



Titre: Factors Affecting the Occurrence and Survival of Opportunistic Pathogens in Water and Biofilms of Premise Plumbing, From the Water Inlet to the Point of Use
Title:

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Date: 2023

Type: Mémoire ou thèse / Dissertation or Thesis

Référence: Cazals, M. (2023). Factors Affecting the Occurrence and Survival of Opportunistic Pathogens in Water and Biofilms of Premise Plumbing, From the Water Inlet to the Point of Use [Thèse de doctorat, Polytechnique Montréal]. PolyPublie.
Citation: <https://publications.polymtl.ca/54139/>

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URL de PolyPublie: <https://publications.polymtl.ca/54139/>
PolyPublie URL:

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Programme: Génie civil
Program:

POLYTECHNIQUE MONTRÉAL

affiliée à l'Université de Montréal

**Factors affecting the occurrence and survival of opportunistic pathogens in
water and biofilms of premise plumbing, from the water inlet to the point of
use**

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

Génie civil

Juin 2023

POLYTECHNIQUE MONTRÉAL

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Cette thèse intitulée :

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présentée par **Margot CAZALS**

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

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ACKNOWLEDGEMENTS

First, I would like to thank my supervisor Dr. Michèle Prévost. Thank you for all the opportunities you have given me during these 4 years. Thank you for always finding ways for me to share my results even during the pandemic. I have learnt a lot during this Ph.D. and you especially taught me to be more audacious to seize opportunities.

To Dr. Emilie Bédard, my co-advisor, thank you for trusting me since the beginning of this whole journey. Thank you for your patience and your availability to answer my questions. Thank you for the coaching, for your guidance and advice in every step of the process.

I would also like to thank Dr. Sarah Dorner, my co-advisor, for believing in me since my first baby steps in research during my Masters.

I am grateful to the members of the examination committee, Dr. Benoit Barbeau, Dr. Olivier Haeck, and Dr. Frederik Hammes, for accepting to review this thesis.

Thank you Dr. Sébastien P. Faucher for sharing your knowledge and expertise, and for all your advice on the different papers we worked on and for the sequencing data. Also thank you Elliston for your help on the analysis of these data, and your patience despite all the questions I asked. I also would like to thank Juan for his help with R and with all the diversity figures.

Many thanks to the IPC and technical teams of the Centre Hospitalier de l'Université de Montréal for trusting me since the beginning of my Ph.D. and for all your help on this project, especially Dr Patrice Savard, Chantal Soucy, Patrick Galipault, and the whole team of plumbers.

I would like to thank the Hot Water System Team, especially Tim Bartrand and Katherine Alfredo, for welcoming me in the team and giving me the chance to participate to the workshop. I learnt a lot from all of you.

Thank you, Mr. Raymond Desjardins, for trusting me and giving me the chance to take my first steps in teaching. You have the art of telling good stories and inspiring students, and I am glad I had the chance to work with you and to learn from you.

I would like to give a special thank to Dr. Margot Doberva. We worked as a team during the first months of my Ph.D. and you taught me a lot. Thank you for trusting me since the beginning with the pilot and thank you for answering my questions even 4 years after.

To all the Chair staff, thank you would certainly not be enough to express my gratitude. Yves, Tetiana, Jacinthe, Julie, and Mélanie, you all have been there throughout my entire project. We spent hours together on field, with the pilot, and in the lab, and even when it got tiring you were always ready to help me and to answer my never-ending questions. Thank you, Loan, for jumping in to help with the lab during the last samplings.

Thank you, Evelyne, Fatemeh and Laura for your help during our group meetings. Thank you, Laura and Adèle, for your help on the administrative part and more.

Thank you, Hana, for your advice, your kindness, and your positivism, you always found a way to make me smile. You were the one who guided me during my decision to start this project, and you kept listening to me and giving me advice until now.

I would also like to thank all the past and present students in water quality, both in drinking water and wastewater, I have had the chance to be around. Thank you for always be ready to help with sampling and to share tips and experience. Amira, Arthur, Jérôme, Liya, Luan, Ludovica, Marianne, Marie-Eve, Thibault, thank you also for our discussions about life, to take our minds off our projects for a little.

To all my friends, in Montreal or elsewhere, thank you, this journey would have not been the same without you. To Solène, Coralie, and Kim, my second family on this side of the ocean, thank you for always looking after me. Thank you for the tears and laughter though this journey, we finally made it! To Juliette, thank you for your enthusiasm and your encouragements during our Korean language learning sessions, 고마워요!

Finally, there are no words meaningful enough to thank and express my gratitude towards my family, especially my parents and my sister, who supported me since the beginning and was there every step of the way. Thank you for always believing in me and supporting me from the day I decided to do a Ph.D. back in high school. Thank you, Maman, Papa and Clémence for always being there, in the good and not so good moments, listening to my never-ending stories and my worries, and encouraging me no matter what.

I would like to acknowledge the FRQNT for the Doctoral (B2X) Research Scholarship.

RÉSUMÉ

Les qualités chimique et microbiologique de l'eau se retrouvent altérées dans les réseaux de plomberie des grands bâtiments, notamment des établissements de santé. En parallèle, de plus en plus d'infections nosocomiales sont reliées aux systèmes d'eau ou à des équipements connectés à ces réseaux qui se retrouvent contaminés par des micro-organismes pathogènes opportunistes. L'OMS recommande depuis plusieurs années aux établissements de santé de mettre en place des plans de gestion de la sécurité sanitaire de l'eau. Ces plans comprennent une définition et évaluation du système complet, ainsi que la définition d'un plan d'échantillonnage et de surveillance de la qualité d'eau. Parmi les micro-organismes pathogènes opportunistes engendrant de plus en plus d'infections nosocomiales liées à l'eau, la bactérie *Legionella pneumophila* est certainement le plus étudié. Cette bactérie est responsable de la légionellose, et est souvent associée aux systèmes de distribution d'eau chaude. Les mycobactéries non tuberculeuses (NTM) sont omniprésentes dans les réseaux de plomberie et sont responsables de nombreuses formes d'infections. *Vermamoeba vermiformis* est une amibe souvent retrouvée dans les réseaux d'eau chaude, et pouvant servir d'hôte pour la multiplication de *L. pneumophila* et certaines espèces de NTM. Finalement, des infections nosocomiales dues à des champignons pathogènes sont également de plus en plus recensées. Les espèces *Candida* sont parmi les espèces fongiques les plus problématiques en milieu hospitalier, avec l'émergence d'espèces multi-résistantes comme *Candida auris*. Ces quatre micro-organismes sont adaptés pour survivre dans les réseaux de distribution d'eau et les appareils qui y sont connectés, et sont difficiles à éliminer une fois la contamination installée.

L'objectif principal de ce projet de recherche est d'identifier des paramètres de conception, d'exploitation, et de maintenance des réseaux de distribution d'eau de grands bâtiments qui préviennent l'apparition ou la persistance de micro-organismes pathogènes opportunistes dans ces installations. L'emphasis a été mise sur *L. pneumophila*, les NTM et leur hôte commun *V. vermiformis* pour les réseaux d'eau potable et d'eau chaude, tandis que la présence de *Candida* spp. a été étudiée aux points d'usage que constituent les machines à glace d'un hôpital. Plus précisément, ce projet vise à : (1) évaluer la survie de *L. pneumophila* dans des chauffe-eaux électriques en fonction de la phase (eau ou sédiments), du régime de chauffe et de la présence d'hôtes dans le système, (2) déterminer l'impact de plusieurs paramètres environnementaux et d'exploitation (matériaux, température, régime d'écoulement) prévenant la persistance de bactéries pathogènes opportunistes (*L. pneumophila*, NTM) et leur hôte *V. vermiformis* dans l'eau et le

biofilm de réseaux d'eau froide et d'eau chaude, et (3) étudier la contamination microbienne de machines à glace en milieu hospitalier et la pertinence des recommandations actuelles concernant ces appareils.

La première phase du projet a consisté en des essais de température dans l'eau et les sédiments de chauffe-eaux électriques afin d'y évaluer la survie de *L. pneumophila* et *V. vermiformis*. Des différences importantes dans les réductions log de *L. pneumophila* ont été observées pour des expositions à 55°C ou 60°C, dans l'eau et les sédiments, rappelant l'importance de respecter les températures réglementaires. Des expositions quotidiennes de 4h à une température de 55°C se sont avérées efficaces pour éliminer les *L. pneumophila* cultivables. Cependant, la co-culture de bactéries *L. pneumophila* exposées à une température de 55°C pendant 4h et d'amibes *V. vermiformis* a entraîné une augmentation de 5 log en 4 jours de la population de *L. pneumophila*.

Un pilote constitué de 12 réacteurs CDC Biofilm inoculés de *L. pneumophila* et *V. vermiformis* et contenant des coupons de 6 matériaux a été utilisé pour la seconde phase du projet. Des concentrations supérieures à la concentration maximale acceptable de 5µg/L de plomb ont été mesurées dans l'eau du réacteur contenant des coupons d'EPDM. Cette étude a également révélé la présence naturelle de NTM et *V. vermiformis* dans l'eau de robinet utilisée pour le remplissage des réacteurs, et les concentrations les plus élevées pour ces deux micro-organismes ont été mesurées dans l'eau et les biofilms en contact avec l'EPDM. Le chauffage à 55°C et 60°C des réacteurs contenant des coupons de cuivre s'est avéré efficace contre tous les micro-organismes mesurés, toutes leurs concentrations tombant sous la limite de quantification. Le chauffage à 40°C ou plus de tous les matériaux a également induit d'importants relargages de carbone organique dissous et sa fraction biodégradable (jusqu'à 13,2 et 3,40 mg/L respectivement). Cette réserve de nutriments a certainement favorisé la survie des micro-organismes pendant les 6 mois de stagnation qui ont suivi. Cette période de stagnation a permis le regain de *L. pneumophila* cultivables dans les réacteurs contenant du PVC, de l'acier inoxydable, et un réacteur cuivre. Le rinçage des réacteurs après la stagnation n'a cependant pas impacté les concentrations en *L. pneumophila* cultivables tandis que la chloration choc réalisée sur tous les réacteurs a permis de les éliminer.

La dernière phase du projet a permis d'établir le potentiel des machines à glace comme réservoirs à micro-organismes pathogènes opportunistes. Une première campagne d'échantillonnage dans un hôpital a démontré une contamination microbienne importante avec de hautes concentrations en

bactéries hétérotrophes aérobies (BHA) et NTM, ainsi que la présence de *Candida* spp. dans l'eau et la glace, et les drains des 36 machines échantillonnées. Ces échantillonnages ont aussi mis en lumière la faible corrélation entre BHA et présence de micro-organismes pathogènes dans ces appareils. Les résultats indiquent que les NTM feraient de meilleurs indicateurs microbiens de l'efficacité de la désinfection des machines à glace. Un échantillonnage séquentiel réalisé dans un second temps sur 5 machines à glace, leurs préfiltres et les robinets adjacents, a démontré que les principaux sites d'amplification microbienne et des NTM sont les préfiltres et les réservoirs d'eau internes des machines à glace. Des dépôts ont également été relevés dans ces mêmes réservoirs et seraient liés au relargage de phosphates par les préfiltres et la stagnation de l'eau dans les réservoirs due à un usage peu fréquent de la glace.

Dans l'ensemble ce projet a permis d'identifier certains paramètres permettant de limiter la prolifération de micro-organismes pathogènes opportunistes dans différents sites des réseaux d'eau de grands bâtiments. Tout d'abord, le respect des températures réglementaires dans les réseaux d'eau chaude est primordial, et l'arrêt des chauffe-eaux en période de pointe de consommation électrique devrait être effectué avec précaution. Les systèmes de distribution d'eau chaude en cuivre semblent aussi efficaces pour contrôler les concentrations en micro-organismes pathogènes opportunistes mais des études au plus long terme sont nécessaires pour le confirmer. Le choix des matériaux dans les réseaux de distribution d'eau et leur listage dans les plans de gestion de la sécurité sanitaire de l'eau sont importants, notamment pour les matériaux polymères des joints d'étanchéité ou des tuyaux flexibles. En effet, ces derniers sont souvent omis mais peuvent fortement impacter la qualité chimique et microbiologique de l'eau. Un rinçage complet du système d'eau est nécessaire après un choc thermique, un chauffage intermittent, ou une chloration choc car ces événements entraînent d'importants relargages de carbone dans l'eau qui pourraient alimenter des communautés microbiennes plus loin dans le réseau et promouvoir leur croissance. Finalement, les machines à glace peuvent être des sites d'amplification microbienne et des réservoirs à micro-organismes pathogènes opportunistes, et doivent donc être intégrées dans les plans de gestion de la sécurité sanitaire de l'eau. Le choix de prétraitements comme des préfiltres doit également être adapté au système et à l'utilisation faite de l'eau.

ABSTRACT

Chemical and microbial water quality are altered in premise plumbing of large buildings, especially healthcare facilities. Concomitantly, an increasing number of hospital-acquired infections are linked to water systems or equipment connected to these systems that become contaminated with opportunistic pathogens. WHO has been recommending for several years that healthcare facilities implement water safety plans. These plans include a definition and assessment of the entire system, as well as the definition of a sampling and monitoring plan for water quality. Among the opportunistic pathogens causing an increasing number of water-related nosocomial infections, *Legionella pneumophila* is certainly the most studied. This bacterium is responsible for legionellosis and is often associated with hot water systems. Non-tuberculous mycobacteria (NTM) are ubiquitous in plumbing systems and are responsible for many forms of infections. *Vermamoeba vermiformis* is an amoeba that is often found in hot water systems and serve as a host for the multiplication of *L. pneumophila* and some NTM species. Finally, nosocomial infections due to pathogenic fungi are also increasingly reported. *Candida* species are among the most problematic fungal species in hospitals, with the emergence of multi-resistant species such as *Candida auris*. All four microorganisms are adapted to survive in water systems and connected devices, and are difficult to remove once contamination is established.

The main objective of this research project is to identify design, operation, and maintenance parameters of large building water distribution systems that prevent the occurrence or persistence of opportunistic pathogens. The focus was on *L. pneumophila*, NTM and their common host *V. vermiformis* for drinking and hot water systems, while the presence of *Candida* spp. was investigated in hospital ice machines. On a more detailed level, this project aims to: (1) evaluate the survival of *L. pneumophila* in electric water heaters depending on the phase (water or loose deposits), the heating regime and the presence of hosts in the system, (2) determine the impact of several environmental and operational parameters (materials, temperature, flow regime) preventing the persistence of opportunistic pathogens (*L. pneumophila*, NTM) and their host *V. vermiformis* in water and biofilm of cold and hot water systems, and (3) to study the microbial contamination of hospital ice machines and the relevance of current guidelines concerning these machines.

The first phase of the project involved temperature testing to assess the survival of *L. pneumophila* and *V. vermiformis* in water and loose deposits from electric water heaters. Significant differences

in log reductions of *L. pneumophila* were observed for exposures at 55°C or 60°C, in water and loose deposits, highlighting the importance of complying with the regulated temperatures. Daily 4-hour exposures at 55°C were effective in eliminating cultivable *L. pneumophila*. However, co-culture of *L. pneumophila* exposed to 55°C for 4 hours with *V. vermiformis* resulted in a 6-log increase in the *L. pneumophila* population over 4 days.

A pilot consisting of 12 CDC Biofilm reactors inoculated with *L. pneumophila* and *V. vermiformis* and containing coupons of 6 materials was used for the second phase of the research project. Concentrations of lead above the maximum acceptable value of 5µg/L were measured in water of the reactor containing EPDM coupons. This study also revealed the natural presence of NTM and *V. vermiformis* in the tap water used to fill the reactors, and the highest concentrations of these two microorganisms were measured in the water and biofilms in contact with EPDM. Heating to 55°C and 60°C of the reactors containing copper coupons was effective against all the target microorganisms, with all their concentrations falling below the quantification limit. Heating all materials to 40°C and above also induced significant releases of dissolved organic carbon and its biodegradable fraction (up to 13.2 and 3.40 mg/L respectively). This supply of nutrients certainly favoured the survival of microorganisms during the 6-month stagnation that followed. This period of stagnation allowed the recovery of culturable *L. pneumophila* in the reactors containing PVC, stainless steel, and one copper reactor. Flushing of the reactors after stagnation did not impact the concentrations of culturable *L. pneumophila*, while shock chlorination of all reactors eliminated them.

The last phase of the project established the potential of water-ice machines as reservoirs for opportunistic pathogens. A first field campaign in a hospital demonstrated significant microbial contamination with high concentrations of heterotrophic plate counts (HPC) and NTM, as well as the presence of *Candida* spp. in ice-water samples and drain biofilms of the 36 machines sampled. These samplings also highlighted the weak correlation between HPC and the presence of opportunistic pathogens in these machines. The results indicate that NTM would make a better microbial indicator of the effectiveness of ice machine disinfection. Sequential sampling of 5 water-ice machines, their pre-filters and adjacent faucets showed that the main sites of microbial amplification and NTM are the pre-filters and ice machine internal water reservoirs. Deposits were also found in these same reservoirs and might be related to the release of phosphates from the pre-filters and the stagnation of water in the reservoirs due to infrequent demand of ice.

Overall, this project identified some parameters to limit the proliferation of opportunistic pathogens in different parts of large building water systems. Firstly, the respect of regulatory temperatures in hot water systems is essential, and water heater shutdown to accommodate peak demand shaving should be done carefully. Copper hot water distribution systems also appear to effectively control concentrations of opportunistic pathogens but studies on the long-term are needed to validate this observation. The choice of materials in water distribution systems and their listing in water safety plans is important, particularly for polymeric materials in gaskets or flexible pipes. They are often omitted but can significantly impact chemical and microbial water quality. A thorough flushing of the water system is necessary after thermal shock, intermittent heating, or shock chlorination as these events result in significant carbon release into water that could feed microbial communities downstream in the system and promote their growth. Finally, water-ice machines can be sites of microbial amplification and reservoirs of opportunistic pathogens and should therefore be included in water safety plans. The choice of pre-treatments such as pre-filters should also be adapted to the system and the use made of the water.

PRECIS IN FRENCH

Le maintien d'une qualité d'eau satisfaisante de la sortie de l'usine de potabilisation au consommateur reste un défi majeur pour les responsables de réseaux de distribution d'eau, notamment ceux dans les grands bâtiments. En effet, les qualités chimiques et microbiologiques de l'eau se retrouvent altérées lors de leur passage dans les réseaux de plomberie des grands bâtiments. Cela est d'autant plus préoccupant dans le cas des établissements de santé abritant des populations plus fragiles. Depuis plusieurs années, de plus en plus d'infections nosocomiales sont ainsi reliées aux systèmes d'eau ou à des équipements connectés à ces réseaux qui se retrouvent contaminés par des micro-organismes pathogènes opportunistes. La mise en place de plans de gestion de la sécurité sanitaire de l'eau est ainsi recommandée par l'OMS, en particulier pour les établissements de santé. Ces plans comprennent une définition et évaluation du système dans son ensemble (donc comprenant tous les appareils reliés aux réseaux de distribution d'eau), ainsi que la définition d'un plan d'échantillonnage et de surveillance de la qualité de l'eau aux points stratégiques.

Parmi les pathogènes opportunistes causant de plus en plus d'infections nosocomiales liées à l'eau, la bactérie *Legionella pneumophila* est certainement le plus étudié. Cette bactérie cause deux types d'infections, une forme bénigne appelée la fièvre de Pontiac, et une forme grave de pneumonie chez les personnes à risque appelée maladie du légionnaire. *Legionella pneumophila* est le plus souvent associée aux réseaux d'eau chaude. Une autre catégorie de bactéries causant de nombreuses infections en milieu hospitalier sont les mycobactéries non tuberculeuses (NTM). Les NTM regroupent plus de 180 espèces de bactéries très résistantes à la désinfection qui sont omniprésentes dans les réseaux d'eau et causent différents types d'infections. Les amibes sont des protozoaires naturellement présents dans les réseaux d'eau et qui jouent un rôle important dans la diversité microbienne de ceux-ci en « broutant » les micro-organismes se trouvant dans les biofilms et en servant d'hôtes pour la multiplication de plusieurs pathogènes opportunistes. Parmi les amibes, l'espèce *Vermamoeba vermiformis* est souvent prédominante dans les réseaux d'eau chaude, et peut servir d'hôte et ainsi favoriser la multiplication de *L. pneumophila* et certaines espèces de NTM. Finalement, les infections nosocomiales dues à des champignons pathogènes sont également de plus en plus recensées. Les espèces *Candida* sont parmi les espèces fongiques les plus problématiques en milieu hospitalier, avec l'émergence d'espèces multi-résistantes comme *Candida auris*. Ces quatre micro-organismes sont particulièrement adaptés à la survie dans les réseaux de distribution d'eau et les équipements qui y sont connectés, notamment grâce à leur

intégration aux biofilms se développant sur les parois internes des tuyaux, et il est particulièrement difficile de les éliminer une fois la contamination installée.

Les paramètres sur lesquels les ingénieurs et responsables de réseaux d'eau ou de bâtiments peuvent jouer sont principalement le design des réseaux et bâtiments, le choix des matériaux, la température de l'eau, et l'ajout ou non d'un désinfectant. Or, l'effet combiné de ces paramètres sur l'occurrence et la survie de micro-organismes pathogènes opportunistes dans les réseaux d'eau chaude et d'eau froide, aussi bien dans la phase liquide que dans les biofilms, reste encore mal compris. De plus, les nombreuses fermetures de bâtiments ayant eu lieu lors de la pandémie de COVID-19, y entraînant des périodes prolongées sans utilisation d'eau, ont engendré de nombreuses réflexions de la part d'experts et des autorités publiques pour émettre des recommandations sur les interventions à mettre en place pour une réouverture sécuritaire de ces réseaux d'eaux et des bâtiments. Néanmoins, une variabilité de l'efficacité des différentes stratégies de remédiation proposées quant aux concentrations en pathogènes opportunistes a été rapportée selon les bâtiments étudiés.

L'objectif principal de ce projet de recherche est d'identifier des paramètres de conception, d'exploitation, et de maintenance des réseaux de distribution d'eau de grands bâtiments qui préviennent l'apparition ou la persistance de micro-organismes pathogènes opportunistes dans ces installations. L'emphasis a été mise sur *L. pneumophila*, les NTM et leur hôte commun *V. vermiformis* pour les réseaux d'eau potable et d'eau chaude, tandis que la présence de *Candida* spp. a été étudiée aux points d'usage que constituent les machines à glace d'un hôpital. Plus précisément, ce projet vise à : (1) évaluer la survie de *L. pneumophila* dans des chauffe-eaux électriques en fonction de la phase (eau ou sédiments), du régime de chauffe et de la présence d'hôtes dans le système, (2) déterminer l'impact de plusieurs paramètres environnementaux et d'exploitation (matériaux, température, régime d'écoulement) prévenant la persistance de bactéries pathogènes opportunistes (*L. pneumophila*, NTM) et leur hôte *V. vermiformis* dans l'eau et le biofilm de réseaux d'eau froide et d'eau chaude, et (3) étudier la contamination microbienne de machines à glace en milieu hospitalier et la pertinence des recommandations actuelles concernant ces appareils.

La première phase du projet a consisté en des essais de température dans l'eau et les sédiments de chauffe-eaux électriques afin d'y évaluer la survie de *L. pneumophila* et *V. vermiformis* (Chapitre

4). Des différences importantes dans les réductions log de *L. pneumophila* ont été observées pour des expositions à 55°C ou 60°C, dans l'eau et les sédiments, rappelant l'importance de respecter les températures réglementaires. Des expositions quotidiennes de 4h à une température de 55°C se sont avérées efficaces pour éliminer les *L. pneumophila* cultivables. Cependant, la co-culture de bactéries *L. pneumophila* exposées à une température de 55°C pendant 4h et d'amibes *V. vermiformis* a entraîné une augmentation de 5 log en 4 jours de la population de *L. pneumophila*.

Un pilote constitué de 12 réacteurs CDC Biofilm inoculés de *L. pneumophila* et *V. vermiformis* et contenant des coupons de 6 matériaux (polypropylène (PP), polychlorure de vinyle (PVC), caoutchouc EPDM, acier inoxydable (SS), polyéthylène réticulé (PEX), et cuivre (Cu)) a été utilisé pour la seconde phase du projet (Chapitres 5 et 6). Celui-ci a été opéré durant 76 semaines en mode semi-batch (avec changements hebdomadaires des $\frac{3}{4}$ du volume d'eau de chaque réacteur). Les 3 premiers mois ont permis la formation de biofilms sur les coupons, puis une souche de *V. vermiformis* a été inoculée, suivie par 2 souches environnementales de *L. pneumophila* 1 mois plus tard. Deux semaines plus tard, la température de chacun des réacteurs a été augmentée pour être représentative des températures retrouvées à différents points des réseaux d'eau chaude des établissements de santé au Québec. Lors du confinement de la pandémie de COVID-19, le pilote a été laissé stagnant à température ambiante. Après ces 6 mois de stagnation les deux stratégies de remise en service des réseaux d'eau les plus recommandées (rinçage et chloration choc) ont été réalisées sur le pilote. Des concentrations supérieures à la concentration maximale acceptable de 5µg/L de plomb ont été mesurées dans l'eau du réacteur contenant des coupons d'EPDM. Cette étude a également révélé la présence naturelle de NTM et *V. vermiformis* dans l'eau de robinet utilisée pour le remplissage des réacteurs, et les concentrations les plus élevées pour ces deux micro-organismes ont mesurées dans l'eau et les biofilms en contact avec l'EPDM. Le chauffage à 55°C et 60°C des réacteurs contenant des coupons de cuivre s'est avéré efficace contre tous les micro-organismes mesurés, toutes leurs concentrations tombant sous la limite de quantification. Le chauffage à 40°C ou plus de tous les matériaux a également induit d'importants relargages de carbone organique dissous et sa fraction biodégradable (jusqu'à 13,2 et 3,40 mg/L respectivement). Cette réserve de nutriments a certainement favorisé la survie des micro-organismes pendant les 6 mois de stagnation qui ont suivi. Cette période de stagnation a permis le regain de *L. pneumophila* cultivables dans les réacteurs contenant du PVC, de l'acier inoxydable, et un réacteur cuivre. Le rinçage des réacteurs après la stagnation n'a cependant pas impacté les concentrations en *L.*

pneumophila cultivables tandis que la chloration choc réalisée sur tous les réacteurs a permis de les éliminer.

La dernière phase du projet a permis d'établir le potentiel des machines à glace comme réservoirs à micro-organismes pathogènes opportunistes (Chapitre 7). Une première campagne d'échantillonnage de 36 machines à glace d'un hôpital de Montréal a démontré une contamination microbienne importante avec de hautes concentrations en bactéries hétérotrophes aérobies (BHA) et NTM, ainsi que la présence de *Candida* spp. dans l'eau et la glace, et les drains des 36 machines échantillonnées. Ces échantillonnages ont aussi mis en lumière la faible corrélation entre BHA et présence de micro-organismes pathogènes dans ces appareils. Les résultats indiquent que les NTM feraient de meilleurs indicateurs microbiens de l'efficacité de la désinfection des machines à glace. Un échantillonnage séquentiel réalisé dans un second temps sur 5 machines à glace, leurs préfiltres et les robinets adjacents, a démontré que les principaux sites d'amplification microbienne et des NTM sont les préfiltres et les réservoirs d'eau internes des machines à glace. Des dépôts ont également été relevés dans ces mêmes réservoirs et seraient liés au relargage de phosphates par les préfiltres et la stagnation de l'eau dans les réservoirs due à un usage peu fréquent de la glace.

Ce projet de recherche visait à améliorer notre compréhension des paramètres influençant la survie de pathogènes opportunistes à différents points des réseaux de plomberie des grands bâtiments. Les principales conclusions de ce projet sont ainsi :

Chauffe-eaux électriques

- L'importance de respecter les températures réglementaires dans les réseaux d'eau chaude et chauffe-eaux, puisqu'une plus grande inactivation de *L. pneumophila* a été observée à 60°C comparativement à celle obtenue à 55°C dans l'eau et les sédiments de chauffe-eaux électriques. De même, une majorité de *V. vermiformis* mortes a été obtenue à 43°C comparativement à 40°C.
- Cependant, la présence d'hôtes au contact de *L. pneumophila* chauffées a entraîné une augmentation de 6-log de cette population au bout de 4 jours, annulant la réduction de 5-log obtenue après 4h de chauffe à 55°C.

Pilote

- Des biofilms peuvent se développer sur tous les matériaux mais à des densités différentes : EPDM > PP > PVC > PEX > Cu – SS
- L'EPDM peut relarguer des concentrations importantes de plomb dans l'eau (dépassant parfois la recommandation canadienne de 5µg/L). Les biofilms d'EPDM accumulent aussi le zinc et le cuivre, tandis que les biofilms de cuivre accumulent le plomb.
- L'addition de *V. vermiformis* a entraîné une augmentation importante des concentrations en NTM dans tous les réacteurs.
- Le chauffage à des températures de 40°C et plus et la chloration choc entraînent tous deux le relargage de concentrations importantes de carbone par tous les matériaux et leurs biofilms.
- Aucun des micro-organismes pathogènes opportunistes étudiés n'a pu être quantifié dans les réacteurs avec des coupons de cuivre chauffés à 55°C et 60°C.
- Un regain de *L. pneumophila* cultivables a été observé après 6 mois de stagnation à température ambiante dans les réacteurs avec des coupons de PVC, SS et un avec du cuivre, mais la réalisation d'une chloration choc sur tous les réacteurs a permis d'éliminer les *L. pneumophila* cultivables.

