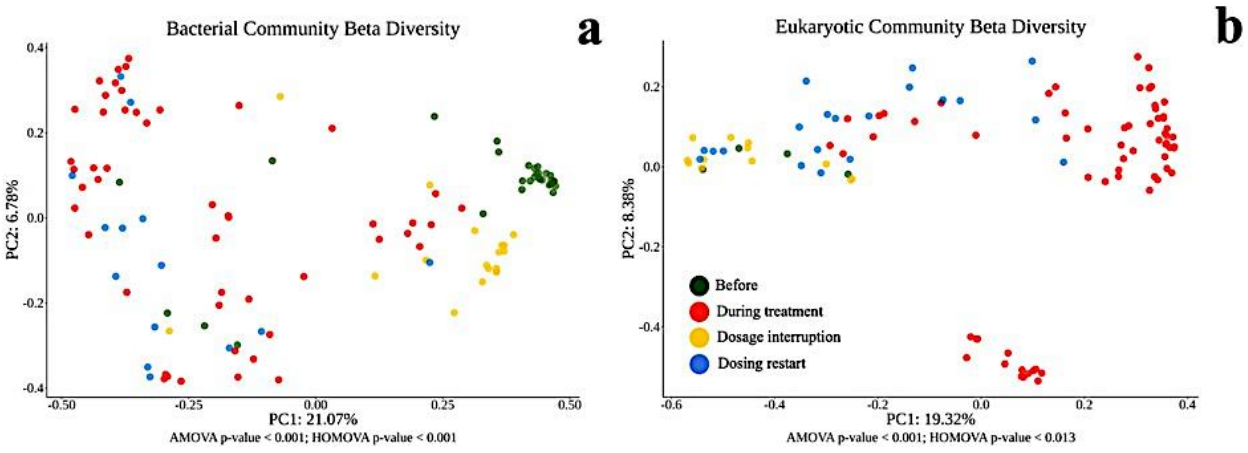

Figure 8.3 Principal coordinates analysis for Beta-diversity among (**a**) Bacterial 16S community (n = 117 samples) and (**b**) Eukaryotic 18S community (n = 103 samples) across the four treatment phases.

8.3.2.2 Bacterial taxa abundance

Figure 8.4 presents the relative abundance of the top 15 most abundant bacterial phyla (Figure 8.4a) and genera (Figure 8.4b) across the four treatment phases. Before the onset of monochloramine treatment, the bacterial communities were generally similar at both the phylum and genus levels, suggesting a well-balanced bacterial ecosystem. Predominant phyla comprised Proteobacteria (27 – 97%), Planctomycetota (1 – 40%), and other unclassified phylum (1 – 31%), whereas most abundant genera included *Gemmata* (0 – 39%), *Burkholderiales* (1 – 30%), and other unclassified genus (0 – 30%). *Legionella* and *Mycobacterium* genera were only minor fractions of the bacterial communities (< 1%). Once monochloramine was introduced into the hospital's hot water system, there was a clear shift towards increased dominance of Proteobacteria over time, reducing the relative abundance of other phyla considerably. At the genus level, substantial differences were observed among sampling locations, thus highlighting the differential impact of monochloramine treatment, although these differences remained relatively stable with prolonged exposure to the

disinfectant. Notably, significant ($p < 0.05$) increases in the relative abundance of *Sphingomonas*, *Acidovorax*, *Phreatobacter*, and *Qipengyuania* were detected at several distal sites over time. Multiple studies highlight the tolerance of *Sphingomonas* spp. to monochloramine treatment. Indeed, Sphingomonas was isolated among the most dominant genera found in biofilms of a municipal drinking water distribution system treated with monochloramine (Pullerits et al., 2020). Additionally, lab-scale monochloramine inactivation treatment demonstrated the increase in the relative abundance of *Sphingomonas* (Chiao et al., 2014), which is further corroborated by a similar observation in a hospital water system with onsite monochloramine treatment (Baron et al., 2014). Altogether, these observations have important implications for hospital plumbing systems, as the genus *Sphingomonas* includes an emerging opportunistic specie, *S. paucimobilis*, which is known to cause infections in susceptible individuals (Ryan et Adley, 2010). In this study, increased relative abundance of *Sphingomonas* was only measured at distal sites, suggesting that the elevated temperatures and higher monochloramine concentrations across the hot water system likely inhibited the growth of this genus in those locations. The application of monochloramine also led to increases in the relative abundance of other minor, or "uncultured" genera, indicating that these previously unidentified bacterial genera became more prevalent within the community under disinfection pressure, which aligns with the rise in ASV counts and Shannon Alpha-diversity (Figure 8.2a).

A clear bacterial community turnover was observed at both the phylum and genus levels once monochloramine dosing was interrupted. This was marked by the resurgence of a few phyla, such as Acidobacteriota, Bacteroidota, and Deinococcota, though in lesser proportions than prior treatment (Figure 8.4a), and a more pronounced increase in some genera like Burkholderiales and Thermus compared to pre-monochloramine samples (Figure 8.4b). In contrast, a near-complete suppression of the phylum Planctomycetota and the genus Gemmata were noticed, as they did not persist across the treatment phases. Once monochloramine treatment was restarted, the bacterial communities shifted back to a composition fairly similar to that observed during the initial weeks to months of application. Interestingly, before the initiation of treatment and during its interruption, the bacterial communities at system and distal sites are quite similar, likely indicating that elevated temperatures across the hot water system or site-specific conditions were not major factors shaping the bacterial communities. However, during phases of monochloramine dosing, there was a manifest divergence in composition between distal and system sites. This was probably because

system sites were exposed to higher and more stable monochloramine levels and temperatures, which favor certain disinfectant-resistant and thermotolerant bacteria. Meanwhile, distal sites experienced lower and more variable disinfectant exposure, thus leading to the development of a distinct bacterial community.

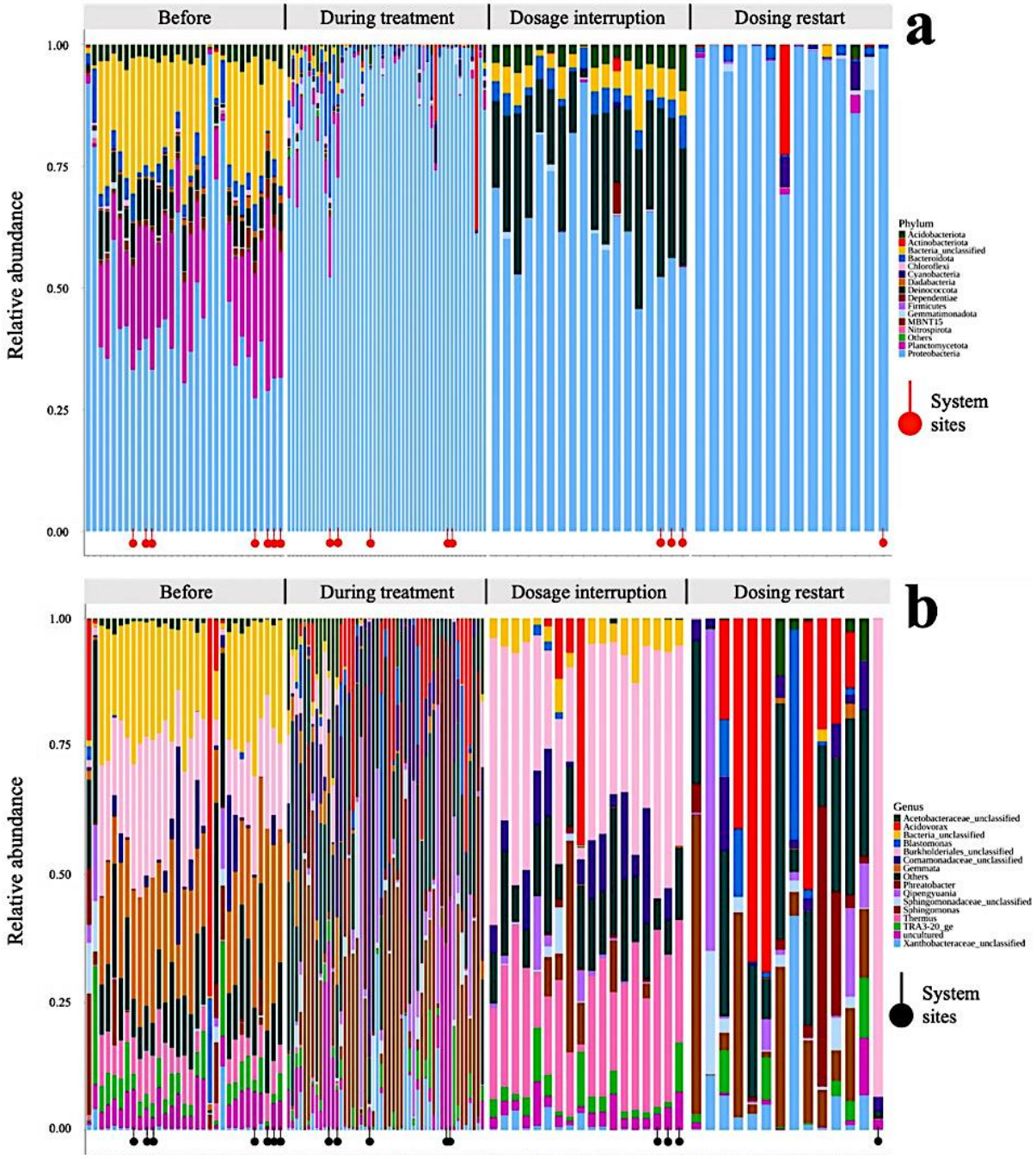

Figure 8.4 Relative abundance of the top 15 most abundant 16S bacterial phyla (**a**) and genera (**b**) across the four treatment phases.

8.3.2.3 Eukaryotic taxa abundance

Before the onset of monochloramine treatment, only four samples were successfully sequenced for 18S rRNA gene amplicon sequencing, all collected from showerheads (Figure 8.5). Despite the small subset of samples, these showerheads (i.e., three showerheads, as the second and fourth samples were taken from the same showerhead at two different dates) showed distinct community compositions, especially at the genus-level (Figure 8.5b), highlighting the unique eukaryotic ecology of each fixture. The introduction of monochloramine resulted in more diversified eukaryotic communities, both at the phylum- and genus-level, as corroborated by the increased number of ASVs (Figure 8.2b). There was a clear dominance of the Dinoflagellate phylum in most samples across the four phases (Figure 8.5a), representing 53% of all eukaryotic sequences. This aligns with observations from longitudinal samplings at cold and hot water taps of a large building (Buse et al., 2013). Similarly to bacterial taxa, once monochloramine treatment was restarted after the dosage interruption period, eukaryotic communities reverted to more diverse states, yet not to their composition during treatment, suggesting some irreversible community changes. For example, the relative abundance of the Gyrista phylum was significantly different ($p < 0.05$) during both phases of active monochloramine dosing, representing 23% of all sequences during the first phase, but only 5% after the dosage stop. Interestingly, the eukaryotic composition among system sites was not drastically different than at distal sites in comparison to bacterial taxa, probably because of their broader ecological niches. Among eukaryotic taxa known to experimentally allow intracellular replication or survival of *L. pneumophila* (Boamah et al., 2017), Tubulinea (6%) (e.g., *Vermamoebae*), Ciliophora (0.6%) (e.g., *Tetrahymena*), and Discosea (0.04%) (e.g., *Acanthamoebae*), represented only minor fractions of the total number of retrieved sequences.

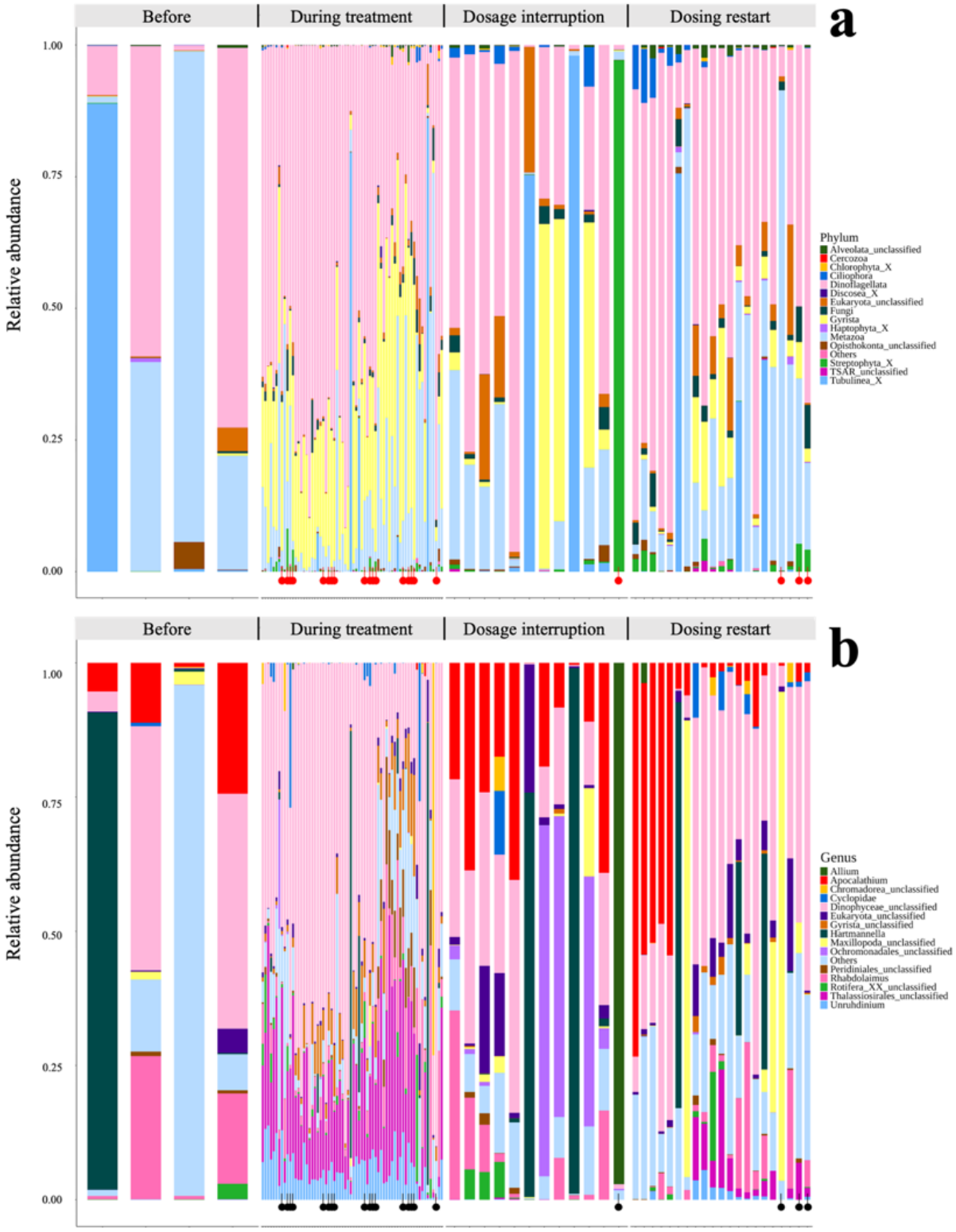

Figure 8.5 Relative abundance of the top 15 most abundant 18S eukaryotic phyla (**a**) and genera (**b**) across the four treatment phases.

8.3.2.4 ASVs analysis

Bubble plots illustrate the logarithmic counts of *Legionella* (Figure 8.6a) and *Mycobacterium* (Figure 8.6b) ASVs sequences collected over time across the four treatment phases: before treatment, during treatment, during dosage interruption, and after the interruption. Distal sites exhibited significantly higher *Legionella* ASVs counts and greater diversity ($p < 0.05$, $n = 15$ ASVs) compared to system sites ($n = 5$ ASVs), regardless of the treatment phase. Although multiple *Legionella* strains were assigned to the dominant ASV00106 (Table E.1), the latter is likely *Legionella anisa*, the non-*pneumophila* species most frequently isolated from building water systems (Crook et al., 2024). *L. pneumophila* was identified as the second most abundant *Legionella* ASV (ASV00207) within samples, but accounted for only 16% of all collected *Legionella* ASVs. Other *Legionella* species known to cause opportunistic infections in vulnerable individuals without being commonly implicated in large outbreaks (e.g., *L. dumoffii*, *L. bozemanii*, *L. cincinnatiensis*, *L. sainthelensi*, etc.) (Muder et Yu, 2002) were also matched with certain ASVs, yet were only present in very low abundances (< 1%).

The introduction of monochloramine in the hospital's hot water system appeared to have triggered the detachment of new, low-abundance *Legionella* ASVs at distal sites, which were not observed before onset of treatment (Figure 8.6a). However, the detection of these new low-abundance ASVs during the application of monochloramine may partially reflect sampling intensity rather than biofilm detachment alone, as a higher number of samples were sequenced, thus leading to the retrieval of more reads and increased likelihood of capturing rare ASVs. While the second (*L. pneumophila*) and third (ASV00219) most abundant *Legionella* ASVs seemed to progressively decrease with monochloramine injection, as they were less frequently isolated from sites than before treatment, the most abundant *Legionella* ASV (likely *L. anisa*) was not suppressed. These findings contrast with those of Duda and colleagues (2014), who observed a shift from *L. pneumophila* to *L. bozemanii* as the dominant species in their hospital water system with the onset of in situ monochloramine treatment. In this study, the clear persistence of ASV00106 during monochloramine application suggests the presence of a strain exhibiting considerable resistance to the disinfectant. As a legacy building with unlined, and probably rusty cast iron pipes, the hospital's plumbing infrastructure may have contributed to the prevalence of *L. anisa*-like ASV00106. Indeed, a previous study demonstrated that cast iron rust in shower mixers significantly promoted the growth of *L. anisa* in a model building plumbing system, leading to increased concentrations

and a higher number of positive samples (van der Lugt et al., 2017). The presence of rubber components in the interior of showerhead thermostatic mixers has also been demonstrated to promote considerably *Legionella* spp. (van Hoof et al., 2014), a plumbing component most often found at tepid distal sites. During the dosage interruption period, some resurgence of ASV00106 and *L. pneumophila* were observed at two different low-use distal sites (one hand washing station, and one showerhead), but were then eliminated when dosage was restarted.

ASV00029 accounted for over 98% of the *Mycobacterium* ASV counts, thus representing a predominant strain. For this same ASV, BLAST analysis identified three closely related slow-growing *Mycobacterium* species, including *M. gordonae*, *M. paragordonae*, and *M. vicinigordonae* (Table E.2), which are highly similar based on their 16S rRNA gene sequences (Kim et al., 2014; Liu et al., 2021). *M. gordonae* is frequently isolated from domestic, public, and hospital water systems (Vaerewijck et al., 2005) and has been associated to several nosocomial pseudo-outbreaks (Prabaker et al., 2015; Zlojtro et al., 2015; Scorzolini et al., 2016). Recent research on the diversity of nontuberculous mycobacteria in drinking water distribution systems (Yang et al., 2024) and shower water (Shen et al., 2022) detected the species *M. paragordonae* as nearly dominating the *Mycobacterium* community. In contrast, the presence and clinical significance of *M. vicinigordonae* is less documented, making it far more likely, without further evidence, that either *M. gordonae* or *M. paragordonae* are the dominant species in this study. However, since short-read sequencing data did not provide sufficient taxonomic resolution (i.e., multiple species aligned with a same ASV), distinguishing between species with certainty becomes unfeasible. Interestingly, ASV00029 was collected from nearly all distal sites, but was absent from system sites, reflecting the influence of site-specific conditions leading to the selection of this particular strain at distal sites. Notably, ASV00029 counts were significantly higher ($p < 0.05$) at low-use tepid water points (hand washing station, showerheads) compared to hot water faucets with higher water usage, which aligns with *M. gordonae* optimal growth temperature in cooler water conditions (< 45 ˚C) (Griffith et al., 2007). Other ASVs were assigned to other clinically relevant *Mycobacterium* species described in (e.g., *M. avium*, *M. mucogenicum*, *M. fortuitum*, *M. xenopi*, etc.), but these were present at low abundances (< 1%).