Machines à glace

- Les machines à glace et les préfiltres installés en amont peuvent être des réservoirs à micro-organismes pathogènes opportunistes, et des sites d'amplification microbienne et d'amplification de NTM.
- Les BHA ne semblent pas être de bons indicateurs de l'efficacité de la désinfection des machines à glace en milieu hospitalier.

Dans l'ensemble ce projet a permis d'identifier certains paramètres permettant de limiter la prolifération de micro-organismes pathogènes opportunistes dans différents sites des réseaux d'eau de grands bâtiments. Les recommandations suivantes ont ainsi pu être émises :

Dans les systèmes d'eau chaude

- le respect des températures réglementaires dans les réseaux d'eau chaude est primordial, et l'arrêt des chauffe-eaux en période de pointe de consommation électrique devrait être effectué avec précaution.
- Les systèmes de distribution d'eau chaude en cuivre semblent aussi efficaces pour contrôler les concentrations en micro-organismes pathogènes opportunistes mais des études au plus long terme sont nécessaires pour le confirmer.

Dans les réseaux de plomberie

- Tous les matériaux présents doivent être listés, même les matériaux d'étanchéité comme l'EPDM, car ils peuvent avoir un impact négatif à la fois sur la qualité chimique et microbiologique de l'eau, et être des zones à risque dans les réseaux.
- Un rinçage complet et approfondi des réseaux d'eau doit être réalisé après chloration choc ou choc thermique afin de prévenir la croissance microbienne.

Dans les machines à glace

- Le design des machines à glace doit être modifié pour réduire la prolifération microbienne. Par exemple, des alternatives pourraient être trouvées aux réservoirs d'eau internes ou un moyen de les faire se vidanger régulièrement dans le drain des machines.
- Le choix du modèle de machine installé, et notamment de la taille du réservoir de glace, doit être adapté à l'utilisation faite de la glace dans l'unité.
- Les pré-traitements comme les filtres à membrane doivent être choisis avec précaution et doivent être adaptés à la qualité et l'utilisation d'eau locale.
- Le besoin de machines à glace dans chaque unité doit être évalué selon la fragilité des patients. Des options de soins sans glace pourraient être envisagées pour les patients les plus vulnérables, avec une distinction de la production d'eau fraîche pour la consommation et la production de glace à partir d'eau stérile pour les soins des patients les plus à risque par exemple.

Il est de plus en plus reconnu que les systèmes de plomberie peuvent abriter de nombreux micro-organismes pathogènes opportunistes et ainsi causer des infections nosocomiales. Cependant, ces systèmes restent des « boîtes noires » microbiennes et des efforts soutenus de recherche

multidisciplinaire sont encore nécessaires pour améliorer notre compréhension de ces systèmes et pour trouver et mettre en œuvre des barrières et des solutions simples et efficaces pour prévenir les infections nosocomiales d'origine hydrique et protéger les patients les plus fragiles.

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LISTE OF SYMBOLS AND ABBREVIATIONS

The list of acronyms and abbreviations presents, in alphabetical order, the acronyms and abbreviations used in the thesis and their meaning. Examples:

AOC Assimilable organic carbon

ARM Amoeba-resisting microorganisms

ASV Amplicon sequence variant

ATP Adenosine triphosphate

BDOC Biodegradable dissolved organic carbon

Ca Calcium

CDC Centres for Disease Control and Prevention

CFU Colony forming unit

CPVC Chlorinated polyvinyl chloride

Cr Chromium

CSA Canadian Standard Association

Cu Copper

DNA Deoxyribonucleic acid

DOC Dissolved organic carbon

EPDM Ethylene propylene diene monomer

F Faucet

F1J Faucet first draw

F30S Faucet after 30s flushing

FCM Flow cytometry

Fe Iron

FLA Free-living amoebae

h	hour
HAI	Hospital-acquired infection
HPC	Heterotrophic plate counts
I	Ice
<i>Lp</i>	<i>Legionella pneumophila</i>
Mg	Magnesium
MIC	Microbially influenced corrosion
min	minute
mip	mip gene
Mn	Manganese
MQ	MilliQ ultrapure water
Ni	Nickel
NTM	Nontuberculous mycobacteria
OP	Opportunistic pathogen
Pb	Lead
PCR	Polymerase chain reaction
PE	Polyethylene
PES	Polyethersulfone
PEX	Cross-linked polyethylene
PFGE	Pulsed-field gel electrophoresis
PI	Propidium Iodide
PP	Polypropylene
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride

qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpm	rotations per minute
s	second
SEM	Scanning Electron Microscopy
SG	SYBRGreen I
SGPI	SYBRGreen I and Propidium Iodide
SS	Stainless steel
TOC	Total organic carbon
UK	United Kingdom
uPVC	unplasticized polyvinylchloride
USA	United States of America
UV	Ultraviolet
VBNC	Viable but non culturable
Vv	<i>Vermamoeba vermiformis</i>
W	Water of water-ice machine
W5m	Water of water-ice machine after 5min flushing
WF	Water from filters
WHO	World Health Organization
WHs	Water heaters
WR	Water from internal reservoir of water-ice machine
WSP	Water safety plan
Zn	Zinc

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CHAPTER 1 INTRODUCTION

1.1 Background

Following improvements in drinking water disinfection processes, most waterborne outbreaks in North America are now related to the contamination of water during its distribution (Craun, 2012). The deterioration of chemical and microbial quality of water in domestic water systems is a recognised source of adverse effects on the health of consumers over the short and long terms. Microbial amplification happening in premise plumbing of large buildings is a growing concern, and more information is needed for water system managers to better control it and maintain acceptable levels of contamination. This is particularly problematic in healthcare and long-term care facilities that house vulnerable populations. Indeed, increasing cases of nosocomial infections are recorded worldwide and the portion of infections that are water-related is also increasing (Anaissie et al., 2002; World Health Organization (WHO), 2011a). According to the World Health Organisation (WHO), nosocomial infections are still one of the leading causes of death for hospitalised patients (World Health Organization (WHO), 2002). The ageing population and larger use of extensive and invasive medical treatments, combined to an increased detection of opportunistic pathogens in premise plumbing is worrying, especially considering the rise in pathogens resistance to both disinfection and medical treatments (Falkinham et al., 2015a). Changes in building plumbing practices to reduce energy and water consumption may also increase the occurrence of waterborne pathogens in water systems and thus accentuate the risk for consumers (Edwards et al., 2014).

Since 2004, WHO promotes the elaboration of water safety plans (WSP) in healthcare facilities as a tool to prevent water-acquired infections (World Health Organization (WHO), 2023). These plans aim to protect not only the patients but also the confidence people have in healthcare facilities and water systems (Decker & Palmore, 2014). This systematic process requires a good understanding of the water system as a whole (including all connected devices) to be able to identify risk areas and efficient solutions to proactively manage them (World Health Organization (WHO), 2023).

Most nosocomial waterborne infections are related to opportunistic pathogens (OPs) such as *Legionella pneumophila* (*Lp*) or nontuberculous mycobacteria (NTM) (Squier et al., 2000). Opportunistic pathogens are microorganisms causing illness in people with predispositions like age (over 50-year-old) or weak immune system (Falkinham et al., 2015a). Opportunistic pathogens are

natural inhabitants of water distribution systems and premise plumbing. Biofilms are recognized niches of opportunistic pathogens who integrate these fixed communities and benefit from the protection they provide (Wingender & Flemming, 2011). They get amplified in these systems and can represent a risk for consumers at the point of use (Falkinham et al., 2015a).

Legionella pneumophila is a Gram-negative bacterium responsible for a serious type of pneumonia called Legionnaires' disease, and a milder infection called Pontiac fever (National Academies of Sciences, 2019). Legionellosis are usually contracted after inhalation of contaminated aerosols and are nationally notifiable diseases in Quebec and in the United States of America (USA). Legionnaires' disease is associated with mortality rates between 15% and 25%, which increase up to 40% for nosocomial cases (Bartram et al., 2007; Fields et al., 2002). In the USA, *Legionella* was responsible for 57% of the 42 drinking-water associated outbreaks that were reported to CDC between 2013 and 2014 (Benedict et al., 2017). When considering all water sources and transmission routes, 11,000 estimated domestically acquired waterborne Legionnaires' disease cases were reported in the USA in 2014, for an estimated direct healthcare total cost of 402 million US dollars (Collier et al., 2021). Because of its ideal growth temperature ranging between 25°C and 43°C, most of the guidelines and regulations regarding the control of *Legionella* risk in healthcare facilities concern hot water systems (BSR/ASHRAE, 2013; Castex & Houssin, 2005; National Academies of Sciences, 2019; Régie du bâtiment du Québec (RBQ), 2014). In addition to hot water systems, *Legionella pneumophila* has also been isolated from cooling towers, drinking water systems, and ice machines (Graman et al., 1997; Lévesque et al., 2016; Sabria et al., 2006).

Nontuberculous mycobacteria (NTM) include more than 150 species which are naturally present in water distribution systems and premise plumbing, and are very resistant to heat, disinfection, and antibiotics. NTM cause different types of infections like pulmonary or dermal infections, and hospital-acquired infections are becoming prevalent (Falkinham, 2016). In 2014 in the USA, 68,900 domestically acquired cases of NTM infections were estimated, for a total healthcare cost of 1,530 million USD (Collier et al., 2021). Free-living amoebae are protozoans widespread in freshwater and water distribution systems. They play a major role in the dynamics of multi-species biofilms as they feed by grazing on various bacteria and contribute to nutrient recycling (Pedersen, 1982; Thomas et al., 2010). Many OPs are amoeba-resisting microorganisms (ARM): they developed the ability to survive phagocytosis and multiply in amoebae instead (Falkinham et al., 2015a). After surviving phagocytosis and multiplying in amoebae, some OPs like NTM and *Lp*

also developed enhanced virulence (Cirillo et al., 1999; Cirillo et al., 1997). In addition of being hosts of OPs, some strains of amoebae are also pathogens like *Acanthamoeba* spp. and *Naegleria fowleri* (da Rocha-Azevedo et al., 2009). Amoebae are also resistant to heating and disinfection as they enter in a highly resistant dormant form called cyst and can at the same time protect intracellular OPs (Cervero-Aragó et al., 2013; Thomas et al., 2004).

Fungi are present in drinking water systems and have been an increasing source of nosocomial infections in the last decades (Alangaden, 2011). Among fungal species, *Candida* species cause invasive infections in healthcare facilities, especially in critically ill and immunocompromised patients (Pfaller, 1996). Most infections (called candidiasis) are endogenous (the infectious fungi was already present in the patient's body but was asymptomatic) but recent studies highlight the rising importance of contamination by environmental sources, especially moist surfaces (Kumar et al., 2019; Pfaller, 1996; Piedrahita et al., 2017). *Candida* species are an increasing concern in healthcare facilities with the multiplication of cases and the emergence of multi-drug resistant species like *Candida auris* (Arendrup & Patterson, 2017).

Temperature, presence of hosts, pipe materials, stagnation, and presence of biofilms are identified key factors impacting OPs growth in buildings water systems but limited information is available on their interplay (Buse et al., 2012). Thus, more information on design, operational, and maintenance parameters of building water systems associated with the presence and survival of these microorganisms in water and biofilms is still needed.

1.2 Structure of the dissertation

This thesis is subdivided in nine chapters. A detailed review of the design and operational characteristics of building water systems, their microbial components, their role in the acquisition of nosocomial infections, and of some of their associated guidelines and regulations is provided in Chapter 2. After a brief statement of the current knowledge's limits, the objectives and experimental approach are presented (Chapter 3).

Chapters 4 to 7 gather findings of the present research project in the form of 4 published or submitted scientific papers. The first paper (Chapter 4, published in *Microorganisms*) investigates the resistance to different heating regimes of *L. pneumophila* and *V. vermiformis* in water and loose deposits of water heaters.

The following two chapters (Chapters 5 and 6, submitted in *Water Research* and *ES&T Water*) summarize the results of a pilot-scale study analysing the interplay between several design, operational, and maintenance parameters of premise plumbing on water chemical and biological quality (Chapter 5), and on occurrence and survival of *L. pneumophila*, NTM and *V. vermiformis* (Chapter 6).

The last paper (Chapter 7, submitted in *Journal of Hospital Infection*) compiles the results of the monitoring and source localisation of the microbial contamination of ice machines in a hospital.

Finally, a general discussion of the results in the perspective of the definition of a water safety plan is provided in Chapter 8, followed by the conclusions and recommendations drawn from this study (Chapter 9).

CHAPTER 2 LITERATURE REVIEW

2.1 Characteristics of building water systems

In the last decades, a lot of progress has been made to ensure drinking water quality and safety from the water treatment plants and throughout the main water distribution networks. Once water reaches the water inlet of a building, it becomes building owner's responsibility to ensure a safe water distribution within the building. The portion of water distribution systems beyond the property lines and inside a building is called premise plumbing (National Research Council, 2006). This includes cold water and hot water lines, as well as all the devices connected to these lines (water heaters, faucets, showers, water and ice dispensers...). Premise plumbing is usually described as a warm and wet environment with periodical stagnation, high surface to volume ratio, and low disinfectant residual, providing ideal conditions for microbial growth (Wang et al., 2017). Premise plumbing systems are very complex with a variety of designs, materials, temperatures, and flow regimes.

2.1.1 Materials in building water systems

A large diversity of materials is used both in cold and hot water lines and their connected devices. Both plastic and metallic materials can be found in premise plumbing systems (Julien et al., 2020). Table 2.1 summarizes the most common uses of polypropylene (PP), polyvinyl chloride (PVC), ethylene propylene diene monomer (EPDM), cross-linked polyethylene (PEX), stainless steel (SS) and copper (Cu) in water systems.

Table 2.1 Common uses of some materials in water plumbing systems (based on Julien et al. (2020) and Waines et al. (2011))

Materials	Plumbing components
Polypropylene (PP)	Drinking water piping and tubing
Polyvinyl chloride (PVC)	Service lines, Drinking water piping and tubing, Fittings, Valves, Small-diameter tubing
Ethylene propylene diene monomer (EPDM)	Sealing gaskets, Flexible plumbing hoses

Table 2.1 Common uses of some materials in water plumbing systems (based on Julien et al. (2020) and Waines et al. (2011)) (continued)

Cross-linked polyethylene (PEX)	Service lines, Piping, Tubing, Small-diameter tubing and Flexible plumbing hoses
Stainless Steel (SS)	Piping, Tubing, Fittings, Valves, Domestic storage
Copper (Cu)	Service lines, Piping, Tubing, Small-diameter tubing. Mostly for hot water systems

While the choice of materials is mostly based on their cost and ease of installation, their impact on water quality should not be omitted. Water quality is affected to some degree by all pipe materials which leach substances into water but the leaching nature and rates depend on material composition, water temperature and pH (National Research Council, 2006). For example, concentrations of metals in water are directly related to materials used in the water system (Gonzalez et al., 2013). Plastics also leach inorganic and organic compounds representing direct or indirect risks for consumers health (Julien et al., 2020; Stern & Lagos, 2008). For instance, PEX pipes leach total organic carbon (TOC), dissolved organic carbon (DOC), and assimilable organic carbon (AOC) (Connell et al., 2016; Inkinen et al., 2014; Lund et al., 2011). This leaching is influenced by temperature, and can be a source of nutrients for microorganisms and opportunistic pathogens in the water systems (Proctor et al., 2017).

Materials also influence the microbial communities of water systems, especially in premise plumbing because of the increased surface-to-volume ratio associated with smaller pipe diameters favouring biofilm formation (Bédard et al., 2015a; Proctor & Hammes, 2015). Pipe materials have been identified as a factor having a significant impact on the microbial composition of water samples from standardized cold water plumbing rigs with pipes in copper, plastic with brass fitting, and copper-lead combined (Ji et al., 2015). Composition of microbial communities and metabolic activity in biofilms are also impacted by materials. Buse et al. (2014) showed different bacterial and eukaryotic communities in biofilms developed on unplasticized polyvinylchloride (uPVC) coupons compared to ones developed on copper coupons by 16S and 18S rRNA gene

pyrosequencing. A more important eucaryotic diversity was noted in uPVC biofilms while more diverse bacterial communities were found on copper coupons (Buse et al., 2014).

Biofilm formation potential is influenced by pipe materials (Yu et al., 2010). It mostly depends on their composition and surface. Thus, materials with high surface roughness or crevices generally provide more attachment sites for bacteria (Waines et al., 2011). Waines et al. (2011) reported a complete coverage of EPDM inserts in a water model distribution system after 28 days, and microcolonies on copper and stainless steel, while PEX showed a thin biofilm layer. The composition of materials directly controls the compounds that can be released by the materials. Migration of organic carbon happens with synthetic polymeric materials because of the additives, antioxidants, and stabilizers they contain (Mao et al., 2018; Stern & Lagos, 2008). Interestingly, the same polymeric materials from different pipe manufacturers can still display completely different carbon release and biofilm formation (Proctor et al., 2016). Finally, materials also influence biofilm formation rate. Lehtola et al. (2004) reported that polyethylene (PE) pipes initially showed higher biofilm concentration than copper pipes, but similar biofilm densities were measured after more than 200 days. Therefore, commercial materials and products in contact with drinking water must comply with several criteria including some on their migration potential and promotion of microbial growth (European Commission, 2021; NSF International Standard et al., 2020). The BioMig method package for polymeric materials was developed recently and combines a migration potential test and a biomass formation potential test (Wen et al., 2015).

Copper is probably the material for which the impact on microbial quality of water and biofilm formation differs the most among studies. Experiments conducted with new copper pipes showed less biofilm development on copper compared to other materials, a result attributed to the bactericidal properties of copper (Gomes et al., 2019; Moritz et al., 2010). However, longer term studies showed little or no differences between copper and plastic biofilm densities, suggesting a slower biofilm formation on copper pipes (Lehtola et al., 2004; Proctor et al., 2017). Similar amount of biofilms (adenosine triphosphate (ATP) levels) were also reported between copper and stainless steel (van der Kooij et al., 2005). Conclusions of studies also differ on the impact of copper on opportunistic pathogens (OPs) concentrations, particularly on the control of *Legionella pneumophila* (Lp) (Cullom et al., 2020). These differences could be explained by the importance of the design of the system, the speciation of copper, the implementation of corrosion control, and

different stagnation times (Cullom et al., 2020; Song et al., 2021). Finally, some microorganisms can tolerate copper, limiting its antimicrobial potential (Bédard et al., 2021; Elguindi et al., 2009).

2.1.2 Temperature in building water systems

Plumbing is not the only factor affecting water quality and biofilm formation in premise plumbing. Temperature influences a lot of chemical reactions and equilibriums taking place in water such as pipe corrosion or disinfectant efficiency. Temperature also affects microbial and biofilm growth and diversity (Julien et al., 2020). Thus, water quality experts and premise plumbing managers acknowledge temperature as a good and reliable indicator of water quality in building water distribution systems (Singh et al., 2020).

The influence of pipe material on water quality also depends on water temperature, as it impacts both metal dissolution (like copper dissolution) and leaching of organic carbon from plastics (Proctor et al., 2017). When comparing metals leaching (nickel, copper and zinc) in a hot and a cold water systems within the same building, more leaching was measured in the hot water system (Inkinen et al., 2014). Hametner (1999) noted an increase of total organic carbon concentration in a polypropylene system when temperature was increased from 23°C to 60°C. Solubility of chemicals in water is also impacted by temperature. For instance, leaching of vinyl chloride monomer from polyethylene pipes is enhanced in warmer water (Stern & Lagos, 2008).

Temperature is also a key determinant of disinfection efficiency. Chlorine decay is significantly impacted by temperature, as chlorine concentrations drop in water when temperature increases (Powell et al., 2000). Partly because of its impact on water chemistry and disinfection efficiency, temperature also impacts water microbial density and composition in premise plumbing. Higher bacterial densities have been monitored in warm and hot water systems as compared to cold water ones. For instance, Bédard et al. (2018) measured culturable cells concentrations 1-log higher in the hot water system (mean temperature of 61.6°C at water heater's outlet) of an hospital compared to the cold water system (mean temperature of 26.2°C at the water inlet). Same observations were made for water samples from an apartment building in Denmark, with mean heterotrophic plate counts (HPC) of 1×10^3 CFU/mL in cold water and 1×10^4 CFU/mL in hot water (Bagh et al., 2004). Density and composition of premise plumbing biofilms are also affected by temperature. The same study in Denmark reported five-fold higher HPC densities fixed on hot water pipes compared to cold water pipes (Bagh et al., 2004). A lower bacterial diversity was also associated with hot water

systems. For instance, 36 mould species have been isolated from samples from cold water taps while only 3 were found in hot water taps in a hospital (Hageskal et al., 2006). In the same study, *Penicillium* spp. was the dominant genera in cold water samples but was not found in hot water samples, highlighting a change of microbial diversity because of temperature (Hageskal et al., 2006).

Temperature is a key parameter in the prevention of opportunistic pathogens (OPs) occurrence and the control of their concentrations in water systems, especially *Legionella pneumophila* (*Lp*) (National Academies of Sciences, 2019). Maintaining elevated temperatures in water heaters and along hot water circuits inactivates microorganisms and prevents their amplification regardless of the type of water heater used (Julien et al., 2020). Electric water heaters are more at risk to be colonized by OPs and *Lp* because of thermal stratification and the formation of loose deposits at their bottom (Dewailly & Joly, 1991). Thus, temperatures in hot water systems are usually defined to avoid *Lp* contamination and proliferation (more details are provided in section 2.3.2). A decrease of *Legionella* diversity from 4 to 1 phylotypes was reported 72h after a heat shock treatment (70°C, 30min) in a pilot-scale hot water distribution system (Farhat et al., 2012). Also, 1h exposure at 55°C induced only a 2-log reduction of the concentrations of 2 environmental *Lp* strains isolated from a hospital hot water system, underlining the ability of *Lp* to adapt to the stress factors of the system (Bédard et al., 2019). A water heater temperature $\geq 55^{\circ}\text{C}$ significantly reduces the presence of NTM in households water systems compared to a water heater temperature $\leq 50^{\circ}\text{C}$ (Falkinham, 2011). Hosts of *Lp* and NTM, especially amoebae, also show resistance to heating and have been isolated from hot water systems with temperatures above 55°C (Rohr et al., 1998). Amoebae were found in 29 out of 56 hot water samples and *Vermamoeba vermiformis* (formerly called *Hartmannella vermiformis*) was the dominant species systemically distributed (Rohr et al., 1998). Kuchta et al. (1993) also showed that a 30min heating at 55°C induced a reduction of less than 2-log of *V. vermiformis* concentrations, while no amoeba resisted a 30min exposure at 60°C. Resistance to heat by hosts is a critical factor to consider when controlling concentrations of OPs in hot water systems as OPs can survive, multiply, and exit hosts, and colonize water system when conditions are more profitable.

Heat shock treatment of building water distribution systems is one of the main remedial tools when confronted to the colonization of the water systems by OPs. For this curative treatment, a temperature of 70°C should be maintained at each point of use for 20 to 30min (ASHRAE, 2020).

However, limited effectiveness over time and adaptation of microorganisms were reported after such treatments (Allegra et al., 2011; Saby et al., 2005). Repeatedly (every 3 to 6 months) raising the temperature to 65°C for a day in a hospital hot water system led to the selection and adaptation of *Legionella* strains which acquired a certain resistance to heat (Allegra et al., 2011). Thus, thermal shocks should be used as an emergency or temporary remedial response (National Academies of Sciences, 2019).

Finally, temperature monitoring can be used as an indicator to evaluate the risk of contamination of hot water systems by *Legionella pneumophila* (Boppe et al., 2016). In France, daily or continuous temperature monitoring at water heater outlet and return loops, and weekly temperature monitoring at service points are mandatory in healthcare facilities (Castex & Houssin, 2005). Bédard et al. (2015b) developed a tool to identify risk areas for *L. pneumophila* contamination and proliferation based on temperature profiling and characteristics of the system.

2.1.3 Flow conditions in buildings water systems

A common saying used to summarize how to prevent decrease of water quality in building water distribution systems is “keep the cold water cold, keep the hot water hot, and keep water flowing”. Indeed, flow conditions impact both chemical and biological water quality. For instance, a 132h stagnation period led to increased release of iron (Fe), manganese (Mn) and nickel (Ni) from corrosion scales of an iron pipe, while active flow conditions led to increased concentrations of aluminium (Al), zinc (Zn) and copper (Cu) (Li et al., 2020). Stagnation led to exponential increase of concentrations of metals (lead (Pb) and copper (Cu)) in water in contact with pipes in lead, brass and copper, especially in the first 20-24h (Lytle & Schock, 2000). Increased leaching of Pb and Zn from brass components was also noted in cold stagnated waters of an office building (Inkinen et al., 2014).

Stagnation is also associated with a degradation of microbiological quality of water in premise plumbing (Inkinen et al., 2014). Thus, higher microbial concentrations are usually found after a period of stagnation in pipes and fixtures. Increase of water age in water-efficient buildings was associated with faster decay and absence of chlorine and chloramine residuals, and higher concentrations of 16S rRNA genes (Rhoads et al., 2016). Longer stagnation periods were also correlated with increased HPC and total cell counts (Ley et al., 2020). Highest total and intact cells concentrations were also measured in the water of infrequently-used taps (Lipphaus et al., 2014).

OPs are also able to survive in stagnation-induced oligotrophic environments with low concentrations of oxygen and nutrients (Falkinham et al., 2015b). Higher concentrations of *Legionella* spp. and *Mycobacterium* spp. measured by qPCR were correlated with longer stagnation times in a water-efficient building (Ley et al., 2020). When sampling a building water system, it is usually advised to sample after a certain stagnation time, usually the first draw in the morning, to be representative of the risk for consumers (Government of Québec, 2022; Wang et al., 2017).

Flow conditions also affect the formation of biofilms and their microbial diversity. Divergent results have been reported concerning the impact of stagnation on biofilm formation. It is usually advised to avoid stagnation periods to prevent biofilm formation, but several studies showed that biofilms had limited growth under stagnant conditions (Chaves Simões & Simões, 2013; Lehtola et al., 2006; Manuel et al., 2007). Lehtola et al. (2006) reported higher growth of biofilms on polyethylene (PE) and copper pipes when water flow rate was increased, highlighting the importance of nutrients transfer. Stagnation times can also lead to the release of planktonic bacteria from biofilms (Bédard et al., 2018). When flow is restarted or increased in the water system, there is also a risk of detachment of biofilm. Thus, rapid changes in water flow rate increased concentrations of bacteria in water by resuspending biofilms (Lehtola et al., 2006).

After the COVID-19 pandemic shutdowns that occurred all around the world, the risk of microbial contamination of building water circuits, especially with *L. pneumophila*, was a major concern. The most frequent recommissioning strategies for the safe reopening of buildings were to flush and to perform shock chlorination (Proctor et al., 2020). Flushing can be sufficient depending on the water quality parameters considered, the integrity of the water system, and the duration of the low/no water use period in the building (American Water Works Association (AWWA), 2020; Proctor et al., 2020; Régie du bâtiment du Québec (RBQ), 2020). However, flushing efficiency is site-dependent and can have limited benefits on the medium- and long-terms (Grimard-Conea et al., 2022). Shock chlorination is performed with high free chlorine concentrations (up to 200ppm) and short contact times (3h), or longer contact times (up to 24h) and lower chlorine concentrations (10ppm) (Canadian Standard Association (CSA), 2016; International Association of Plumbing and Mechanical Officials (IAPMO), 2021; Proctor et al., 2020).

2.1.4 Disinfection in building water systems

An essential objective in water supply is to maintain a good water quality during distribution throughout the entire system to the consumers (van der Kooij et al., 1999). There are two approaches on how to achieve this goal. The first one, most common in Europe, is to prevent chemical and microbial contamination using biostable materials and biostable drinking water (van der Kooij et al., 1999). The second one, applied in North America, is to maintain a disinfectant residual in the distribution system. For instance, in Quebec, a free chlorine residual of at least 0.3mg/L must be measured at the exit of water treatment plants (Ministère de l'Environnement et de la Lutte contre les changements climatiques, 2014). However, it is challenging to maintain a disinfectant residual at all points of the distribution system as disinfectants react with a lot of organic and inorganic components present in water and at the surface of pipes and biofilms (Prest et al., 2016a). Disinfectant decay is increased in pipes with higher surface to volume ratios (smaller diameters) because of an increased contact with biofilms and pipe materials (Prest et al., 2016a; Prévost et al., 1998). As a result, disinfectant residuals are usually quite low in water distribution systems and premise plumbing (Bédard et al., 2015b; Rhoads et al., 2016).

Most used secondary disinfectants are chlorine and chloramines. In Canada, most drinking water treatment plants use chlorine, and free chlorine concentrations in water distribution systems range from 0.04 to 2mg/L (Health Canada, 2009). Chloramines, in particular monochloramines, grant longer-lasting disinfection and are more stable than chlorine. For these reasons they are usually used as secondary disinfectant in distribution systems. However, both chlorine and monochloramines react with organic matter and form disinfection by-products, some of which have been shown to be carcinogenic and genotoxic (Villanueva et al., 2015). Therefore, a balance should be found between the dose of disinfectant needed to prevent microbial growth and the risk represented by disinfection by-products.

The use of a disinfectant as well as its nature impact water chemistry and microbial composition and can affect the pipe materials. For instance, chlorine increases total organic carbon (TOC) migration from PEX and EPDM (Mao et al., 2018). Also, a greater chlorine demand was associated with ductile-iron pipes as compared to PVC pipes (Haas et al., 2002). The fastest chlorine decay was observed with copper pipes as compared to brass, PEX and chlorinated polyvinyl chloride (CPVC) when performing a shock chlorination at 200mg/L Cl_2 (Raetz, 2010). Type and levels of

disinfectant affect the composition of suspended bacterial communities. For instance, relatively stable microbial profiles are found in unchlorinated drinking water distribution systems. In chlorinated systems, changes in the range of disinfectant residual were linked to variations in microbial communities (Dias et al., 2019). Unfortunately, disinfection strategies are based on the sensitivity of faecal indicators like *E. coli* but are less effective against OPs because of their higher resistance (Falkinham et al., 2015a). For instance, NTM are resistant to both chlorine and chloramines, and the presence of a disinfectant residual inhibits growth of competitive microorganisms, benefiting NTM (Dowdell et al., 2019; Falkinham et al., 2015a). Free-living amoebae like *V. vermiformis* are also resistant to disinfectants frequently used in hot water systems (Fouque et al., 2015a).

A decrease of disinfectant residual in premise plumbing is usually associated with the formation or growth of biofilms. Biofilms are resistant to disinfection, especially thanks to the extracellular polymeric substances (EPS) they contain, and are niches of hygienically relevant microorganisms (faecal indicators, opportunistic pathogens...) (Wingender & Flemming, 2011). Biofilms resistance to disinfection is influenced by the composition of the biofilm and by the disinfection strategy applied. For example, *Legionella* released from biofilms that were not subjected to a disinfectant residual during their development were less inactivated and had a higher infectivity (Shen et al., 2017). Conclusions differ regarding which disinfecting agent to use against biofilms, as the efficiency depends on microbial populations present in biofilms, the structure of the biofilms, and the quality of water surrounding biofilms. Chemical disinfectants with long-lasting residuals (such as hydrogen peroxide or monochloramines) are generally more effective in reducing biofilm density than other disinfectants such as chlorine, UV or ozone (Eboigbodin et al., 2008).

2.2 Microbial components of building water systems

As depicted by recent applications of molecular methods in premise plumbing systems, these systems are home to highly diverse microbial ecosystems with viruses, protozoa, fungi, and bacteria (Douterelo et al., 2014). As mentioned previously, microbial diversity of water systems highly depends on the parameters presented in section 2.1.

2.2.1 Importance of fixed life

Four different phases are generally considered in water systems : (1) the liquid phase (or bulk water), (2) the pipe wall biofilms, (3) the suspended solids, and (4) the loose deposits (Liu et al., 2013). These four microenvironments are not completely independent from each other, but interactions between these phases are still unclear.

Most microorganisms in water distribution networks are in fixed phase. Biofilms represent nearly 95% of the total microbial biomass present in distribution systems, only 5% remaining in liquid phase (Flemming et al., 2002). Densities of 10^6 to 10^8 CFU/cm² are usually reported for biofilms in drinking water distribution systems (Batté et al., 2003a). However, some studies showed that the importance of the sedimented phase might have been underestimated (Zacheus et al., 2001). Biofilms can form on any surface in contact with non-sterile water. In the United Kingdom (UK), biofilms have been found in every part of a domestic plumbing, including drains, washing machines and dishwashers, shower heads and shower curtains (Eboigbodin et al., 2008). An increase in temperature or a decrease in the concentration of disinfectant in household systems often leads to biofilm formation. Biofilms can develop in water systems because of the presence of biodegradable organic matter, nutrients and bacteria in the water and the ability of certain transported bacteria to adhere to pipe walls and multiply (Block et al., 1997).

Biofouling refers to the unwanted accumulation of biotic material on a surface (Chaves Simões & Simões, 2013). Biofilms are dynamic ecosystems attached on surfaces including a large variety of live and dead microorganisms surrounded by a protective matrix of macromolecules of biological origin (World Health Organization (WHO), 2002). These macromolecules are called extracellular polymeric substances (EPS). They provide microorganisms with protection against mechanical stresses and many disinfectants, since they limit the penetration of the disinfectant through the matrix and consume part of the residual by reacting with it (Chaves Simões & Simões, 2013; Shen et al., 2017). EPS and extracellular enzymes also serve as an external digestion system for the biofilm, trap and concentrate nutrients, and facilitate intracellular communication, horizontal gene transfer, and metabolic interactions between bacteria with physiological differences (Block et al., 1997; Chaves Simões & Simões, 2013; Wingender & Flemming, 2011).

Biofilms are the main source of microorganisms in drinking water distribution systems that are supplied with treated water and without pipe breaks, and are particularly problematic in ageing

networks (Chaves Simões & Simões, 2013). Many factors influence the formation and growth of biofilms in drinking water systems, such as pipe materials, water flowrate, temperature and disinfectants used, but conclusions on their impact differ between studies (Eboigbodin et al., 2008). Biofilms age also influences the structure and diversity of these bacterial communities (Liu et al., 2013; Martiny et al., 2003). Surface materials influence the composition of the biofilms formed and their resistance to disinfection. Some materials can provide nutrients to microorganisms and cause point source contamination (Rogers et al., 1994). However, there is still no consensus on which materials to preferentially use in water systems, with studies leading to contradictory results on biofilm formation depending on the operating conditions applied and the detection methods used. Biofilms also influence the materials they develop on. Microbially influenced corrosion (MIC) or biocorrosion is the accelerated deterioration of a material related to the presence and activity of microorganisms (Vargas et al., 2017). In case of copper pipelines, extensive biofilm growth in corroding surfaces demonstrated the implication of microorganisms in accelerated corrosion of copper and increased release of copper particles in water (Pavissich et al., 2010; Vargas et al., 2017). Other studies highlighted the importance of flow regime (continuous flow or stagnation) over materials or reactors conception on biofilms growth. Manuel et al. (2007) reported enhanced biofilm growth under flow conditions compared to stagnant water. However, when considering MIC, increased flowrate led to increased biofilm growth and higher copper and bacteria release in water (Lehtola et al., 2006; Pizarro & Vargas, 2016).

Resistance of biofilms to disinfection strategies depends on the composition of the biofilms themselves and the disinfection strategy used. Thus, single-species biofilms are less resistant to hydrogen peroxide than multispecies biofilms which can modify their community composition to resist exposure to hydrogen peroxide (Burmølle et al., 2006). Costerton et al. (1999) outline two mechanisms involved in biofilms resistance to disinfection. First is the inability of the disinfectant agent to penetrate deep into biofilms due to the many layers of cells and EPS. The disinfectant will therefore only be effective on the surface, on the first layers of bacteria, and the other bacteria remain viable. It concurs with a study showing that the majority of bacteria were released from biofilms subjected to disinfection within the first 5 minutes of treatment (Shen et al., 2017). The second mechanism is related to the slow-growth state of the bacteria in biofilms due to the lower availability of nutrients for the cells in the biofilms. Bacteria in a slow-growth state or starved are

more resistant to disinfection (Du et al., 2015). Also, the exposure of biofilms to disinfectant is not uniform in real systems, which can lead to different results than in a controlled environment.

Several solutions have been proposed to prevent, or at least control, biofilm formation in drinking water systems. Parameters that can be controlled by water system managers are the content of nutrients in water, the hydrodynamics in the systems, the type and concentration of disinfectant, pipe materials and maintenance, and other environmental factors such as temperature or pH. Chaves Simões and Simões (2013) proposed realistic actions that can be taken to limit biofilms in water distribution systems:

- (1) minimise the concentration of organic matter entering the system;
- (2) use chemically and biologically stable pipe and fitting materials;
- (3) prevent water stagnation and accumulation of sediments;
- (4) maintain a disinfectant residual.

Regarding the disinfection strategy to adopt, monochloramines would theoretically be more effective than free chlorine because of their deeper penetration into the EPS matrix. To further improve the effectiveness of chemical disinfection, increasing the shear stresses would increase the flow of disinfectant into the biofilm but could at the same time increase the release of microorganisms into water (Shen et al., 2017). Another study concluded that UV disinfection did not remove biofilms from copper and polyethylene pipes, while free chlorine effectively removed biofilms from plastic pipes but only removed those from copper pipes at the point of chlorine application (Lehtola et al., 2005). A last approach would be to consider the microbial populations present in each network and to modify them to reduce the number of OPs, for example by reducing the concentration of amoebae that could no longer serve as hosts (Falkinham et al., 2015a). The latter approach requires further research and testing of all networks to determine the best strategy on a case-by-case basis, which is currently not feasible.

The presence of biofilms in premise plumbing cannot be prevented and does not constitute such an issue in itself, but rather the microorganisms and metals they release into the liquid phase. Indeed, when a biofilm reaches a certain bacterial density, or when faced with some environmental changes (increase in flowrate, change in temperature, pH, disinfectant, vibrations...), portions of the biofilm break off and enter the liquid phase (Wingender, 2011). Detachment is initially an advantageous

process for biofilm microorganisms to achieve greater dispersal in the system (Chaves Simões & Simões, 2013). For instance, differences in communities observed between samples from 5 sectors of a chlorinated water distribution system could be due to sporadic releases of cells from biofilms developed on the pipes and to the resuspension of deposits and sediments, themselves linked to changes in hydraulics (Dias et al., 2019). Such detachment can also be problematic as biofilms can capture metals like Pb and Cu which are then released with detached biofilms (Beech & Sunner, 2004; Belcher & Zhang, 2018). Finally, the capacity of biofilms to harbour opportunistic pathogens is a cause for concern. Indeed, opportunistic pathogens can integrate and grow in biofilms which become temporary or long-term reservoirs. Opportunistic pathogens are then released in water and can lead to an outbreak (Wingender & Flemming, 2011; World Health Organization (WHO), 2002).

2.2.2 Opportunistic pathogens in premise plumbing

Opportunistic pathogens (OPs) are microorganisms that can cause infectious diseases in hosts with depressed resistance (age $\geq$ 50-year-old, immunocompromised, underlying disease as cystic fibrosis or AIDS...) (Falkinham et al., 2015a). Their presence in hospital water systems is of particular concern for vulnerable patients who are at high risk of contracting a waterborne infection (Williams et al., 2013). Costs associated with domestically acquired waterborne infections is estimated to be at least US \$3.3 billion in 2014 in the USA. Authors estimated that OPs (*Legionella*, NTM and *Pseudomonas*) caused 101,560 of these infections for a total cost of US \$2.39 billion the same year, making OPs a major emerging health problem (Collier et al., 2021). This is especially true given the joint increase in the population at risk of OP infection and in the concentrations of OPs in drinking water currently observed (Falkinham et al., 2015a).

OPs are indigenous inhabitants of water distribution systems and are adapted to persist and grow in these environments. As a result, their presence and concentrations are not well-correlated with the concentrations of faecal indicators such as *E. coli* or enterococci, which are generally the only microbial indicators measured at the effluent of water treatment plants (Lu et al., 2015; Wang et al., 2017). As water treatment was optimized to suppress faecal pathogens, the disinfection strategies developed for faecal organisms are less effective for OPs, and sometimes even allow them to predominate (Falkinham et al., 2015a).

OPs share some common characteristics which allow them to persist and grow in premise plumbing as they can (1) adhere to surfaces and form biofilms, (2) grow with low oxygen and organic carbon concentrations (oligotrophic environment) usually found under stagnation, (3) grow in amoebae, and (4) resist disinfection (Falkinham, 2015a). Among OPs, the present research focused mainly on *L. pneumophila*, NTM, the yeasts *Candida* spp., and their common host the amoeba *V. vermiformis*.

Legionella pneumophila

Legionella pneumophila (*Lp*) is probably the most studied opportunistic pathogen. *Lp* causes two forms of diseases. The first is a mild form called Pontiac fever with flu-like symptoms, the second is a severe form of pneumonia called legionellosis or Legionnaires' disease (National Academies of Sciences, 2019). In North America and Europe, *Legionella pneumophila* represents most of *Legionella* isolates from sporadic community-acquired legionellosis, with serogroup 1 being predominant (National Academies of Sciences, 2019; Yu et al., 2002). Patients with Pontiac fever generally do not require treatment to recover, while mortality rates from 15 to 25% are attributed to legionellosis (Fields et al., 2002). Collier et al. (2021) estimated a total of 995 deaths in the USA in 2014 attributed to domestically acquired Legionnaires' disease. Since 2001, when legionellosis was first listed as a reportable disease, *L. pneumophila* has become the leading cause of waterborne disease in the US (Lu et al., 2015). From the late 1990s to the mid-2000s, an increase in cases of Legionnaires' disease was observed in France and several European countries regulating the monitoring of *Lp* in domestic water (Ashbolt, 2015; Lu et al., 2015). Yet, the numbers of infections by *Legionella* are still underestimated as they only represent infections involving hospitalisation, and therefore do not include infections treated outside hospitals (National Academies of Sciences, 2019).

People get infected by *Lp* after inhaling aerosols or breathing in droplets of contaminated water. Many devices connected to water distribution systems generate such aerosols (taps, showers, humidifiers...) (National Academies of Sciences, 2019). Faucet aerators also contribute to the development and proliferation of *L. pneumophila* (World Health Organization (WHO), 2002). *Lp* is mainly isolated from hot water systems where temperatures are within its optimal growth range (25°C to 45°C) (National Academies of Sciences, 2019). Several studies also reported temperature-resistant environmental strains of *Lp* isolated from hot water systems (Allegra et al., 2011; Bédard

et al., 2019). *L. pneumophila* is also resistant to disinfection strategies applied in drinking water. Environmental *Lp* strains adapted to life in water have shown a seven-fold increase in resistance to chlorine compared to strains grown on rich media (Kuchta et al., 1985).

L. pneumophila resists to grazing by certain protozoa and mostly replicates intracellularly. *Lp* can infect and multiply in both ciliates and free-living amoebae, ciliates being its preferred hosts in cold water systems and amoebae its preferential hosts in hot water systems (Falkinham, 2015a; Paniagua et al., 2020). Cyst-forming protozoan hosts protect *Lp* from extended environmental stresses (Nisar et al., 2020). Cells from intracellular replication also show enhanced virulence and resistance to disinfection (Buse et al., 2013).

L. pneumophila has been detected in biofilms developed on pipes made of many materials, both plastics (PVC, silicone, rubber), metals (copper, stainless steel) or glass (Falkinham, 2015a). The association of *Lp* with drinking water biofilms provides greater resistance to disinfection and high flow rinsing, and the necessary nutrients for growth (Falkinham, 2015a). The disinfection of *Lp* in biofilms occurs in 2 steps: (1) a rapid inactivation during the first 5 minutes of contact with the disinfectant when *Lp* accompanies the detachment of biofilm, and (2) the reaching of a plateau (Shen et al., 2017).

Nontuberculous mycobacteria (NTM)

The group of nontuberculous mycobacteria (NTM), also known as atypical mycobacteria, includes nearly 180 species of opportunistic pathogenic mycobacteria from soil and water (Dowdell et al., 2019; Falkinham, 2016). NTMs cause different pathologies depending on the strain and the infected individual, and multi-mycobacillary infections are often reported with NTM-infected patients (Falkinham, 2016; Wallace Jr et al., 2002). In adults, NTM mostly cause pneumonia and skin infections, while in young children they usually cause cervical lymphadenitis, and bacteraemia in immunocompromised individuals (Dowdell et al., 2019; Falkinham, 2016). NTM infections are of particular concern in AIDS patients as they represent the second cause of death for these individuals (Covert et al., 1999). Species of NTM causing infections include those from the *M. avium* complex and *M. abscessus* complex, as well as *M. chelonae*, *M. kansasii* and *M. fortuitum* (Dowdell et al., 2019). Reported NTM infections are increasing worldwide. For instance, positive rate of NTM specimens doubled (from 8.2 to 16 per 100,000 persons) in 20 years (from 1994 to 2014) in 4 US states (Donohue, 2018). However, cases are still underestimated due to the

difficulties in identifying NTM from clinical samples, and the lack of reporting requirements in different countries (Dowdell et al., 2019).

CDC lists 3 transmission routes of NTM, namely (1) direct contact, (2) ingestion (of contaminated water or ice), and (3) indirect contact (e.g. touching a medical device previously contaminated with water) (Centers for Disease Control and Prevention (CDC), 2019). Indeed, NTM are present both in water distribution systems and pipes in buildings, and also in aerosols from humidifiers, and water and ice from ice machines (Falkinham, 2016; Millar & Moore, 2020; Wallace Jr et al., 1998). Depending on the species, NTM can survive in water from several months to several years (Klanicova et al., 2013). Since patients who have been once infected by NTM are more likely to be reinfected, it is important to consider and implement measures to ensure a water supply that limits their exposure to NTM (Falkinham, 2015b).

As other OPs, NTM are also able to survive phagocytosis and multiply in some amoebae, with occurrences of both microorganisms being highly associated in drinking water (Dowdell et al., 2019). Studies also showed that NTM grown in amoebae were more virulent and had better resistance to disinfectants (Cirillo et al., 1997; Sarink et al., 2023).

The main biological characteristic of NTM is their outer membrane composed of long-chain fatty acids (C60 to C80), also called mycolic acids (Falkinham, 2018). This membrane composition provides NTM with a hydrophobic surface allowing good adhesion to surfaces, increased resistance to disinfectants and antibiotics, and an easier aerosolization (Dowdell et al., 2019; Falkinham, 2018). NTM lipid-rich membrane increases their resistance to chlorine, chloramines and ozone (Falkinham, 2015b). However, NTM membrane also causes slower growth, lower proportion of cells in suspension in water and an impermeability to nutrients (Falkinham, 2016).

Even if NTM are generally slow-growing (with 1 generation per day at 37°C), they are also very resistant to chemical disinfectant, heat and antibiotics (Falkinham, 2016; Rastogi et al., 1981; Taylor et al., 2000). NTM are resistant to both chlorine and chloramine, but an enhanced frequency of detection was reported in distribution systems with chloramine as residual disinfectant (Donohue et al., 2019; Taylor et al., 2000). Moreover, disinfection and water treatment strategies influence NTM concentrations at the tap, but differences in inactivation between species and growth conditions complicate laboratory inactivation simulations and increase the need for experiments in full-scale systems (Dowdell et al., 2019).

NTM are usually outcompeted by other microorganisms in nutrient rich environments, but after drinking water treatment process and disinfection, NTM have no more competitors for the available nutrients (Falkinham 2016). Conditions in water distribution systems are then ideal for NTM growth as pipes offer large surfaces to attach and form biofilms and disinfectant residual prevents the growth of competing microorganisms (Dowdell et al., 2019; Falkinham, 2015b, 2016). Stagnation in building water system is not a problem either since NTM can live with oxygen concentrations as low as 6 to 12% (Lewis & Falkinham, 2015). Therefore, NTM concentrations increase along the water distribution systems, with doubled NTM concentrations noted between the outlet of the treatment plant and consumer's tap (Falkinham et al., 2001). NTM concentrations of 10^5 CFU/cm² and 10^6 to 10^8 cells/L are usually measured in drinking water networks (Dowdell et al., 2019; Falkinham, 2016). NTM diversity, abundance and growth in water systems are also influenced by pipe materials, dissolved metal concentrations, and water age (Dowdell et al., 2019; Haig et al., 2018; Mullis & Falkinham, 2013).

To limit NTM numbers and to control their proliferation in water distribution systems, some realistic measures have been proposed for managers of building water systems: (1) increase water heater temperature to 55°C, (2) monthly drain water heaters and building water systems, and (3) identify and if possible remove dead-end pipes (Falkinham, 2016).

Prevention of NTM infections is particularly important in healthcare and long-term care facilities because of the vulnerability of their populations. Thus, all recommendations made for building water systems should be considered, and shock chlorination and heat treatment can be implemented periodically (Falkinham, 2016). Unfortunately, authors did not provide further information on the temperatures or chlorine concentrations and contact times to be applied for these treatments to be effective.

2.2.3 *Vermamoeba vermiformis*, a free-living common host

Free-living amoebae (FLA) are predatory heterotrophic protozoa ubiquitous of water systems where they interact with other microorganisms such as fungi, viruses, and bacteria (Cateau et al., 2014; Scheid, 2019a). FLA are grazing on biofilms and feeding on fungi and bacteria via phagocytosis (Scheid, 2019a). Some microorganisms, like *Lp* and NTM, can survive phagocytosis and replicate in FLA. Amoebae present in hospital water systems can harbour OPs and protect them against unfavourable conditions, offering them a vehicle of dissemination, and thus be

indirectly related to nosocomial infections (Cateau et al., 2014). More than 20 FLA species are now recognized to be supportive of OPs intracellular growth (Buse et al., 2013). Among those FLA, *Vermamoeba vermiformis* (Vv) is one of the most prevalent and probably one of the most studied.

Vermamoeba vermiformis

Vermamoeba vermiformis (formerly called *Hartmannella vermiformis*) is a FLA often encountered in water systems and biofilms. Depending on the environmental conditions, it is present under two forms: (1) a worm-like active form called trophozoite, and (2) a small round and double-walled dormant form called cyst (Delafont et al., 2018; Fouque et al., 2015a; Fouque et al., 2015b). *V. vermiformis* encystment process takes around 9h (Delafont et al., 2018). Cases of keratitis due to a conjunction of *V. vermiformis* and another amoeba have been previously reported, but the discussion about the medical and public health relevance of *V. vermiformis* is still ongoing (Scheid, 2019b; Siddiqui et al., 2021). Several studies highlighted the need to implement strategies to limit the proliferation of FLA in hospital water networks (Delafont et al., 2019).

V. vermiformis is ubiquitous of water distribution systems and has been frequently isolated from hospital water systems (Delafont et al., 2018; Samba-Louaka et al., 2019). Several studies reported *V. vermiformis* as the predominant amoeba in hot water systems (Thomas et al., 2006). The colonization of hot water systems by *V. vermiformis* has been linked to its thermotolerance (Delafont et al., 2018; Thomas et al., 2006). Indeed, *V. vermiformis* can survive to 30min heat exposure at 70°C (Farhat et al., 2012). *V. vermiformis* was also the predominant species in water samples taken from 6 hospital hot water systems contaminated with *Legionella* spp. (Rohr et al., 1998).

Like other FLA, *V. vermiformis* is described as a “trojan horse” because it can be an intracellular reservoir of bacteria, fungi, and viruses (Siddiqui et al., 2021). OPs can survive in both trophozoite and cyst forms of *V. vermiformis* (Cateau et al., 2014; Kuchta et al., 1993). Thus, *V. vermiformis* positively influences the proliferation of numerous microorganisms and OPs like *Legionella pneumophila* and nontuberculous mycobacteria (Kuiper et al., 2004; Sarink et al., 2023). Delafont et al. (2019) also showed that the presence of *V. vermiformis* increased *Aeromonas hydrophila* growth in the first 48h of contact and protected the bacteria against unfavourable conditions for 28

days. *V. vermiformis* also helps the proliferation of *Candida albicans* in tap water (Barbot et al., 2012).

2.2.4 Fungi and yeasts in water systems: example of *Candida* spp.

Fungi are natural inhabitants of water distribution systems but the isolation of allergenic and pathogenic strains in water and their possible aerosolization is particularly problematic (Hageskal et al., 2009).

Candida species

Candida spp. are one of the most common human fungal pathogens causing both systemic (bloodstream infections, invasive candidiasis) and superficial (cutaneous and mucosal) infections (Papon et al., 2013; Turner & Butler, 2014). Many *Candida* species are widely distributed in nature and common members of the normal human microflora (Papon et al., 2013). However, after modifications in the host environment, some of these species can transition from a commensal to a pathogenic phase (Papon et al., 2013). From 1997 to 2007, 31 species of *Candida* were identified in clinical samples from 41 countries (Pfaller et al., 2010). Among those species, *Candida albicans* was associated with more than 65% of candidiasis and *Candida parapsilosis* with 6% (Pfaller et al., 2010). Even if *C. albicans* remains the most frequently isolated agent causing candidiasis, the proportion of non-*albicans* *Candida* species from clinical isolates is increasing worldwide (Papon et al., 2013). Among the non-*albicans* *Candida* species, the incidence of *C. parapsilosis* is higher in North America (Turner & Butler, 2014). In the USA, *Candida* spp. are the fourth most important cause of nosocomial bloodstream infections accounting for 9% of these infections (Wisplinghoff et al., 2004). Estimates of mortality attributed to candidemia in the USA remains high (between 10% and 49%) (Pfaller & Diekema, 2007).

Fungi grow in water distribution systems, especially in low-circulation parts of the systems (Arvanitidou et al., 1999). Fungi were isolated by culture from more than 95% of community water samples, and more than 76% of hospital water samples (Arvanitidou et al., 1999). Recently, contaminated sinks were proved to be an important source for propagation of *Candida* spp. in healthcare facilities (Jencson et al., 2017). Fungi, especially *Candida* species, are able to form and adhere to biofilms (Arvanitidou et al., 1999; Seneviratne et al., 2008). Slow release of organic matter from materials, as well as the presence of cracks, pitting or dead ends in water systems favour formation of fungal biofilms (Niemi et al., 1982; Paterson & Lima, 2005). *Candida* spp.

from biofilm acquire enhanced resistance to antifungal agents (Seneviratne et al., 2008). Hawser and Douglas (1995) reported a resistance to multiple antifungal agents 30 to 2000 times higher from *Candida albicans* biofilms compared to planktonic cells. Some *Candida* species also display good resistance to disinfectants used in healthcare facilities. In vitro assays on laboratory media showed that *C. glabrata* was resistant to hydrogen peroxide, while *C. parapsilosis* and *C. guilliermondii* were resistant to a combination of salt and oxidative stress while it was efficient against *C. albicans* (Heaney et al., 2020). Another study reported that *C. parapsilosis* resisted to chlorine-based cleaners (Trofa et al., 2008). However, sodium hypochlorite seems efficient against *C. auris*, *C. albicans* and *C. glabrata* (Heaney et al., 2020; Ku et al., 2018).

2.2.5 Methodologies for surveillance of opportunistic pathogens in premise plumbing

Sampling strategies

Currently, there is no consensus regarding sampling strategies and detection or quantification methods to use for the monitoring of OPs. Key elements for the definition of an efficient sampling plan are the selection of the sampling sites and frequency, the type and size of sample (aqueous phase, biofilm...), and the flow patterns (first draft, after flushing, flushing duration...) (Wang et al., 2017). Also, sampling plans differ depending on the purpose of the study/monitoring (outbreak investigation, routine monitoring, risk assessment...). Current water quality policies mainly focus on main water distribution systems and do not apply to water across property lines while conditions in premise plumbing are particularly favourable for OP proliferation (National Research Council, 2007; Wang et al., 2017). Sampling for OPs at treatment plant outlets is less relevant as their concentrations are at their lowest, and it would be more relevant to sample stagnant water in buildings (Falkinham et al., 2015a). Due to the multiple and complex configurations of plumbing systems, it is difficult to define sampling strategies that would provide representative samples of OP contamination and disease transmission risks (Wang et al., 2017).

The two main niches for OPs in drinking water systems are biofilms and water. The concentrations of OPs are generally higher in biofilms, which would increase the probability of detection compared to water. However, bulk water samples are more accessible and representative of the dispersion and exposure to OPs, and thus remain the most common type of sample collected for OP monitoring in plumbing systems (Buse et al., 2012; Wang et al., 2017). If monitoring for

different OPs, it is important to consider their different known exposure pathways. As microbial concentrations are generally higher after stagnation, sampling water after a period without utilisation (for instance the first draw in the morning) maximises chances of OP detection. For biofilm sampling, biofilms are often collected from taps, drains, filters and shower heads, by scraping a certain surface area with a swab or sterile knife (Wang et al., 2017). For fungi, biofilm sampling is usually performed with swabbing or scraping a surface area of 1cm² (Kauffmann–Lacroix et al., 2016).

Standards and guidelines for OP monitoring in water systems of healthcare facilities vary a lot between countries, and there is no universal consensus on when and how to sample these systems. For instance, annual, semi-annual, quarterly and monthly sampling frequencies have been proposed for the monitoring of *P. aeruginosa* and *L. pneumophila*, with sampling frequencies also varying according to the water end usage (Castex & Houssin, 2005; Wang et al., 2017).

Analytical methods

There is currently no consensus on the methods and protocols to use for OP monitoring. Because of the large diversity of species included in this group and their different ecological and physiological characteristics, simultaneous monitoring of multiple OPs is complicated (Wang et al., 2017).