Upon the start of monochloramine dosage into the hospital's hot water system, ASV00029 counts increased (Figure 8.6b). However, the introduction of monochloramine led to the emergence of a greater diversity of *Mycobacterium* ASVs, which were mostly isolated from system sites, though

ASV00029 remained dominant. In a study comparing the diversity of nontuberculous mycobacteria in one municipal drinking water distribution system without disinfectant to another supplied with monochloramine, biofilm samples collected from the latter consisted almost solely of the species *M. gordonae*, and especially at high monochloramine concentrations around 3.5 mg/L (Waak et al., 2019). During the dosing interruption period, ASV00029 continued to be highly abundant, while the newly emerged strains were eliminated. Since ASV00029 is most likely linked to slow-growing nontuberculous mycobacteria, previous rebounds observed in *Mycobacterium* species during the 5-day interruption period (Grimard-Conea et al., 2025, Submitted to Science of the Total Environment) are most probably to detachment occurring from biofilms. Interestingly, once monochloramine dosage was restarted, the composition of the *Mycobacterium* population remained unchanged from the previous phase, still dominated by the single ASV00029. The continued presence and abundance of ASV00029 throughout each treatment phase suggests that the strain was not significantly impacted by the introduction of monochloramine and may have already possess specific adaptations, including high tolerance to monochloramine, allowing it to thrive independently.

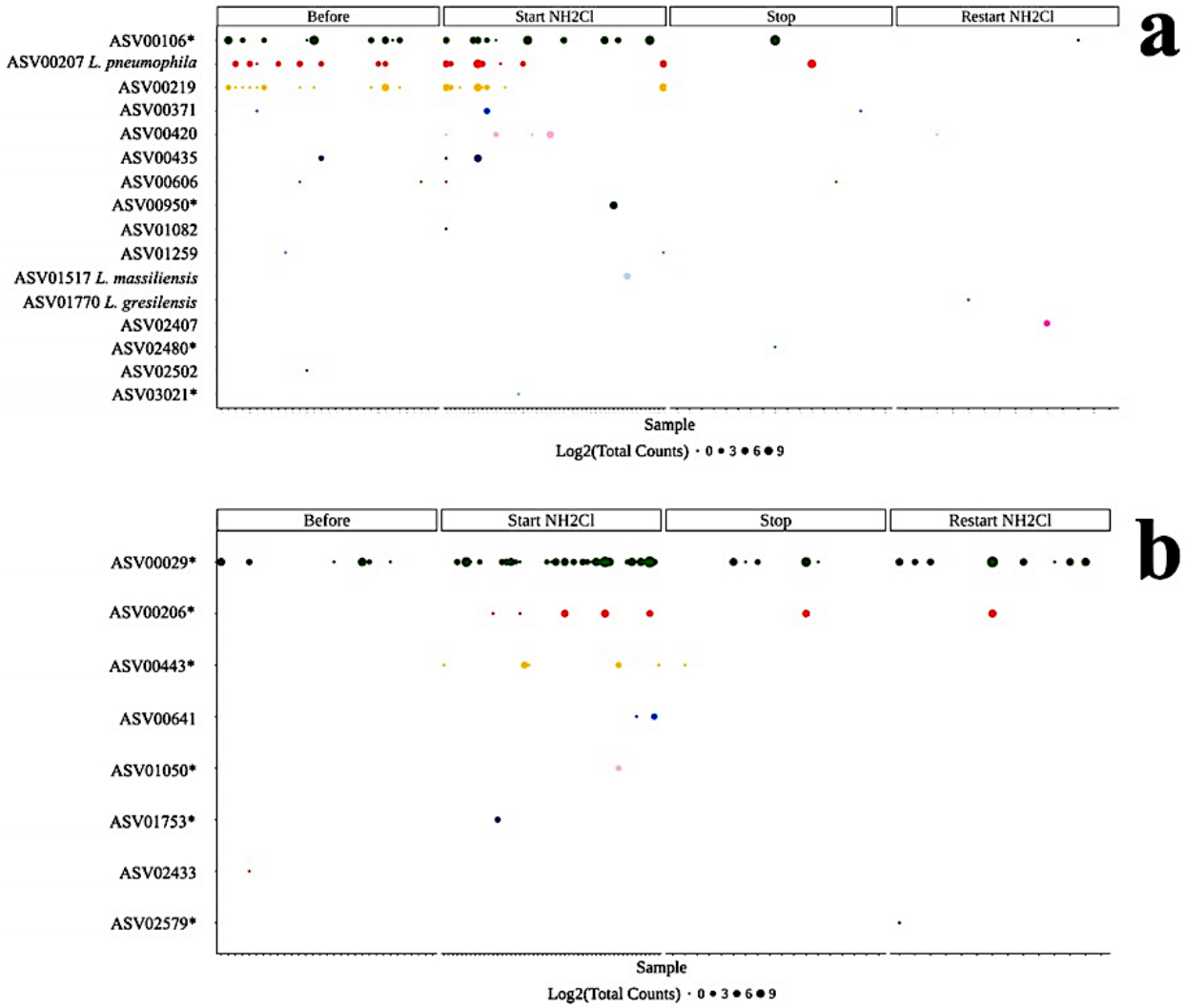

Figure 8.6 ASV sequences logarithmic abundance plots for (**a**) *Legionella* genus (n = 16 ASVs and 494 counts) and (**b**) *Mycobacterium* genus (n = 8 ASVs and 6,270 counts) across the four treatment phases: before the introduction of monochloramine (n = 31 samples), during application of monochloramine (n = 49 samples), during dosage interruption period (n = 18 samples), and when monochloramine dosage was restarted (n = 14 samples). Legend: Colored bubble – Logarithmic counts per ASV, Asterix – Multiple species producing significant alignment (> 99% identity) with a same ASV.

8.4 Conclusions

The impact of onsite monochloramine treatment on microbial communities is complex, involving important shifts in bacterial and eukaryotic community composition over time, increased ASVs diversity, and the potential emergence of more pathogenic strains. Findings highlight the need for longitudinal monitoring to account for spatial and temporal variability across sampling sites and

public health-focused interventions. The communities composition changes after the onset of treatment could indicate ecological niche differentiation occurring in response to changing environmental conditions, or detachment from biofilms. Some strains, emerging specifically during monochloramine exposure, showed a transient occurrence pattern. Overall, monochloramine exerted selective pressures that promoted the persistence of certain strains and suppressed more susceptible ones, indicating a highly dynamic and evolving microbial community under disinfection stress. Community turnover driven by monochloramine application was reflected by taxa benefiting from the disturbances caused by the disinfection pressure, while others dominated only during interruption of dosage. Species-level identification of *Legionella* and *Mycobacterium* genera is valuable for targeted surveillance of the most clinically relevant species in hospital water systems since *Legionella* and *Mycobacterium* species differ widely in terms of pathogenicity, routes of exposure, and clinical manifestations (Muder et Victor, 2002; Haworth et al., 2017). In this study, *Legionella* and *Mycobacterium* were not among the most abundant genera, although occurred at low abundances compared with other bacterial genera. While monochloramine substantially reduced *L. pneumophila*, the alteration of microbial populations may lead to the emergence of other clinically relevant strains. These unintended consequences may pose a risk to vulnerable populations and should not be overlooked, but the trade-off between a significant reduction in *L. pneumophila* and increased relative abundance of less harmful *Sphingomonas* spp. is considered a net gain for patient safety.

The temporal variability observed in the data demonstrated that different bacterial or eukaryotic phylum and genus were occasionally detected across the distinct treatment phases as time progressed, thus highlighting the critical nature of sampling timing to fully capture the diversity and dynamic nature of microbial communities. Spatial heterogeneity among sampling sites also yielded considerably different results, underscoring the complexity of monitoring and more particularly of pathogen surveillance and outbreak investigation. The diversity across sites and the possibility of low-abundance strains slipping through typical surveillance efforts mean that routine monitoring may not always detect clinically relevant strains before an outbreak occurs. Findings from this study evidenced that the hot water system had a strong influence on the selection of bacterial and eukaryotic taxa, driven by selective environmental pressures including elevated temperatures, consistent flow, and higher monochloramine concentrations. Indeed, communities composition before monochloramine treatment and during dosage interruption were similar at

system and distal sites, suggesting that the absence of disinfection pressure allowed for more uniform microbial communities development throughout the hospital's water system. However, once monochloramine was applied, the communities diverged with distal sites showing more ASVs due to more hospitable conditions. This also makes distal sites potential reservoirs for a wide variety of microorganisms which are more difficult to control once they are established in those environments. Therefore, while longitudinal sampling helps understanding temporal dynamics, the effectiveness of interventions, and long-term trends, transversal sampling can complement this by providing a comprehensive spatial assessment, identifying risk-prone areas, and informing on corrective actions to ensure water safety.

Finally, results also emphasize the limitations of general microbial indicators, such as ATP and flow cytometry cell counts, in revealing shifts in diversity and evaluating risks. In this study, ATP and flow cytometry counts were moderately good surrogates for *Mycobacterium* spp. and *V. vermiformis*. However, given the absence of correlation with *L. pneumophila*, integrating pathogen-specific methods alongside general microbial monitoring remains the most reliable approach for ensuring comprehensive water quality monitoring. The value of microbial indicators lies in their ability to provide rapid detection of changes in treatment conditions (e.g., monochloramine dosing interruptions), to identify potential problematic microbial proliferation niches (e.g., low-use sites). More importantly, both indicators detected significant and sustained biofilm detachment events, providing valuable information for water safety planning.

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Supplementary materials

Supplementary material is available with this article in Appendix E.

CHAPTER 9 GENERAL DISCUSSION

This chapter discusses key findings of the doctoral project, linking them back to the overarching main objective and placing them within the broader context of water safety plans for the effective management and mitigation of OPs in large building water systems. Using novel tools to investigate the occurrence of specific OPs (*Legionella*, *Pseudomonas*, and NTMs) and evaluate the effectiveness of preventive and corrective measures intended to lower the user's exposure risk, the primary objective of this research was to provide evidence-based recommendations to improve the monitoring, operation, and design of plumbing systems in large buildings (Chapter 3).

9.1 Overview of the thesis outcomes

To achieve the specific objectives presented in Chapter 3 and test the corresponding hypotheses, the experimental approach of this thesis was structured into four main parts (Figure 9.1).

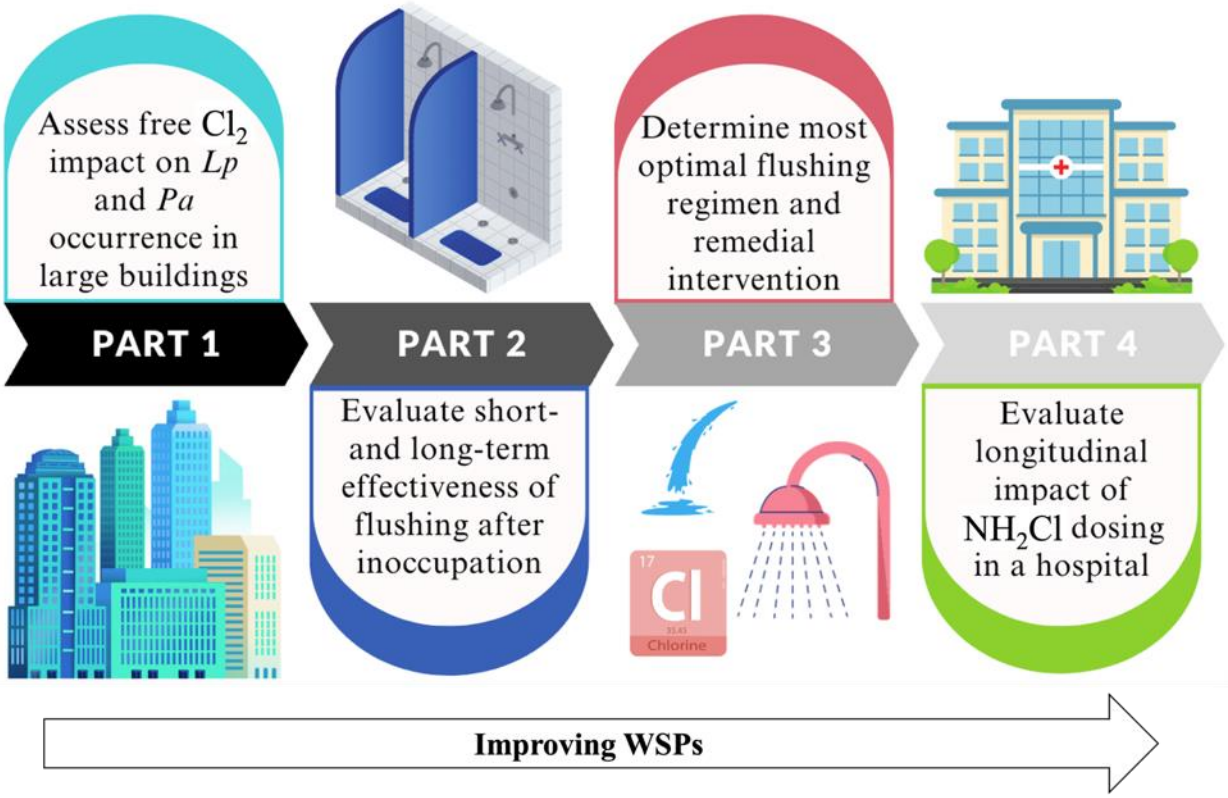

Figure 9.1 Schematic overview of the four main parts of the thesis.

The first investigative part of this thesis aimed to assess the impact of maintaining free chlorine residuals across nine large buildings on the occurrence of culturable *Legionella pneumophila* and

Pseudomonas aeruginosa. The second part examined the effectiveness of device recommissioning flushing at 23 showerheads in a large sports complex, evaluating both the short-term (24h) and long-term (4-week, distal water stagnation) impacts following a period a 16-week inoccupation on the presence of *Legionella pneumophila*. Furthermore, aggregated data from the first and second part of this thesis were used in Dowdell and colleagues (2023) as part of a larger scale study investigating the occurrence of *Legionella pneumophila* from 26 low-occupancy buildings across 11 cities in the United States, Canada, and Switzerland (published in Environmental Science: Water Research & Technology, July 2023). The third investigative focus was to determine the most optimal combination of preventive flushing, whether daily, weekly, or none, along with remedial interventions, such as device recommissioning flushing or shock chlorination, to mitigate the occurrence of *Legionella pneumophila* in two grouped shower systems. Finally, the fourth part evaluated the longitudinal impact (> 1-year) of *in situ* monochloramine disinfection in a large hospital's hot water system on microbial communities and the prevalence of *Legionella* species, nontuberculous mycobacteria, and *Vermamoeba vermiformis*. Table 9.1 provides an overview of the key outcomes of this thesis in relation to the research hypothesis presented in Chapter 3.

Table 9.1 Overview of the key thesis outcomes for each research hypothesis and experimental approach.

Research hypothesis	**Experimental approach**	**Thesis outcomes**
1) Water temperature is a major predictor of the maintenance of free Cl_2 residuals in large building plumbing systems (Chapter 4)	Data gathering from past sampling campaigns (2012 – 2022) using already published data sets (aggregated data) and novel (unpublished) results in nine large buildings in the province of Québec (hospitals, sports complex, elementary schools, university buildings) <u>*Article 1:*</u> *Published in Science of the Total Environment*	Moderate inverse (R = -0.53) and significant ($p < 0.001$) correlation between free Cl_2 concentrations and water temperatures Decreasing compliance rates to the 0.2 mg/L guide value with increasing water temperature in flushed samples (cold = 83%, tepid = 15%, hot = 1%), and even more so in first draws
2) Free Cl_2 concentrations above 0.2 mg/L can significantly reduce the occurrence of *Lp* and *Pa*, and the overall microbial biomass (Chapter 4)		No culturable *Lp* was detected among 809 samples when free chlorine was above 0.2 mg/L, and only 2 out of 286 samples were positive for culturable *Pa* under the same conditions Weak, yet significant inverse correlations observed between free Cl_2 and ATP (R = -0.16), TCC (R = -0.14) and ICC (R = -0.17)
3) Device recommissioning flushing conducted after a prolonged period of low occupancy can significantly lower *Lp* exposure risk (Chapter 5)	Collection of first draws and 5-min flushed samples at 20 – 22 showers of a sports complex following a 16-week building closure, 24h and four weeks (no water use) after the intervention <u>*Article 2:*</u> *Published in Frontiers in Water*	Device recommissioning flushing reduced culturable *Lp* below the common alert level of 1,000 MPN/L at short-term, then rebounds Device recommissioning flushing lowered per exposure risks for Pontiac fever and LD endpoints by 2-log at first draws, and per exposure DALY for a LD endpoint by 2-log
4) The combination of daily flushing and shock chlorination is the most optimal strategy to reduce significantly the abundance of *Lp* at showerheads (Chapter 6)	Collection of first draws at six showerheads per shower system before and weekly after conducting either device recommissioning flushing (5-min flush) or shock chlorination (20 – 25 mg/L, 16h), and implementing preventive flushing (daily, weekly, stagnant) at pairs of showerheads per shower system <u>*Article 3:*</u> *Published in Microorganisms*	The combination of shock chlorination and daily flushing led to a 2-week suppression of culturable *Lp* (down 2-log) and to the greatest decreases in its gene copies (qPCR) compared to the other combination of remedial and preventive flushing tested

Table 9.1 Overview of the key thesis outcomes for each research hypothesis and experimental approach (continued).

5) *In situ* dosing of NH_2Cl in a hospital's hot water system can selectively eliminate *Lp* long-term (> 1-year), but it is less effective on the abundance of NTM, *L*spp, and *Vv* (Chapter 7)	Collection of first draws at 22 distal sites (showerheads, hand washing stations, faucets) and 2-min flushed samples at 10 system sites representative of the main hot water flowing system before and after introducing NH_2Cl at target concentrations ranging 1.5 to 3.5 mg/L <u>*Article 4:*</u> *Submitted to Water Research* <u>*Article 5:*</u> Submitted to *Science of the Total Environment*	Monochloramine rapidly eliminated culturable *Lp* reservoirs (< 24h) and progressively reduced its gene copies over time (< 4-week) (up to 3-log reductions), except at one site with persistent contamination Persistence of NTM, *L*spp, and *Vv* in biofilms due to large rebounds in their gene copies observed over dosage interruption periods, but monochloramine still achieved 2-log mean reductions in < 6-month
6) The introduction of NH_2Cl results in speciation of *Legionella* and NTMs due to changes in microbial communities over time (Chapter 8)		NH_2Cl induced significant shifts in bacterial and eukaryotic community composition over time, increased ASVs diversity, and community turnover NH_2Cl temporarily triggered the detachment of new, low-abundant *Legionella* and *Mycobacterium* ASVs, with clear persistence of potentially clinically relevant strains long-term
7) Surrogate parameters, such as ATP, HPC, and flow cytometry cell counts, can reliably predict the presence and abundance of *Lp*, *Pa*, NTMs, and *Vv* (Chapters 4 to 8)	Correlation analysis (Spearman rank) between general microbial parameters (ATP, HPC, flow cytometry cell counts) and specific microorganisms <u>*Articles 1, 2 & 5:*</u> *Unifying theme across these studies, when general microbial parameters were compared with targeted organisms*	General microbial parameters demonstrated large variability in their predictive value for the presence of *Lp*, *Pa*, NTM, and *Vv*, yet followed similar trends during intervention studies ATP, ICC, and TCC were relatively strongly correlated with NTMs (R = 0.48 – 0.60) and moderately correlated with *Vv* (R = 0.32 – 0.50), but showed only weak or negligible correlations with *Lp* and *Pa* (R < 0.39, including HPC)

9.2 Targeted contributions to water safety plans

The following discussion links thesis outcomes and other results directly to their relevance in enhancing WSPs, thereby detailing key findings that refine both the definition and implementation of effective prevention and control strategies. More specifically, the thesis findings impact some components of WSPs, including the evaluation and control of water-related risks, as well as the validation step of the WSP (Section 2.2.1). They provide targeted and actionable guidance to support building managers in effectively implementing and optimizing their WSPs.