Culture remains the reference technique for the identification and typing of infectious agents in healthcare facilities, and recommendations, intervention guidelines, and standard methods are still based on culture techniques. However, OPs are mostly present under viable but non culturable (VBNC) state in water distribution systems, and are therefore not detected by culture (Wingender & Flemming, 2011). Culture methods are also labour-intensive, require trained staff to correctly identify target microorganisms, and provide delayed response because of the long incubation times, which is an issue in outbreak situations (Wang et al., 2017). Culture methods can also give an overview of cultivable fungal pathogens present, but results vary significantly because of the choice of growth media, and the incubation temperature and time (Novak Babič et al., 2017).

As a result, molecular techniques are increasingly used to assess microbial diversity in both biofilms and water from premise plumbing. Molecular techniques are based on the analysis and manipulation of proteins, RNA or DNA, and present high specificity and selectivity, relatively short analysis times and detect VBNC cells (Girones et al., 2010). Molecular techniques developed

for detection and quantification of OPs include polymerase chain reaction (PCR), quantitative polymerase chain reaction (qPCR), and sequencing. Lu et al. (2015) recommend performing PCR for initial screening and to compare the results with those of culture or microscopy or both. Sequencing of 16S and 18S rRNA genes has provided a finer understanding of the compositions and structures of the communities present in biofilms and water in water distribution systems, and next-generation sequencing technologies may bring new perspectives (Buse et al., 2014; Buse et al., 2013; Douterelo et al., 2018). However, cell characteristics and extraction methods influence the quantity and quality of recovered nucleic acids, and thus influence results of molecular techniques.

Optical techniques constitute the final category of detection and quantification methods for OPs. Several microscopy techniques have been developed to study biofilms, such as fluorescence or confocal microscopy and scanning electron microscopy (SEM) (Surman et al., 1996). Another optical technique is flow cytometry (FCM). FCM analyses suspended particles passing through a laser beam based on how they fluoresce or scatter light (Safford & Bischel, 2019). FCM has been used both to study changes in microbial water quality depending on the type and presence of disinfectant, and to monitor microbial community changes and growth throughout drinking water distribution systems (Gillespie et al., 2014; Hammes et al., 2010; Prest et al., 2016b). Prest et al. (2014) also highlighted the application of FCM to identify areas within distribution systems harbouring microbial contamination or excessive bacterial growth.

Ultimately, the objective of the study drives the choice of the technique used to detect and/or quantify OPs. For outbreak investigations, culture-based methods remain the gold standard. For risk mitigation and prevention, and for rapid screening to find outbreak sites, physiological and molecular methods are most recommended (Wang et al., 2017).

2.3 Water systems in healthcare facilities

2.3.1 Waterborne nosocomial infections

A hospital-acquired infection (HAI) or nosocomial infection is an infection contracted by a patient, a visitor, or a healthcare worker during the process of care in a healthcare facility and which was not present or incubating at the time of admission (World Health Organization (WHO), 2011a). Based on data provided between 1995 and 2010, the WHO estimated that 7 out of 100 patients in acute-care hospitals from high-income countries will acquire at least one HAI, and this percentage

increases in intensive care units, especially among neonates (World Health Organization (WHO), 2011a). Pseudo-infections (or pseudo-outbreaks) also happen when patients are colonized by an infectious agent but do not show any sign of disease or when clinical samples are contaminated. Both cases have detrimental impacts on patients as they can lead to misdiagnosis and inappropriate treatment (Williams et al., 2013).

Water and water-containing medical devices serve for many different care practices. In the past few decades, several studies have highlighted the increasing occurrence of waterborne infections in healthcare facilities and the need for clearer water safety guidelines (Anaissie et al., 2002; Williams et al., 2013). Contamination of water usually occurs within the water distribution system of the facility (Williams et al., 2013). Proximal (in the central pipes) and distal (at the point of use) contamination of hospital water supply can induce waterborne infections (Decker & Palmore, 2014). Biofilms have long been acknowledged as niches for growth of waterborne microorganisms like NTM or *L. pneumophila* in water distribution systems and points of use in healthcare facilities (Decker & Palmore, 2014). Various modes of transmission of waterborne pathogens to patients exist: direct or indirect contact with contaminated water, aspiration or ingestion, inhalation of contaminated aerosols (Chinn & Sehulster, 2003). In healthcare facilities, the complex water distribution systems with diverse plumbing materials, disinfectant types and levels, temperatures, and stagnation promote the development of biofilms, and occurrence and growth of opportunistic pathogens like *Lp* and NTM (Perkins et al., 2019). NTM were involved in 40 out of 134 CDC consultations on potential water-related HAIs performed between 2014 and 2017 and involving 1,380 patients (Perkins et al., 2019). However, outbreak investigations are challenging, and a high level of suspicion is usually needed to acknowledge that an infection might be related to water exposure (Perkins et al., 2019).

Many pieces of water-containing hospital equipment have been linked to HAI. Yiek et al. (2021) listed 8 water-containing devices with semi-closed water circuits and which were associated with at least one outbreak in the literature. In the past few years, several studies have reported outbreaks of invasive *M. chimaera* infections related to contaminated heater-cooler units used during cardiac surgeries worldwide (Sommerstein et al., 2017). *Lp*-related outbreaks have been linked to many devices including neonatal incubators, hydrotherapy pools, steam towel warmers, decorative fountains (Haupt et al., 2012; Higa et al., 2012; Marrie et al., 1987; Yiallourous et al., 2013).

Contaminated showers have also been linked to infections by NTM and *Lp* in hospitals and retirement homes (Bauer et al., 2008; Cooksey et al., 2008; Kauppinen et al., 1999).

A category of devices connected to water systems but often over-looked in water safety plans and guidelines is ice machines. Yet, colonisation and infection of patients with various microorganisms have been linked to ice machines. Table 2.2 summarises case studies of contaminated ice machines in hospitals or healthcare centres reported in the literature and related to NTM and *L. pneumophila*.

Microbial contamination of ice machines mainly results from the drinking water used to make ice and from hands when using the machine (Centers for Disease Control and Prevention (CDC), 2019). Common causes of contamination identified in several studies are: (1) heating by heat exchangers of water volumes in the machine, (2) the implementation of an oversized machine compared to the usage resulting in stagnation of water and ice, (3) retro-contamination of the machine by users or from drains, and (4) the installation of activated carbon filters upstream of the ice machine. Regarding transmission of pathogens from ice machines to patients, the three main transmission routes identified are (1) ingestion of contaminated ice cubes, (2) aspiration of contaminated droplets or aerosols when sucking on ice, and (3) contact on the machine or on a recipient contaminated by the machine when ice was collected (Walker et al., 2023b). Sampling of ice and water from ice machines also identified contamination by fungi (*Candida* spp.) and other bacterial opportunistic pathogens (*P. aeruginosa*) and Gram-negative bacteria (Kanwar et al., 2018; Yorioka et al., 2016).

Table 2.2 Nosocomial infections and pseudo-infection related to ice machines

Organism(s)	Patient population	Environmental study	Detection method	Remediation	Reference
<i>M. fortuitum</i>	Pseudo-outbreak 19 patients colonized	2 ice machines involved Ice, ice bins, machine water intake lines, and ice discharge funnels contaminated	Culture	Removal of the ice machines	LaBombardi et al. (2002)
<i>M. fortuitum</i>	Pseudo-outbreak 47 patients colonized HIV ward	1 ice machine involved Feed water, water in filter, water between filter and reservoir, water in reservoir, ice in augur and collection bins contaminated	Culture PFGE	Removal of the ice machine	Gebo et al. (2002)
<i>M. porcinum</i>	Pseudo-outbreak 16 patients in ICU	2 ice machines and 1 water dispenser contaminated	Culture	Removal of ice machines and revision of cleaning protocols	Kloth et al. (2019)
<i>M. paraffinicum</i> (unofficial taxon)	Pseudo-outbreak 10 patients	Ice machine located on the patient care unit contaminated.	Culture PFGE 16S rRNA genes amplification	<i>Not Mentioned</i>	Wang et al. (2008)

Table 2.2 Nosocomial infections and pseudo-infection related to ice machines (continued)

Organism(s)	Patient population	Environmental study	Detection method	Remediation	Reference
Rapidly growing mycobacteria (<i>M. chelonae</i> , <i>M. abscessus</i>)	15 pediatric hematopoietic stem cell transplant patients: 10 colonized and 5 infected	Amplification within the hospital water distribution system, highest concentrations in drinking water and ice from the machines.	Culture	Prohibition of consumption of water and ice for patients, distribution of bottled water. Replacement of all the machines with smaller models, silver-coated components, 0.005µm filters, copper piping. Plan to add UV disinfection devices.	Guspiel et al. (2017) & Tam et al. (2014)
<i>L. pneumophila</i> (serogroup 6)	ICU ventilator-dependent 66-year-old patient infected (no potable water intake, only ice chips from the ICU ice machine)	Ice, cold water and water inlet of the ICU ice machine contaminated.	Culture	Disinfection of the ice machine (2h-flush of 2.625% sodium hypochlorite), replacement of machine's supply line, disinfection cold water pipe (83ppm sodium hypochlorite, 48h)	Graman et al. (1997)

Table 2.2 Nosocomial infections and pseudo-infection related to ice machines (continued)

Organism(s)	Patient population	Environmental study	Detection method	Remediation	Reference
<i>L. pneumophila</i> (serogroup 1)	ICU 79-year-old patient with a swallowing disorder infected	All water samples, swabs and ice cubes from the central ice machine were positive. The ice from this machine was originally not intended for consumption.	Culture Fingerprinting	<i>Not Mentioned</i>	Bencini et al. (2005)

2.3.2 Regulations and guidelines for water systems in healthcare facilities

In Quebec, drinking water quality is regulated by the Regulation on drinking water quality (“Règlement sur la qualité de l’eau potable”) (Ministère de l’Environnement et de la Lutte contre les changements climatiques, 2014). Among the different rules cited, there is the need to maintain a free chlorine residual of at least 0.3mg/L at the outlet of the treatment plant, and water must be exempt of pathogens and faecal indicators like *E. coli* and enterococci (Ministère de l’Environnement et de la Lutte contre les changements climatiques, 2014).

In Canada, the Canadian Standard Association (CSA) developed specific standards for plumbing systems in healthcare facilities specifying that cold water should be kept below 20°C (Canadian Standard Association (CSA), 2021). Other countries have defined guidelines relative to drinking water systems in such facilities. In all these guidelines it is stated that water must first meet local drinking water standards. In France, different categories of water and their quality criteria are defined based on their usage in healthcare facilities (Castex & Houssin, 2005). Water intended for human consumption must be exempt of *E. coli* and enterococci, and a maximum variation of 1-log of concentrations of revivable aerobic germs (22°C and 37°C) must be met (Castex & Houssin, 2005). The optimal temperature defined for drinking water is 25°C (Castex & Houssin, 2005). In the UK, cold water should be maintained at a temperature below 20°C throughout the system to meet the criteria of being delivered at less than 20°C at the point of use after 2min of flushing (Department of Health (DH) et al., 2016b; Walker et al., 2023a). In healthcare facilities in the USA, CDC also specifies that distributed cold water should be below 20°C (Centers for Disease Control and Prevention (CDC), 2019).

Since temperature has been identified as a key factor limiting the presence and proliferation of *Legionella* spp., it is regulated in Quebec for hot water systems (Régie du bâtiment du Québec (RBQ), 2014). In all large buildings, including healthcare and long-term care facilities, water should be $\geq 60^{\circ}\text{C}$ at the water heater outlet, and $\geq 55^{\circ}\text{C}$ in the recirculation loops. On the other hand, in healthcare and long-term care facilities, water at shower heads and bathtub taps should be at a maximum of 43°C to avoid scalding (Régie du bâtiment du Québec (RBQ), 2014). CSA also specifies that conditions in hot water systems must be sufficient to inactivate or kill pathogens, and that additional treatment or filtration process can be implemented to provide additional warranty of antimicrobial activity (Canadian Standard Association (CSA), 2021). In France, a temperature

above 55°C should be measured at the water heater outlet. It should be >50°C in return loops and systems, but ≤50°C for personal care (Castex & Houssin, 2005). Sampling points and frequency for measurements of temperature and *Legionella* concentrations are specified. This guideline also defines an alert level of 10³ CFU/L and an action level of 10⁴ CFU/L of *L. pneumophila* (Castex & Houssin, 2005). In the UK, hot water leaving water heater should be ≥60°C, and then be ≥55°C in the distribution system and at all outlets (Department of Health (DH) et al., 2016a). Hot water returning to the water heater should be ≥50°C (Department of Health (DH) et al., 2016a). In the USA, minimal temperature for hot water production and storage is 60°C, and hot water should be maintained at a minimal temperature of 51°C in the system and return loop (Centers for Disease Control and Prevention (CDC), 2019). However, at points of use, hot water should be 49°C or less in patient-care areas, and 43.3°C or less in nursing-care facilities (Centers for Disease Control and Prevention (CDC), 2019).

Regarding ice machines, especially their maintenance, cleaning and disinfection, guidelines are ambiguous. Table 2.3 summarizes some guidelines specific to ice machines in healthcare facilities in Canada, France, UK, and USA.

No specific regulations exist for ice machines in healthcare facilities in Quebec and Canada, but some provinces have published guidelines (Alberta Health Services (AHS), 2020; Provincial Infection Control Network of British Columbia (PICNet), 2016; Public Health Ontario & Provincial Infectious Diseases Advisory Committee (PIDAC), 2018). In Quebec, the only regulation applying to ice is the *Quebec Food Regulation* and it specifies that ice must be made from drinking water (Government of Québec, 2012). According to the *Guidelines and standards for the interpretation of analytical results in food microbiology*, ice must be exempt of pathogens and *E. coli* in a 100mL sample (MAPAQ, 2019). The maximum acceptable value of HPC in ice is 1 x 10³ CFU/mL (MAPAQ, 2019). All guidelines refer to manufacturer's recommendations regarding maintenance, cleaning, and disinfection of ice machines, which often are not adapted to the specificities of the healthcare environment. Alberta and CDC guidelines both propose the same alternative 15 step-procedure for the cleaning and disinfection of ice machines. There is no monitoring requirement following the cleaning or disinfection of ice machines as a follow up on the efficiency of the proposed procedures. Several studies mention the lack of maintenance records and standards for ice machines and stress the need to establish them (Guspiel et al., 2017; Kanwar

et al., 2018). In addition, several authors suggest that the design of these machines should be modified to reduce the risk of contamination (Burnett et al., 1994).

Table 2.3 Guidelines on cleaning and maintenance of ice machines in healthcare facilities

	Type of Maintenance & Frequency	Procedure	Reference
Alberta, Canada	Regular cleaning, disinfection and maintenance (exterior weekly, interior monthly, maintenance quarterly)	According to manufacturer's instructions. 15 general steps proposed for cleaning and maintaining ice machines. Use of sodium hypochlorite to sanitize.	Alberta Health Services (AHS) (2020)
British Columbia, Canada	Cleaning, de-scaling and disinfection – at least quarterly	According to manufacturer's instructions.	Provincial Infection Control Network of British Columbia (PICNet) (2016)
	Daily	Visual inspection and disinfection of the scoop.	
	Quarterly	Thorough internal cleaning with water and detergent, remove scale, sanitize with sodium hypochlorite.	

Table 2.3 Guidelines on cleaning and maintenance of ice machines in healthcare facilities (continued)

	Type of Maintenance & Frequency	Procedure	Reference
Ontario, Canada	Cleaning, de-scaling and disinfection – at least quarterly	According to manufacturer’s instructions.	Public Health Ontario and Provincial Infectious Diseases Advisory Committee (PIDAC) (2018)
	Daily	Visual inspection and disinfection of the scoop.	
	Quarterly	Thorough internal cleaning with water and detergent, remove scale, sanitize with sodium hypochlorite.	
France	Maintenance – monthly to quarterly	Defrosting, cleaning and disinfecting with food grade products. Rinse before putting back into service.	Castex and Houssin (2005)
United Kingdom	Maintenance of the ice machine	According to manufacturer’s instructions. Frequently change ice.	Department of Health (DH) et al. (2016a)
	Maintenance of inlet filters	According to manufacturer’s instructions	

Table 2.3 Guidelines on cleaning and maintenance of ice machines in healthcare facilities (continued)

	Type of Maintenance & Frequency	Procedure	Reference
USA	Cleaning and/or maintenance	According to manufacturer's instructions. Use of an EPA-registered disinfectant. 15 general steps proposed for cleaning and maintaining ice machines. Use of sodium hypochlorite to sanitize.	Centers for Disease Control and Prevention (CDC) (2019)

2.3.3 Water safety plans

A water safety plan (WSP) is a systematic proactive approach for assessing and managing risk associated to drinking water safety in the entire drinking water supply (Gunnarsdottir et al., 2020; World Health Organization (WHO), 2023). Recommended by the WHO since 2008 for large buildings like healthcare facilities, WSP gathers and systematizes the best management practices historically adopted in the water sector and is acknowledged as the most reliable and effective tool to consistently ensure safety of drinking water and preserve consumers' health (World Health Organization (WHO), 2017, 2022). WSPs are now implemented in 93 countries, with 46 countries having policies or regulations promoting or requiring WSP, and 23 countries currently developing such documents (World Health Organization (WHO), 2017).

The three minimum key components of a WSP are: (1) the assessment of the water system, (2) an effective operational monitoring of the system, and (3) communication and management (World Health Organization (WHO), 2022). WHO defined 10 sub-steps constituting the WSP approach (World Health Organization (WHO), 2023). The assessment of the system includes its full description, the identification of risk areas and hazards and their assessment, the validation of existing control measures and the planning for their improvement (World Health Organization (WHO), 2023). The monitoring of the system includes the monitoring of the control measures and the verification of the effectiveness of the WSP (World Health Organization (WHO), 2023). Management and communication aim to strengthen the management procedures for normal and abnormal conditions, and to strengthen WSP supporting programs (World Health Organization (WHO), 2023). Finally, as WSP is an iterative process, it should be continuously reviewed and updated (World Health Organization (WHO), 2023).

In healthcare facilities, risks come from both the complexity of the plumbing systems and the multiplicity of water networks with different purposes (producing distilled water, ice...), and the vulnerability of the individuals (Bonadonna et al., 2017). Moreover, plumbing systems of large buildings are often poorly documented (especially with old, modified, or extended buildings), and the presence of many branch pipes induces large variations in flowrate and makes it difficult to maintain an effective control over water distribution systems (World Health Organization (WHO), 2011b). In the multidisciplinary WSP team, which is usually a subgroup of the infection-control team, maintenance and hospital engineers are usually in charge of the implementation and the

monitoring component of WSPs and ensure that water systems and the connected devices function as required at all times (World Health Organization (WHO), 2011b).

CHAPTER 3 OBJECTIVES AND METHODOLOGY

3.1 Critical review of the literature

Plumbing systems in large buildings are highly complex, with a large diversity of environmental and operational conditions (temperature, materials, flow...), making it hard to fully understand these systems and phenomena observed regarding water quality. Despite numerous studies on these systems, they remain chemical and microbiological black boxes, and the consequences of the interplay of conditions like temperature, materials and flow on water quality remain obscure (Neu, 2020). There is a wide body of literature reporting the impact of some of these parameters on water quality, in laboratory-, pilot- or real-scale systems. However, few studies consider both temperature, materials, and stagnation, while all these parameters interact in real water distribution systems. Because of differences in methodology and water composition, it is also difficult to compare results of different studies focusing on one parameter at a time. Indeed, these differences often lead to contradictory results, making it difficult for managers of building water distribution systems and WSP teams to correctly identify areas at risk and ways of dealing with decreased water quality. With the emphasis on WSP as a tool to assure safe drinking water in healthcare facilities, there is a need for studies providing feedback on how to identify and correctly assess risks in water distribution systems and connected elements, and on the monitoring of these systems (what to look for ? where ? how ?). Research on effective corrective measures and barriers to implement is also necessary.

Biofilms are key components in the understanding of OPs behaviour and fate in premise plumbing as they constitute niches of OPs (Wingender & Flemming, 2011). However, for practical reasons many studies are based on recombined biofilms with lab strains or a limited number of environmental strains (Biedroń et al., 2017). Although such studies are necessary to better understand the interactions between microorganisms, their representativeness of natural drinking water biofilms and survival of OPs in them is limited. A better understanding of eukaryotic and prokaryotic species associated with *L. pneumophila* in premise plumbing biofilms is necessary (Farhat et al., 2012). Also, many studies focus on the impact of the choice of materials on biofilms, but other parameters like temperature or water flow should be considered as well (Eboigbodin et al., 2008). Regarding plumbing materials, more information is needed on their suitability for contact with water, especially because of structural and chemical changes that occur over time

(Bonadonna et al., 2017). Specific regulations and update of existing ones based on the latest technological and knowledge advances are required (Bonadonna et al., 2017).

COVID-19 pandemic led to a remarkable number of building closures for extended periods of time. This unprecedented situation prompted building owners, utilities, health officials and specialists to provide new guidelines for the safe reopening of these buildings and their safely occupation (Proctor et al., 2020). This situation also highlighted the lack of understanding of parameters governing both chemical and microbial water quality under extended periods of stagnation, and of the effectiveness of remediation strategies proposed (mostly flushing and shock chlorination) (Proctor et al., 2020).

Despite several studies highlighting their ubiquity in water distribution systems, some microorganisms are still overlooked. Indeed, the presence of fungi and free-living protozoa such as amoebae in hospital water systems is still poorly documented (Arroyo et al., 2020; Thomas et al., 2006; Wingender & Flemming, 2011). Fungal nosocomial infections have been increasing in the last decades, especially invasive infections by *Candida* species which can survive for long periods on dry and moist surfaces (Kumar et al., 2019). Free-living amoebae are ubiquitous of hospital water systems where they also offer protection to OPs, indirectly causing nosocomial infections (Cateau et al., 2014). A recent study even reports protection and proliferation of the emerging fungal pathogen *Candida auris* in free-living amoebae (Hubert et al., 2021). Thus, more research on these microorganisms is needed in order to better understand the global microbial ecology of water distribution systems and maybe identify ways to modify microbial communities present as to avoid OPs survival in the systems (Cavallaro et al., 2022; Wang et al., 2013).

While norms and recommendations made for hot water systems are commonly precise, with temperature being regulated at different points of the system to prevent proliferation of *Legionella*, other components of water distribution systems are less regulated. Thus, there is a lack of guidelines for maintenance and cleaning of ice machines in healthcare facilities, despite them having been linked to outbreaks and pseudo-outbreaks (Graman et al., 1997; Guspiel et al., 2017). Current guidelines, when mentioning ice machines, always refer to manufacturer's guidelines for cleaning and maintenance. However, there is no consensus on the frequency of cleaning and disinfection (external and internal components) of ice machines. Also, there is currently no

guidelines for the monitoring of the efficiency of cleaning procedures of ice machines and of the quality of water and ice produced by these machines.

3.2 Research objectives and hypotheses

The main objective of this research project is to identify design, operation, and maintenance parameters preventing the occurrence and persistence of opportunistic pathogens in large buildings premise plumbing and connected devices.

On a more detailed level, this research project will aim to:

- Assess the survival of *L. pneumophila* in water heaters depending on the phase (water vs loose deposits), the heating regime, and the presence of hosts;
- Determine environmental and operational factors (materials, temperature, flow) preventing the persistence of opportunistic pathogens (*L. pneumophila*, NTM) and their hosts (*V. vermiformis*) in water and biofilms of cold and hot water systems of large buildings;
- Assess the impact of devices on the occurrence of opportunistic pathogens in water, ice, and biofilms at the point of use.

The previous objectives are derived from the 8 following research hypotheses:

1. Daily heating of four hours at sufficient temperature can prevent the proliferation of *Lp* in water heaters. *With the need to reduce energy consumption and financial costs associated with water heating in households, new models of water heaters are developed to allow interruptions of heating during peak electricity demand while always maintaining sufficient temperatures. Because of the design of such devices the risk of proliferation of *Lp* is mainly at the bottom where lower temperatures and loose deposits are found, and it has not been assessed yet. Thus, the inactivation of *Lp* in water heaters loose deposits programmed to heat 4 hours per day needs to be assessed.*
2. **Lp* better survive heating in loose deposits than in water. Temperature assays will be conducted in both simulated drinking water and loose deposits from 2 electric water heaters to assess the reduction of an environmental *Lp* strain in both phases.*
3. The presence of hosts in hot water systems accentuates the risk of proliferation of *Lp* in these systems. *While most of regulated temperatures of hot water systems in buildings and healthcare facilities have been defined as to avoid the optimal growth range of *Lp*,*

little attention has been paid to hosts presence and resistance in these systems. Several studies also report inefficiency of temperature to control Lp proliferation, but the presence of hosts has not been systematically investigated. The proposed work will evaluate Lp regrowth potential in presence of hosts downstream in the hot water distribution system.

4. Materials of cold and hot water systems influence water quality (metal and nutrient concentrations) and the survival of opportunistic pathogens and hosts in water and biofilms. *Previous studies describe the formation of biofilms on a diversity of materials but none of them compare biofilms developed on 6 different materials under the same conditions and in contact with the same water. To date, no study has compared the water quality of a pilot simulating cold then hot water systems made of 6 different materials. This work will provide practical insight into the potential for biofilm formation of 6 different materials, and the impact of these materials on the ability of Lp and V. vermiformis to survive in the system.*
5. The persistence of Lp and V. vermiformis in hot water systems with copper pipes is affected by the temperature set point. *Quebec regulations for hot water systems and their connected components (such as water heaters, showerheads, etc.) in healthcare facilities specify 3 regulated temperatures: below 43°C at taps to avoid scalding, 55°C in the recirculation loop, and 60°C at the water heater outlet. Although copper pipes are increasingly used because of their antibacterial effect, their higher cost hinders their installation. It is therefore important to quantify the benefits of using copper. However, no studies have yet compared the short- and medium-term persistence of Lp and V. vermiformis in copper pipes or coupons at the different temperatures indicated for hot water systems in large buildings and healthcare facilities.*
6. Prolonged (several months) stagnation leads to changes in water quality and a resurgence of opportunistic pathogens and hosts in water. *The COVID-19 pandemic confronted buildings managers with a new difficulty, namely the management of a prolonged shutdown (several months) of the water systems of these buildings during the confinements declared in many countries. To allow the reopening of these buildings to users and safe access to their water systems, several research teams around the world*

have conducted water quality tests before and after the application of local guidelines. However, no pilot-scale studies have been carried out to apply long stagnation to systems in operation. Demonstrating the impact of such stagnation on culturable and total Lp and on host presence will be particularly novel.

7. Ice machines are sites of microbial amplification in healthcare facilities. *Several studies have reported outbreaks and pseudo-outbreaks related to ice machines. However, no regulations exist in Quebec or Canada on the maintenance of ice machines in healthcare facilities. Only recommendations have been published, and most of them refer to the recommendations made by the manufacturers of these machines. Also, no study has focused on the microbial contamination of these devices. The present work will provide a first glance at microbial niches in ice machines of a hospital healthcare and the impact of current cleaning procedures.*
8. NTM are better indicators of the presence of opportunistic pathogens compared to HPC. *To date, the bacterial indicators used are not representative of the effectiveness of the disinfection procedures with respect to the opportunistic pathogens causing nosocomial infections. This work proposes to use NTM as indicator organisms to optimise the cleaning procedures of ice machines currently in place.*

3.3 Methodology

The experimental approach was conducted in 3 main parts (Figure 3.1):

- (1) Temperature assays in water heaters loose deposits and water to assess the risk of proliferation of *L. pneumophila* in hot water systems;
- (2) Pilot-scale study to investigate the interplay between materials, temperature and flow regime on water quality, biofilm formation, and survival of opportunistic pathogens;
- (3) Monitoring of microbial contamination in ice machines of a large hospital.

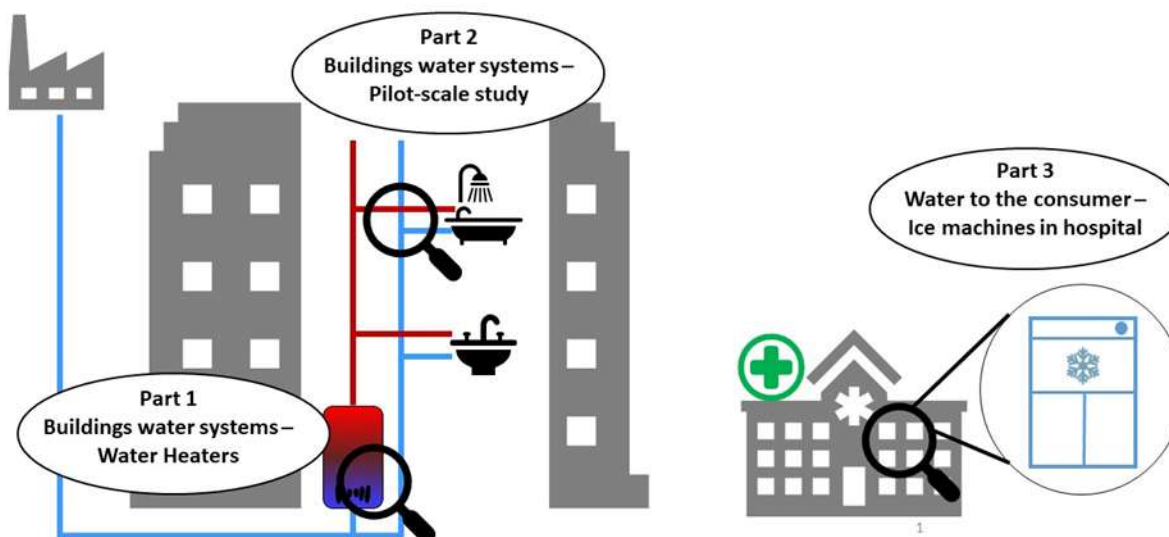


Figure 3.1 Schematic of the 3 parts of the research project

The methodology and experimental approach of each part of the project is then described.