9.2.1 Evaluation of water-related risks

The evaluation of water-related risks in a building is a crucial step in developing a WSP, as it provides objective information on control and prevention targets, helps prioritize resources for implementing effective control measures, and ensures compliance with standards and regulations.

9.2.1.1 Defining microbial targets

Throughout this research project, *Legionella pneumophila* was present in the hot water systems of various large buildings, including hospitals, sports complex, and university buildings across the province of Québec (Chapter 4), but at varying occurrence rates, thus reflecting differences in operational practices and flawed design considerations fostering their growth. In a large sports complex, after a 16-week shutdown during the COVID-19 lockdown, the pathogen was highly prevalent, with culture-based and qPCR methods detecting it in 81% and 90% of first draw samples, respectively (Chapter 5). Despite flushing interventions conducted to safely reopen the sports complex before reoccupation, *Legionella pneumophila* remained strikingly persistent at the showerheads even 12-week later (Chapter 6). A cross-sectional study during the pandemic analyzing 26 reduced-occupancy buildings across 11 cities in the United States, Canada (including aggregated data from Chapter 4 and Chapter 5), and Switzerland further demonstrated that the occurrence of *Legionella pneumophila* can be specific to each building, with certain plumbing characteristics likely contributing to its proliferation (Dowdell et al., 2023). In Chapter 7, the high culturable (61%) and molecular (98%) positivity to *Legionella pneumophila* at distal sites in the hospital's building plumbing system prior to the introduction of monochloramine was also evidenced. Similar to observations in the sports complex, these elevated occurrence rates were

associated with high *Legionella pneumophila* abundances in both culture-based and qPCR methods (up to 10^5 – 10^6 MPN/L or gc/L) at sentinel sections of these buildings.

- Based on results from Chapters 4 to 7, buildings with documented *Legionella pneumophila* positivity tended to have widespread contamination across most of their hot water system, indicating that the presence of the pathogen was most often extensive rather than localized, likely due to systemic factors related to operational and design features.

Although the occurrence of *Pseudomonas aeruginosa* was not systematically assessed during all phases of the thesis, findings from Chapter 4 indicated that positive samples to culturable *Pseudomonas aeruginosa* were detected far less frequently (2.4%, 5/206) than were for *Legionella pneumophila* (26%, 207/802) across cold, tepid, or hot water systems. Additionally, Chapter 8 revealed that the *Pseudomonas* genus was only a very rare taxon in terms of relative abundance in the hospital's hot water system, either before or after the onset of monochloramine treatment. While *Pseudomonas aeruginosa* is also a significant OP, it does not have the same level of regulatory oversight as *Legionella pneumophila* because its occurrence is tightly influenced by environmental factors, such as warm water environments and surface colonization for biofilm formation (Bédard et al., 2016a), making it rarely associated with widespread contamination events in the United States (Gerdes et al., 2023) or in Québec (INSPQ, 2023a). Other potential pathogens were further detected at very low abundances through 16S rRNA gene amplicon sequencing in Chapter 8, although their ASV sequences were not clearly matched to a singular *Legionella* or *Mycobacterium* species.

- While these clinically relevant bacteria (e.g., *Pseudomonas aeruginosa*, *Legionella anisa*-like, *Mycobacterium gordonae*-like) may not be dominant in a building plumbing system, their presence can still have implications for risk management.

9.2.1.2 Identification of risks

A distinct risk assessment for each target chosen by the WSP team must subsequently be conducted to systematically identify locations or conditions that present or increase potential hazards. This is a crucial step to ensure the integrity of water systems. To this end, identifying risks associated with microbiological targets requires specific knowledge on the ecology of the pathogens, their route of exposure, and the type of susceptible clientele, whereas contamination sources and exposure pathways are further necessary for chemical targets (WHO, 2011). Generally, risk identification by

the WSP team allows the (1) determination of risks to patients/occupants, (2) compliance with building codes, standards and regulations, and (3) demonstration of due diligence. Three key areas of concern contributing to increased risks arise based on findings from Chapters 5 to 7, including prolonged stagnation events and the design and operation of shower and hot water production systems.

Stagnation. Stagnation in building plumbing systems can lead to considerable degradation in water quality (Section 2.1.5 Water usage patterns). This is particularly problematic in systems that experience extended intermittent use, such as during prolonged building closures or low-occupancy periods (e.g., COVID-19 pandemic lockdowns, off-season closures, summer holidays), and over the course of construction and renovation activities. In Chapter 5, the 16-week closure of the sports complex revealed amplified mean concentrations of general microbial measurements in first draw samples, including elevated ATP (10.0 pg ATP/mL), total cell counts (1.7 X 10^6 cells/mL), intact cell counts (5.2 X 10^5 cells/mL), and widespread contamination by *Legionella pneumophila* (culture-based mean of 5,487 MPN/L and qPCR mean of 68,822 gc/L) across shower systems. These concentrations represented the highest means observed throughout the study, exceeding values previously documented in large building plumbing during normal occupancy (Siebel et al., 2008; Lipphaus et al., 2014; Buse et al., 2020). Following the targeted recommissioning flushing intervention, the subsequent 4-week period of distal stagnation resulted in considerable rebounds of all microbial measurements to levels similar to those measured after the initial 16-week closure, thus underscoring the severe impact of extended stagnation on water quality. In Chapter 6, observations from the same sports facility showed that a 12-week period of distal stagnation in shower systems also led to elevated concentrations of ATP, TCC, ICC and *Legionella pneumophila*, reinforcing findings from the Chapter 5. Even more concerning was the impact of interventions after which a period of 3-week distal stagnation promoted complete regrowth (and more) of ATP, TCC, and ICC after shock chlorination, and of *Legionella pneumophila* after targeted recommissioning flushing. This further highlights that stagnation post-intervention can increase risks associated with microbial regrowth and potential resurgence of *Legionella pneumophila*. Finally, points of use with extreme low water usage (< 2h per month), as assessed with flushing monitors and specifications from the infection prevention and control team of the hospital setting, had significantly higher ($p < 0.05$) qPCR concentrations of *Mycobacterium* species, *Legionella* species, and *Vermamoeba vermiformis* (Chapter 7).

Collectively, these findings demonstrate the critical need to address water stagnation in water safety planning by:

- Mapping out low-use areas and infrequently used sites during hazard assessment to understand water usage patterns in the building;
- Developing specific strategies to replenish water after prolonged no water use (> 3-week) or interventions carried out in the water system;
- Ensuring stagnant sites are included in monitoring efforts to prevent them from becoming persistent reservoirs of OPs.

Shower systems. Showerheads present greater risks than other common indoor points of use, especially for the transmission of opportunistic drinking water pathogens whose route of exposure is through the inhalation of fine water droplets (aerosols). For example, Hamilton and colleagues (2019) showed that shower exposure systematically resulted in higher annual infection and per exposure infection risks (1- to 2-log in median values) than sinks and toilets. Indeed, shower systems operate at temperatures that are typically ideal for microbial growth (30 – 40 ˚C), feature complex designs with diverse plumbing materials, are used intermittently (i.e., distal stagnation), and generate an important quantity of respirable size range aerosols. Therefore, shower systems are high-exposure points for vulnerable populations and they create unique conditions favorable for microbial growth and transmission of inhalable pathogens. In Chapters 5 to 8, showerheads were included in samplings at locations serving vulnerable populations. In the sports complex setting (Chapters 5 and 6), the elderly population tends to visit early in the morning, making them the first to use water that has stagnated overnight, whereas in the hospital setting (Chapters 7 and 8), showerheads were located either in rooms of immunocompromised patients or used as shared showerheads for patients within the same wing. In legacy buildings, where modern ventilation systems are often absent or insufficient, the risk associated with exposure to pathogens is further compounded by poor air circulation and prompt aerosols removal. Finally, large group shower systems investigated in Chapters 5 and 6, where a single thermostatic mixing valve supplied water to more than 20 showerheads at once, had volumes of mitigated water averaging 200 to 300 liters compared to smaller grouped (or not) shower systems with less than 6 liters of mitigated water.

- Large tepid grouped shower systems are a flawed design from a water safety perspective due to their high volume of stagnant water at ideal water temperatures (25 – 40 ˚C) and their increased surface area to volume ratio contributing to biofilm development.

Hot water production systems. Risk identification in hot water production systems is essential as these systems often serve as prime reservoirs for growth of OPs like *Legionella pneumophila* (Bédard et al., 2016c). Identifying vulnerabilities and deficiencies related to these complex networks of water heaters, recirculation loops, and temperature control devices (e.g., mitigators) helps mitigate conditions that favor optimal microbial growth, thus ensuring the safe delivery of hot water. For example, one study gathering over 200,000 data points showed that ideal cut-off points where occurrence of *Legionella* spp. is more probable were of 48 ˚C, 53 ˚C, and 56 ˚C for sentinel sites, recirculated water, and the hot water supply, respectively (Kistemann et al., 2024). The study of the sports complex (Chapters 5 and 6) highlighted numerous design and operational considerations that likely contributed to the systemic occurrence of *Legionella pneumophila* across the facility. Indeed, the characterization of the centralized hot water system through sampling and temperature monitoring revealed several vulnerabilities, including:

1) One primary risk factor stemmed from the configuration and operation of the water heaters. Of the four heaters in series, the furthest heater from the hot water supply point was out of service but not taken off line by closing valves. This heater was at 30 ˚C for an extended period, likely creating a conducive environment for *Legionella pneumophila*.

- This nursery effect presents a tangible risk when inactive (decommissioned) heaters are left within a system without proper isolation, emphasizing the need for deficient or unused elements to be adequately isolated to prevent important microbial amplification.

2) Further compounding this risk, recent modifications to the hot water feed to increase energy efficiency inadvertently reduced the overall water temperature. Specifically, recirculated hot water initially meant to return to the first heater was instead partly blended with the hot water supply from the final heater in the series. Most critically, this implies that not all returning hot water was submitted to elevated temperature. Furthermore, this blending resulted in hot water being supplied to the building at temperatures of 53 – 55 ˚C, rather than 60 ˚C (i.e., temperature at which heaters were set), and returning to the heater series at 48 – 51 ˚C, falling within a range that allow survival of *Legionella pneumophila*. These temperature ranges were previously associated with increased

probability of *Legionella* spp. occurrence and density (Kistemann et al., 2024), which are not sufficient for reliable control.

- This balancing act between energy conservation and OPs control adds a layer of complexity to water safety planning. Moving forward, careful risk assessments and integrated designs will be essential to ensure that energy-efficient measures do not compromise water quality and user safety. It is crucial that recirculated water be maintained at a minimum temperature of 55 ˚C at all times without mixing to effectively control microbial proliferation.

3) Additional thermal risks were noted in the episodic temperature drops within a small plate heat exchanger used to preheat returning hot water, in which temperatures fell as low as 25 ˚C, potentially creating yet another niche where *Legionella pneumophila* could thrive intermittently.

- Such temperature inconsistencies in heat exchangers pose substantial risks in hot water systems by providing temporary but frequent conditions favorable to microbial growth. Where possible, or in high-risk settings like healthcare facilities, system designs should prioritize direct heating or alternative design ensuring consistent hot temperatures.

4) Lastly, the system's overall layout contributed to increased water age. The central hot water recirculation loop fed secondary piping to shower rooms across the building, but these represented dead volumes of uncirculated water that reached up to 300 liters in some areas, thus causing significant delays (> 30 minutes) in reaching target temperatures.

- Therefore, designing piping layouts to minimize dead stagnant volumes and ensuring quicker temperature recovery at points of use, especially in distant or low-use areas, are essential for preventing pathogen growth.

9.2.2 Control of water-related risks

Based on risk prioritization, the WSP team identifies locations of control measures, and their tolerable value limits, to ensure both systemic (at the system level) and distal (at the points of water use) risk prevention. The thesis results are discussed specifically focus on temperature regulation, the maintenance of biocide residuals, and preventative flushing, which are core control strategies outlined in WSPs.

Temperature regulation. In Chapter 4, a comprehensive review of 809 data points on culturable *Legionella pneumophila* from five buildings across Québec revealed that growth was most

prominent between 20 and 60 ˚C, with the highest proportion of positive samples between 40 and 50 ˚C (48%). Mean concentrations remained relatively uniform among categorized temperature ranges from 20 to 60 ˚C, ranging from 1.73 X 10^3 to 2.81 X 10^3 MPN/L (or CFU/L). Temperatures above 60 ˚C and below 20 ˚C were critical for inhibiting the presence of *Legionella pneumophila* in these water systems, consistent with statistical models estimating the odds of detecting *Legionella* in hotel water systems (Rasheduzzaman et al., 2020; Chochlakis et al., 2023). In Chapter 7, the impact of temperature was assessed by considering results before and after the onset of monochloramine separately. In pre-monochloramine samples, positivity approached 100% for qPCR concentrations of *Legionella pneumophila*, *Legionella* species, *Mycobacterium* species, and *Vermamoeba vermiformis* across all temperatures (10 to 60 ˚C). The outstanding persistence of *Legionella pneumophila* in the hospital's hot water system regardless of the maintenance of adequate thermal regime control most probably reflects the adaptation of the main sequence-based typing (SBT) strain. This resistance shows the risk of developing resistance to heat after repeated thermal heat shock and the application of continuous thermal control (Liang et al., 2023). Then, the combination of elevated water temperatures (> 55 ˚C) and monochloramine exposure led to large reductions in the abundances and occurrences of all targeted organisms, with the exception of *Mycobacterium* species, which, while remaining ubiquitous, were also found in lower concentrations. Furthermore, in Chapters 5 and 7, continuous temperature monitoring with probes at key locations within the hot water circulation system revealed episodic system vulnerabilities and deviations that could support *Legionella pneumophila* growth.

- WSPs guidelines largely focus on temperature control as the primary barrier to prevent growth of *Legionella* in hot water systems. For this approach to be effective, target temperatures need to be maintained across the hot water distribution systems. Temperature monitoring becomes essential to verify these conditions at critical system sites, including the heater outlet (> 60 ˚C), and through principal and secondary hot water return loops (> 55 ˚C), and at distal points of use (> 50 ˚C). Combined with the management of thermal regime, this allows for real-time tracking of temperature deviations, which are crucial to enable timely interventions before conditions become conducive to OPs growth.
- WSPs further focus on temperature control regimes mostly targeting *Legionella* bacteria, and more directly *Legionella pneumophila*. The longitudinal hospital study indicated that this strategy alone may be insufficient against *Legionella pneumophila* strains that have

adapted to withstand well implemented thermal regimes over a prolonged period of time. Furthermore, in buildings serving vulnerable populations, waterborne infection control measures often prioritize other OPs than *Legionella*. Findings from this thesis indicated that the effectiveness of thermal control varies across different OPs and may be less effective against more resistant organisms (e.g., NTMs). This highlights the importance of adopting comprehensive strategies that address a broader range of OPs to ensure effective control.

Maintenance of disinfectant residuals. Although free chlorine concentrations greater than the 0.2 mg/L threshold were nearly all associated with undetectable levels of culturable *Legionella pneumophila* and *Pseudomonas aeruginosa*, maintaining this target was challenging in tepid water systems and nearly impossible in hot water systems due to the rapid depletion with increasing temperatures (Chapter 4). Similarly, highest proportion of positive samples by qPCR to *Legionella pneumophila* and *Mycobacterium* were measured when free chlorine concentrations were below 0.5 mg/L (Donohue et al., 2019). Therefore, the limitation of maintaining adequate residuals in building plumbing underscores the need for WSPs to account for temperature-related disinfectant decay and explore alternative or supplemental control strategies to ensure consistent microbial control across varying water temperatures. As demonstrated in Chapters 7 and 8, onsite monochloramine application was an effective control measure (supplemental disinfection) to ensure mitigation of diverse OPs in a facility housing vulnerable populations. Monochloramines are more stable compared to free chlorine, regardless of temperature variations, and over stagnation periods, thus ensuring more consistent protection varying conditions. Despite targeted microorganisms exhibiting varying levels of tolerance to monochloramine, supplemental monochloramine residuals ranging 2 – 3 mg/L were the most beneficial to minimize to the greatest extent concentrations of *Legionella pneumophila*, *Legionella* species, *Mycobacterium* species, and *Vermamoeba vermiformis*.

- Achieving and sustaining effective free chlorine thresholds from incoming municipal water proved challenging in tepid water systems, and nearly impossible in hot water due to rapid residual decay, thus underscoring the necessity for WSPs to account for temperature-induced disinfectant depletion;
- Implementing dosages of monochloramine ranging from 2 to 3 mg/L coupled with thermal control almost immediately provided a robust barrier against *Legionella pneumophila* and

progressively against *Legionella* spp., NTMs, and *Vermamoeba vermiformis* in the hospital setting, thus supporting its use as a key supplemental disinfection strategy in facilities with susceptible individuals.

Preventive flushing. In Chapter 6, daily flushes (Monday to Friday) of showerheads after shock chlorination and device recommissioning flushing resulted in significantly ($p < 0.05$) lower concentrations of ATP and TCC than weekly flushes over the course of three weeks. Additional benefits of daily flushes over weekly flushes were not statistically observed when measuring culturable and qPCR concentrations of *Legionella pneumophila* post-interventions. Nonetheless, implementing routine flushing generally produced larger decreases of *Legionella pneumophila* than showerheads that were left stagnant during the study period. Furthermore, daily flushing of large group shower systems over two consecutive months during another temporary building closure (lockdown procedures) led to a considerable 3-log reduction in the abundance of culturable *Legionella pneumophila* in rear-end showerheads flushed for 15-30 minutes. However, upstream showerheads, which were sequentially flushed for 30-sec, showed a persistent *Legionella pneumophila* occurrence rate of 41% and a mean culturable concentration of positive samples of 3,163 MPN/L, highlighting the importance of adequate flushing duration for effective reduction. This was also illustrated in a model plumbing system operated at similar conditions and in which only prolonged (28 days) daily flushing (about 20 minutes at 37 ˚C to simulate shower usage) achieved significant reductions in planktonic culturable and qPCR *Legionella pneumophila* after the colonization stage (Nisar et al., 2024).

- Current evidence is lacking to formulate definitive minimum flushing frequency and duration. While recent guidance requires weekly preventive flushing, findings from this thesis showed that daily flushing was more beneficial. Hence, there is clear need for additional studies to provide more specific recommendations that are balanced with water conservation measures.

9.2.3 Validation and verification of the WSP

The validation phase typically involves environmental monitoring of water quality to confirm that the methodology implemented as part of the WSP (e.g., control measures) is effective at reducing water-related risks. On the other hand, the verification stage includes the documentation of all activities related to the WSP (e.g., monitoring control parameters, tracking corrective actions,

environmental sampling results, etc.) to demonstrate that the plan has been genuinely implemented within a building system (McCoy and Rosenblatt, 2015).

9.2.3.1 Water quality monitoring

Environmental monitoring of water quality involves sampling a range of physico-chemical and microbiological parameters to confirm the effectiveness of control measures implemented in WSPs. This section aims to provide insights on the benefits and limitations of key aspects of environmental monitoring, including what, how, where, and when to sample/measure/monitor.

What to measure. The first question is WSPs application is what to measure (e.g., selection of target OPs). However, the feasibility of implementing a comprehensive monitoring approach targeting several microbial hazards is often constrained by financial and energy costs, time demands, and the need for specialized technical expertise (Proctor et al., 2022). In healthcare facilities, infection prevention and control teams should identify the pathogens of concerns to tailor WSPs planning.