3.3.1 Survival of *L. pneumophila* in water heaters

The first part of the research project is to assess the survival of *L. pneumophila* and the amoeba *V. vermiformis* in water heaters liquid and solid phases depending on the heating regime applied. It is aimed at evaluating the efficiency of a decrease of the water heater set point temperature or of repeated heating cycles on the risk of proliferation of *Lp* in hot water systems. The impact of the presence of hosts like the amoebae in hot water systems and water heaters on the resistance of *Lp* to the different heating regimes is also assessed. This first part addresses the hypotheses #1, #2 and #3.

An environmental strain of *L. pneumophila* serogroup 5 (CEAEQ isolate ID Q076826-03) and the ATCC 50237TM *V. vermiformis* were used for the different temperature assays conducted. The resistance to heat of *Lp* was assessed in two phases: the water in water heaters and the loose deposits found at the bottom of electric water heaters. For the assay in the liquid phase, 1×10^8 cells/mL of starved *Lp* were inoculated in simulated drinking water. Suspensions were exposed for 1h at 55°C, 60°C or 70°C and then stored in the dark at $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$. *Lp* densities were evaluated by culture on BCYE agar after 1h, 6h, 1 day, 2 days, 3 days, 1 week, 2 weeks, and 1 month of storage.

Lp resistance to heat was also assessed in water heaters loose deposits. Sediments were collected at the bottom of 2 electric water heaters connected to two different municipal water distribution

systems. Loose deposits were free of *Lp* as evaluated using culture-based enzymatic test (IDEXX, USA) when sampled, and were then inoculated with 10^6 CFU/mL of *Lp*. Six different temperatures were applied (40°C, 45°C, 50°C, 55°C, 60°C and 65°C) and samples were taken after 1h, 2h, 4h, 6h and 24h of heating. Again, the Legiolert™ culture-based enzymatic test (IDEXX, USA) was used to estimate *Lp* concentrations.

The efficiency of repeated daily 4h heating exposures on the survival of *Lp* in water heaters loose deposits was also assessed. Thus, daily 4h-exposures at 55°C followed by 20h at 40°C in the dark were performed on the same water heaters loose deposits inoculated with 10^6 cells/mL of *Lp* 5 days in a row. Before each heating cycle, *Lp* concentrations were estimated using the Legiolert™ culture-based enzymatic test (IDEXX, USA).

To evaluate the probability of presence of *Lp* hosts in water heaters, resistance to temperature of the ATCC *V. vermiformis* strain used in this research was also assessed. Suspensions of 10^5 cells/mL of *V. vermiformis* in autoclaved tap water were heated for 4h at 8 different temperatures: 25°C, 30°C, 37°C, 40°C, 43°C, 50°C, 55°C and 60°C. A sample heated at 100°C was used as a control of dead amoebae. Flow cytometry combined with SYBRGreen I (SG) and Propidium Iodide (PI) staining was used to enumerate and differentiate viable and dead amoebae.

Finally, to address the hypothesis #3 of this research, the impact of the presence of hosts on *Lp* resistance to heat was assessed. Loose deposits samples from the same two water heaters were inoculated with 10^6 cells/mL of *Lp* and heated for 4h at 40°C, 55°C and 60°C. Half of the samples were then inoculated with *V. vermiformis* at a final concentration of 10^6 cells/mL while the others were kept free of amoebae. All samples were incubated in the dark at $36.5^\circ\text{C} \pm 0.5^\circ\text{C}$ for 4 and 7 days, and culturable *Lp* concentrations were estimated using the Legiolert™ culture-based enzymatic test (IDEXX, USA).

3.3.2 Pilot-scale study

A pilot of 12 CDC Biofilm Reactors® (Biosurface Technologies Corporation, USA) was set up to assess the interplay between materials, temperature, and flow regime on the formation of biofilms and the survival of some opportunistic pathogens and their hosts in premise plumbing (hypotheses #4, #5 and #6).

The reactors were filled with 600mL of dechlorinated tap water under controlled temperature and mixing conditions (80rpm). Each reactor had a polytetrafluoroethylene (PTFE) baffled stir bar. For dechlorination, a 10L container filled with water from the same faucet of the CREDEAU lab (Polytechnique Montréal) was let open for at least 3 days. Pilot was operated in semi-batch with twice-weekly change of $\frac{3}{4}$ water volume (later changed to weekly changes from week 24). Each reactor contained 24 coupons of the same material (among the 6 materials studied) distributed on 8 polypropylene coupon holders. Each side of a coupon was 1.27cm², and when considering the thickness of the coupon, it represents a total surface of 3.24cm². In this project, 6 different materials were considered: 4 plastics (polypropylene (PP), polyvinyl chloride (PVC), ethylene propylene diene monomer (EPDM) rubber, and cross-linked polyethylene (PEX)), and 2 metals (stainless steel (SS) and copper (Cu)).

The pilot was operated for a total of 76 weeks as illustrated on Figure 3.2.

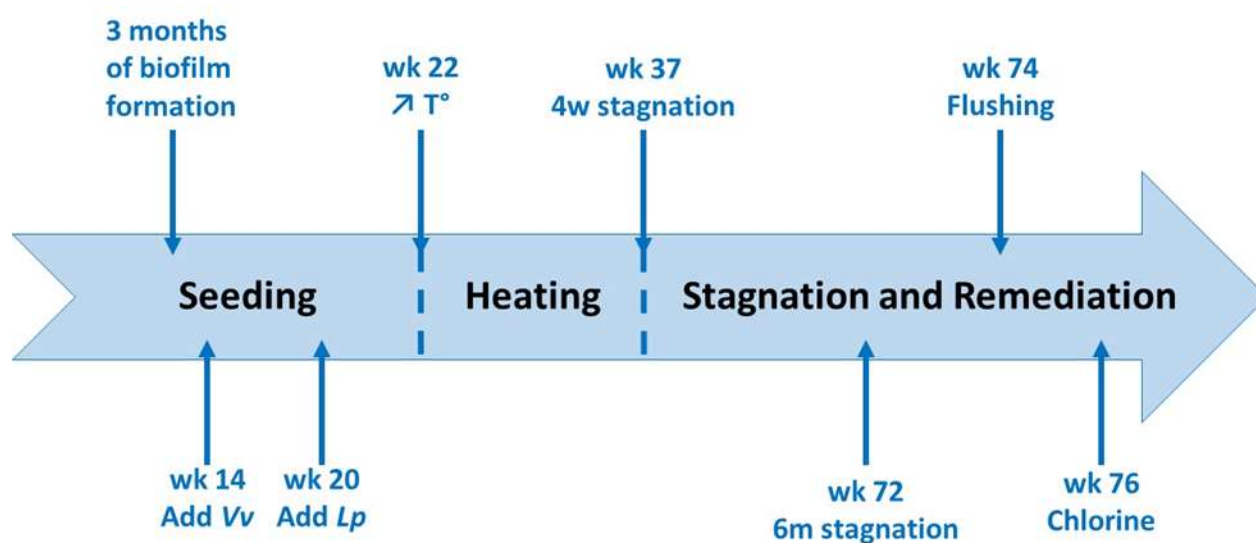


Figure 3.2 Chronology of operation of the pilot

The first 3 months, pilot was operated with twice-weekly changes of water to allow biofilms to form on the coupons. On week 13, the same ATCC strain of *V. vermiformis* as the one used in the first part of the experimental approach was added to the reactors at a final concentration of 1.5×10^3 cells/mL. Six weeks later, 11 reactors were inoculated with two starved environmental *Lp* strains (serogroup 1 (ID Q067501-02) and serogroup 5 (ID Q076826-03)) at final concentrations of 1.5×10^7 cells/mL of serogroup 5 and 2×10^7 cells/mL of serogroup 1. The only exception was one reactor with PP coupons (later called PP_A) which was inoculated with the serogroup 5 strain only.

Temperature of all the reactors except PP_A was increased 2 weeks after the addition of *Lp* in the reactors as to simulate conditions of hot water systems in the Quebec province. Materials, temperature and seeding characteristics of each reactor are summarised in the Table 5.1 in Chapter 5.

On week 32, mixing was stopped, and all reactors were brought back at room temperature for 4 weeks without water change, as to simulate the stagnation of water in hot water distribution systems of a household during holidays. Once stagnant water had been sampled, mixing and heating were restarted but had to be stopped again on week 45 because of the COVID-19 shutdown. Pilot was let stagnant at room temperature for the whole closure period of the university (6 months). On week 72 water and biofilms of all reactors were sampled and a flushing as recommended for the recommissioning of buildings water systems was simulated in the reactors (Régie du bâtiment du Québec (RBQ), 2020). To do so, reactors were completely emptied, filled with 600mL of dechlorinated tap water with mixing at 80rpm for 5min, then emptied again and refilled with dechlorinated tap water. This water was let in the mixed reactors for 2 weeks. After 2 weeks, reactors were sampled again, and a shock chlorination was performed. After emptying reactors for the sampling, reactors were filled with a 20ppm chlorine solution for 24h without mixing. After 24h, chlorine concentrations were measured, and all reactors were emptied and refilled with dechlorinated tap water prepared as from the beginning of the study. Reactors were sampled for the last time 2 weeks after the shock chlorination. Simulated flushing and shock chlorination were performed on the pilot as to evaluate the efficiency of these two most advised strategies for the recommissioning of water systems of large buildings after the COVID-19 shutdown (hypothesis #6).

For each sampling event, 450mL of the water of each reactor were collected in a sterile PP container. For biofilms, the 3 coupons of a coupon holder were sampled on the same sampling event. Each coupon was collected in 2mL of autoclaved MilliQ Ultrapure Water (MQ) filtered on 0.2µm. To loosen biofilm, coupons in MQ water were manually scratched with a sterile plastic inoculation loop for 1min, then vortexed for 1min, and finally sonicated for 1min in a sonication bath. After each sampling event, clean coupons from the previous sampling were put again on the coupon holder as to always maintain 24 coupons in each reactor, except reactors PP_A and PP_B where a coupon holder had to be replaced by a PP blank rod on week 18 due to a lack of coupons. The protocol for the cleaning of the coupons is provided in Appendix A.

At each sampling event, physico-chemical parameters were measured on water samples (pH, dissolved oxygen, turbidity, conductivity). More details on the devices used are given in Chapter 5. Total (DOC) and biodegradable dissolved organic carbon (BDOC) were measured following previously published methods (American Public Health Association (APHA) et al., 2012; Servais et al., 1989).

Concentrations of metals (Ca, Cr, Cu, Fe, Mg, Mn, Pb and Zn) were estimated in water and biofilms using a PerkinElmer NexION 300x mass spectrometer (PerkinElmer, USA).

Pictures of coupons before and 3 months after the launch of the pilot were taken using a JSM-7600F Schottky Field Emission Scanning Electron Microscope (JEOL, Japan). Elementary composition of each coupon was determined using energy-dispersive X-ray spectroscopy. Specimens were sputter coated with gold and double-sided conductive tape was used to fix them on a standard specimen holder.

As for general microbiological parameters, total and intact cells densities were estimated using flow cytometry with SG and SGPI staining (Gatza et al., 2013). Culturable *Lp* concentrations were determined using the Legiolert™ culture-based enzymatic test (IDEXX, USA) following the 100mL potable water and 1mL non potable water manufacturer's protocols. During each sampling event, 250mL of water of each reactor were filtered on 0.2µm polyethersulfone membranes (Pall Corporation, USA). Filters and 500µL of each biofilm sample were frozen at -25°C until DNA extraction. DNA extraction was performed following the bead beating method adapted from Yu and Mohn (1999) and described in Bédard et al. (2015b). Briefly, samples were inserted into 2mL extraction tubes containing a ¼-inch ceramic sphere and garnet matrix (Lysing Matrix A, MP Biomedicals, USA). A lysing buffer (EDTA-2Na pH8, SDS 3%, Tris HCl pH8) with RNase (20µg/mL) was added in each tube before the bead beating step being performed on a FastPrep-24™ classic bead beating grinder (MPBio, USA). Bead beating was followed by an ammonium acetate (2mol/L) precipitation step and an overnight concentration step with cold 70% isopropanol and glycogen. Washes with cold 70% ethanol were performed twice, and DNA was finally resuspended in 100µL DNA-free water.

qPCR analyses were then performed on each sample to estimate *Lp*, NTM and *V. vermiformis* concentrations. For *Lp*, the iQ-Check® Quanti *L. pneumophila* Real-Time PCR Quantification Kit (Bio-Rad, USA) based on the amplification of the *mip* gene was used. NTM concentrations were

estimated using qPCR primers targetting the *atpE* gene developed by Radomski et al. (2013), and the qPCR protocol optimised for CFX Opus 96 Real-Time PCR System (Bio-Rad, USA) developed by Haig et al. (2018). Briefly, each 20µL reaction mixture was composed of 10µL of SsoFast EvaGreen Supermix (Bio-Rad, USA), 0.5µL of bovine serum albumin (Thermo Fisher Scientific, Canada), 1µL of each primer, and 2µL of DNA. A 5min initial denaturation step preceded 40 cycles of denaturation (95°C for 20s), annealing (57°C for 30s) and elongation (72°C for 30s). The qPCR protocol developed by Kuiper et al. (2006) was used to estimate *V. vermiformis* concentrations. Each 20µL reaction was composed of 10µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), 1µL of each primer and 1µL of DNA. A predenaturation step at 95°C for 3min was followed by 40 cycles of denaturation (95°C, 20s), annealing (56°C, 30s) and elongation (72°C, 40s). A final elongation step was performed at 72°C for 10min.

Finally, sequencing of the V4 region of the 16S rRNA gene and the V9 region of the 18S rRNA gene was performed on a MiSeq platform (Illumina, Inc.) on DNA extracted from all water samples. For the 16S V4 region, 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers were used. For the 18S V9 region, 1391F (5'-GTACAC ACC GCC CGT C-3') primers were used as described in Paranjape et al. (2020). Sequencing data was processed using the Mothur pipeline (Schloss et al., 2009). Taxonomic abundance profiles and alpha- and beta-diversity analyses were performed using The MicrobiomeAnalyst web-based platform (Chong et al., 2020).

3.3.3 Microbial contamination of ice machines in healthcare facilities

The last part of the research aimed at determining if ice machines could represent reservoirs of opportunistic pathogens in healthcare facilities, and to assess the efficiency of current disinfection procedures of these devices. The potential of NTM as indicators of the maintenance efficiency of these machines was also evaluated.

This part began with the sampling of 36 ice machines located in the same building of a 772-bed hospital in Montreal (Canada). Two different models of ice machines were present in the building, and the specificities of each model as well as the distribution of machines on the floors are gathered in the Appendix B and Appendix F Table F.1. A 5µm membrane filter and a 1µm membrane filter containing phosphate beads were placed upstream of each ice machine. Figure 3.3 is a schematic representation of the kitchenettes where most of the ice machines were located in the building.

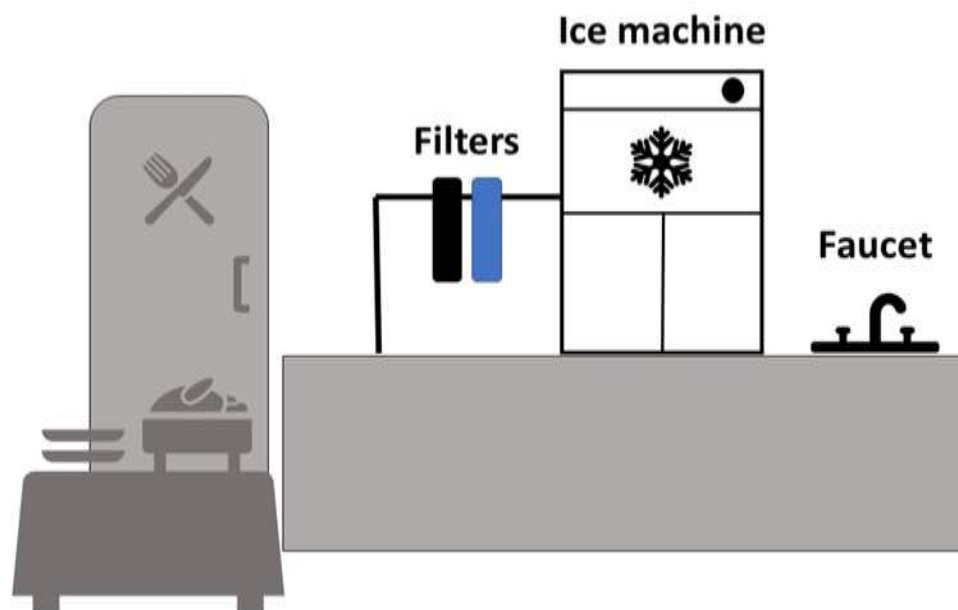


Figure 3.3 Schematic representation of a typical kitchenette in the hospital and the positions of the different elements

On a daily base, the external parts of ice machines were cleaned using the Oxivir® Plus Disinfectant Cleaner Concentrate (Diversey, USA) using Accelerated Hydrogen Peroxide (AHP®) technology. Weekly, the Suma® Cal-X Descaler (Diversey, USA) was used on the external components of the machines.

Internal components of ice machines were disinfected once every 6 months. First, the ice reservoir and the internal water reservoir of the machines were emptied manually. The air filter was cleaned, and the drain unclogged. A first 30min cleaning cycle was then performed using the Nickel-Safe Ice Machine Cleaner (Nu-Calgon, USA). It is a food grade, USEPA-accepted liquid cleaning product for all models of ice machines. It is composed of citric and phosphoric acids and removes scale deposits. Then the internal circuit of the machine was rinsed with cold water for around 5 min. A second cleaning cycle of 30min was then operated with the Stera-Sheen® Green Label Machine Sanitizer (Purdy Products Company, USA) which is a USEPA registered and FDA approved sanitizer for ice machines. It is also classified as a D2 sanitizer (not requiring rinse) by NSF. The active molecule of this sanitizer is the sodium dichloro-s-triazinetrihydrate (for an equivalence of available chlorine of 1.34%). This molecule is also used to treat water of swimming pools or cooling towers (National Center for Biotechnology Information (NCBI), 2023). Finally, the water circuit of the machines was rinsed again by circulating cold water for 5 min. The first ice

formed (approximately 20 min after the disinfection ended) was discarded because medical staff had complained before about small deposits in ice after cleaning of the machines.

During this monitoring survey, 2 samples were taken at each ice machine: a sample of water (0.5L) and ice (1.5L) collected in a sterile container, and a swab of the biofilm (19.2cm²) taken at the drain inlet and resuspended in 2mL of saline solution. Physico-chemical parameters (pH, temperature, total and free chlorine, conductivity, dissolved oxygen) were measured on site while turbidity was measured on a 250mL water sample of each machine at the CREDEAU lab with a Hach TL2300 turbidimeter (Hach, USA). For general microbiological parameters, total and intact cells densities were estimated using a BD AccuriTM C6 flow cytometer (BD Biosciences, USA), and heterotrophic bacteria (HPC) were enumerated by culture on R2A agar. Culture-based enzymatic tests were used to evaluate *Pseudomonas aeruginosa* and enterococci concentrations (IDEXX, USA). *Candida* spp. presence was verified by culture using the CHROMagar *Candida* media (CHROMagar, France). The API *Candida* test (BioMerieux, USA) was used for confirmation of colonies of a subset of samples. For molecular analyses, 1L of each water-ice sample was filtered on 0.2µm polyethersulfone membrane (Pall Corporation, USA), and 500µL of each resuspended biofilm sample were frozen at -25°C before extraction. A phenol-chloroform protocol improved for NTM detection was used for DNA extraction (Haig et al., 2018). qPCR was performed on a Rotor-Gene Q thermocycler (Qiagen, USA) following the protocol developed by Radomski et al. (2013) to estimate NTM concentrations.

The aim of the second act of this field study was to identify the source of the microbial contamination observed in ice machines. Profiling samplings were performed in 5 rooms with ice machines. First, three 1L-cold water samples were taken at the adjacent faucet of each machine: (1) the first draw (F1J), (2) after a 30s flushing (F30S), and (3) after water temperature was stabilized for 30s (F). Then, the water contained in the filters placed upstream of each machine was sampled (WF). Feed water (W) and water in the internal water reservoir (WR) of each machine were collected. This time, ice (I) was sampled from the ice chute and was separated from water, and a 1L water sample was collected after a 5min flush (W5m) at the water chute. To evaluate the microbial amplification occurring in the building's premise plumbing, a 10L sample was taken at the building's water inlet. The same physico-chemical parameters were measured on the 3 samples collected at each faucet. In all samples, total and intact cells densities were estimated using a BD AccuriTM C6 Plus flow cytometer (BD Biosciences, USA) while HPC concentrations were

estimated by culture on R2A agar. Samples were filtered on 0.2µm polyethersulfone membranes (Pall Corporation, USA) and the FastDNA™ SPIN kit (MP Biomedicals, USA) was used for DNA extraction. NTM qPCR were performed on a CFX96 BioRad Thermocycler (BioRad, USA) following the version of the protocol adapted for this thermocycler and developed by Haig et al. (2018). Ca, Mg, Fe, Cu, Mn, and Ni concentrations were estimated in all water samples using a PerkinElmer NexION 300x mass spectrometer (PerkinElmer, USA).

During these samplings, precipitates were observed in the internal water reservoirs of 3 ice machines. SEM analyses as well as anions and cations analyses were performed on these precipitates to determine their nature. The filters located upstream of one of the machines with a precipitate and which had been used for 4 months were also collected and the same analyses were performed.

Finally, the efficiency of the current cleaning procedures was assessed over 4 months as the filters are changed every 4 months. Ice machines throughout the building were gathered in 3 groups of 5 ice machines and the following disinfection procedures were applied:

Group 1 – No change : the same disinfection procedure of the internal components of the machines was applied, and only the filters were changed.

Group 2 – Change of components : the same disinfection procedure of the internal components of the machines was applied, but the internal water reservoirs and the flexible braided hose between the filters and the machine were replaced.

Group 3 – Improved disinfection : disinfection was performed with the double dose of disinfectant, and the internal water reservoirs and the flexible braided hose between the filters and the machine were replaced.

Samplings of the ice machines were performed 4 times: (1) before disinfection, (2) 10 days after disinfection, (3) 2 months after disinfection, and (4) 4 months after disinfection and before filters were changed. During each sampling event, 4 1L-samples were taken per machine: (1) the water after filters (water inlet of the ice machine (EF), (2) the first draw at the water chute of the ice machine (1J), (3) ice at the ice chute (I), and (4) 1L of water after a 5min flushing at the machine (5M). On each sample, total and intact cells densities were estimated using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences, USA). This time, HPC concentrations were not estimated. Instead, ATP concentrations were measured using the Quench-Gone™ Aqueous Test Kit

(LuminUltra Technologies, Canada). Concentrations of Mg, Ca, Mn, Fe, Ni, Cu and Pb in ice samples were measured using a PerkinElmer NexION 300x mass spectrometer (PerkinElmer, USA). DNA concentration and extraction, as well as NTM qPCR were performed as previously.

The experimental approach implemented for each hypothesis as well as the expected results and the corresponding scientific paper are summarised in the Table 3.1.

Table 3.1 Experimental approaches implemented to address research hypotheses and corresponding scientific papers

Hypothesis	Experimental Scale	Experimental Approach	Expected Results	Scientific Paper
#1 - Daily heating of four hours at sufficient temperature can prevent the proliferation of <i>Lp</i>	Laboratory – loose deposits from water heaters	Loose deposits from 2 electric water heaters inoculated with an environmental strain of <i>Lp</i> and heated at 55°C for 4h every day for 5 days.	Inactivation logs of <i>Lp</i> throughout the heating cycles.	#1 – Published in <i>Microorganisms</i> (Chapter 4)
#2 - <i>Lp</i> better survive heating in loose deposits than in water.	Laboratory – loose deposits from water heaters	Inoculation of an environmental strain of <i>Lp</i> in simulated drinking water and in loose deposits of 2 electric water heaters. Heating of the samples at different temperatures and durations.	Inactivation logs of <i>Lp</i> when exposed to different heating temperatures in water or in loose deposits.	#1 – Published in <i>Microorganisms</i> (Chapter 4)
#3 - The presence of hosts in hot water systems accentuates the risk of proliferation of <i>Lp</i> in these systems.	Laboratory – loose deposits from water heaters	Loose deposits from 2 electric water heaters inoculated with an environmental strain of <i>Lp</i> and heated for 4h at 40°C, 55°C and 60°C. Inoculation post-heating with <i>V. vermiformis</i> .	Concentrations of culturable <i>Lp</i> in loose deposits after exposure to heat, in presence and in absence of <i>V. vermiformis</i> .	#1 – Published in <i>Microorganisms</i> (Chapter 4)

Table 3.1 Experimental approaches implemented to address research hypotheses and corresponding scientific papers (continued)

Hypothesis	Experimental Scale	Experimental Approach	Expected Results	Scientific Paper
#4 - Materials of cold and hot water systems influence water quality (metal and nutrient concentrations), microbial diversity, and the survival of opportunistic pathogens and hosts in water and biofilms.	Pilot-scale study	12 CDC Biofilm reactors with coupons of 6 materials, inoculated with <i>Lp</i> and <i>V. vermiformis</i> in dechlorinated tap water, and operated for 76 weeks with a period at room temperature and a period heated at $\geq 40^{\circ}\text{C}$.	Temporal monitoring of physico-chemical parameters (metals, DOC, BDOC), general microbial indicators (Total and Intact cells), concentrations of <i>Lp</i> (culture and qPCR), NTM (qPCR) and <i>V. vermiformis</i> (qPCR).	#2 – Submitted in <i>Water Research</i> (Chapter 5) And #3 – Submitted in <i>ES&T Water</i> (Chapter 6)
#5 - The persistence of <i>Lp</i> and <i>V. vermiformis</i> in hot water systems with copper pipes is affected by the temperature set point	Pilot-scale study	6 CDC biofilm reactors with copper coupons inoculated with <i>Lp</i> and <i>V. vermiformis</i> in dechlorinated tap water and heated at 40°C , 55°C and 60°C .	Concentrations of <i>Lp</i> (culturable and qPCR) and <i>V. vermiformis</i> in water and biofilm samples of the reactors.	#3 – Submitted in <i>ES&T Water</i> (Chapter 6)
#6 - Prolonged (several months) stagnation leads to a change in microbial composition and a resurgence of opportunistic pathogens and hosts in water	Pilot-scale study	12 CDC biofilm reactors inoculated with <i>Lp</i> and <i>V. vermiformis</i> in dechlorinated tap water let stagnant at room temperature for 6 months. Simulation of a flushing and a shock chlorination.	Temporal evolutions of concentrations of <i>Lp</i> , NTM and <i>V. vermiformis</i> before and after stagnation, after flushing and after chlorination.	#3 – Submitted in <i>ES&T Water</i> (Chapter 6)

Table 3.1 Experimental approaches implemented to address research hypotheses and corresponding scientific papers (continued)

Hypothesis	Experimental Scale	Experimental Approach	Expected Results	Scientific Paper
#7 - Ice machines are sites of microbial amplification in healthcare facilities.	Ice machines in a hospital	Field study in a Montreal hospital. Analysis of the quality of ice-water and drain biofilms of 36 ice machines and research of <i>Candida</i> spp., enterococci, <i>Pseudomonas aeruginosa</i> and NTM.	Measurements of different general microbial indicators (HPC, Total and Intact cells) and concentrations of OPs (NTM, <i>Candida</i> spp. or <i>P. aeruginosa</i>) during the monitoring of microbial contamination in 36 ice machines.	#4 – Submitted in <i>Journal of Hospital Infection</i> (Chapter 7)
#8 - NTM are better indicators of the presence of opportunistic pathogens compared to HPC.	Ice machines in a hospital	Field study in a Montreal hospital. Monitoring of HPC and NTM concentrations in 36 ice machines, and then 5 ice machines and their pre-filters and adjacent faucets.	Concentrations of HPC, NTM and other OPs measured during the monitoring of microbial contamination in 36 ice machines. Concentrations of HPC and NTM during a profiling sampling performed on 5 ice machines, their pre-filters and their adjacent faucets.	#4 – Submitted in <i>Journal of Hospital Infection</i> (Chapter 7)

CHAPTER 4 ARTICLE 1: COMPROMISED EFFECTIVENESS OF THERMAL INACTIVATION OF *LEGIONELLA PNEUMOPHILA* IN WATER HEATER SEDIMENTS AND WATER, AND INFLUENCE OF THE PRESENCE OF *VERMAMOEBIA VERMIFORMIS*

Legionella pneumophila is mostly found in hot water systems and previous studies showed that electric water heaters were preferentially colonized by *Legionella* compared to other models of water heaters. The impact of the phase, the temperature regime and the presence of hosts on the survival of *L. pneumophila* in water heaters was assessed by performing temperature assays on both *L. pneumophila* and its host *V. vermiformis* in water and in loose deposits sampled from 2 electric water heaters. This paper was published February 15th 2022 in *Microorganisms*. Supplementary information is provided in Appendix C.

Compromised Effectiveness of Thermal Inactivation of *Legionella pneumophila* in Water Heater Sediments and Water, and Influence of the Presence of *Vermamoeba vermiformis*

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Abstract:

Intermittent reduction of temperature set-points and periodic shutdowns of water heaters have been proposed to reduce energy consumption in buildings. However, the consequences of such measures on the occurrence and proliferation of *Legionella pneumophila* (*Lp*) in hot water systems have not been documented. The impact of single and repeated heat shocks was investigated using an environmental strain of *L. pneumophila* and a reference strain of *V. vermiformis*. Heat shocks at temperatures ranging from 50°C to 70°C were applied for 1 h and 4 h in water and water heaters loose deposits (sludge). The regrowth potential of heat-treated culturable *L. pneumophila* in presence of *V. vermiformis* in water heaters sludges was evaluated. A 2.5-log loss of culturability of *L. pneumophila* was observed in simulated drinking water at 60°C while a 4-log reduction was reached in water heaters loose deposits. Persistence of *Lp* after 4 h at 55°C was shown and the presence of *V. vermiformis* in water heater's loose deposits resulted in a drastic amplification (6-log). Results show that thermal inactivation by heat shock is only efficient at elevated temperatures (50°C) in both water and loose deposits. The few remaining organisms can rapidly proliferate during storage at lower temperature in the presence of hosts.

Keywords: *Legionella pneumophila*; *Vermamoeba vermiformis*; hot water distribution system; water heater; thermal treatment; temperature; flow cytometry

4.1 Introduction

Legionella reside in many aquatic environments and can develop in water systems from buildings, including those harbouring vulnerable populations such as healthcare facilities and long-term care facilities (Boppe et al., 2016; Marchesi et al., 2011; National Academies of Sciences, 2019). *L. pneumophila* causes most of the Legionnaires' disease cases, a severe form of pneumonia associated with high morbidity for at risk populations such as the elderly or immunocompromised (ASHRAE, 2020; Falkinham, 2020; National Academies of Sciences, 2019). *L. pneumophila* is particularly adapted to hot water environments, with reported optimal temperatures for growth ranging between 25 °C and 45 °C, while temperatures of 50 °C and over have been shown to decrease the culturable *L. pneumophila* populations (ASHRAE, 2020; National Academies of Sciences, 2019; van der Kooij et al., 2016). Optimal growth temperature as well as resistance to heat may vary significantly between strains of *L. pneumophila* studied. Temperature-resistant environmental strains have been isolated from hot water systems and from systems subjected to repeated thermal shocks (Allegra et al., 2011; Bédard et al., 2019; Ministère de la Santé et des Services sociaux (MSSS), 2015). For instance, Allegra et al. reported that 6 out of 12 clinical and environmental strains of *Legionella* maintained viability for 10% to 25% of the cell population after 30 min at 70 °C (Allegra et al., 2008).

In Quebec, water heaters, as well as institutional, commercial or residential hot water distribution systems, are known as reservoirs involved in the dispersion of *Legionella* bacteria (Ministère de la Santé et des Services sociaux (MSSS), 2015). Indeed, it was reported that 33% of electrical water heaters were colonized by *Legionella*, with concentrations from 10 to more than 100 CFU/mL (Dewailly & Joly, 1991).

Thermal control to prevent and remediate *Legionella* in hot water systems. Maintaining temperature outside *Legionella*'s preferred growth range is the paramount and well demonstrated *Legionella* preventative control strategy in building water systems (National Academies of Sciences, 2019). To prevent the risk of legionellosis, temperatures in water heaters, hot water distribution systems and associated devices are regulated in many countries, especially in healthcare facilities and retirement homes. Thermal control, often by maintaining temperatures over 55 °C across the hot water system, can be successful in controlling *Legionella* and *L. pneumophila* in hot water systems (National Academies of Sciences, 2019). Longitudinal studies

in hospital water systems have shown that increasing temperature at distal points from 50°C to 55°C significantly decreases positivity and concentrations of *Legionella* (Bédard et al., 2016a; Gavalda et al., 2019). Temperature monitoring in hot water systems is considered a useful control tool to evaluate the risk of contamination by *Legionella* and more specifically *L. pneumophila* (Bédard et al., 2015b; Boppe et al., 2016). The Quebec construction code requires a minimal temperature of 60°C to be maintained at the outlet of the water heater, and of at least 55°C in the recirculation loop (Régie du bâtiment du Québec (RBQ), 2014). However, to prevent scalding in healthcare facilities and retirement homes, the temperature of hot water at the bathtub faucets and showers should not exceed 43 °C (Régie du bâtiment du Québec (RBQ), 2014).

The efficiency of thermal control mainly relies on the maintenance of high temperatures at distal ends of the hot water distribution systems. A study with more than 30,000 water samples from 4,600 German public buildings over 7 years revealed that mean temperatures measured at distal points were 7 °C and 11 °C lower than those measured in recirculation loops and flushed samples respectively, which corresponded to 10-fold higher concentrations of *Legionella* (Kistemann & Wasser, 2018). Efficient temperature control can only be achieved in hydraulically balanced systems without dead-end pipes and faulty devices. It can be difficult to reach target control temperatures at each outlet in large buildings where hydraulic balancing is not optimal (ASHRAE, 2020; Blanc et al., 2005; Boppe et al., 2016; Marchesi et al., 2011). Furthermore, temperatures in water heaters, reservoirs and building water systems vary in space and time as a function of system architecture, water demand, and hydraulics. In the distal part of the distribution system where recirculation is not present, temperatures oscillate between recirculation/post thermal mixing valve temperatures and room temperature between water uses.

Thermal shocks as an emergency response to *Legionella* contamination. Curative heat shock treatment at 70 °C can be used to disinfect water system (ASHRAE, 2020; National Academies of Sciences, 2019; Whiley et al., 2017). A temperature of 70 °C or more should be maintained for 20 to 30 min at each point of use (ASHRAE, 2020; Ministère de la Santé et des Services sociaux (MSSS), 2015). Yet, sporadic thermal treatments have shown limited efficiency against *L. pneumophila* in time (Allegra et al., 2011; Saby et al., 2005). Similarly, thermal disinfection was conducted twice a day for two weeks by flushing hot water (60–70 °C) at taps and showers for several minutes in 4 buildings water systems in Finland. *Legionella* recolonized these systems within 4 months, despite maintaining the hot water system at 55 °C between and after thermal

disinfection (Zacheus & Martikainen, 1996). Therefore, thermal shocks are considered a temporary remedial or emergency response, not a preventive measure because of their limited impact over time, and their potential for the selection of heat resistant *L. pneumophila* strains (National Academies of Sciences, 2019). More importantly, Allegra and colleagues showed that repeated heat shock treatments in a health care facility selected for heat resistance of *L. pneumophila* strains. *L. pneumophila* strains from a hot water circuit where temperature was frequently increased to 65 °C for a day were more heat resistant than those from a hot water system with sporadic heat treatments. Their results suggest that *Legionella* strains can adapt and become heat resistant after repeatedly applying super heat shock treatments (Allegra et al., 2011). To further support the short term benefits of shock heat treatment, it was shown that an elevated temperature set point at the water heater has a stronger impact on *Legionella* concentrations at distal taps than a one-time 30 min-heat shock at 60 °C (Ji et al., 2018). More recently, Whiley and colleagues have hypothesized that superheat and flush thermal tolerant *Legionella* have a greater public health significance (Whiley et al., 2017).

Impact of hosts. The regrowth of *Legionella* bacteria after heat treatment has been attributed to necrotrophic growth, their integration into biofilm, and their capacity to resist phagocytosis and grow within heat-resistant forms of protozoans (Atlas, 1999; Bartram et al., 2007; Saby et al., 2005; Temmerman et al., 2006). Amoebae are the preferential *L. pneumophila* hosts in hot water systems (Paniagua et al., 2020; Rohr et al., 1998). Particularly, *L. pneumophila* can infect amoebae and even survive within them when they transform into cysts, which are known to be more resistant to disinfectants and temperature. For instance, Storey and colleagues showed that *L. pneumophila* can survive in *Acanthamoebae* cysts after 10 min at 80 °C (Storey et al., 2004). *Vermamoeba vermiformis* are free-living amoebae ubiquitous in water environments, including in hospitals where they can survive to temperatures above 55 °C (Masangkay et al., 2018). *V. vermiformis* is the most often associated host of *L. pneumophila* in warm- and hot-water distribution systems (Buse et al., 2017; Ji et al., 2017). *L. pneumophila* residing in *V. vermiformis* cysts can resist temperatures from 50 °C to 70 °C (Cervero-Aragó et al., 2013; Fouque et al., 2015a). Farhat et al. reported that cysts of *V. vermiformis* survive a heat shock of 70 °C for 30 min (Farhat et al., 2012). The survival of hosts is a critical factor for regrowth, as there is now a consensus that the control of eucaryotic hosts of *Legionella* is necessary to achieve effective *Legionella* control in water

systems, as the ability of *Legionella* to grow without hosts is limited (National Academies of Sciences, 2019).

Alternative temperature management for energy conservation. In 2017, water heating represented 19.3% of the energy consumed in the average Canadian home (Natural Resources Canada, 2012). In the context of energy conservation to reduce building's carbon footprint, there is increased pressure to operate water heaters and building hot water systems at lower temperature set-points. However, lower temperature steady state operation increases the risk of *Legionella* in hot water heaters and distribution systems (Kistemann & Wasser, 2018; National Academies of Sciences, 2019). To reduce the energy consumption due to water heating, incentivized customer shutdowns of water heaters (WHs) have been applied to shave off peak demands. Thus, alternating cycles of elevated temperature and lower temperature operations during peak demand periods occur, and the high temperature cycles are analogue to heat shock treatments.

Electric water heaters are by design thermally stratified, unlike oil and gas water heaters and are therefore more susceptible to *Legionella* contamination (Alary & Joly, 1991; Dewailly & Joly, 1991; Marrie et al., 1994; Mathys et al., 2008; Stout et al., 1992; Wallet et al., 2016). This is attributed to the lower temperatures in the bottom of the water heater caused by thermal stratification and the presence of sediments. Indeed, sediments accumulate in the conical bottom sections of electric WHs, and potentially provide nutrients and growth-promoting conditions for multiplication of *L. pneumophila* and host cells and turn these devices into preferential niches for *Legionella* bacteria (Alary & Joly, 1991; Dewailly & Joly, 1991).

The main objective of this study was to investigate the effects of single and repeated 1 h- and 4 h- heat treatments at temperatures from 50 to 70 °C on the survival and growth of *V. vermiformis* and an environmental strain of *L. pneumophila* isolated from a health care facility faucet biofilm (Bédard et al., 2016b). The impact of repeated short-term exposures to higher temperatures followed by stagnation at lower temperatures was investigated to simulate conditions in hot water systems submitted to curative heat shock and water heater peak demand shutdowns. The dynamics of *V. vermiformis* and *Legionella* decay and growth were investigated in water and in water heater loose sediments (sludge) to evaluate the survival in the different phases present in water heaters and hot water systems. Water heater loose deposits were preferred to the liquid phase because more

hosts were expected, and the lower temperature encountered at the bottom of electric stratified water heaters would make these deposits a more susceptible niche for *L. pneumophila* to grow.

This study aims to provide evidence to support the risk assessment of periodical shutdowns of water heaters in order to allow electric demand peak shaving for energy conservation, while avoiding unintended consequences on water safety.

4.2 Materials and Methods

4.2.1 Preparation of Inocula

Amoeba Culture of the ATCC 50237TM strain of *Vermamoeba vermiformis* was performed using Falcon[®] T75 cell-culture vented flasks (Fisher Scientific, ON, Canada) with 25 mL of modified PYNFH media incubated at 30 °C. *V. vermiformis* trophozoites and cysts were enumerated using a haemocytometer. A volume of 100 µL was mixed with 300 µL of clean modified PYNFH media and 100 µL of 0.4% methylene blue, and 10 µL of this mix were put on the haemocytometer. The cells were then enumerated using an Olympus BX51 microscope using 100X magnification.

Flow cytometry was also used to enumerate amoebae and to differentiate viable and dead cells. All samples were diluted by a factor of 10, and each staining method was performed in duplicates with 300 µL of sample and 3 µL of SYBRGreen I (SG, 100x concentrated) or a mix of SYBRGreen and Propidium iodide (SGPI, end concentration of 6 µM of PI). Before the addition of the dye, the samples were incubated for 3 min at 37 °C. Once the plate was ready with samples and dyes, it was incubated in the dark for 10 min at 37 °C. The flow cytometry assay was then conducted using a BD Accuri-C6 flow cytometer and data were analysed with the Accuri sampler software (BD Biosciences, NJ, United States of America). Flow cytometer readings were conducted using FL1 (530–533 nm) and FL3 (>670 nm) filters. The FL1 threshold was set at 130,000 and the SSC threshold at 90,000.

For the flow cytometry data analysis, the sample heated at 100 °C was considered as the control for dead *V. vermiformis* and its graph of the red fluorescence (FL3) in function of the green fluorescence (FL1) was used to define the gate gathering all the dead amoebae in the SGPI-stained samples. Then, the same cytograms of FL3 vs FL1 were used to estimate the number of dead *V. vermiformis* (events in the gate) in SGPI-stained samples. For each temperature, the mean value of the percentage of dead cells of the duplicates was calculated.

Legionella An environmental strain of *Legionella pneumophila* serogroup 5 (CEAEQ isolate ID Q076826-03) was extracted from the biofilm of a faucet from a healthcare facility (Bédard et al., 2016b). The conservation and culture methods were those described in Bédard et al. (2021). Briefly, after storage at -80°C in 60% glycerol, it was cultured for 3 days at 36°C on buffered charcoal yeast extract (BCYE, Oxoid, ON, Canada). Resulting colonies were directly inoculated into sterile yeast extract broth with the Oxoid growth supplement SR0110 (Oxoid, ON, Canada) and incubated at 36°C for 18 h. Suspensions were centrifuged at $3,000\text{ g}$ for 30 min to harvest the cells, which were then washed twice with sterile water (simulated drinking water or $0.22\text{ }\mu\text{m}$ filtered water from water heaters, as presented in 4.2.3) and resuspended in the same medium at 1×10^8 cells/mL. Cells were starved for 5 to 10 days at room temperature.

4.2.2 Collection and Concentration of Water Heaters' Loose Deposits

Sediments were collected from two water heaters located in two different municipalities (A and B). The 2 water heaters were completely emptied, then partially refilled to resuspend all the deposits accumulated in the bottom. The water heaters were connected to a sediment trap system to collect the resuspended deposits. With this system, between 30 and 40 L were collected from each water heater. The deposits were then concentrated. For the water heater A, successive volumes of 250 mL of the mix of water and deposits were centrifuged 15 min at $1,500\text{ g}$, to recover 400 mL of sediments. The sediments were then equally distributed in 10 bottles and resuspended in a final volume of 500 mL with filtered water heater's water. For the water heater B, an additional step was required due to the presence of very light sediments that were not captured by centrifugation. Following centrifugation, supernatant was filtered on $0.45\text{ }\mu\text{m}$ to recover fine particles. Deposits' characterisation is provided in Table C.1.

4.2.3 Temperature inactivation testing

Temperature inactivation of *V. vermiformis*. Cells from two volumes of 25 mL of *V. vermiformis* culture were washed three times as follows: initial cultures were centrifuged 10 min at 200 g , the supernatant was then discarded, and the pelleted amoebae were resuspended in 25 mL of autoclaved tap water filtered on $0.22\text{ }\mu\text{m}$ for a final concentration of 1×10^5 cells/mL. The temperature assay was conducted on 5 mL of *V. vermiformis* suspension at 1×10^5 cells/mL. A total of 8 temperatures were tested with a contact time of 4 h: 25°C , 30°C , 37°C , 40°C , 43°C ,

50 °C, 55 °C and 60 °C. A test was also conducted at 100 °C and was considered as the control for dead amoebas.

Temperature inactivation of *L. pneumophila* in water (hot water distribution system conditions). Starved *L. pneumophila* cells were resuspended in 50 mL sterile tubes containing 20 mL of simulated drinking water (final concentration = 1×10^8 cells/mL). Simulated drinking water and starvation were performed as described in Bédard et al. (2021). Suspensions were treated for 1 h at 55 °C, 60 °C or 70 °C. After 1 h of thermal treatment, tubes were stored at 36.5 ± 0.5 °C in the dark and samples were taken for enumeration after 1 h, 6 h, 1 day, 2 days, 3 days, 1 week, 2 weeks and a month. A temperature of 36.5 °C was chosen as it is representative of the temperature encountered in temperature-controlled faucets like electronic faucets and thermostatic mixing valves (Bédard et al., 2016c; Sydnor et al., 2012). Negative (without *L. pneumophila*) and positive controls (*L. pneumophila* without thermal treatment, left in the dark at 36.5 ± 0.5 °C for the duration of the experiment) were also monitored. *L. pneumophila* concentrations were estimated by culture on BCYE agar, with an incubation in the dark at 36.5 ± 0.5 °C for 7 days, with a first enumeration after 3 days.

Temperature inactivation of *L. pneumophila* in water heater loose deposits. The short term (1 to 24 h) impact of the thermal stress on *L. pneumophila* was also assessed in the loose deposits collected from two water heaters located in two different municipalities (Table C.1). Both loose deposits were free of *L. pneumophila* when sampled (tested with the Legiolert enzymatic test (IDEXX, ME, United States of America)) and no hosts were observed by microscopy. Duplicate volumes of 18 mL of homogenised sludge were heated at the desired temperature and then inoculated with 2 mL of *L. pneumophila* suspension for a final concentration of 10^6 CFU/mL. Testing was conducted at six temperatures (40, 45, 50, 55, 60 and 65 °C) for 5 different durations (1 h, 2 h, 4 h, 6 h and 24 h). Negative control samples were monitored at $t = 0$ h and $t = 24$ h to evaluate the natural decay of *L. pneumophila* concentration without any thermal stress. The Legiolert enzymatic test was used to quantify culturable *L. pneumophila* using the 1 mL non-drinking water protocol and dilutions of the loose deposits.

To assess the impact of a daily exposure of 4 h at the target temperature within the water heater, repeated 4 h exposures at 55 °C followed by 20 h at 40 °C were performed on water heater sludges inoculated with *L. pneumophila*. Inactivation of *L. pneumophila* after repeated exposure to heat

was determined using duplicates of 100 mL of each sludge inoculated with a final concentration of 1×10^6 cells/mL and left in the dark at 40 °C for 72 h. Then, samples were exposed to a temperature of 55 °C for 4 h and then to 40 °C for 20 h. Deposit samples were then exposed daily to 55 °C for 4 h, for 5 heating cycles in total. Between cycles, samples were kept in the dark at 40 °C. Samples were taken before each heating cycle to quantify the culturable *L. pneumophila* concentration using the Legiolert enzymatic test 1 mL non-drinking water protocol following the manufacturer's instructions.

Temperature inactivation of *L. pneumophila* in water heater loose deposits in the presence of *V. vermiformis*. For this assay, 100 mL of each loose deposits containing 1×10^6 cells/mL of *L. pneumophila* were treated for 4 h at 40 °C, 55 °C and 60 °C. Following treatment, for each suspension and each temperature, samples were divided in four identical volumes of 2 mL. The first two volumes were spiked with *V. vermiformis* to a final concentration of 1×10^6 cells/mL and the two remaining volumes were kept as controls without amoeba. Tubes were then incubated in the dark at 36.5 ± 0.5 °C for 4 days and 7 days, and the concentrations of culturable *L. pneumophila* were measured using the enzymatic Legiolert test.

4.3 Results

4.3.1 Thermal Inactivation of *L. pneumophila* and *V. vermiformis* in Hot Water

4.3.1.1 Impact of a Short Duration Heat Shock on *L. pneumophila* in Water

The aim of the first assay was to evaluate the impact of a short duration (1 h) heat shock (55 °C, 60 °C and 70 °C) applied to *L. pneumophila* before its storage at a temperature of 36.5 ± 0.5 °C for up to a month. This combination simulates the conditions faced by bacteria during a short heat shock in a dead-end of a building water system: high temperature in the water network for 1 h, then the temperature comes back to its previous value (Figure 4.1).

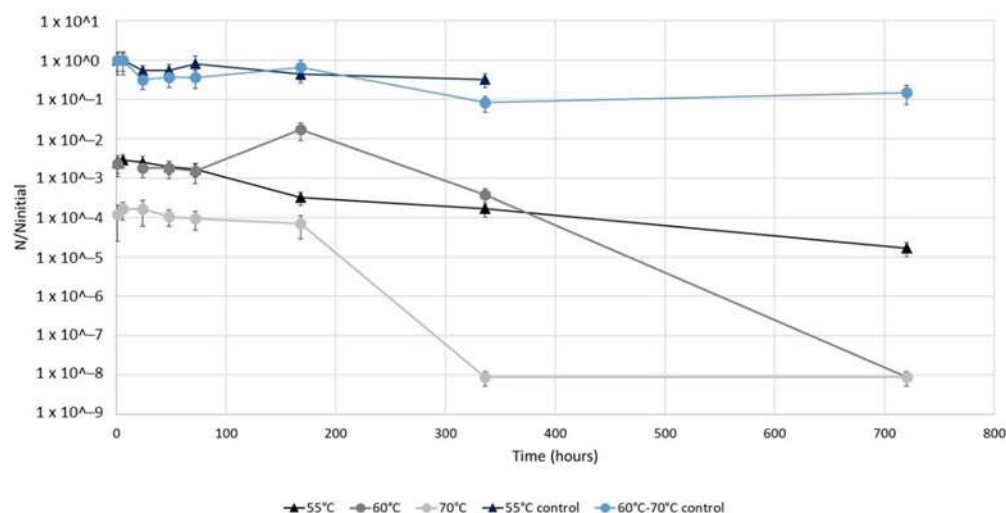


Figure 4.1 Temporal evolution of the ratio of the concentrations of culturable *L. pneumophila* in samples after a 1 h heat treatment at 55 °C, 60 °C or 70 °C on the initial concentrations of culturable *L. pneumophila* in respective controls left at 36.5 ± 0.5 °C. Experiments were conducted on an environmental *L. pneumophila* strain previously starved in simulated drinking water at room temperature. After the 1 h heat shock, bacteria were stored at 36.5 ± 0.5 °C for up to 1 month.

L. pneumophila concentrations in control suspensions left at 36.5 ± 0.5 °C during the experiment decreased by one log only after 336 h (for exact values, see Table C.3). The first point of each curve represents the initial impact of the 1 h heat shock at each temperature after just 1 h of storage. Thus, the heat shocks at 55 °C and 60 °C resulted in an immediate 2.5 log reduction in the culturable *L. pneumophila* populations, while at 70 °C the culturable *L. pneumophila* population was reduced by 4 logs. Then, for the first 168 h (7 days) of storage, culturable *L. pneumophila* suspensions remained constant, except for the sample at 60 °C for which an increase of a log was observed after 168 h. Finally, after 168 h of storage, the concentrations of culturable *L. pneumophila* decreased in all the heat-treated samples, with an absence of these bacteria reached after 720 h (5-log reduction) and 336 h (4-log reduction) of storage at 60 °C and 70 °C, respectively. After a 1 h treatment at 55 °C, a reduction of only 2 logs was reached at the end of the experiment (720 h of storage).

4.3.1.2 Impact of Elevated Temperatures on the Form and Decay of *V. vermiformis* in Hot Water

This assay aimed to evaluate the survival of *V. vermiformis* in water at temperatures representative of different parts of drinking water and hot water networks. The survival of amoebae *V. vermiformis* was evaluated by exposing them to different temperatures for 4 h, followed by their enumeration by microscopy and flow cytometry.

Microscopic measurements revealed a relatively constant total population of *V. vermiformis* (between 2×10^4 and 2×10^5 cells/mL, exact values given in Table C.2) with minimal lysis. However, a shift between the trophozoite and cysts forms of *V. vermiformis* is observed at temperatures above 40 °C, with cysts becoming predominant at 55 °C (Figure 4.2).

Flow cytometry analyses were performed on the same heat-treated *V. vermiformis* suspensions (Figure 4.3). The cytograms obtained for temperatures from 25 °C to 40 °C show similar partitioning between the viable and dead amoebae, as well as those obtained from 43 °C to 60 °C. The sample heated at 100 °C was considered as the dead control. Because the only population obtained from 43 °C to 60 °C on FL1-FL3 cytograms seemed equivalent to the one observed at 100 °C, it can be assumed that this population is representative of the dead amoebae *V. vermiformis*. The proportion of dead amoebae estimated by flow cytometry on Figure 4.3 shows a drastic shift between 40 °C and 43 °C. Indeed, the increase of 3 °C induced a transfer from 25% of dead *V. vermiformis* to more than 90% of dead amoebae.

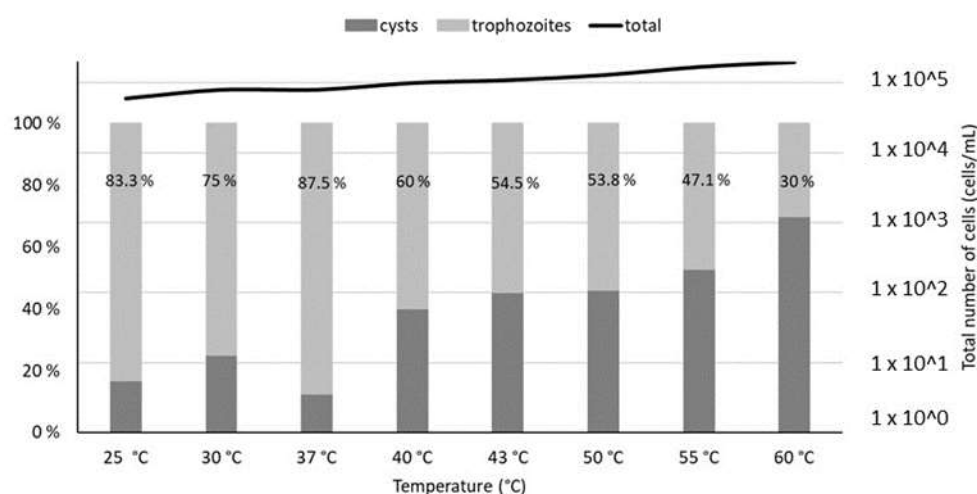


Figure 4.2 Trophozoites and cysts percentages estimated by optical microscopy (100X magnification), after a 4 h thermal treatment of *V. vermiformis* suspensions at temperatures from 25 °C to 60 °C. The percentages of trophozoites in the total *V. vermiformis* population are indicated on the graph. The line represents the total population of amoebae in each sample.

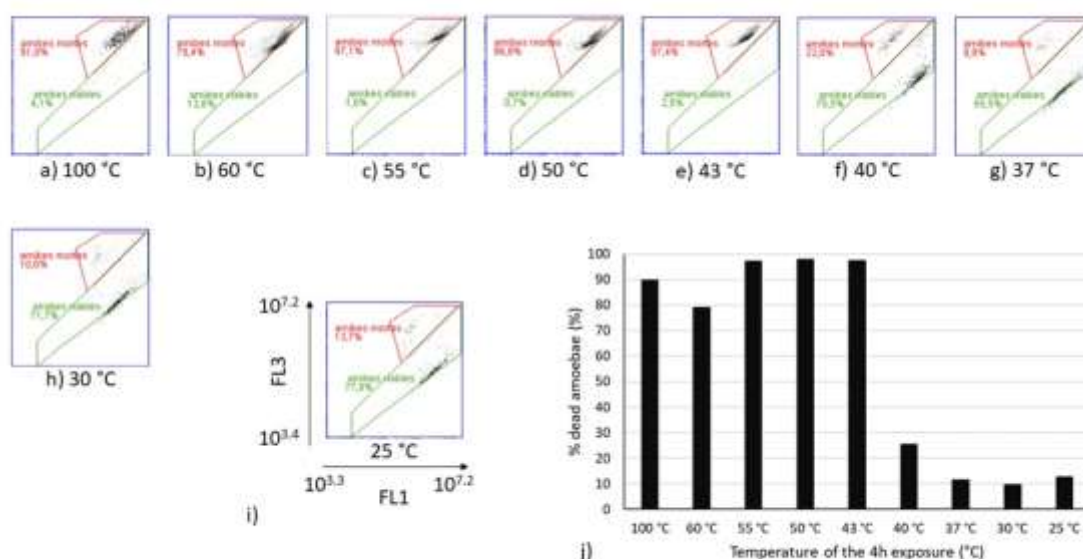


Figure 4.3 Cytograms of heat-treated *V. vermiformis* samples with SYBRGreen I and Propidium iodide (SGPI) staining (Figure 3 a) to h)), and evolution of the percentage of dead *V. vermiformis* depending on the temperature of the 4 h treatment (Figure 3 j). Figure 3 i) is a zoom on one of the cytograms with the specification of the scales of the axes. The gate in red dot lines delimited the population of dead amoebae. “amibes mortes” means dead amoebae and “amibes viables” means viable amoebae. For the percentages indicated on the cytograms, a comma separates the decimals.