In this thesis, both culture-based and molecular methods (qPCR and next-generation sequencing [NGS]) were employed for the detection and quantification of OPs as they provide valuable, yet distinct insights into their dynamics. In intervention studies such as those presented in Chapters 5, 6, and 7, the complementary use of culture-based and qPCR methods was critical to evaluate the long-term effectiveness of preventive and corrective actions. For *Legionella pneumophila* in particular, intervention-induced stress may drive the pathogen into a VBNC state, suppressing rapidly culturability, while qPCR would still detect persistent residual DNA over time thereby offering essential feedback on the actual duration and effectiveness of the intervention's impact. For example, shock chlorination suppressed *Legionella pneumophila* culturability for two weeks before small rebounds were observed, yet qPCR allowed the quantification of gene copies ranging $10^2 – 10^3$ gc/L in the meantime (Chapter 6). Similarly, the introduction of monochloramine in the hospital hot water system quickly reduced culturable *Legionella pneumophila* concentrations within 24 hours, whereas reductions in qPCR concentrations were more progressive (four weeks). Additionally, the application of qPCR to monitor NTMs, although lacking specificity, and *Vermamoeba vermiformis* proved useful to track temporal trends and to provide more insights into their dynamic responses with monochloramines or over interruption of dosage (Chapter 7).

The relatively strong relationship between culture-based and qPCR methods is beneficial to guide risk assessment frameworks, which have traditionally relied solely on culturable concentrations to

ensure compliance with regulations (Sylvestre et al., 2024). In this thesis, moderate to strong correlations between culture-based and qPCR concentrations of *Legionella pneumophila* were observed in both first draws (R = 0.64) and 5-min flushed samples (R = 0.51) among samples collected during the device recommissioning flushing intervention (Chapter 5), as well as with samples from before and after the onset of monochloramine treatment (Chapter 7). Additionally, in Chapter 8, NGS Illumina MiSeq revealed detailed microbial community dynamics during the shift from free chlorine to monochloramine, highlighting changes in the relative abundance and diversity of OPs, even when these pathogens were not dominant taxa. NGS was particularly valuable for detecting low-abundant taxa, emerging genera containing potential pathogens (e.g., *Sphingomonas*), or other clinically relevant species (e.g., *Legionella anisa*-like, *Mycobacterium gordonae*-like) that may persist or proliferate post-intervention. However, its elevated cost, long turnaround time, bioinformatics expertise requirements, and inability to deliver absolute quantification without additional methods make it less practical for routine or rapid assessments.

- In intervention studies, culture-based and qPCR methods should be used in tandem for the effective quantification of OPs over time, thus capturing both viable and total loads which are crucial for more accurate risk assessments. For environmental monitoring within WSPs, where budget and time constraints are typical considerations, the choice between culture-based methods, specific qPCR, or both, should be strategically made based on specific monitoring goals and resource availability. For example, incubation times for many NTMs species can extend up to eight weeks with solid culture, making qPCR a more practical alternative due to its more rapid processing time (Pfyffer et Wittwer, 2012);
- While most WSPs emphasize on the control of *Legionella*, other OPs should be considered to reflect infection control priorities and due diligence liability concerns.

Surrogate parameters, including ATP, HPC, and flow cytometry counts, are often used as indirect indicators to assess the presence of OPs or as early warning indicators for rising microbial risks, thus providing a rapid and cost-effective means to monitor microbial activity as part of WSP. Results from this thesis highlight the variability in the predictive value of such microbial indictors for different targeted organisms or OPs. In Chapter 4, both culturable *Legionella pneumophila* and *Pseudomonas aeruginosa* showed weak correlations with ATP (R = 0.12 – 0.39) and no correlations with HPC and ICC (R < 0.1), despite large datasets including hundreds of samples

from different large buildings. Similarly, in Chapter 5, weak relationships were measured between ATP and culturable *Legionella pneumophila* in both first draws (R = 0.17) and 5-min flushed samples (R = 0.25) from shower systems, as well as between qPCR *Legionella pneumophila* and this same parameter (R = 0.21 in first draws, R = 0.28 in 5-min flushed samples). ICC and TCC did not show any better correlations with culturable (first draws: ICC – R = 0.18, TCC – R < 0.1; 5-min flushed samples: ICC and TCC – R < 0.1) or molecular *Legionella pneumophila* concentrations (first draws: ICC and TCC – R = 0.14; 5-min flushed samples: ICC and TCC – R < 0.1). Finally, ATP, ICC, and TCC demonstrated relatively strong correlations with NTMs (R = 0.48 for ATP, R = 0.60 for ICC, and R = 0.56 for TCC), indicating their potential as surrogate indicators (Chapter 8). Conversely, ATP, ICC, and TCC showed negligible correlations (R < 0.1) with culturable and qPCR *Legionella pneumophila*, limiting their applicability for assessing *Legionella* burden, whereas ICC showed moderate correlations with *Vermamoeba vermiformis* (R = 0.50), suggesting its utility in tracking viable amoebae populations, while TCC was less predictive (R = 0.38).

- These findings emphasize that while microbial indicators can effectively reflect global changes and microbial dynamics in the context of intervention studies, they cannot serve as standalone proxies for specific pathogens. Targeted methods remain essential for reliable detection and quantification of OPs as part of water quality monitoring in WSPs.

How to sample. The second question refers to choosing a sampling methodology, as it directly influences the reliability and relevance of the data being collected to assess microbial risks and guiding water safety management decisions.

In this work, both discrete and profile samplings were carried out, serving different objectives. Discrete sampling, such as the collection of first draws and flushed samples, is widely used for environmental water quality monitoring to capture specific conditions at distinct points in time and place. While first draw samples are typically collected after a short stagnation period to maximize the likelihood of gathering higher microbial concentrations, flushed samples are taken after running a tap for a set duration to assess the upstream water quality in flowing systems (Wang et al., 2017). In contrast, profile sampling involves collecting multiple sequential samples from a same site, thus providing a detailed understanding of microbial dynamics and water quality along the flow path (Bédard et al., 2018) to pinpoint areas of concern and improve flushing protocols. However, the

resource-intensive nature of profile samplings makes it less suitable for routine monitoring in the context of WSPs. Instead, it is better suited for research studies or specific investigations, such as identifying contamination sources or optimizing remediation strategies, rather than serving as a routine tool for ongoing water safety planning.

In this thesis, first draw samples provided insights into elevated concentrations of *Legionella pneumophila* and other OPs in shower systems after prolonged distal stagnation due to building closures (Chapters 5 and 6) or across various distal points of use during normal building occupancy and water demand patterns (Chapters 4, 7, and 8). Flushed samples captured the effectiveness of different flushing times, ranging two to 60 minutes in cold, tepid, and hot water systems, demonstrating significant reductions in microbial concentrations, including OPs and microbial indicators (Chapters 4 – 8). Moreover, profile samplings revealed key microbial dynamics and water quality patterns along plumbing systems. In Chapter 4, free chlorine profiles in cold water showed that even after 30 minutes of flushing, site-specific factors like flow rates and pipe lengths often prevented chlorine levels from matching those at the building entry. In Chapter 5, total and intact cell counts amplification in distal sections of small and large shower systems after stagnation demonstrated the need for substantial flushing to reduce microbial loads effectively.

- Results underscore the importance of tailoring sampling strategies to the specific objectives of environmental water quality monitoring in WSPs, balancing practicality and resource availability with the need for detailed insights into microbial risks and system dynamics. For WSPs application, first draw samples should be recommended as a default for distal points of use to capture risks associated with user exposure, whereas flushed samples are better suited to monitor system (upstream) water quality. Finally, profile sampling, while resource-intensive, is valuable to identify contamination issues or optimize flushing.

Where to sample. Effective environmental water quality monitoring within WSPs relies on the selection of strategic sampling locations that accurately reflect the water system's vulnerability to contamination and potential exposure risks, enabling timely interventions, if needed.

In all studies conducted throughout this thesis, samples were collected from distal points of use (e.g., showerheads, faucets, hand washing stations) and within the main flowing systems (e.g., building cold water point of entry, water heater outlet/inlet, hot water return loops, remotely located sites from the hot water production after a brief flush) to gain a comprehensive overview of each

investigated building. In Chapter 4, the collection of first draw samples, representing distal sections of building plumbing systems, showed the highest concentrations of ATP, HPC, and flow cytometry counts, as well as greater occurrence rates of culturable *Legionella pneumophila* and *Pseudomonas aeruginosa* than flushed samples. Surprisingly, even within a same grouped shower system, showerheads located beside one another displayed different levels of *Legionella pneumophila* and microbial indicators, thus suggesting that colonization can be highly localized. In this same sports complex, discrete samples collected at key locations from the building's point of entry and the hot water production system to the thermostatic mixing valves using cold and hot water to supply tepid water to large group shower systems and their corresponding showerheads, revealed considerable microbial amplification occurring along the plumbing system from the point of entry to the point of use (Chapter 6). Indeed, 3-log and 100-fold increases in total cell counts and ATP were measured between the building's point of entry and the hot water production system, respectively. Additionally, cell counts increased by another log at the showerheads, coinciding with the detection of *Legionella pneumophila*.

Finally, in Chapters 7 and 8, the introduction of monochloramine into the hospital's hot water system had varying impacts on distal and system sites. Notably, larger reductions in all targeted organisms and microbial indicators were measured at system sites, attributable to higher temperatures (> 55 ˚C), greater monochloramine residuals (> 2 mg/L), and consistent flow comparatively to distal sites where site-specific conditions led to considerable variability in results. Results from the 16S and 18S rRNA genes amplicon sequencing in Chapter 8 highlighted a clear divergence in bacterial community composition between system and distal sites during monochloramine dosing. System sites, exposed to higher and more stable disinfectant concentrations, were more likely to favor monochloramine-resistant and thermotolerant bacteria in contrast to distal sites where distinct bacterial and eukaryotic communities were longitudinally observed among locations. Moreover, distal sites supplying tepid water (showerheads, hand washing stations) and those with extremely low water use (< 30-sec use within a week) were associated to greater microbial abundances, as well as a larger mobilization of plumbing metals like lead and iron from leaded brass fittings post-treatment.

- Results from this thesis underscore the importance of including both system and distal sites for the environmental water quality monitoring to capture spatial variability during routine surveillance, in the aftermath of corrective interventions or for outbreak investigations.

System sites across the most vulnerable hot water system, such as the water heater outlet and hot water return loops, are essential to monitor in order to ensure proper control of parameters like temperature and residual disinfectants (if other than free chlorine). Given their larger volumes, system sites should focus on maintaining consistent temperature thresholds and disinfectant levels (cold water systems) to limit OPs amplification. However, as they were not systematically predictive of microbial risks at distal locations, developing a WSP focusing solely or mainly on system sites would overlook critical reservoirs of pathogens amplification at points of use to which users are exposed. Therefore, a comprehensive WSP should also prioritize monitoring at points of use based on specific risk factors, including vulnerability of the population, aerosol generation potential, and suboptimal temperature control or stagnation;

- In this thesis, distal sites were selected based on their potential for aerosolization (showerheads for the assessment of *Legionella pneumophila* in Chapters 5 and 6), or based on the presence of highly susceptible patients (e.g., oncology, transplant, pneumology, NICU, ICU, COVID-19 unit), and previous confirmed nosocomial LD and/or positive *Legionella pneumophila* measurements (Chapters 7 and 8). While monitoring numerous distal sites to account for the large heterogeneity of results among points of use would be ideal to capture representativeness of the building plumbing system, logistical challenges, such as financial, labor, and laboratory resources and costs, make this sampling approach unrealistic (Wang et al., 2017).

When to sample. Determining the timing and frequency of environmental water quality monitoring is essential to refine monitoring practices and improve accuracy and statistical reliability to support more effective risk evaluation and mitigation strategies (Wang et al., 2017).

In this thesis, continuous monitoring of water temperature (30-sec to 10-min intervals) at system and distal sites was applied using probes and temperature data loggers installed on specific pipe segments without thermal insulation (Chapters 5 to 7). Hatam and colleagues (2024) integrated EPANET-MSX with field data from Chapter 6 to model temperature variations and *Legionella pneumophila* concentrations, for a case application. These simulations showed good alignment with actual measurements during daily flushing of showers, but stagnation events highlighted the

probable influence of additional factors on concentrations because of discrepancies between field and modeled data.

- This example demonstrates the use of continuous temperature monitoring to support risk assessment by linking temperatures and microbial dynamics at distal points of use;
- While it would be invaluable to continuously monitor microbial trends over time to gain deeper knowledge into system dynamics, this appears unfeasible in the context of routine water quality monitoring due to the high cost, complexity of technology, invasive procedures, and narrow range of microbial parameters. Such approaches are currently more suited for research purposes (Pereira et al., 2021), whereas temperature monitoring proves a practical and reliable proxy for assessing conditions that may influence microbial growth.

Finally, even monthly samplings at the same sites showed large temporal variations (> 1-log) in microbial measurements during routine monitoring of the hospital's water system before the introduction of monochloramine (Chapter 7). Culturable *Legionella pneumophila* cells were transiently detected at some sites, thus reinforcing the need of repeating samplings rather than relying on a single snapshot to capture microbial dynamics over time. This is corroborated by prior studies reflecting the sporadic occurrence and spatiotemporal variability of *Legionella pneumophila* in large building water systems (Donohue et al., 2018; Buse et al., 2020), which are largely tied to the selected sampling methodology as well. Additionally, in the context of remediation efforts (Chapters 6 and 7), where repeated samplings were carried out, the importance of follow-up samplings to verify the efficacy of interventions was demonstrated. While remedial flushing at one shower system resulted in minimal changes in culturable concentrations of *Legionella pneumophila* over three weeks of weekly sampling, shock chlorination showed rebounds in concentration between the first and the third week post-intervention (Chapter 6). Similarly, different trends were distinguished among targeted microorganisms in response to monochloramine (Chapter 7). For instance, *Legionella pneumophila* reservoirs were rapidly reduced in culturable concentrations (< 24h) and more progressively in qPCR concentrations (< four weeks), while *Vermamoeba vermiformis* also showed significant reductions within the first day of treatment, demonstrating the need for frequent initial sampling (monthly) to capture short-term changes. Conversely, NTMs and *Legionella* species had more gradual declines, stabilizing over six months and four weeks, respectively. Temporality of data was also observed with different

bacterial and eukaryotic phylum and genus that were only occasionally detected across the distinct treatment phases (Chapter 8).

- Hence, these observations emphasize the importance of tailoring sampling frequencies to assess both the immediate impacts of interventions and long-term trends in microbial dynamics, while balancing the need for comprehensive monitoring with practical constraints such as costs, resources, and the operational feasibility of implementing more frequent follow-up samplings in comparison to routine samplings;
- Pre- and post-implementation monitoring of the same investigated sites of corrective actions is essential to evaluate their effectiveness, and samplings should be conducted at intervals that reflect the expected microbial response dynamics (e.g., weekly during the first month following an intervention, and every other months thereafter to track long-term stability).

CHAPTER 10 CONCLUSIONS AND RECOMMENDATIONS

The primary objective of this doctoral project was to provide evidence-based recommendations to improve the monitoring, operation, and design of large building plumbing systems, and most specifically within the framework of water safety plans. Using novel tools, this research examined the occurrence of OPs and evaluated preventive and corrective measures to reduce exposure risks. The findings aim to guide building managers in implementing proactive water management strategies that prioritize occupant safety while minimizing liability risks. To address the objective, the following key questions were explored: Can building managers rely on incoming free chlorine residuals from the utility to prevent microbial growth? How effective is recommissioning flushing in the short- and long-term at reducing the occurrence of *Legionella pneumophila* after prolonged building closure? What is the most effective combination of preventive flushing regimes and remedial interventions to prevent regrowth of *Legionella pneumophila* at showerheads? Can *in situ* disinfection with monochloramine in a hospital's hot water system reduce the prevalence of multi-pathogen species on the long-term?

The following conclusions can be drawn regarding the monitoring of building plumbing systems:

- There is a need for comprehensive monitoring of microbial hazards that extend beyond *Legionella*-centric strategies to address diverse microbial risks effectively. This refinement should prioritize a tailored monitoring framework that reflects the unique priorities and occupant risk profiles of each building. Nonetheless, in the context of remediation efforts and research, the combination of culture-based and qPCR approach for the quantification of *Lp* is valuable to track treatment efficacy and to provide insights into both the viability of the pathogen and its total load.
- Discrete sampling along the sports complex's plumbing system revealed large microbial amplification, progressing from the building's PoE to the hot water production system (3-log and 100-fold increases in TCC and ATP, respectively), and ultimately from the cold and hot water supplying TMVs to the showerheads (2-log and 10-fold increases in TCC and ATP, respectively). While *Lp* was only detected at background levels in the hot water returning to the production system, culture-based and qPCR concentrations at showerheads were 2-log higher (Chapter 6). Despite fairly similar (< 1-log) mean qPCR concentrations of *Lp*, *L*spp, NTMs, and *Vv* between system and distal sites in the hospital plumbing system,

system sites exhibited much larger mean reductions post-monochloramine treatment than distal locations. Additionally, culturable *Lp* cells were detected more frequently at distal sites (61%) compared to system sites (13%) (Chapter 7), and bacterial communities were significantly different between system and distal sites, regardless of treatment (Chapter 8).

  - System sites are not systematically predictive of pathogen positivity and microbial composition at distal points of use. Risk-based strategies must not be limited to traditional control points located across the cold and hot water systems, but should also consider distal plumbing sections where factors like flow patterns and fixtures design contribute to significant distal microbial amplification.

- Microbial indicators demonstrated variability in their predictive value for the presence of OPs or other targeted microorganisms in all studies. While ATP, ICC, and TCC were relatively strongly correlated with NTMs ($R = 0.48 – 0.60$) and moderately correlated with *Vv* ($R = 0.32 – 0.50$) (Chapter 8), these same surrogate parameters, as well as HPC, showed only weak or most often negligible correlations with culturable *Lp* and *Pa* ($R < 0.39$) (Chapters 4, 5, and 8).

  - Even with large datasets (> 200 samples) or within a same building plumbing system (sports complex or hospital), no consistent correlations could be established across studies between cost-effective microbial indicators (ATP, HPC, FC counts) and targeted pathogens (*Lp*, *Pa*, NTMs) or hosts (*Vv*). While these general microbial parameters have proven valuable for assessing overall microbial dynamics, particularly in the context of remediation efforts, they should not be substitutes for pathogen-specific testing during routine surveillance. They remain fair indicators of conditions supporting general microbial growth (e.g., nutrient availability, low disinfectant residuals, warm temperatures, stagnation), thus helping to identify potential risks and optimize control strategies aimed at mitigating OPs.

- The integration of continuous (real-time) data for temperature monitoring and the use of flushing sensors to assess recent water demand patterns at distal sites (Chapters 5-7) can considerably enhance proactive monitoring and water safety planning by providing instant insights into system dynamics, allowing early detection of operational/design anomalies, and low-use areas, by generating a robust dataset that supports WSP decision-making.

  - Temperature probes should be installed at critical points within the hot water system, such as heater outlets, and main and secondary hot water recirculation lines, or any other sections prone to stagnation or suboptimal temperatures. Temperature reading should be taken at regular intervals that capture meaningful variations, considering that slower rate of temperature changes occur in hot water systems. Data interpretation should be guided by threshold values (> 55 ˚C for hot water) to identify deviations possibly indicative of potential microbial risks.

- The significant spatial and temporal variability in the occurrence of specific pathogens and microbial composition observed both within a same point of use and among different ones located throughout a same building implies that a one-size-fits-all approach to monitoring may fail to detect transient risks or changes over time (Chapters 7-8). This variability suggests that microbial risks cannot be reliably predicted based on isolated sampling events or single locations, emphasizing the need for a monitoring approach that includes repeated sampling across multiple time points and diverse locations.
  - While water safety planning typically provides detailed guidance on characterizing plumbing systems and water use patterns, an initial, thorough characterization of microbial risks through a comprehensive baseline sampling campaign would provide critical insights into site-specific microbial dynamics and risk hotspots. This step would ensure that subsequent monitoring and mitigation efforts are better informed and tailored to the actual microbial hazards within a water system.
- Despite having identical design and receiving the same mitigated water, measurements at two neighboring showerheads from large group shower systems revealed notably different *Legionella pneumophila* levels, with one showing detectable concentrations and the other none (Chapter 5).
  - Group distal sites served by tepid water (e.g., large shower systems) should be banned or redesign due to their inherent microbial risks (e.g., elevated dead water volumes, stagnation, tepid water temperatures). In cases where such systems cannot be immediately replaced, they must be closely monitored, with sampling conducted at a minimum of two water points to account for variability. Furthermore, automatic

flushing devices should be installed to reduce stagnation and maintain consistent water quality.

The following conclusions emerge from the operation of building plumbing systems:

- Temperature played a critical role in controlling *Lp* presence in large building water systems, with culturable detection most prominent between 20 and 60 ˚C, and peaking at 40 – 50 ˚C (48% of samples positive), while temperatures above 60 ˚C and below 20 ˚C seemed effective at inhibiting its occurrence (Chapter 4). However, qPCR analysis of pre-monochloramine samples in the hospital study revealed the ubiquitous detection of *Lp*, *L*spp, NTMs, and *Vv* across all temperature ranges (10 – 60 ˚C) (Chapter 7).
    - These results underscore the importance of maintaining water temperatures outside the critical 20 – 60 ˚C range to inhibit, at least, *Lp*, particularly through consistent water heating at a minimum of 60 ˚C. Despite optimized thermal regimes in some buildings, effective WSPs should integrate complementary control strategies that extend beyond temperature management (e.g., supplemental disinfection, flushing) to reduce the abundance of multi-organisms.
- Free chlorine residuals were largely undetectable in hot water, hardly maintained in tepid water, and predominantly detectable in cold water, particularly after flushing (2-60 min). A comprehensive review of 1,737 data points from nine large buildings revealed that the commonly suggested 0.2 mg/L threshold for WSPs was met only in 26%, 6%, and 2% of first draws in cold, tepid, and hot water, respectively. Flushing distal points of use only increase this ratio at cold water sites (83%), but profile samplings highlighted important variability in the time and volume required to achieve steady free chlorine concentrations or concentrations from the building's PoE. Nonetheless, free chlorine above 0.2 mg/L were generally associated with the absence of culturable *Lp* and *Pa* cells, as well as lower ATP, HPC, and FC counts, but this trend was most observed in cold water (Chapter 4).
    - These observations demonstrate the significant challenges in maintaining free chlorine residuals throughout large buildings, and most particularly in hot and tepid water systems. The inability to reach compliance with the 0.2 mg/L threshold at most distal sites indicates that the residual provided by utilities often cannot be reliably maintained within buildings due to factors such as temperature, design, and

inter-use stagnation. Building managers should not rely on incoming free chlorine residuals for microbial growth control in hot water systems. Therefore, a 0.2 mg/L threshold in WSPs remains suitable for cold water points, but it should not be recommended for tepid and hot water points due to rapid chlorine depletion at higher temperatures, making it impractical and ineffective in those conditions.

- Device recommissioning flushing carried out after prolonged building closure (16-week) significantly reduced culturable *Lp* concentrations (> 1-log down) and occurrence (81% to 48%) within first draws collected from showerheads, but only short-term (24h) (Chapter 5). The combination of shock chlorination (20 – 25 mg/L, 16h) and preventive flushing (daily or weekly) at showerheads suppressed *Lp* culturability down 2-log for two weeks, before small rebounds were measured (20 – 84 MPN/L) (Chapter 6). Then, introducing monochloramine at 1.5 – 3.5 mg/L in a hospital's hot water system effectively eliminated *Lp* reservoirs long-term (> 1-year), rapidly reducing culturable concentrations within 24h and progressively decreasing qPCR concentration (< four weeks of treatment) (Chapter 7).
    - In the event of *Lp* contamination, recommissioning flushing alone was insufficient for control. However, building managers can use locally applied shock chlorination combined with preventive flushing as a temporary measure to reduce *Lp* loads while awaiting the implementation of appropriate engineering controls. For long-term control, especially in buildings with vulnerable occupants, dosing monochloramine at 1.5 – 3.5 mg/L is more effective to eliminate *Lp* reservoirs rapidly (< 3-week).
- The introduction of monochloramine (1.5 – 3.5 mg/L) led to a 2-log reduction in mean *Vv* concentrations within 24h, whereas NTMs and *L*spp concentrations stabilized after achieving 2-log mean reductions over six months and four weeks, respectively, thus demonstrating their persistence in biofilms. Additionally, a prolonged interruption in monochloramine dosing (4-week) caused significant increases in *Vv*, NTMs, and *L*spp at all system and distal sites, while a shorter interruption period of five days resulted in comparatively smaller regrowth (Chapter 7).
    - Monochloramine was effective at reducing NTMs, *Vv*, and *L*spp, although not to the same extent than *Legionella pneumophila*. However, to ensure sustained microbial control across this wide range of OPs, consistent monochloramine dosing

is crucial, along with rapid responses to operational alerts from the monochloramine generator that should be well detailed in WSPs.

- Both culture-based and qPCR *Lp* concentrations rebounded to post-16-week building closure levels in the four weeks after device recommissioning flushing without showerhead usage (Chapter 5). Moreover, 3-week distal stagnation following hyperchlorination led to larger microbial regrowth at showerheads than with targeted device recommissioning flushing performed at two distinct grouped shower systems, such as measured by ATP ($RFs_{chlor} > 4.3$ vs. $RFs_{flush} > 1.2$), TCC ($RFs_{chlor} > 3.5$ vs. $RFs_{flush} > 0.8$), and ICC ($RFs_{chlor} > 4.7$ vs. $RFs_{flush} > 0.8$) (Chapter 6).
    - These results emphasize the importance of proactive water management during periods of low water use to prevent microbial regrowth. Device recommissioning flushing alone or shock chlorination, without regular water usage or normal building re-occupancy, are not sufficient to sustain reductions in *Lp*, nor in any other general microbial parameters. Therefore, periods of low water use should be specifically addressed in WSPs, incorporating measures to maintain water circulation and minimize stagnation and microbial amplification (e.g., flushing).
- Daily flushing of showerheads significantly reduced ATP and TCC concentrations in comparison to weekly flushing over three weeks, though no additional benefits were observed for culturable or qPCR *Lp*. Moreover, daily flushing of rear-end showerheads of large grouped shower systems for 15-30-min achieved a 3-log reduction in culturable *Lp*, while upstream showerheads flushed only for 30-sec had a persistent *Lp* occurrence rate of 41% (Chapter 6).
    - Short 30-sec daily flushing is insufficient to prevent persistent *Lp* contamination, highlighting the need for sustained and adequately timed interventions to maintain water quality and safety during building low-occupancy periods. Nonetheless, daily flushing are likely to reduce exposure risk to *Lp* by reducing abundances.

Finally, the following conclusions were reached for the <u>design</u> of building plumbing systems:

- Results from Chapters 4 to 7 demonstrated that first draw samples consistently exhibited higher microbial concentrations (ATP, HPC, or FC counts) and greater likelihood of OPs

presence compared to flushed samples, highlighting significant distal amplification. In contrast, upstream water showed comparatively lower microbial loads, emphasizing the role of distal fixture design and water usage patterns in fostering these conditions. Profile samplings of TCC at small and large grouped shower systems also demonstrated that distal amplification typically occurs within the first one to two liters of water (Chapter 5). This observation was even more pronounced in extreme low-use distal sites, predominantly showerheads and some unused faucets, as evidenced by the significantly higher *Vv*, NTMs, and *L*spp concentrations than sites with at least one continuous 30-sec use within the last seven days (Chapter 7).

  - To minimize microbial amplification and the presence of OPs at distal sites, distal plumbing sections and connecting fixtures should be designed to reduce stagnation and limit biofilm development, including simple fixture with minimal surface and more uniform materials, as well as promoting regular water circulation during more prolonged inter-use stagnation (e.g., auto-flush devices, flushing of taps). Moreover, adopting the "1L rule" in system design widely applied in European countries and maintenance can help reduce the buildup of elevated microbial concentrations.

- Chapter 5 highlighted several vulnerabilities in the design and operation of hot water production systems that likely contributed to systemic *Lp* contamination. Key risks included one inactive heater left without proper isolation from the heaters, which operated at conducive temperatures for microbial growth, and energy-efficiency modifications that inadvertently lowered hot water temperatures supplied to the building to 53 – 55 ˚C. Additional risks arose from occasional temperature drops, as low as 25 ˚C, in a small plate heat exchanger, and the presence of significant dead volumes (up to 300 liters) in the piping system, thus delaying temperature delivery and increasing consequently water age.

  - Therefore, building managers should prioritize isolating inactive heaters to prevent "nursery effects", maintaining consistent hot water temperatures above 60 ˚C, and avoiding blending practices that lower supply hot water temperatures. System designs should minimize dead volumes to ensure rapid temperature recovery at distal sites, and replace plate heat exchangers with direct heating systems where possible, especially in high-risk settings like healthcare facilities.

This research project has opened new avenues for future investigations, including:

- **Development of evidence to improve standards and guidance:** (1) Establish evidence-based alert and action thresholds for OPs using robust, rapid, and cost-effective analytical monitoring tools, (2) Define standardized procedures for building (re)commissioning, disinfection protocols, and fixture-specific design standards (e.g., sinks, showers, etc.).
- **Pathogen-specific insights:** (1) Obtain data on monochloramine susceptibility among clinically relevant *Legionella* species and NTMs (e.g., *Legionella anisa*, *Mycobacterium gordonae*) to better understand their persistence under prolonged disinfectant exposure, (2) Explore the validation of integrated multi-pathogen monitoring tools capable of detecting and quantifying key OPs to enable high-throughput monitoring at system and distal sites, (3) Investigate markers of temperature resistance and conditions leading to the potential selection of more resistant and possibly virulent OPs strains.
- **Stagnation and amplification risks:** (1) Determine critical duration thresholds for water stagnation events that promote significant microbial growth to inform (re)commissioning and construction activities management, (2) Investigate microbial amplification dynamics in distal sections of plumbing systems, focusing on how design factors such as pipe length, dead volumes, and usage patterns contribute to stagnation risks.
- **Balancing disinfection and sustainability:** (1) Perform system-scale life cycle analysis that integrate onsite chemical disinfection strategies with energy and water conservation measures to assess the combined environmental and operational impacts of balancing microbial safety with sustainability goals, (2) Evaluate the economic and exposure risk trade-offs of implementing energy-efficient and water-saving designs alongside additional disinfection measure to optimize building plumbing operations and ensure safety without compromising conservation efforts.
- **Sampling and surveillance strategies:** (1) Evaluate the feasibility of pooled sampling to expand spatial monitoring coverage to reduce allocated resource, acknowledging potential trade-offs in site-specific insights, (2) Explore how sampling frequency can be tailored to specific operational and design features to better capture transient microbial risks and guide targeted interventions effectively.

The ongoing challenges in achieving and maintaining water safety highlight the interplay between operational practices, system design, and pathogen control measures. Future efforts must address the complexities of balancing microbial risk management with energy and water conservation strategies, particularly as greener buildings with low-flow fixtures, energy-efficient water heating systems, and innovative water recycling practices become the norm. This calls for versatile research across diverse fields of expertise to bridge existing limitations and open broader discussions enabling the development of holistic strategies to manage OPs

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APPENDIX A SUPPLEMENTARY MATERIAL, ARTICLE 1: CAN FREE CHLORINE RESIDUALS ENTERING BUILDING PLUMBING SYSTEMS REALLY BE MAINTAINED TO PREVENT MICROBIAL GROWTH?

Journal: Science of the Total Environment

Title: Can free chlorine residuals entering building plumbing systems really be maintained to prevent microbial growth?

Authors: Marianne Grimard-Conea, Emilie Bédard, Michèle Prévost

Number of figures: 2 (Figure A.1, Figure A.2)

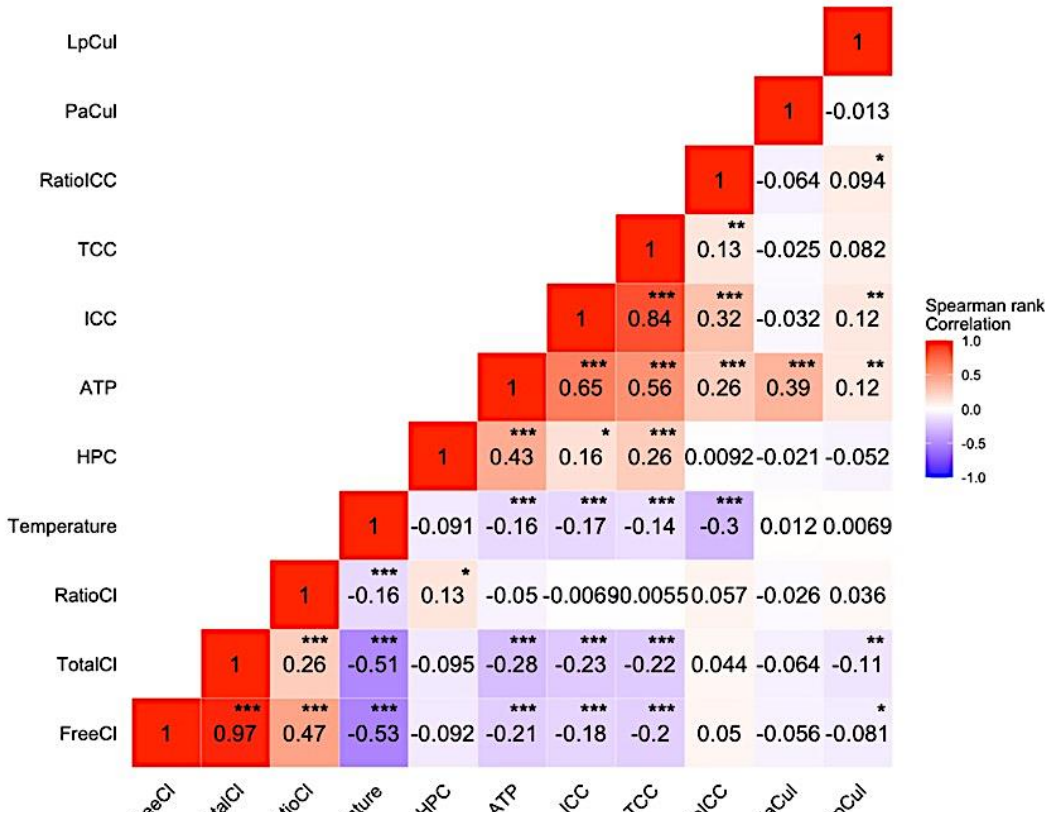

Figure A.1 Spearman's rank test correlation results (rho values, R) using all samples (first draws and flushed samples). Significance levels (p-value): One star if $p < 0.05$, two stars if $p < 0.01$ and three stars if $p < 0.001$. Legend: FreeCl – free chlorine, TotalCl – total chlorine, RatioCl – ratio of free chlorine on total chlorine, HPC – Heterotrophic plate count, ATP – Intracellular adenosine triphosphate, ICC – Intact cell count, TCC – total cell count, RatioICC – ratio of intact cell counts, PaCul – culturable *Pseudomonas aeruginosa*, LpCul – culturable *Legionella pneumophila*.

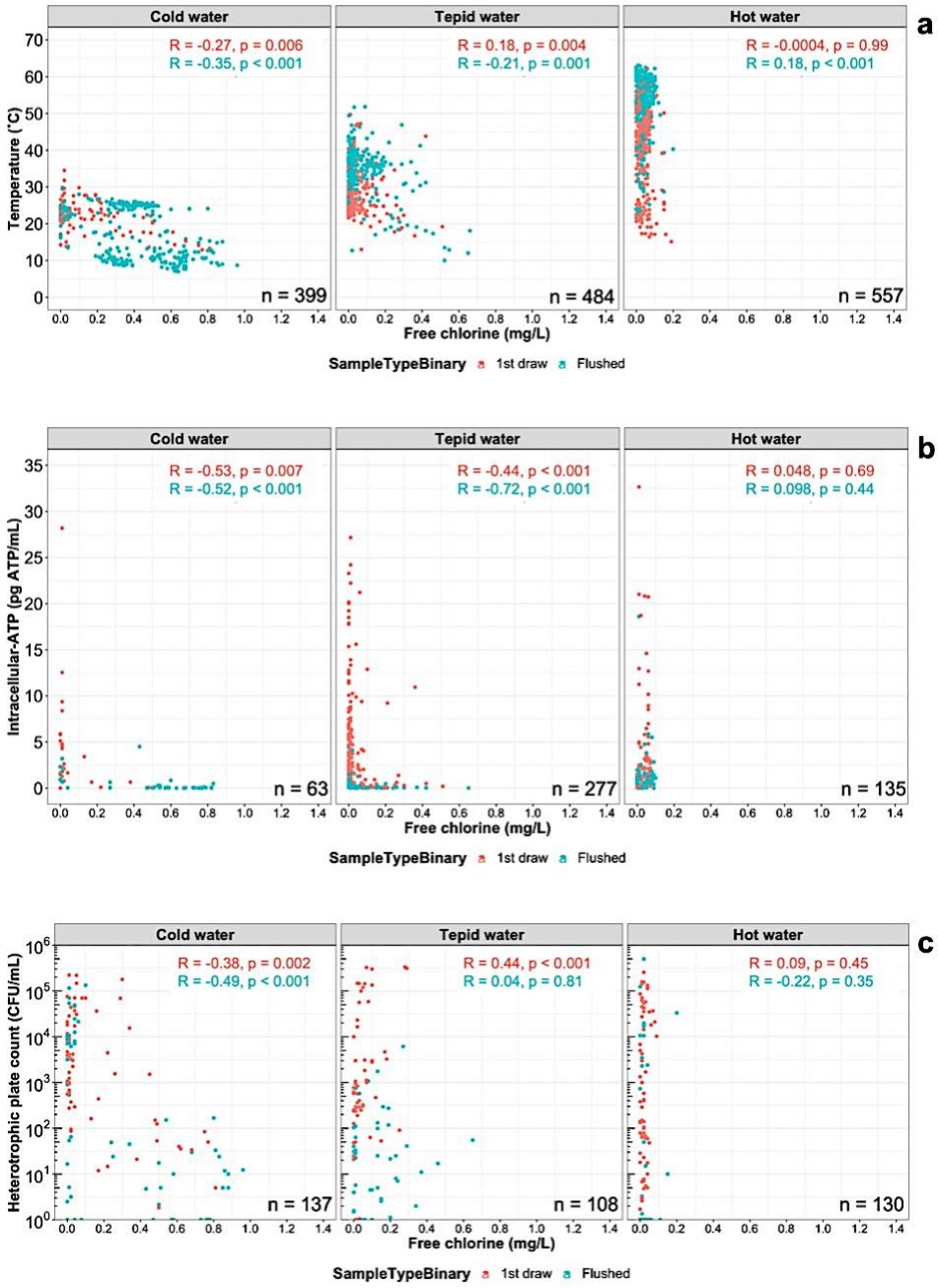

Figure A.2 Scatter plots and Spearman rank correlation coefficients (R) of free chlorine (x-axis) against (**a**) Temperature (n = 1,440, 9 buildings), (**b**) Intracellular-ATP (n = 475, 4 buildings), (**c**) Heterotrophic plate count (n = 375, 6 buildings), (**d**) Total cell counts (n = 574, 7 buildings), (**e**) Intact cell counts (n = 563, 7 buildings), (**f**) Culturable *Legionella pneumophila* (n = 809, 5 buildings), and (**g**) Culturable *Pseudomonas aeruginosa* (n = 286, 3 buildings) in first draws (red) and flushed samples (blue).