4.3.2 Thermal Inactivation of *L. pneumophila* in Loose Deposits of Water Heaters

Different contact times, as well as repeated short heat exposures, were performed on a hot water system-adapted *L. pneumophila* strain to evaluate its survival in water heater sludge.

4.3.2.1 Heat Exposures of Different Durations

Most of the microorganisms present in electric water heaters are found in the loose deposits at the bottom of the water heater, a niche providing particles for attachment, nutrients, and lower temperatures. To investigate the impact of a thermal treatment on *L. pneumophila* in resuspended loose deposits of electric water heaters, heat treatments of up to 24 h were conducted on two samples of resuspended water heater loose deposits inoculated with 1×10^6 CFU/mL of a starved environmental strain of *L. pneumophila*.

Culturable *L. pneumophila* were detected in the resuspended sludges at various levels (from 2.49×10^2 to 1.27×10^6 MPN/100 mL) after a 24 h exposure to temperatures between 40 °C and 55 °C (Figure 4.4). A 3-log reduction was achieved at 50 °C with a long exposure of 24 h, while the culturable population remained constant at 45 °C, and showed an almost 1-log increase at 40 °C. On the other hand, after 24 h at 55 °C a few bacteria were still detected. At 60 °C and 65 °C, short two- and one-hour contact times were sufficient to completely abate the culturable population.

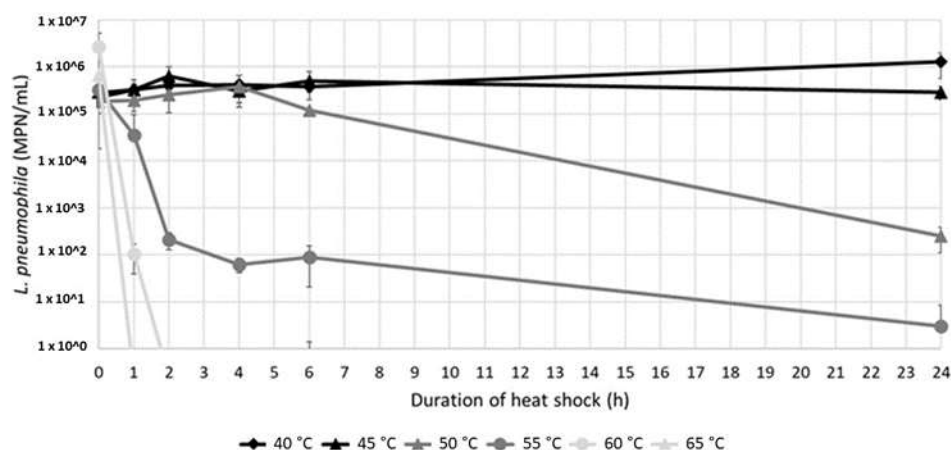


Figure 4.4 *L. pneumophila* concentrations (mean value of sludges A and B, n = 4) depending on the contact time and temperature. The detection limit of the enzymatic test is 1 MPN/mL.

Two different inactivation kinetics were observed at 50 °C and 55 °C. At 50 °C, no inactivation was observed in the first 4 h, followed by a progressive decrease after 4 h (Figure 4.4). On the contrary, at 55 °C, culturable *L. pneumophila* rapidly decreased after a contact time of 2 h, followed by a slower rate of decrease.

4.3.2.2 Impact of Repeated Short Heat Shocks

Once present within the recirculating hot water system, bacteria undergo periodic heat exposures every time they flow through the water heater. This was simulated by repeating the exposure to heat assay performed on the environmental strain of *L. pneumophila*. In this assay, a drastic decrease of 5 logs of the culturable *L. pneumophila* population after the initial 4 h heat shock at 55 °C was observed (Figure 4.5). The following four heat shocks gradually further reduced the culturability of this population, and no more culturable *L. pneumophila* could be found on day 4 in both sludges. A final measurement performed 72 h after the final heat shock showed no reactivation of the bacteria population left for 20 h at a favourable temperature of 40 °C after heat shock.

4.3.3 Resuscitation of *L. pneumophila* by *V. vermiformis* in Water Heater Loose Deposits

Finally, *L. pneumophila* cells were exposed to *V. vermiformis* following temperature inactivation to assess the potential for regrowth downstream in the distribution system, in the presence of hosts. Here, the role of amoebae in the regrowth of *L. pneumophila* after a heat shock was evaluated in resuspended water heater loose deposits. The addition of *V. vermiformis* 4 days after a 4 h exposure at 40 °C did not impact the culturable *L. pneumophila* concentrations (difference of less than 0.5 log), the latest remained relatively constant at more than 1×10^6 cells/mL (Figure 4.6A). However, at 55 °C, although a small increase of less than a log was observed for culturable *L. pneumophila* concentrations 4 days after the heat shock, *L. pneumophila* concentrations in sludges inoculated with *V. vermiformis* increased by 5 logs as compared to sludges without hosts, so there was an important regrowth in the presence of amoebae (Figure 4.6B). Moreover, the concentration remained constant for the following 72 h. At 60 °C the culturable *L. pneumophila* concentrations remained null with or without hosts (data not shown).

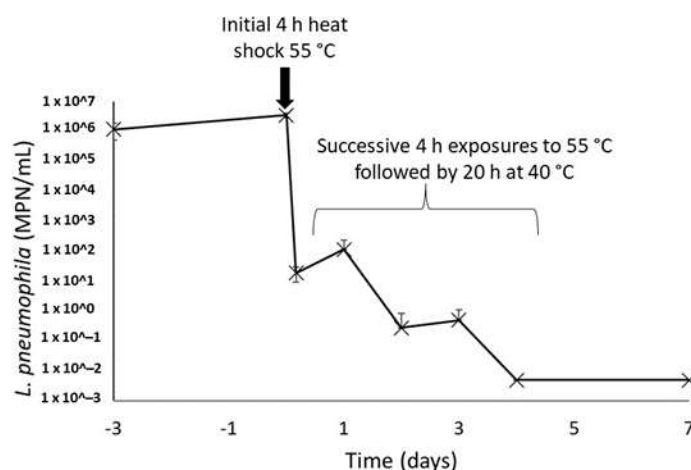
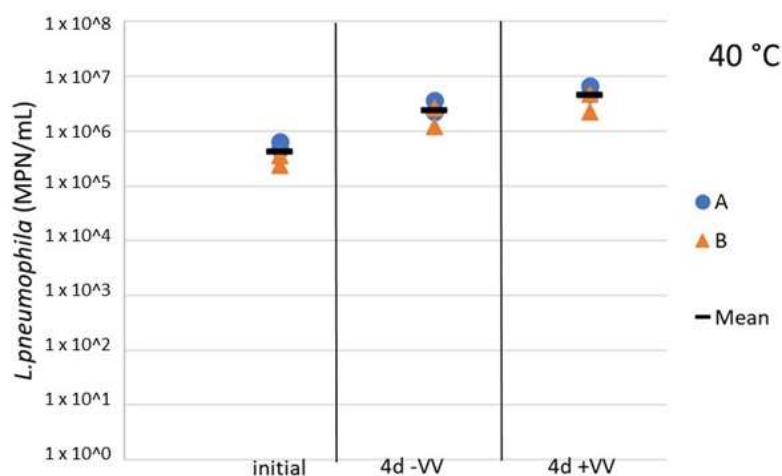
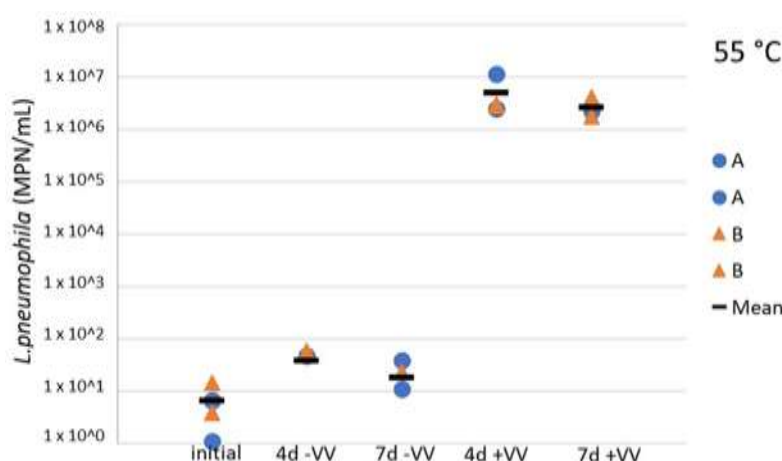


Figure 4.5 Concentrations of culturable *L. pneumophila* in water heater sludges after repeated 4 -h thermal shocks at 55 °C. The concentrations on days 4 and 7 were under the detection limit of the enzymatic test in all samples, duplicate of each water heater loose deposits.



(A)



(B)

Figure 4.6 Concentrations of culturable *L. pneumophila* during storage at 37 °C in water heater loose deposits subsequent to a thermal shock of 4 h at 40 °C (A) and 55 °C (B), with and without the presence of amoebae *V. vermiformis*. Blue dots are for sludge A samples, orange triangles represent results from sludge B samples, and black horizontal lines illustrate the mean values of all samples. +VV = sludges inoculated with *V. vermiformis*, -VV = sludges free of *V. vermiformis*.

4.4 Discussion

4.4.1 Thermal Heat Shock Inactivation of *L. pneumophila* and *V. vermiformis* in Water Heaters and Hot Water Systems

Curative heat shock treatments at temperatures of 60–75 °C have been used by many healthcare facilities as temporary curative actions in response to elevated concentrations of *Legionella* spp. or *L. pneumophila* (ASHRAE, 2020; National Academies of Sciences, 2019). Notably, the

effectiveness of shock heat treatment is limited in time and recolonization of the treated systems is often observed (ASHRAE, 2020; Epalle et al., 2015).

In this study, we found that a 1 h heat exposure led to an immediate 4-log reduction of culturable *L. pneumophila* at 70 °C and 2.5-log reduction at 55 °C and 60 °C in simulated drinking water. Following the 1 h exposure at 60 °C and 70 °C, complete loss of culturability was reached when left stagnant at 36.5 °C after 1 month and 2 weeks respectively. Indeed, after the initial decrease, concentrations of culturable *L. pneumophila* remained constant for the first 168 h (1 week) of storage, before starting to decrease again. The constant phase following the first decrease may be due to the necrotrophic ability of *L. pneumophila* (Temmerman et al., 2006). In comparison, a 1 h exposure at 65 °C led to a reduction in culturable *L. pneumophila* greater than 6 logs in resuspended loose water heater deposits, while 4-log and 1.5-log reductions were reached after a 1 h exposure at 60 °C and 55 °C respectively in the same type of sample. These results suggest that some *L. pneumophila* can survive a thermal shock of 30 min at 70 °C in water and sludge and can persist in a water heater or a hot water network and could then seed distal parts of the distribution systems.

Resistance of *V. vermiformis* to heat A rapid shift from a predominantly trophozoite form to a predominantly cyst form is observed between 50 °C and 55 °C as shown by microscopy. Flow cytometry confirms a shift from viable to dead amoebae occurring at lower temperatures between 40 °C and 43 °C. These results suggest an elevated susceptibility to temperatures greater than 43 °C associated with a significant loss of membrane integrity as determined by PI staining. The reference strain used in this study (*Hartmannella vermiformis* Page–ATCC 50237) was isolated from the drain of a hospital cooling tower. Cooling tower water temperatures typically do not exceed 48 °C. Our observations are in agreement with a previous study showing that *V. vermiformis* in biofilms were 2-log lower at 42 °C than at 38 °C and that the lack of a thermotolerant host at the highest temperature may prevent the proliferation of *L. pneumophila* in the studied system (van der Kooij et al., 2016). The results also concurred with the hypothesis that thermotolerance is strain dependent. Thus, depending on the origin of the strain of *V. vermiformis* used for the assays, studies showed absence of *V. vermiformis* in biofilms at 41 °C (van der Kooij et al., 2016) while others reported 1-log and 2-log reductions after an exposure at 50 °C for 30 min (Cervero-Aragó et al., 2013) and 60 min (Fouque et al., 2015a).

The resistance to heat of amoebae can be attributed to the strain intrinsic resistance, the form in which it is present (trophozoite or cyst) and the method to assess its persistence. Indeed, amoebae have 2 stages in their life cycle: the mobile form of trophozoite, and a dormant form emerging under prolonged unfavourable conditions, the cyst (Scheid, 2019b). The cysts are known to be more resistant to numerous disinfectants and to temperature (Cervero-Aragó et al., 2013; Scheid, 2019b). However, depending on the enumeration technique used and the strain studied, reported levels of heat resistance of *V. vermiformis* vary. Thus, studies using sequencing or qPCR to enumerate *V. vermiformis* after heat treatment (Farhat et al., 2012) or thermal stresses (Rhoads et al., 2015), and studies based on culture (Fouque et al., 2015a; Rohr et al., 1998) reported various heat resistance.

Detection and quantification of *V. vermiformis* Although the presence of hosts in a water system is considered as the determining factor for the proliferation of *L. pneumophila*, the methods available for the detection of protozoan hosts are cumbersome. In this study, flow cytometry was used to enumerate amoebae. Flow cytometry (FC) presents numerous advantages over the standard culture and microscopy methods. First, it provides a faster response than culture, as the results can be obtained hours after sampling. Moreover, a significant advantage when dealing with environmental samples, detection of amoebae by culture is difficult to achieve while it is more convenient using flow cytometry. The staining and flow cytometry protocols used in this study for the monitoring of *V. vermiformis* were adapted from protocols for the differentiation and enumeration of viable and dead bacteria in water and biofilm samples (Gatza et al., 2013). Flow cytometry has been previously used to study amoebae, especially to follow the encystation process and to differentiate viable and dead cells (Borazjani et al., 2000; Mi-ichi et al., 2018). However, this study is, to our knowledge, the first to adapt an FC-staining procedure for the quantification of bacteria to the enumeration of amoebae in water samples. The results obtained showed that the staining and FC protocols used allowed the differentiation of viable and dead amoebae in water. Still, further investigations are needed to confirm which life cycle state of *V. vermiformis* compose each population observed on the cytograms, and to assess whether the gates defined here can be accurately applied in environmental samples where higher densities of other microorganisms are present.

4.4.2 Thermal Inactivation of *L. pneumophila* in Water and Hot Water Loose Deposits

The resistance of the environmental strain isolated from a healthcare facility in Québec was assessed in two types of loose deposits. The strain appears to be well adapted to warm water temperatures considering the absence of inactivation at 40 °C and 45 °C, as compared to the inactivation constants reported in previous studies (Sanden et al., 1989; Stout et al., 1986), and its persistence at 50 °C and 55 °C. Inactivation kinetic constants were calculated for each temperature based on the inactivation curves of the 24 h exposure of *L. pneumophila* in water heater's loose deposits, and were compared to those obtained in previous studies (Table 4.1). The graphical method proposed by Körmendy and Körmendy (1997) was applied (Kormendy & Kormendy, 1997). The inactivation of *L. pneumophila* at 55 °C does not follow a simple first order kinetics as proposed in the Bigelow's method (Kormendy & Kormendy, 1997) (data shown in Figure C.1). Moreover, for all the temperatures tested, the inactivation kinetic constants were lower than those reported by Sanden et al. and Stout et al., somehow indicating that the current tested strain is more resistant to heat than those tested by these authors (Sanden et al., 1989; Stout et al., 1986) or is more acclimatized to its environment prior to the application of heat stress.

Table 4.1 Inactivation kinetic constants of *L. pneumophila* for temperatures between 40 °C and 60 °C. Sanden et al. (1989) is for a strain of *L. pneumophila* serogroup 1 isolated from cooling tower water, inactivation measured in chlorine-free water. Stout et al. (1986) is for 3 *L. pneumophila* strain of serogroup 5, inactivation measured in Buffered Yeast Extract Broth (BYEB).

Temperature and Time Lapse	k in This Study (h ⁻¹)	k in Previous Studies
From 0 to 24 h, 40 °C	0 *	
From 0 to 24 h, 45 °C	0 *	k = 0.024 h ⁻¹ [50]
From 0 to 4 h, 50 °C	0 *	
From 4 to 24 h, 50 °C	0.16	k = 0.16 h ⁻¹ [50]
From 0 to 2 h, 55 °C	1.6	k = 4.3 h ⁻¹ (measured on 20 min) [50]
From 2 to 24 h, 55 °C	0.034	
From 0 to 1 h, 60 °C	4.27	k = 81 h ⁻¹ [50] k from 17.6 to 26 h ⁻¹ (measured on 20 min) [51]

* These constants were considered null because there was no inactivation.

Heat resistance of *L. pneumophila* has been shown to vary between strains (National Academies of Sciences, 2019) and environmental strains of *L. pneumophila* isolated from hot water systems can develop a greater resistance to temperature, even above 60 °C (Allegra et al., 2008; Bédard et al., 2019). During heat treatment experiments conducted on 4 *L. pneumophila* strains (2 references and 2 environmental isolated from hot tap water), Cervero-Aragó et al. noted significant differences between the inactivation patterns by culture depending on the strain and the temperature applied (50 °C, 55 °C, 60 °C, 65 °C and 70 °C) (Cervero-Aragó et al., 2015). Similarly, Allegra et al. obtained different thermal inactivation curves for 12 *Legionella* strains at 70 °C using flow cytometry (Allegra et al., 2008). Indeed, after 30 min at 70 °C, the authors were still able to detect between 10% and 25% of viable but nonculturable (VBNC) cells of 6 of the 12 tested strains of *Legionella*.

The general resistance of *Legionella* to temperature is typically assessed in laboratory experiments using suspensions of cultured *Legionella* exposed to a constant temperature. However, the results of such assays and the resulting resistance to temperature are influenced by the state of the bacteria. Indeed, it was shown that starving bacteria prior to temperature exposure can increase their resistance to environmental stress (Chang et al., 2007). Thus, Chang and colleagues showed that the heat disinfection efficiency on *L. pneumophila* cells starved for 1 to 2 months decreased, revealing the resistance of long-term starved *L. pneumophila* against thermal disinfection (Chang et al., 2007). It must be noted that the strain used in this study was washed and acclimatized to low nutrient conditions before exposure to heat. This can be one of the factors explaining the lower inactivation kinetic constants as well as the resistance to temperature up to 55 °C found in the present work.

Implications for dynamic hot water systems *Legionella* and their hosts present in recirculating hot water distribution systems undergo repeated heat exposures associated with:

- (1) passage through the water heater and reservoirs, before returning in the hot water distribution system;
- (2) storage in the water heater or reservoirs during periods of low or no use. Temperature conditions will vary depending on the water heater type, hot water demand and stratification. For instance, thermal stratification typically results in 10 to 15 °C lower temperatures in the bottom part of the reservoir of electric water heaters;

- (3) exposure in the recirculation loops. In a well-balanced system, a temperature of more than 55 °C should be maintained.

When water heaters are operated dynamically to conserve energy during peak demand periods, *Legionella* will be exposed to lower temperatures during shutdown periods followed by periods of elevated temperatures during normal usage. Our experimental results of heat shock simulations show that, when heated at 60 °C in presence of water heater loose deposits, it took between one and two hours for suspended *L. pneumophila* to completely lose cultivability, while less than an hour was required at 65 °C. Thus, a 4 h- exposure to temperatures equal or higher than 60 °C could inactivate *Legionella* that is present in the water.

Our results also provide some insight in the potential benefits from repeated cycles of elevated temperature to provide a barrier for the proliferation of *Legionella* in water and sludge of water heaters. The repetition of 4 h exposures at 55 °C on water heaters sludges inoculated with *L. pneumophila* followed by 20 h at 40 °C simulated night-time temperature exposure followed by lower temperatures during the day. Results revealed an absence of culturable *L. pneumophila* after 3 cycles of 4 h exposure at 55 °C, and no regrowth was observed after 72 h at 40 °C. Thus, comparatively to single exposures where culturable *L. pneumophila* were still detected after 24 h at 55 °C, repetitive heating appeared more effective against the studied environmental strain.

It should be noted that this experiment was conducted in laboratory conditions and not in a real water heater, and that no measurement was made on the medium term (several weeks) to verify if repeated heating was still efficient or if the strain was able to adapt. On the other hand, a 4 h- exposure at 60 °C and 65 °C was sufficient to eliminate all culturable *L. pneumophila* in water heaters' loose deposits, suggesting that fixing the regulated temperature of water heaters to 60 °C would efficiently prevent *L. pneumophila* proliferation in these devices if the temperature is also reached at the bottom of the water tank. These observations are in accordance with a study where the hot water temperature at the water heater outlet was increased from 55 to 60 °C. They reported a gradual decrease of *L. pneumophila* concentrations and positivity over 18 months (Bédard et al., 2016a). Rhoads and colleagues also showed that the temperature setting (from 39 °C to 58 °C) of continuously recirculating water heaters was a crucial factor for the prevention of *L. pneumophila* growth in recirculating water lines and distal faucets (Rhoads et al., 2015).

4.4.3 Resuscitation of *L. pneumophila* by *V. vermiformis* after a 4 h-Heat Treatment in Water Heater Loose Deposits

The co-cultivation of heated *L. pneumophila* with *V. vermiformis* in water heater sludges clearly highlighted the role hosts can play in reviving *L. pneumophila* after thermal treatments. As other *Legionella* species, *L. pneumophila* can survive phagocytosis and multiply in many protozoans like ciliates and amoebae (Best & Kwaik, 2018). Integration and replication of *L. pneumophila* in *V. vermiformis* has already been observed in several studies (Fields et al., 1993; Thomas et al., 2006).

Here, 4 days after a 4 h treatment at 55 °C, *L. pneumophila* concentrations were 5-log higher in presence of *V. vermiformis* than in the absence of amoeba, and the concentrations remained stable for 3 more days. However, the presence of amoebae was not sufficient to increase the concentrations of *L. pneumophila* previously treated at 60 °C for 4 h, either by resuscitation or growth of survivors. These findings are similar to those of Cervero-Aragó et al. who reported that the association amoeba-bacteria between *Acanthamoeba* spp. and *L. pneumophila* had the most detrimental impact on the effectiveness of thermal treatments at 50 °C and 55 °C compared to the control without amoeba (Cervero-Aragó et al., 2015).

VBNC *L. pneumophila* can invade and replicate in amoebae, leading to the resuscitation of these bacteria and the release of more viable bacteria in the immediate environment (García et al., 2007; Thomas & Ashbolt, 2011). In fact, protocols have been defined to resuscitate VBNC *Legionella* cells via the co-culture with amoebae (Steinert et al., 1997). However, when *L. pneumophila* is exposed to higher temperatures, cells are no longer viable and cannot be resuscitated by the presence of hosts. Results from Cervero-Aragó et al. support this, since the presence of a host did not increase the exposure time required to achieve 4-log reduction at higher temperatures (60 °C, 65 °C and 70 °C) (Cervero-Aragó et al., 2015). In addition to helping *Legionella* bacteria to survive heat treatments, the presence of amoebae also influences the resistance to disinfection and the infectivity of these bacteria. Indeed, it was underlined by several studies that *L. pneumophila* were more resistant to antibiotics and other disinfectants after their passage through amoebae and were also more infectious (Barker et al., 1995; Cirillo et al., 1994). Epalle et al. showed that heat-treated environmental *L. pneumophila* resuscitated in *A. polyphaga* were able to infect U937 and HL-60 macrophage-like cells while they could not infect these same cells right after the heat treatment at 70 °C for 30 min (Epalle et al., 2015). The authors underlined that amoebae present in hot water

systems may facilitate the resuscitation of VBNC *L. pneumophila* induced by thermal treatments and may possibly lead to outbreaks (Epalle et al., 2015).

Because of the key role played by protozoans in the persistence of *L. pneumophila* in water distribution systems, and in its resistance to many remediation strategies, it should be a priority to better understand the impact of disinfectants and temperature on all the microorganisms associated with *L. pneumophila* in water systems. This is especially the case for protozoan hosts, as host control appears to be the best approach to tackle more effectively the proliferation of *L. pneumophila* in such systems (Buse et al., 2013; Falkinham et al., 2015a; National Academies of Sciences, 2019).

4.4.4 Limitations of the Study

To our knowledge this work is the first to monitor the impact of temperature on *L. pneumophila* in loose deposits collected from the bottom of water heaters and in hot water, and to evaluate the influence of the presence of a host after thermal treatments. Nevertheless, only a single *L. pneumophila* environmental strain was tested, and it would be interesting to investigate the fate of other strains in similar conditions. Moreover, in this study *L. pneumophila* were added to the water and the sludges samples which originally did not contain any *Legionella* bacteria. The microbial diversity present at first in the loose deposits may affect the survival of *L. pneumophila* in these samples during the study and the results of similar assays conducted on different deposits samples may lead to different conclusions. Moreover, biofilms and hosts present in plumbing can help protect *L. pneumophila* against temperature, but these factors were not present in our lab experiment.

In this study, only culturable *L. pneumophila* were monitored, using culture on BCYE media and an enzymatic test. However, several studies using other techniques such as flow cytometry mentioned the presence of viable but non culturable (VBNC) *Legionella* bacteria after heat treatments. For instance, VBNC *L. pneumophila* of 3 different strains (one reference, one clinical and one environmental) were still detected by flow cytometry after 30 min at 60 °C and 70 °C (Epalle et al., 2015). Moreover, even if culture is considered the best indicator of risk of infection, the loss of culturability does not necessarily equate to the loss of infectivity. Indeed, it was shown that VBNC *L. pneumophila* cells produced by heat exposure at 55 °C, 60 °C and 70 °C were able

to infect THP-1 macrophages and *Acanthamoeba castellanii* cells for 85 days at 55 °C and 60 °C, and 8 days at 70 °C (Cervero-Aragó et al., 2019).

Finally, only one strain of amoeba was used in this study. Thus, different results may be obtained with another strain or another genus of amoebae if used in a thermal inactivation assay. Indeed, Cervero-Aragó and colleagues showed that an exposure of 30 min at 50 °C of a lab strain of *V. vermiformis* induced a reduction of more than 2 logs of its trophozoite form and a reduction of less than 1 log of its cyst form, while the reduction of cysts of an environmental strain of *V. vermiformis* subjected to the same test was higher than 1 log (Cervero-Aragó et al., 2013). The authors conducted the same assay on a lab and an environmental strain of *Acanthamoeba* and reported a higher decrease of the concentrations of their trophozoites (3- log reductions) but similar reductions of their cysts concentrations (Cervero-Aragó et al., 2013). Other studies reported reductions of 1 log of cysts of *Acanthamoeba castellanii* after 10 min at 55 °C (lab strain) (Storey et al., 2004), and a reduction of 5 logs after 60 min at 60 °C (clinical isolate) (Coulon et al., 2010). Thus, it may be interesting to repeat a similar assay as the one described in the present study with other hosts of *L. pneumophila* found in water systems to assess their thermal resistance and the risk they may represent in the context of energy saving measures.

4.4.5 Considerations for the Operation of Residential Water Heaters and Hot Water Distribution Systems

The results of the present study provide additional evidence that reducing the set point temperature or sporadic shutdown of water heaters in buildings to conserve energy should be subjected to a risk analysis to carefully establish its benefits. Reducing water usage and lowering hot water temperature may promote the proliferation of opportunistic premise plumbing pathogens, such as *Legionella* (Dewailly & Joly, 1991; Falkinham, 2020).

Domestic water heaters have been shown to be frequently positive for *Legionella*, especially in the loose deposits found at their bottom, and positivity has been clearly correlated with temperature. *L. pneumophila* was detected in loose sediments of 45% of devices set at temperatures lower than 40 °C, while it was detected in only 14% of devices set at higher temperatures (Dewailly & Joly, 1991). Water heater shutdown to accommodate peak demand periods should be implemented with compensating measures, such as increased base line temperature and improved design, to minimize thermal stratification and the accumulation of loose deposits during normal operations.

In residential and large building hot water systems, sporadic lower temperature conditions are likely to increase the potential for *Legionella* growth and the associated exposure risk for the occupants, especially in buildings housing at-risk populations such as healthcare facilities, long term care and retirement homes containing aerosol-producing devices. To reconcile energy conservation and infection prevention, measures should be taken to limit the additional risk associated with these periods of vulnerability. Optimizing the operation of the water heater and water system can be achieved through multiple actions such as improving hydraulics, maintaining optimal thermal regime, increasing temperature set-points, and adding on-site disinfection.