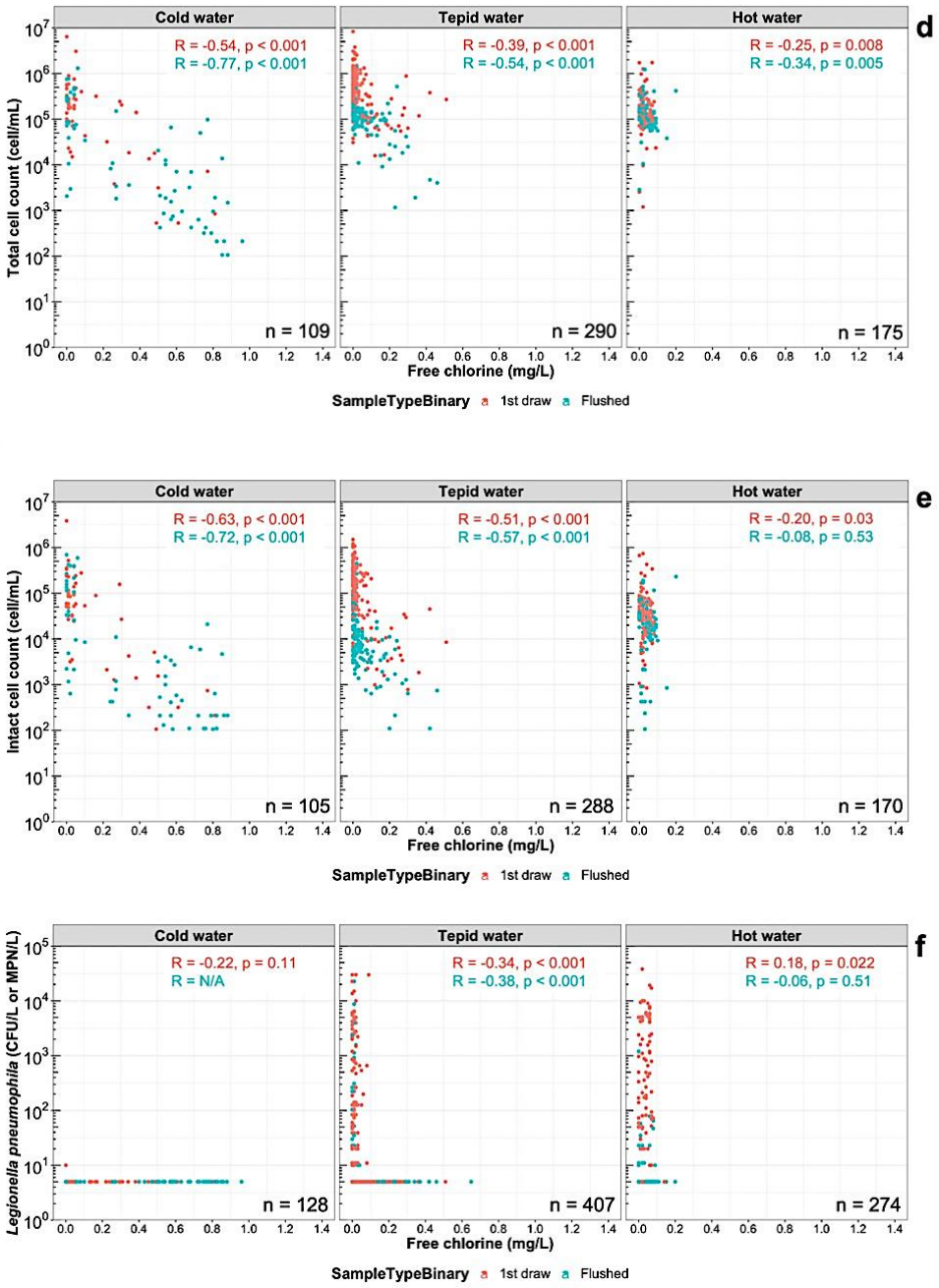

Figure A.2 Scatter plots and Spearman rank correlation coefficients (R) of free chlorine (x-axis) against (**a**) Temperature (n = 1,440, 9 buildings), (**b**) Intracellular-ATP (n = 475, 4 buildings), (**c**) Heterotrophic plate count (n = 375, 6 buildings), (**d**) Total cell counts (n = 574, 7 buildings), (**e**) Intact cell counts (n = 563, 7 buildings), (**f**) Culturable *Legionella pneumophila* (n = 809, 5 buildings), and (**g**) Culturable *Pseudomonas aeruginosa* (n = 286, 3 buildings) in first draws (red) and flushed samples (blue) (continued).

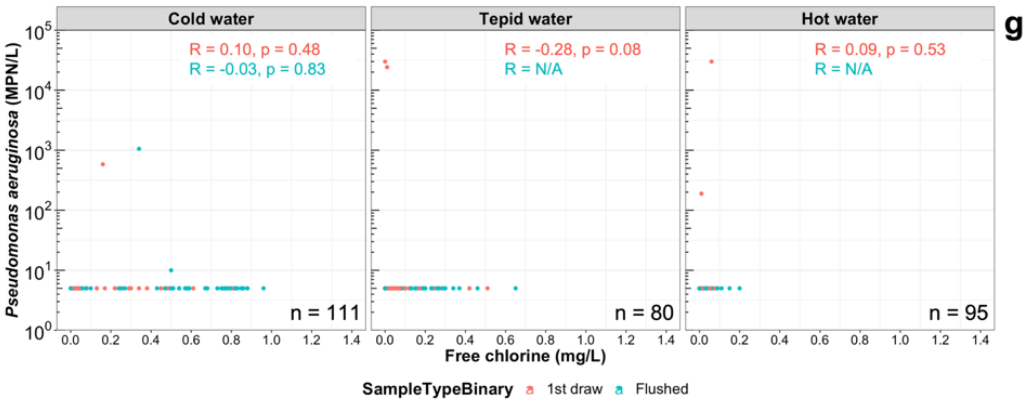

Figure A.2 Scatter plots and Spearman rank correlation coefficients (R) of free chlorine (x-axis) against (**a**) Temperature (n = 1,440, 9 buildings), (**b**) Intracellular-ATP (n = 475, 4 buildings), (**c**) Heterotrophic plate count (n = 375, 6 buildings), (**d**) Total cell counts (n = 574, 7 buildings), (**e**) Intact cell counts (n = 563, 7 buildings), (**f**) Culturable *Legionella pneumophila* (n = 809, 5 buildings), and (**g**) Culturable *Pseudomonas aeruginosa* (n = 286, 3 buildings) in first draws (red) and flushed samples (blue) (continued).

APPENDIX B SUPPLEMENTARY MATERIAL, ARTICLE 2: IMPACT OF RECOMMISSIONING FLUSHING ON *LEGIONELLA PNEUMOPHILA* IN A LARGE BUILDING DURING THE COVID-19 PANDEMIC

Journal: Frontiers in Water, Sec. Water and Human Health

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

Authors: Marianne Grimard-Conea, Elise Deshommes, Evelyne Doré, Michèle Prévost

Number of figures: 3 (Figure B.1, Figure B.2, Figure B.3)

Number of tables: 2 (Table B.1, Table B.2)

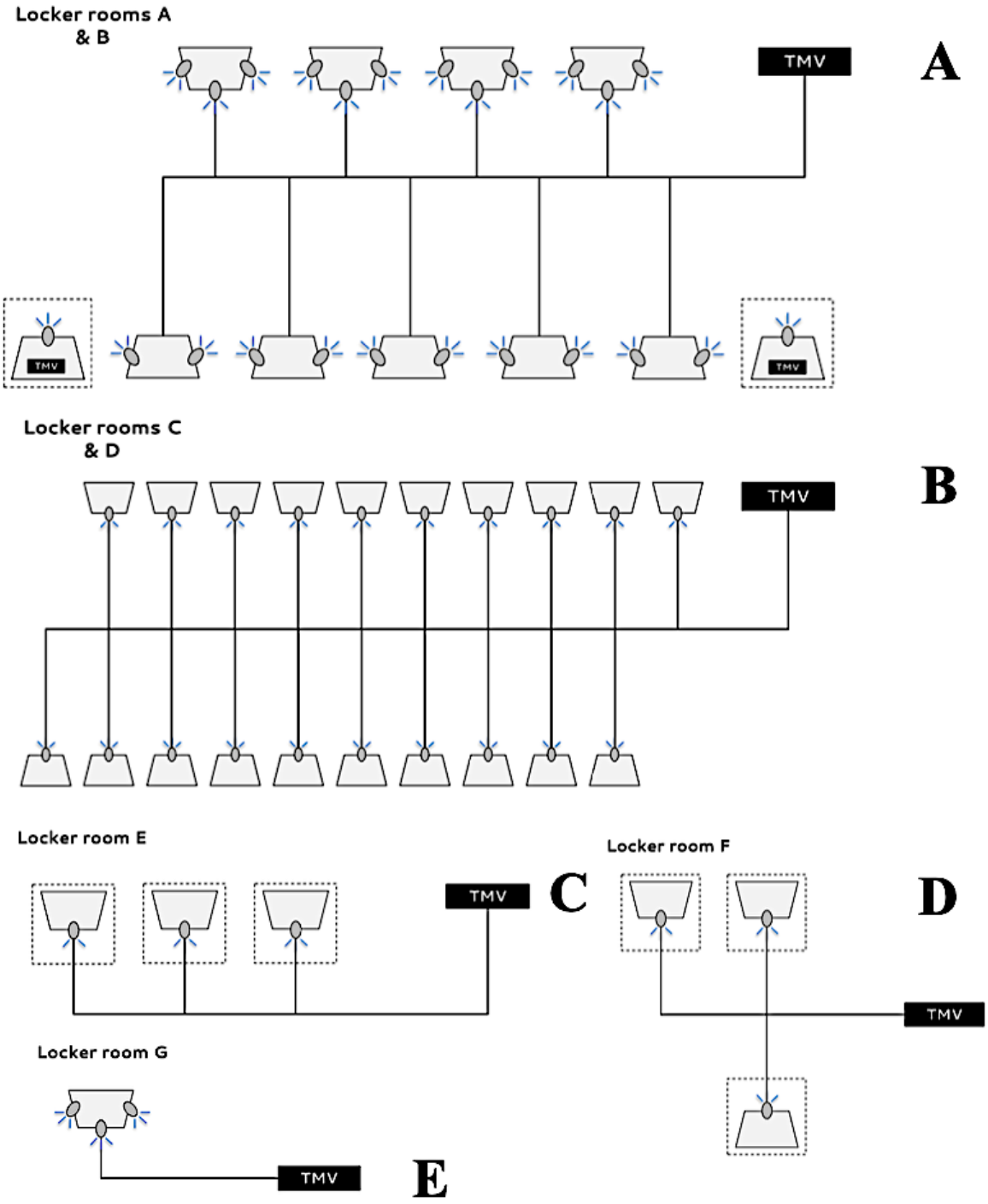

Figure B.1 Schematic of piping configurations and showerheads from each investigated locker room (n = 7), including (**A**, **B**) large grouped shower systems and (**C**, **D**, **E**) small shower systems. Legend: grey ovals – showerheads; black line – piping; TMV – thermostatic mixing valve; dotted line – showers separated with physical panels.

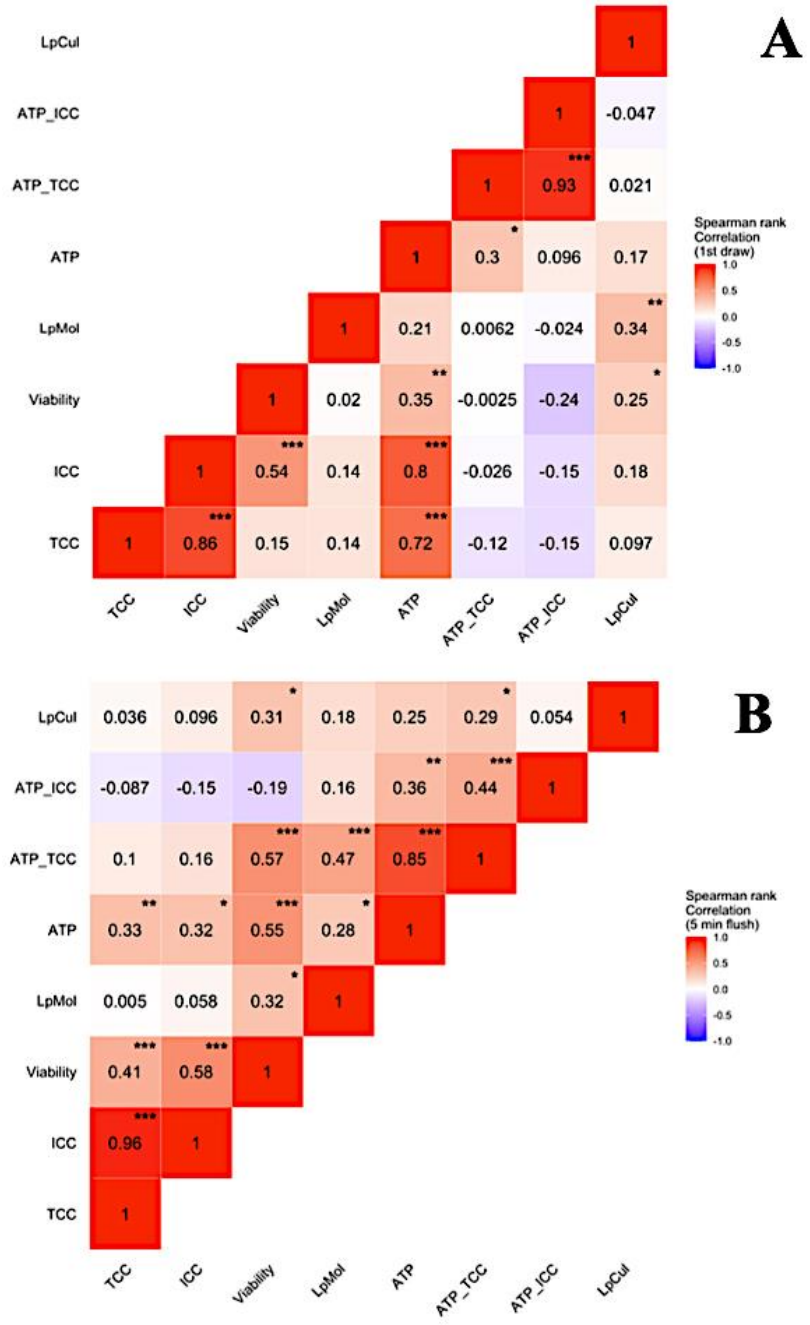

Figure B.2 Spearman's rank test results (rho values) for (**A**) first draws and (**B**) five-min flush samples. Significance levels: One star if $p < 0.05$, two stars if $p < 0.01$, three stars if $p < 0.001$. Legend: LpCul – Culture-based *L. pneumophila*; ATP_ICC – Bacterial-ATP over intact cell counts; ATP_TCC – Bacterial-ATP over total cell counts; ATP – Bacterial-ATP; LpMol – qPCR *L. pneumophila*; Viability – Viability ratios; ICC – Intact cell counts; TCC – Total cell counts.

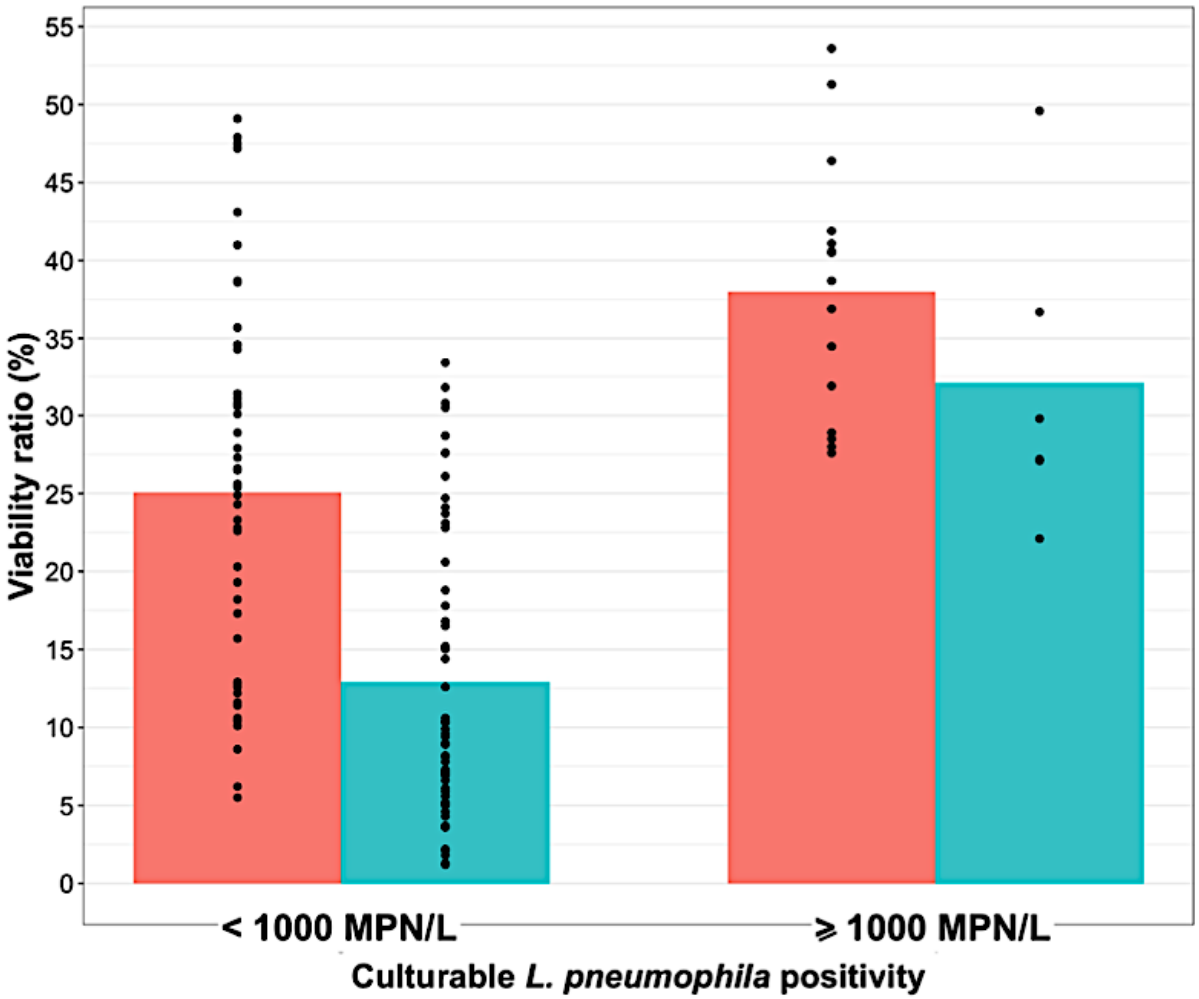

Figure B.3 Results of viability ratios of first draw (red) and five-min flushed samples (blue) categorized into dichotomous culture-based *Legionella pneumophila* concentrations (< 1,000 MPN/L or ≥ 1,000 MPN/L. Notes: Bar – Mean of viability ratios; Black dots – Raw data.

Table B.1 Summary of statistical results of microbiological measurements. Note: results are presented as mean value (min value – max value).

Parameter	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
Bacterial ATP (pg ATP/mL)	10.0 (0.7 – 24.2)	0.7 (0.1 – 3.0)	1.8 (0.2 – 9.9)	0.3 (0 – 1.9)	5.0 (1.7 – 10.1)	0.4 (0 – 2.3)
Total cell counts (cell/mL)	1.7e+06 (1.3e+05 – 3.8e+06)	2.4e+05 (6.1e+04 – 1.3e+06)	4.8e+05 (1.7e+05 – 1.2e+06)	1.6e+05 (9.4e+04 – 3.2e+05)	7.2e+05 (4.2e+05 – 1.2e+06)	1.1e+05 (4.5e+04 – 2.6e+05)
Intact cell counts (cell/mL)	5.2e+05 (8.0e+03 – 1.1e+06)	4.8e+04 (7.6e+02 – 4.5e+05)	9.1e+04 (1.7e+04 – 4.2e+05)	2.2e+04 (3.4e+03 – 9.1e+04)	2.4e+05 (1.5e+05 – 4.4e+05)	2.7e+04 (4.1e+03 – 1.1e+05)
Viability ratio (%)	32 (6 – 54)	13 (1 – 33)	18 (6 – 35)	11 (4 – 29)	35 (17 – 48)	21 (9 – 50)
ATP/ICC (pg ATP/cell)	6.8e-05 (2.9e-06 – 1.1e-03)	3.2e-05 (7.2e-07 – 6.6e-05)	2.3e-05 (3.2e-06 – 7.3e-05)	1.1e-05 (1.4e-06 – 3.6e-05)	2.1e-05 (7.9e-06 – 4.4e-05)	1.3e-05 (3.1e-06 – 5.3e-05)
ATP/TCC (p ATP/cell)	8.5e-06 (5.3e-07 – 6.7e-05)	2.9e-06 (2.4e-07 – 8.4e-06)	4.4e-06 (3.4e-07 – 1.3e-05)	1.3e-06 (2.1e-07 – 8.7e-06)	7.2e-06 (2.1e-06 – 1.4e-05)	3.2e-06 (2.8e-07 – 1.9e-05)
Culture-based *L.pneumophila* (MPN/L)	4 487 (5 – 30 000)	1 622 (5 – 22 726)	100 (5 – 723)	23 (5 – 264)	2 368 (5 – 30 000)	450 (5 – 3 971)
qPCR *L.pneumophila* (gu/L)	63 822 (10 – 1 160 000)	3 461 (10 – 17 200)	6 839 (10 – 54 800)	686 (10 – 2 730)	47 007 (132 – 808 000)	3 158 (169 – 18 200)

Table B.2 Mean per exposure risk values for both Legionellosis outcomes (Pontiac fever and Legionnaires' disease - LD) in conventional (> 13 lpm) and low-flow (< 7 lpm) showerheads.

Health outcome	Shower type	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
Pontiac fever endpoint with a *per exposure risk*	> 13 lpm	1.4×10^{-2}	4.1×10^{-3}	2.7×10^{-4}	7.3×10^{-5}	6.2×10^{-3}	1.3×10^{-3}
	< 7 lpm	2.6×10^{-3}	7.8×10^{-4}	5.3×10^{-5}	1.4×10^{-5}	1.2×10^{-3}	2.7×10^{-4}
LD endpoint with a *per exposure risk*	> 13 lpm	1.4×10^{-5}	4.7×10^{-6}	2.9×10^{-7}	7.5×10^{-8}	6.6×10^{-6}	1.4×10^{-6}
	< 7 lpm	2.6×10^{-6}	8.8×10^{-7}	5.7×10^{-8}	1.5×10^{-8}	1.3×10^{-6}	2.8×10^{-7}
LD endpoint with a *per exposure DALY*	> 13 lpm	1.3×10^{-5}	4.5×10^{-6}	2.8×10^{-7}	7.3×10^{-8}	6.4×10^{-6}	1.4×10^{-6}
	< 7 lpm	2.6×10^{-6}	8.6×10^{-7}	5.5×10^{-8}	1.4×10^{-8}	1.2×10^{-6}	2.7×10^{-7}

APPENDIX C SUPPLEMENTARY MATERIAL, ARTICLE 3: CONTROLLING *LEGIONELLA PNEUMOPHILA* IN SHOWERHEADS: COMBINATION OF REMEDIAL INTERVENTION AND PREVENTIVE FLUSHING

Journal: Microorganisms

Title: Controlling *Legionella pneumophila* in showerheads: Combination of remedial intervention and preventative flushing

Authors: Marianne Grimard-Conea, Michèle Prévost

Number of figures: 1 (Figure C.1)

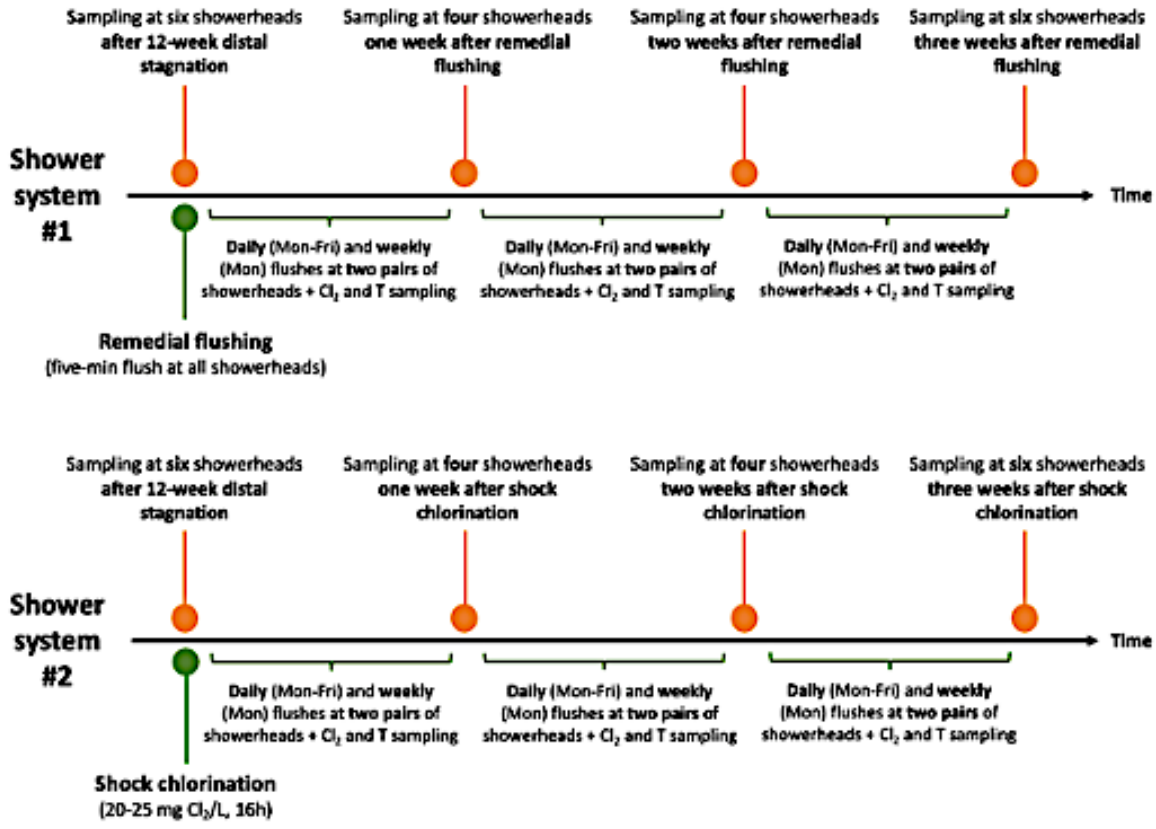

Figure C.1 Chronological steps of the sampling events (orange) and interventions (green) carried out in both shower systems.

APPENDIX D SUPPLEMENTARY MATERIAL, ARTICLE 4: MITIGATION OF OPPORTUNISTIC DRINKING WATER PATHOGENS BY ONSITE MONOCHLORAMINE DISINFECTION IN A HOSPITAL WATER SYSTEM

Journal: Submitted to Water Research on January 23rd, 2025

Title: Mitigation of opportunistic drinking water pathogens by onsite monochloramine disinfection in a hospital water system

Authors: Marianne Grimard-Conea, Xavier Marchand-Senécal, Sébastien P. Faucher, Michèle Prévost

Number of figures: 5 (Figure D.1, Figure D.2, Figure D.3, Figure D.4, Figure D.5)

Number of tables: 2 (Table D.1, Table D.2)

D.1 Hospital setting configuration and sampling sites location

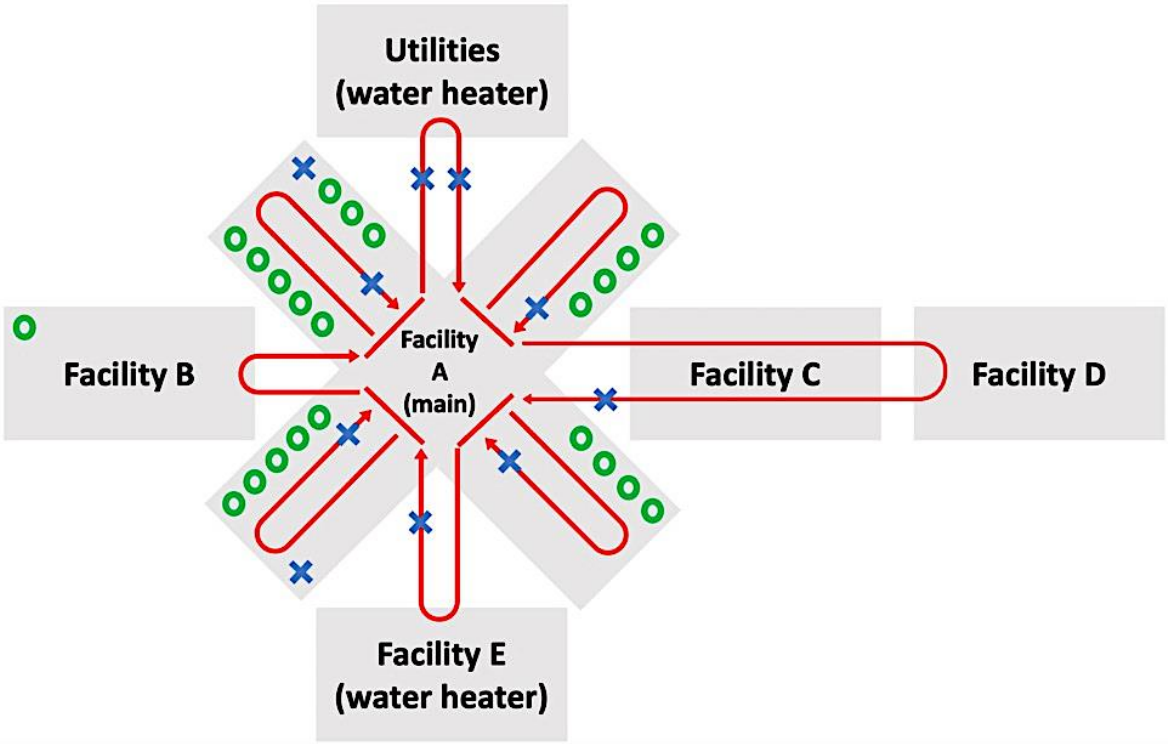

Figure D.1 Hospital setting layout and sampling site location (distal sites [n= 22]: green circles, system sites [n = 10]: blue crosses, horizontal hot water loops: red arrows).

D.2 Physico-chemical analysis

Temperature, pH, conductivity, dissolved oxygen, free and total chlorine were measured as described by Grimard-Conea and Prévost (2023). Total organic carbon (TOC) was measured in duplicates (2 X 20 mL) according to the standard method 5310C (APHA et al., 2005) by conductivity on the Sievers M5310C Laboratory TOC Analyzer (Veolia, Boulder, CO, USA). Metals concentrations were analyzed in triplicates (3 X 5 mL, with nitric acid 0.15% v:v) by inductively coupled plasma mass spectrometry (ICP-MS) on the Perkin Elmer NexION 5000 instrument (PerkinElmer Inc., Woodbridge, ON, Canada) according to the manufacturer's instructions. ICP-MS detection limits (µg/L) were of 0.0009, 0.0150, 0.0060, and 0.0003 for manganese, iron, copper, and lead, respectively. Ammonium ions were assessed by colorimetry with sodium salicylate in compliance with the published analysis method MA. 300-N 2.0 (CEAEQ, 2014), whereas nitrite and nitrate concentrations were assessed by ion chromatography according to the published analysis method MA. 300 – Ions 1.3 (CEAEQ, 2020). Reported limits of detection for ammonium, nitrite and nitrate were of 0.020 mg/L.

D.3 Microbiological analysis

Culturable *L. pneumophila* concentrations were determined as specified in Grimard-Conea and Prévost (2023), through an enzymatic culture-based method. Gene copies (gc) quantification of *Legionella* species, *L. pneumophila*, *L. pneumophila* serogroup 1, *Mycobacterium* species, and *V. vermiformis* was conducted in triplicates by real-time quantitative polymerase chain reaction (qPCR) according to specifications provided in Table D.1. For all qPCR assays, template DNA and master mix were assembled in hard-shell 96-well PCR plates (Bio-Rad, catalog number HSR9905), and fluorescence curves were retrieved on the Bio-Rad CFX Opus 96 Real-Time PCR instrument. Amplification efficiencies between 75% and 125%, and correlation coefficients (R^2) greater than 0.99 recovered from standard curves for DNA quantification were systematically ensured for each targeted organism and qPCR assay.

Table D.1 Overview of qPCR protocols and standard curve specifications for each organism.

Organism	Targeted gene	Manufactured kit or referenced protocol	Amplification efficiencies	Standard curve R^2
Legionella species	Not specified	Triplex protocol as described in the commercial laboratory microproof® *Legionella* Quantification LyoKit (Biotecon Diagnostics, R 602 45)	84.1-98.1%	0.995-0.999
Legionella pneumophila	Not specified		92.0-100.8%	0.994-0.999
Legionella pneumophila sg 1	Not specified		95.6-104.5%	0.994-1.000
Mycobacterium species	*atpE*	Adapted laboratory protocol from Haig et al. (2018)	84.9-123.2%	0.993-1.000
Vermamoeba vermiformis	18S rRNA	Laboratory protocol from Kuiper et al. (2006)	75.0-106.3%	0.990-0.999

Legionella DNA quantification, including *Legionella* species, *L. pneumophila* and *L. pneumophila* serogroup 1, was performed as a multiplex assay using the commercial microproof® *Legionella* Quantification LyoKit based on recommendations from the experience of the Ontario Public Health (Canada) environmental microbiology laboratory. The manufacturer's kit procedure was thoroughly followed for the program setup (cycling conditions, fluorescence channels), the preparation of the PCR mix, the data interpretation and quantification, in addition to the verification for the presence of inhibiting agents.

Nontuberculous mycobacteria (NTMs) were quantified with an adapted protocol from Haig et al. (2018) which targets with a high specificity the *atpE* gene in the *Mycobacterium* species genomes from environmental samples (Radomski et al., 2013). NTMs standards were prepared with genomic DNA from *Mycobacterium avium* subspecies *paratuberculosis* strain K-10 (ATCC BAA-968D™) and sterile PCR water in order to get a series of dilutions ranging from 2.0E+01 gc/L to 2.0E+05 gc/L. The qPCR master mix for one 96-well PCR plate (25 samples in triplicates) was composed of 605 µl of sterile PCR water, 1100 µl of the SsoFast™ EvaGreen® Supermix (Bio-Rad, catalog number 1725201), 55 µl of bovine serum albumin (20 mg/mL) (Thermo Scientific™, catalog number B14), 110 µl of a 100-fold diluted forward *atpE* primer (5'-CGG YGC CGG TAT CGG YGA-3'), and 110 µl of a 100-fold diluted reverse *atpE* primer (5'-CGA AGA CGA ACA RSG CCA T-3'), with both sets of primers synthesized with custom IDT oligos. PCR plates (Bio-Rad, catalog number HSR9905) were filled with a total reaction volume of 20 µl (18 µl of the qPCR master mix, 2 µl of sampled DNA extract or NTMs standard or sterile PCR water [negative controls]), using triplicates of each content. Cycling conditions (40 cycles) included an initial

denaturation (5 min, 95 ˚C), followed by a denaturation (20s, 95 ˚C), and an annealing (30s, 57 ˚C) and elongation phases (30s, 72 ˚C).

Finally, targeting the 18S rRNA gene in *Vermamoeba vermiformis*, the DNA of this protozoan was quantified following the qPCR procedure briefly outlined in Cazals and colleagues (2023), which was previously developed by Kuiper and colleagues (2006). Concentrations were expressed as cell equivalents (CE), considering that 1,330 copies of the targeted 18S rRNA gene were approximatively present in each *V. vermiformis* cell (Kuiper et al., 2006).

D.4 Data analysis

For both statistical and graphical curation, samples with culturable concentrations of *L. pneumophila* below the detection limit (10 MPN/L) were assigned a value of 5 MPN/L. Contrastingly, the sole sample with a concentration exceeding the upper detection limit (22,726 MPN/L) was capped at 30,000 MPN/L. For each qPCR assay (*Legionella*, NTMs, *V. vermiformis*), concentrations that were undetectable (< 20 gc/L) were set at 10 gc/L. For concentrations falling between the limit of detection and the lower limit of quantification, or for concentrations above the upper limit of quantification, values were kept at their estimated value or capped at the upper limit of quantification, respectively.

D.5 Results and discussion

Table D.2 Mean total chlorine concentrations (mg/L) per sampling site location. Note: * Dosage was restarted right before sampling during the first prolonged 4-week stop.

Sampling site location	Before monochloramine	Before dosage increase	After dosage increase	Dosage interruption periods
Target value (mg/L)	N/A	1.5	2.5	N/A
System sites [2-min flush] (*range*)	0.09 (*0.05 – 0.15*)	1.10 (*0.40 – 1.60*)	2.09 (*0.11 – 3.40*)	0.35 (*0.08 – 1.20*)
Heater outlet	0.11	1.30	2.22	0.19
Combined return	0.11	1.10	1.99	0.13
Main cross-shape building 1	0.09	1.20	2.21	0.59*
Main cross-shape building 2	0.08	1.20	2.08	0.47*
Main cross-shape building 3	0.09	1.10	2.10	0.59*
Main cross-shape building 4	0.09	1.10	2.04	0.57*
Facility C/D	0.09	1.10	2.05	0.68*
Facility E	0.08	0.80	1.57	0.12
Remote hot water faucets	0.09	1.30	2.32	0.11
Distal sites [first draws] (*range*)	0.12 (*0.05 – 0.58*)	0.70 (*0.10 – 1.50*)	1.62 (*0.21 – 3.60*)	0.17 (*0.06 – 0.75*)
Hot water faucets	0.09	0.90	1.91	0.17
Showerheads [tepid]	0.23	0.30	0.90	0.17
Hand washing stations [tepid]	0.19	0.20	0.77	0.23

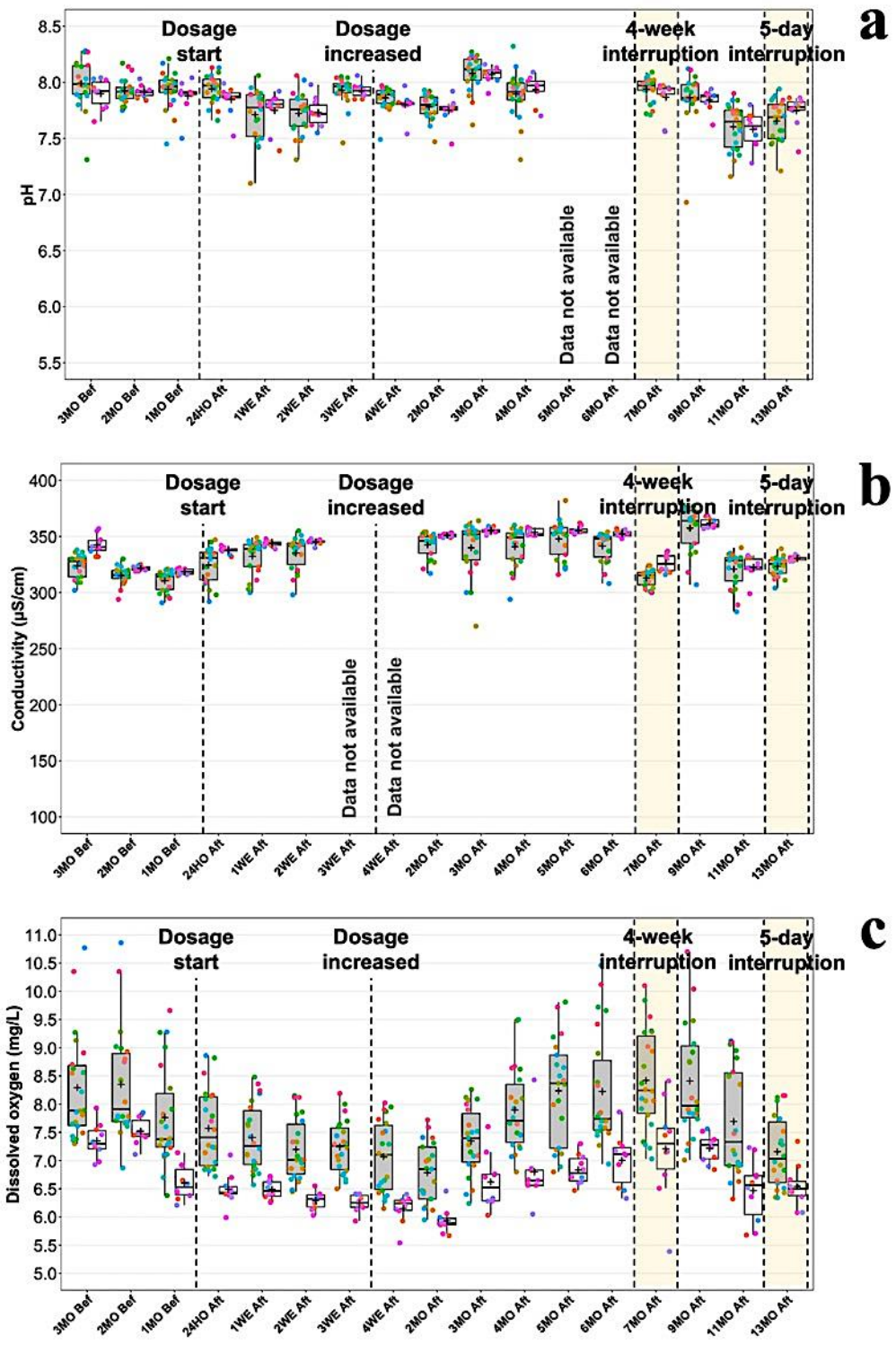

Figure D.2 Box plots of (**a**) pH, (**b**) Conductivity, and (**c**) Dissolved oxygen per samplings (x-axis) at distal (grey boxes, n = 22) and system sites (white boxes, n = 10). Legend: Black cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Colored dots – Raw data per sampling site, MO – Month, WE – Week, Bef – Before, Aft – After.

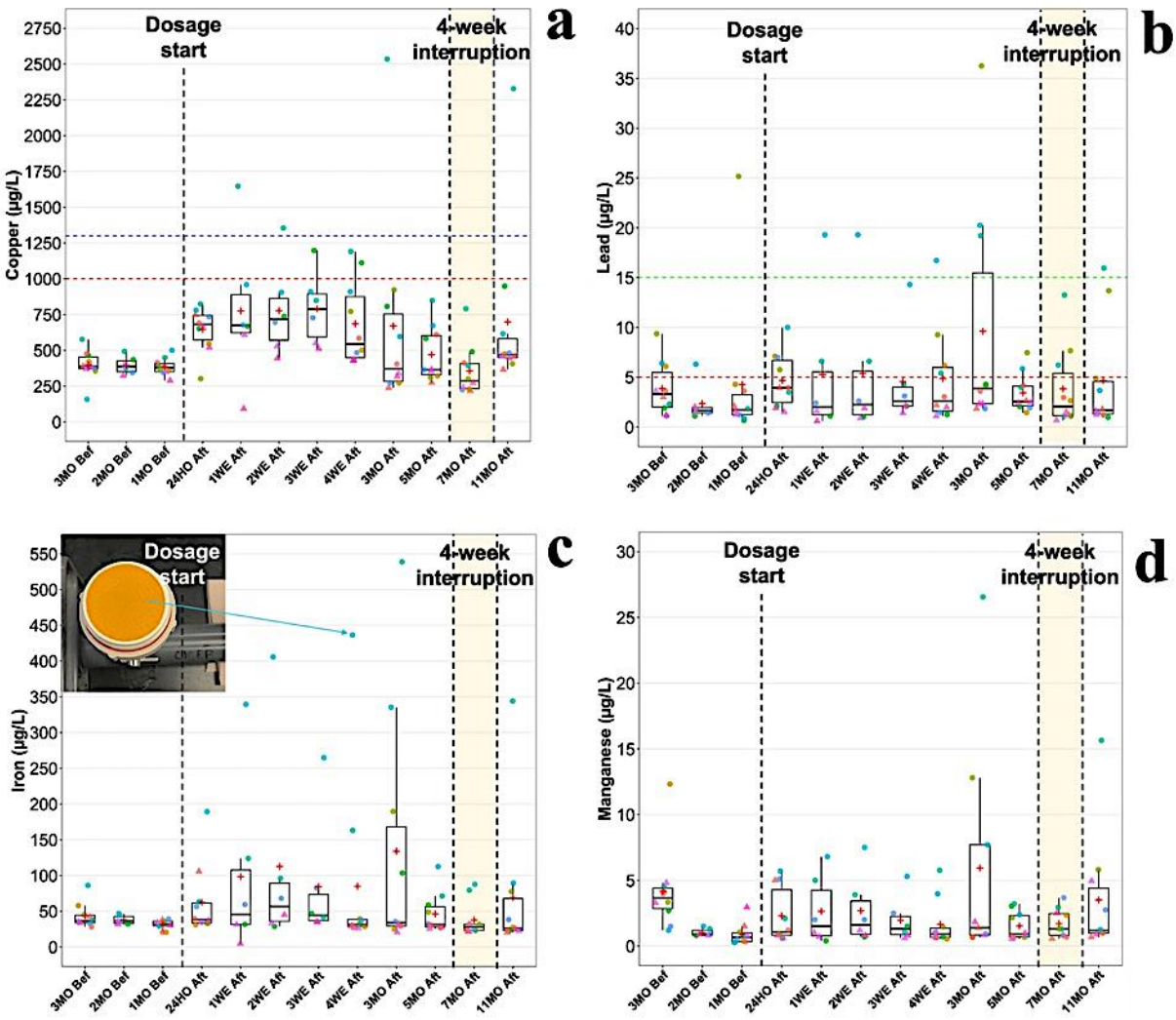

Figure D.3 Box plots of (**a**) Copper, (**b**) Lead, (**c**) Iron, and (**d**) Manganese per samplings (x-axis) at a subset of ten sampling sites. Legend : Red cross – Mean, Horizontal black line – Median, Boxes – 25th and 75th percentiles, Colored dots – Raw data per sampling site, Circle points – Distal sites (n = 7), Triangle-shaped points – System sites (n = 3), Red lines – regulatory concentration in Québec water quality guidelines, Blue line – U.S. EPA regulatory concentration for copper, Green line – U.S. EPA regulatory concentrations for lead, MO – Month, WE – Week, Bef – Before, Aft – After.

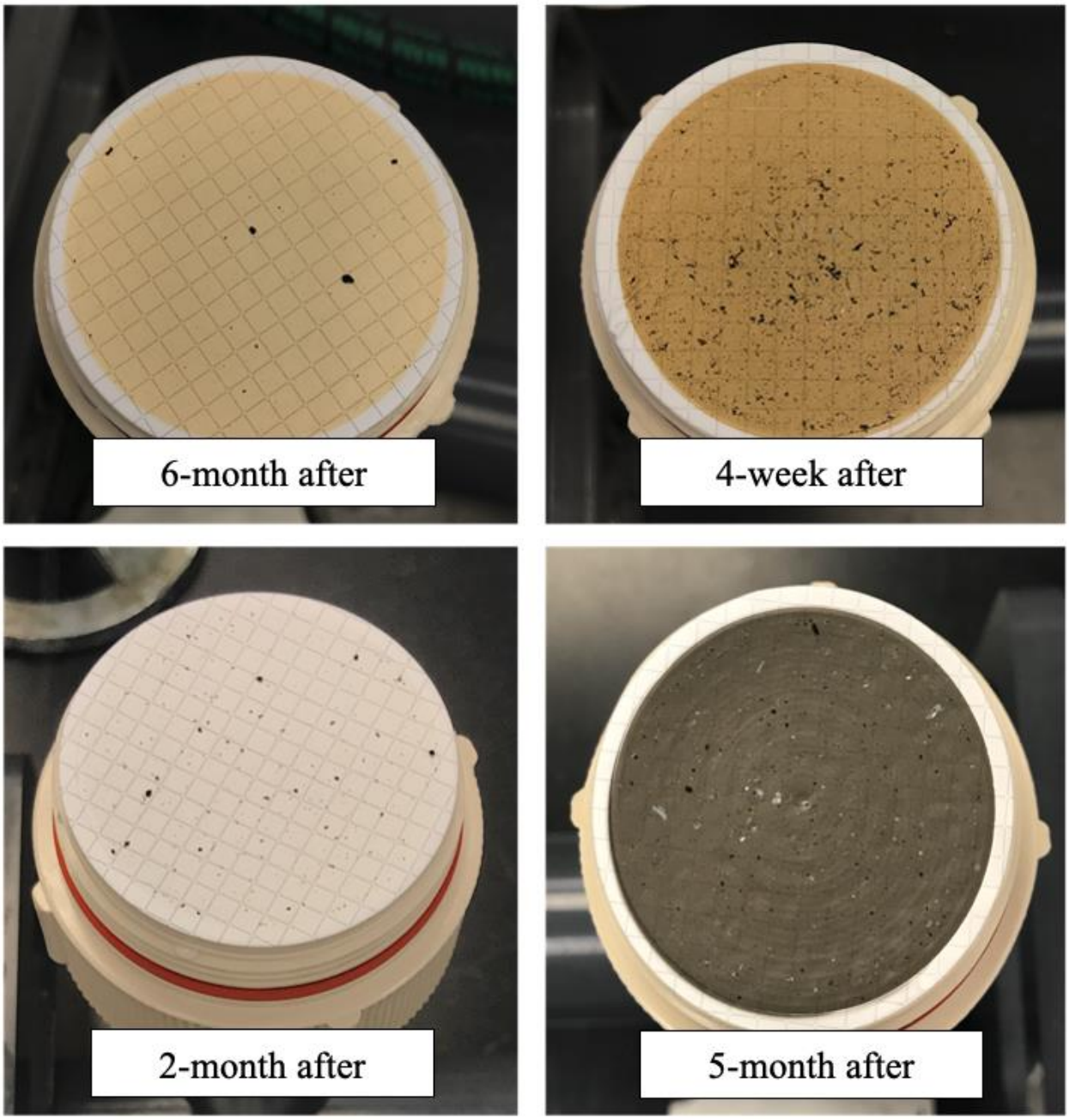

Figure D.4 Examples of plumbing metals in particulate and dissolved forms collected on 0.2 µm membranes during vacuum-filtration after the onset of monochloramine treatment in the hospital's hot water system.

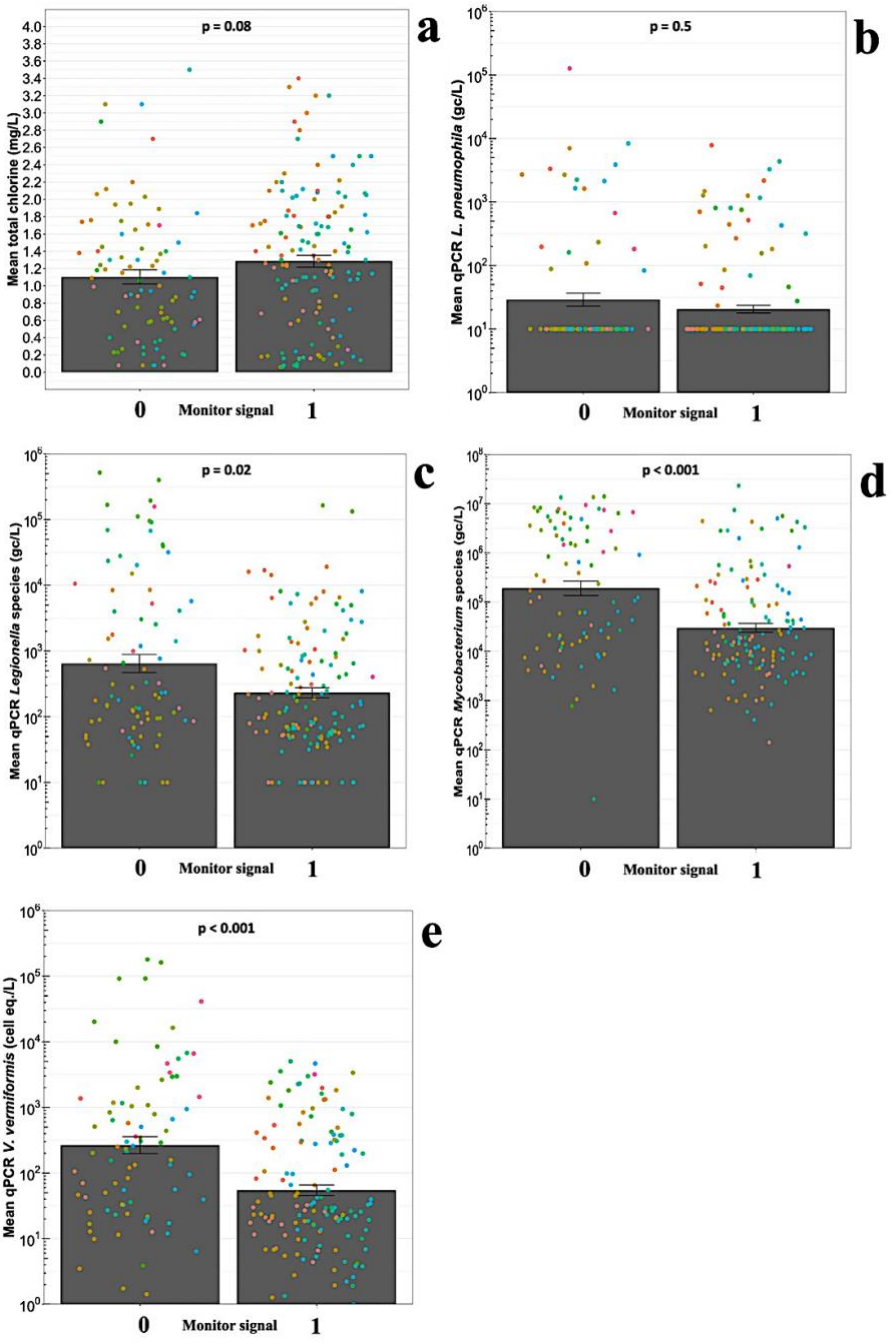

Figure D.5 Bar plots of mean (**a**) Total chlorine, (**b**) qPCR *Legionella pneumophila*, (**c**) qPCR *Legionella* species, (**d**) qPCR *Mycobacterium* species, and (**e**) qPCR *Vermamoeba vermiformis* according to the flushing monitor signal installed following onset of monochloramine treatment (0: less than 30-sec use within the last week; 1: at least one 30-sec of continuous use within the last week). Legend: Bar plot – Mean values, Bracket – Error bars, Colored dots – Raw data per sampling site.

APPENDIX E SUPPLEMENTARY MATERIAL, ARTICLE 5: *IN SITU* DOSING OF MONOCHLORAMINE IN A HOSPITAL HOT WATER SYSTEM RESULTS IN DRASTIC MICROBIAL COMMUNITIES CHANGES

Journal: Submitted to Science of the Total Environment on February 24th, 2025

Title: Introduction of onsite monochloramine in a hospital hot water system results in drastic microbial communities changes

Authors: Marianne Grimard-Conea, Elliston Vallarino Reyes, Xavier Marchand-Senécal, Sébastien P. Faucher, Michèle Prévost

Number of tables: 2 (Table E.1, Table E.2)

Table E.1 Description of *Legionella* ASV sequences producing at least 99.00% percent identity alignment using the BLAST search tool.

ASV	Number of counts	Description
ASV00106	301	*Legionella longbeachae*, *Legionella anisa*, *Legionella parisiensis*, *Legionella cherrii*, *Legionella oakridgensis*, *Legionella gingyii*, *Legionella norrlandica*, *Legionella dumoffii*, *Legionella resiliens*, *Legionella santicrusis*, *Legionella taurinensis*, *Legionella sainthelensi*
ASV00207	77	*Legionella pneumophila*
ASV00219	58	None
ASV00371	13	None
ASV00420	11	None
ASV00435	9	None
ASV00606	5	None
ASV00950	5	*Legionella sainthelensi*, *Legionella fallonii*, *Legionella steelei*, *Legionella dumoffii*
ASV01082	4	None
ASV01259	4	None
ASV01517	2	*Legionella massiliensis*
ASV01770	1	*Legionella gresilensis*
ASV02407	1	None
ASV02480	1	*Legionella bozemanii*, *Legionella santicrusis*, *Legionella cincinnatiensis*
ASV02502	1	None
ASV03021	1	*Legionella longbeachae*, *Legionella anisa*, *Legionella parisiensis*, *Legionella cherrii*, *Legionella oakridgensis*, *Legionella gingyii*, *Legionella norrlandica*, *Legionella dumoffii*, *Legionella resiliens*, *Legionella santicrusis*, *Legionella taurinensis*, *Legionella sainthelensi*

Table E.2 Description of *Mycobacterium* ASV sequences producing at least 99.00% percent identity alignment using the BLAST search tool.

ASV	Number of counts	Description
ASV00029	6,174	*Mycobacterium gordonae*, *Mycobacterium paragordonae*, *Mycobacterium vicinigordonae*
ASV00206	69	*Mycolicibacterium insubricum*, *Mycolicibacterium phocaicum*, *Mycolicibacterium phlei*, *Mycolicibacterium llatzerense*, *Mycolicibacterium mucogenicum*, *Mycolicibacterium houstonense*, *Mycolicibacterium chubuense*, *Mycolicibacterium poriferae*, *Mycolicibacterium smegmatis*
ASV00443	14	*Mycobacterium avium* subs. *paratuberculosis*, *Mycobacterium avium* subs. *hominissuis*, *Mycobacterium marseillense*, *Mycobacterium tuberculosis*, *Mycobacterium marinum*, *Mycobacterium basiliense*, *Mycobacterium saskatchewanense*, *Mycobacterium kansasii*, *Mycobacterium pseudoshottsii*
ASV00641	5	None
ASV01050	3	*Mycolicibacterium frederiksbergense*, *Mycolicibacterium helvum*, *Mycolicibacterium sarraceniae*, *Mycolicibacterium sediminis*, *Mycolicibacterium hackensackense*, *Mycolicibacterium mucogenicum*, *Mycolicibacterium peregrinum*, *Mycolicibacterium gilvum*, *Mycolicibacterium helvum*, *Mycolicibacterium fluoranthenivorans*, *Mycolicibacterium fortuitum* subsp. *fortuitum*
ASV01753	3	*Mycobacterium xenopi*, *Mycobacterium heckeshornense*
ASV02433	1	None
ASV02579	1	*Mycobacterium cookii*, *Mycobacterium paraterrae*