4.5 Conclusions

- Exposure of an environmental strain of *L. pneumophila* to 60 °C for 1 h led to a 2.5-log to 4-log loss of culturability in simulated drinking water and resuspended water heater loose deposits.
- Exposure to temperatures over 43 °C decreases the viability of *V. vermiformis* and causes a rapid shift to the dead form.
- Successive daily exposures at 55 °C for 4 h can prevent the growth of culturable *L. pneumophila* after one week.
- After a 4 h exposure to 55 °C, *L. pneumophila* concentrations with *V. vermiformis* are 5 log higher than without host 4 days after the treatment, and concentrations remain constant for at least 3 more days.
- The relevance of meeting regulated, and recommended water heaters and hot water system minimal set temperatures were confirmed.

Acknowledgments: The authors would like to acknowledge Tristan Marrié, Océane Perras, and the industrial chair technical staff for their help, as well as Jacinthe Mailly for her help in the analysis of the flow cytometry measurements. They also would like to thank local utility for providing data on residential hot water usage.

CHAPTER 5 ARTICLE 2: WHAT HAPPENS IN YOUR WATER SYSTEM? IMPACT OF MATERIALS, TEMPERATURE, STAGNATION AND CHLORINATION ON WATER QUALITY AND BIOFILM FORMATION

In premise plumbing a better understanding of the interplay between design and operational factors and its impact on chemical and microbial water quality as well as biofilms formation is needed. In this chapter, the impact of materials, temperature, stagnation, and shock chlorination on water chemical (metals, dissolved organic carbon) and microbial (total and intact cells) quality, and on biofilm formation (structure and total and intact cells) is assessed through a pilot-scale study. This paper was submitted on April 7th 2023 to *Water Research*. Supplementary information is presented in Appendix D.

What happens in your water system ? Impact of materials, temperature, stagnation and chlorination on water quality and biofilm formation

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Keywords: drinking water, plumbing materials, water temperature, stagnation, chlorination, dissolved organic carbon

Highlights:

- EPDM leaches Pb in drinking water ($>5\mu\text{g/L}$) while copper biofilms accumulate Pb
- Materials show diverse biofilm densities : EPDM > PP > PVC > PEX > Cu – SS
- Heating at 40, 55 and 60°C leads to striking leaching of DOC/BDOC by all materials
- Limited cells recovery after 6-month stagnation in copper previously at 55-60°C
- High BDOC release followed shock chlorination, highlighting the need of flushing

Abstract

Materials used in water distribution systems of large buildings are diverse and their impact on water quality depends on nutrients, stagnation, and temperature. The main objective of our study was to investigate the interplay between material type, temperature and stagnation on water quality and the potential for biofilm formation. CDC biofilm reactors were used to compare concentrations of metals, total (DOC) and biodegradable dissolved organic carbon (BDOC), and total and viable cells densities in water and biofilms in contact with coupons of 6 materials: polypropylene (PP), polyvinyl chloride (PVC), EPDM, cross-linked polyethylene (PEX), stainless steel (SS) and copper (Cu). All materials were colonized by bacteria, with denser biofilm observed on EPDM > PP > PVC > PEX > Cu – SS. Copper and EPDM reactors showed different dynamics of accumulation and release of Cu and Pb, with bulk Pb concentrations exceeding $5\mu\text{g/L}$. Increasing temperatures from 25°C to 40, 55 and 60°C resulted in an increase by at least a factor 2 of DOC and BDOC, a temporary decrease in cell viability (%) and contrasting trends for suspended total bacteria (up to 1.4-log). Biofilm densities remained lower for all reactors after two months of constant heating at 40°C, and even further for copper reactors heated to 55°C (2.5-log) and 60°C (2.7-log). A 4-week stagnation at room temperature promoted recovery of total bacterial densities in bulk water and biofilm for all materials except copper. Extended stagnation to 6 months (COVID-19 shutdown) further increased total bacteria and the viable fraction in water, except in copper reactors.

5.1 Introduction

Water distribution systems are complex systems composed of a large diversity of materials, both metals and plastics, in service lines and fixtures (Waines et al., 2011). Copper (Cu) pipes are often used in water distribution systems because of their potential antimicrobial properties (Moritz et al., 2010; Waines et al., 2011). Stainless steel (SS) represents a good alternative to copper pipes because of its long period of resistance to corrosion (Proctor et al., 2020; Waines et al., 2011). Plastics like polypropylene (PP) and polyvinyl chloride (PVC) are mostly found in DWDS, while ethylene propylene diene monomer (EPDM) rubber and cross-linked polyethylene (PEX) are found in flexible plumbing hoses and sealing gaskets (Proctor et al., 2020; Waines et al., 2011). While the choice of the materials relies mostly on their cost and ease of installation, it is important to keep in mind that materials greatly impact the water quality in the DWDS. The concentration of metals in water is directly related to the materials used in the distribution system (Gonzalez et al., 2013). Also, some plastic materials like PEX leach inorganic and organic compounds that can be hazardous for the consumers (Stern & Lagos, 2008). Finally, because of their surface structure or their leaching potential (metals, nutrients), materials influence the growth of biofilms on their surfaces (Lehtola et al., 2004; Waines et al., 2011). Materials also release carbon that can be used as nutrient by microorganisms and favour the formation of biofilms (Mao et al., 2018).

Temperature influences the solubility of chemicals in water, with higher vinyl chloride monomer leaching from polyethylene pipes being reported in warmer water for instance (Stern & Lagos, 2008). Also, Hametner showed that the increase of water temperature from 23°C to 60°C in a polypropylene system led to an increase of total organic carbon (Hametner, 1999). Microbial quality of water is also dependent to temperature, with 1-log higher concentrations of culturable cells being monitored in hot water distribution systems compared to cold water in some buildings (Bédard et al., 2018). In the Canadian province of Quebec, temperature in hot water distribution systems is regulated as follow: (1) at least 60°C at the water heater outlet, (2) at least 55°C in the recirculation loop, (3) less than 43°C at the tap in healthcare and long term care facilities to prevent risk of scalding (Régie du bâtiment du Québec (RBQ), 2014).

Stagnation alters water quality, especially in buildings water distribution systems. However, factors controlling both microbial and chemical water quality under prolonged stagnation periods as well as the effectiveness of the remediation strategies proposed are still not well understood (Proctor et

al., 2020). Among the multiple recommendations issued for the safe reopening of buildings after the COVID-19 pandemic shutdown flushing and shock disinfection were the most advised corrective actions (Canadian Standard Association (CSA), 2020; Grimard-Conea et al., 2022; Proctor et al., 2020; Régie du bâtiment du Québec (RBQ), 2020). Depending on the duration of the building closure, the integrity of the plumbing system, and the water quality parameters considered (chemicals like copper or lead, or microbial), recommissioning flushing can be sufficient (American Water Works Association (AWWA), 2020; Canadian Standard Association (CSA), 2020; Proctor et al., 2020; Régie du bâtiment du Québec (RBQ), 2020). Yet, the efficiency of flushing remains site-dependent and there is still a lack of data concerning the benefits of flushing on the short- and long-term (Grimard-Conea et al., 2022). For shock chlorination, both low free chlorine concentrations (10ppm) with prolonged contact time (up to 24h) and high free chlorine concentrations (200ppm) with short contact times (3h) are recommended, but the latter can damage plumbing systems (Canadian Standard Association (CSA), 2016; International Association of Plumbing and Mechanical Officials (IAPMO), 2021; Proctor et al., 2020)

The main objective of this study was to investigate the interplay between material type, temperature and stagnation on water quality and the potential for biofilm formation. The specific objectives of this study were to : (1) assess the variation of water quality and biofilm formation potential among reactors containing coupons of 6 different materials in contact with dechlorinated tap water, (2) evaluate the impact of an increase in temperature on the water quality and biofilm, and (3) monitor the evolution of water quality and biofilm formation after an extended period of stagnation (6 months) and as a result of the application of remediation measures. Thus, this study provides insight on the evolution of water quality and biofilm densities in contact with different materials under various operating conditions common in building water systems.

5.2 Material and methods

5.2.1 Pilot set up and operation

In this study, a pilot composed of 12 CDC Biofilm Reactors (BioSurface Technologies Corporation, USA) was operated from May 2019 to October 2020 (17 months). Reactors contained 600mL of tap water and were operated at 80rpm in semi-batch with semi-weekly change of 75% of the water volume. Tap water was left for at least 72 hours in a tank for natural dechlorination before use in the pilot. In each reactor, 24 coupons (1,27cm² of surface for each side) of a specific

material were inserted in 8 polypropylene rods immersed in water (See Figure D.1). The reactors associated to the 4 plastics (polypropylene (PP), polyvinyl chloride (PVC), EPDM rubber, and cross-linked polyethylene (PEX)) and the 2 metals (stainless steel (SS) and copper (Cu)) tested are summarised in Table 5.1.

Table 5.1 Characteristics of the 12 CDC Biofilm reactors

Reactor ID	Material	<i>L. pneumophila</i>	T° after increase of the set point
PP_A	Polypropylene	1 strain	25°C
PP_B	Polypropylene	2 strains	40°C
PVC	Polyvinyl chloride	2 strains	40°C
EPDM	EPDM rubber	2 strains	40°C
SS	Stainless steel	2 strains	40°C
PEX	Cross-linked Polyethylene	2 strains	40°C
Cu_A	Copper (2 reactors)	2 strains	40°C
Cu_B	Copper (2 reactors)	2 strains	55°C
Cu_C	Copper (2 reactors)	2 strains	60°C

After 3 months of operation, the ATCC 50237TM CDC-19 strain of the amoeba *Vermamoeba vermiformis* was added at a final concentration of 1.5×10^3 cells/mL. Two environmental starved strains of the bacteria *Legionella pneumophila* (*Lp*) (one of serogroup 1 (ID Q067501-02) and one of serogroup 5 (ID Q076826-03)) previously extracted from biofilms in two hospitals in Quebec were added 6 weeks later in 11 out of 12 reactors (Bédard et al., 2021). Final concentrations in reactors were 1.5×10^7 cells/mL of the serogroup 5 strain and 2×10^7 cells/mL of the serogroup 1 strain. One PP reactor (PP_A) was inoculated with only 1 *Lp* strain at a final concentration of 1.5×10^7 cells/mL. All microorganisms were cultured as described in Cazals et al. (2022) before addition into the reactors. Two weeks after the inoculation with *Lp*, the set point temperature of the stirrer hotplates (VWR, USA) was increased for all the reactors as described in Table 5.1, except for reactor PP_A which was left at 25°C for the entire experiment. The temperatures were chosen to reproduce the typical temperatures found in hot water distribution systems. Two months later, reactors were brought back to ambient temperature and mixing was stopped for 4 weeks, to simulate stagnation occurring in hot water pipes during holidays. In February 2020, mixing and weekly change of water were resumed but had to be halted again in March 2020 because of the COVID-19 shutdown. Thereafter, the pilot was kept stagnant at room temperature for 6 months. At the end of September 2020, the reactors were sampled again. After, the reactors were rinsed

twice with naturally dechlorinated tap water, with a 5min agitation at 80rpm between each rinsing to simulate a flushing event in a building water distribution system, as recommended by the Canadian Standard Association after a stagnation of at least 2 weeks (Canadian Standard Association (CSA), 2016). The reactors were left at 80rpm mixing without water change for two weeks after the simulated flush and were sampled again. Shock chlorination was simulated by filling the reactors with a 20ppm chlorine solution for 24h without mixing. The reactors were then emptied, refilled with naturally dechlorinated tap water at 80rpm and left without water changes. The last samples were collected 2 weeks after the shock chlorination. The chronology of the pilot operation is summarised in Figure 5.1.

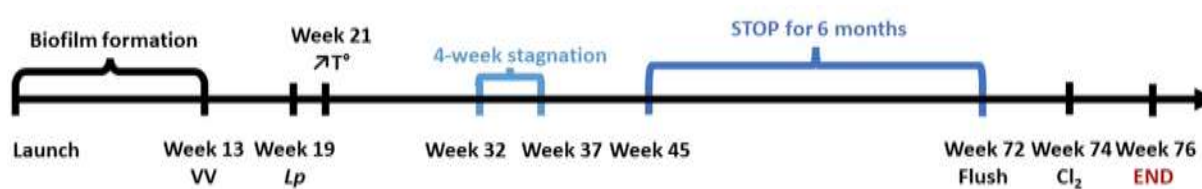


Figure 5.1 Chronology of the operation of the pilot

Water samples were collected in sterile polypropylene bottles. For each biofilm sampling, the 3 coupons were removed from a single rod, and each coupon was immersed in 2mL of sterile 0.2µm-filtered ultrapure water (MilliQ). The coupons were then manually scraped on both sides with sterile inoculation loops for 1min, then vortexed for 1min, and finally sonicated for 1min in a sonication bath. The volumes recovered from the 3 coupons were pooled into a single sample (final volume of 6mL), equivalent to a 7,62 cm² surface.

5.2.2 Analytical methods

General physicochemical parameters - Once a week, pH, conductivity, turbidity, and dissolved oxygen concentrations were measured at the time of water change. pH was measured using the Hach 186 Multi-parameter HQ40d and the PHC301 probe (Hach, USA), conductivity was assessed with a ECTestr Low conductivity meter (ThermoScientific, USA), turbidity was measured using Hach TL2300 turbidimeter (Hach, USA), and dissolved oxygen concentration was measured with the Hach 186 Multi-parameter HQ40d and the 187LDO101 probe (Hach, USA). Temperature in each reactor was monitored with a RTD temperature probe provided with the reactors.

Dissolved organic carbon (DOC) was determined following the method of the American Public Health Association (American Public Health Association (APHA) et al., 2012) and its biodegradable fraction (BDOC) was measured using a previously published method (Servais et al., 1989). Concentrations of Ca, Cr, Cu, Fe, Mg, Mn, Pb and Zn were measured using a PerkinElmer NexION 300x mass spectrometer (PerkinElmer, USA).

SEM pictures - A JSM-7600F Schottky Field Emission Scanning Electron Microscope (JEOL, Japan) was used to characterize the surface of coupons from each material before and 3 months after the launch of the pilot. Specimens were sputter coated with gold and double-sided conductive tape was used to maintain them on a standard specimen holder. Their elementary composition was estimated using energy-dispersive X-ray spectroscopy.

Flow cytometry - Total and viable bacterial densities were estimated by flow cytometry with a BD AccuriTM C6 flow cytometer until September 2020, and a BD AccuriTM C6 Plus flow cytometer after (BD Biosciences, USA). Staining with SYBRGreen I (SG, 100x concentrated) or a mix of SYBRGreen I and Propidium Iodide (SGPI, final concentration of 6µM of propidium iodide) was used, and samples were analysed according to the protocol published by Gatza et al. (2013).

5.2.3 Data analysis

All figures were created using Excel software (Microsoft, USA) and RStudio version 4.2.2. A Mann-Whitney U test was conducted in pairs on the copper concentrations measured in copper reactors before the increase of temperature.

5.3 Results

5.3.1 General water quality parameters

Physicochemical parameters

Physicochemical parameters measured in the feed water were stable throughout the study (Table D.1). A notable increase of the turbidity was observed in all the reactors after the spiking of *V. vermiciformis* as well as after the temperature increase, especially in the copper reactors at 55°C and 60°C in which the turbidity reached 6.5 and 7.9NTU respectively. Dissolved oxygen levels were relatively constant and decreased by 1.5mg/L in the reactors after the increase of the temperature at 40°C and above. pH followed the same trends in all reactors and remained relatively constant at 8.48 ± 0.21 . During shock chlorination, chlorine decay measured after 24h in the EPDM reactor

was much faster compared to other reactors, with a final free chlorine concentration of 3,4mg/L compared to 15mg/L in other plastic reactors, and a mean free chlorine residual of 11,5mg/L in copper reactors.

Concentrations of metals

Concentrations of copper and lead were measured in water and biofilm samples (Figure 5.2).

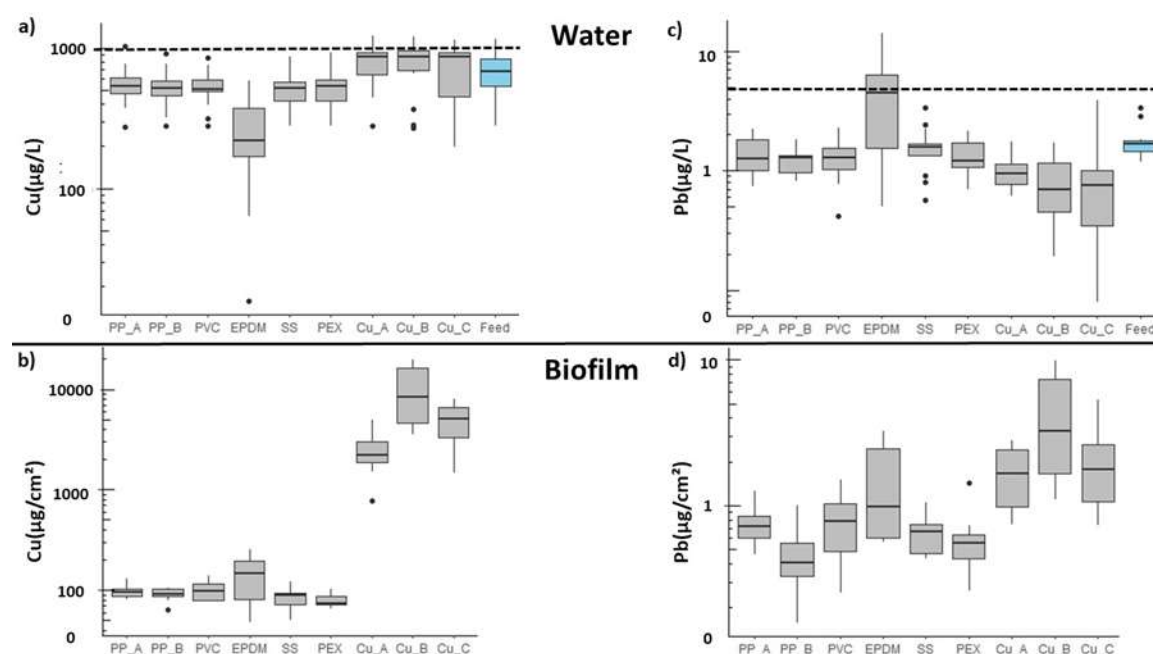


Figure 5.2 Concentrations of copper and lead in water (a and c respectively) and biofilm samples (b and d respectively). The dotted lines represent the limits of 1mg/L for copper and 5µg/L for lead as recommended by Health Canada (Health Canada, 2018, 2019). For copper reactors, the mean result is presented for duplicates at the same temperature. See Table 5.1 for detailed reactors description.

Reactors with plastic or stainless-steel coupons showed an average bulk copper concentration of $492 \pm 191 \mu\text{g/L}$, almost twice as low as in the feed water ($735 \pm 208 \mu\text{g/L}$) (Figure 5.2). Contrarily, average copper concentrations were 30% higher than feed water before heating in reactors with copper coupons ($905 \pm 135 \mu\text{g/L}$ versus $711 \pm 202 \mu\text{g/L}$). After the temperature increase, an average copper concentration of $393 \pm 36 \mu\text{g/L}$ was measured in copper reactors compared to $686 \pm 193 \mu\text{g/L}$ in feed water. Average copper concentration in biofilms from copper coupons was $5939 \pm 3280 \mu\text{g/L}$, more than 10 times higher than those from other reactors ($100 \pm 23 \mu\text{g/L}$). While the lowest copper concentrations in water were measured in the EPDM reactor, it harboured the highest average biofilm copper concentration ($145 \pm 73 \mu\text{g/L}$) amongst the non-copper reactors. Heating at

40°C and above led to an increase of the copper concentrations in biofilms from copper reactors, with the ones heated at 55°C being 4-fold and 2-fold higher than the ones at 40°C and 60°C respectively. Bulk copper concentrations in copper reactors doubled after the 4-week stagnation at room temperature. Shock chlorination led to a reduction by 50% of copper concentrations measured in biofilms of copper reactors.

EPDM was a significant source of lead in water, with an average concentration of $5.12 \pm 4.22 \mu\text{g/L}$. The lowest Pb concentrations in water were measured in copper reactors, with an average concentration of $0.94 \pm 0.19 \mu\text{g/L}$ compared to $2.07 \pm 1.70 \mu\text{g/L}$ in plastic reactors. It appears that reactors with the lowest concentrations of Pb in water (the copper ones) were the ones with the highest concentrations in the biofilms (Figure 5.2). For example, the highest average concentration of Pb in biofilm from plastic reactors was measured in EPDM biofilms ($1.49 \pm 1.08 \mu\text{g/L}$ compared to an average of $0.67 \pm 0.16 \mu\text{g/L}$ in other plastic biofilms). Pb concentrations in copper biofilms increased by 2-fold when heated compared to when reactors were set at room temperature. Concentrations of Fe, Cr and Zn were measured during the operation of the reactors, as shown in Figure D.2.

Dissolved Organic Carbon (DOC) and Biodegradable Organic Carbon (BDOC)

DOC and BDOC concentrations were measured in all the reactors and the feed water (Figure 5.3).

DOC (mg/L)	wk 12	wk 16 (+Vv)	wk 19	wk 20 (+Lp)	wk 22 (ΔT)	wk 27	wk 31	wk 37 (4ws)	wk 72 (6ms)	wk 74 (F)	wk 76 (Cl ₂)
	Seeding				Temperature Change			Stagnation and Remediation			
Feed	2.10	1.62	1.92	1.67	1.82	2.10	2.23	2.15	1.70	1.62	1.82
PP_A	2.44	3.05	2.65	2.12	2.48	2.35	2.60	2.79	4.32	2.06	2.30
PP_B	3.02	2.41	2.40	1.87	4.18	4.28	4.12	4.42	3.62	1.96	1.97
PVC	2.37	2.01	2.61	2.09	4.76	3.72	4.33	4.70	3.27	2.00	2.44
EDPM	2.75	2.65	2.55	2.72	8.12	5.10	6.36	5.10	4.90	2.07	2.61
SS	2.46	2.33	2.77	2.06	4.03	3.80	4.78	4.57	3.04	2.00	2.04
PEX	2.37	1.95	2.33	1.90	4.80	11.2	4.20	3.83	3.13	1.97	2.11
Cu_A	2.29	1.93	2.05	2.12	4.04	4.10	4.00	3.88	3.48	1.91	2.05
Cu_B	2.18	1.78	1.88	1.86	7.79	7.59	7.33	5.99	2.92	1.81	2.28
Cu_C	2.10	1.91	2.66	2.90	11.6	8.49	7.19	7.02	3.14	1.85	2.64
BDOC (mg/L)	wk 12	wk 16 (+Vv)	wk 19	wk 20 (+Lp)	wk 22 (ΔT)	wk 27	wk 31				
	Seeding				Temperature Change			Stagnation and Remediation			
Feed	0.22	0.12	0.16	0.11	0.09	0.14	0.17	0.10	0.06	0.04	
PP_A	0.19	0.38	0.61	0.30	0.45	0.18	0.27	0.25	0.06	0.26	
PP_B	0.60	0.20	0.46	0.09	0.70	0.59	0.97	0.17	0.12	0.24	
PVC	0.25	0.16	0.55	0.10	1.09	0.60	1.19	0.10	0.12	0.48	
EDPM	0.33	0.11	0.28	0.10	1.53	/	2.79	0.19	0.11	0.50	
SS	0.21	0.27	0.77	0.27	0.72	0.38	1.45	0.12	0.12	0.24	
PEX	0.37	0.12	0.36	0.21	0.92	1.30	1.07	0.21	0.15	0.29	
Cu_A	0.31	0.21	0.28	0.23	0.60	0.44	0.89	0.34	0.12	0.34	
Cu_B	0.17	0.18	0.30	0.27	1.94	2.76	2.58	0.42	0.12	0.44	
Cu_C	0.20	0.2	0.55	0.84	1.40	1.33	2.62	0.40	0.08	0.75	

Figure 5.3 Heatmaps of the concentrations of dissolved organic carbon (upper) and biodegradable dissolved organic carbon (lower) in water of all reactors and feed water. Lowest values are in blue while higher ones are in red. Samples were collected three days after interventions (+Lp, +Vv and ΔT) and 2 weeks after flushing (F) and chlorination (Cl₂).

DOC and BDOC concentrations in feed water were stable, with average values of 1.89 ± 0.23 mg/L, and 0.12 ± 0.05 mg/L. DOC and BDOC concentrations were higher in all reactors compared to feed water. In general, addition of microorganisms in the reactors was linked to the decrease of DOC and BDOC. However, this was not observed after the addition of *L. pneumophila* in EPDM and Cu reactors. Maximal DOC and BDOC concentrations were measured in all reactors after the increase of the temperature. During the heating phase, DOC and BDOC concentrations higher than those of most of plastic reactors were assessed in copper reactors, especially at 55°C and 60°C. BDOC concentrations increased by at least 3-fold in copper reactors after heating to 55°C and 60°C. After the 6-month stagnation, the BDOC concentrations measured were at least a factor 2.7-fold lowered in all the reactors except PP_A, which remained stable throughout the study. Finally, shock chlorination led to an increase of BDOC concentrations in all reactors, especially in copper reactors where they were at least multiplied by 3.

5.3.2 Biological parameters

Biofilm structures

SEM pictures of the coupons were taken before the pilot start up and after 3 months of inoculation with feed water (Figure 5.4).

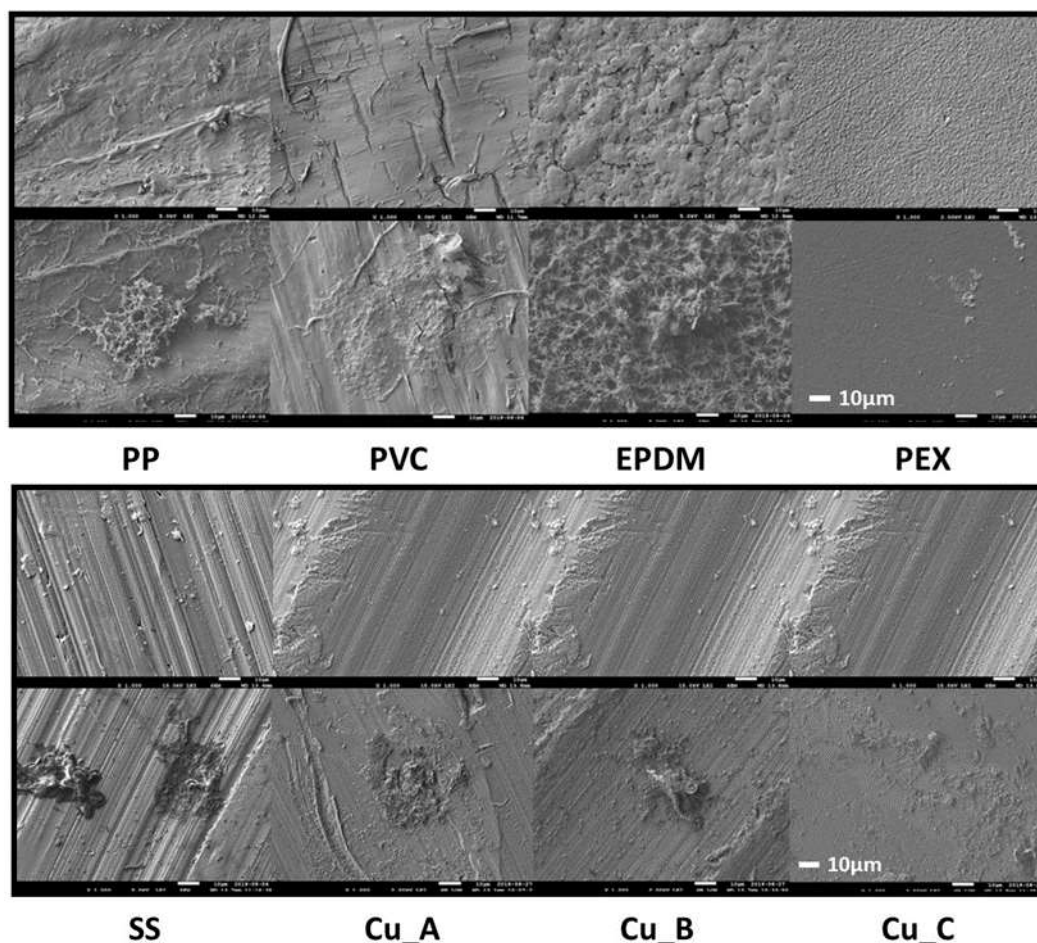


Figure 5.4 Pictures of coupons used in the pilot-scale study before (upper lines) and 3 months after the launch of the pilot (lower lines) taken with a scanning electron microscope (SEM), 1,000X.

New EPDM coupons showed a preferential structure for biofilm development, with many pores available for the attachment and development of bacteria. New PVC coupons also presented crevices where bacteria could adhere, while new PEX coupons presented a grainy structure. Interestingly, new PP coupons seemed to already have some biomass or a smooth substance on them, while regular surfaces were observed on metallic coupons. After 3 months of biofilm development, differences in biofilms coverage and structure were noticed among the materials. Scattered biofilms with a thin layer of extracellular polymeric substances (EPS) were observed on PVC and PEX while more complex and dense biofilms were observed on EPDM and PP. The

thickest biofilm was observed on EPDM coupons. Dispersed microcolonies were observed with some scattered biofilms on SS and copper coupons.

Bacterial densities

Total and viable bacterial populations in water and biofilm samples were estimated using flow cytometry (Figure 5.5).

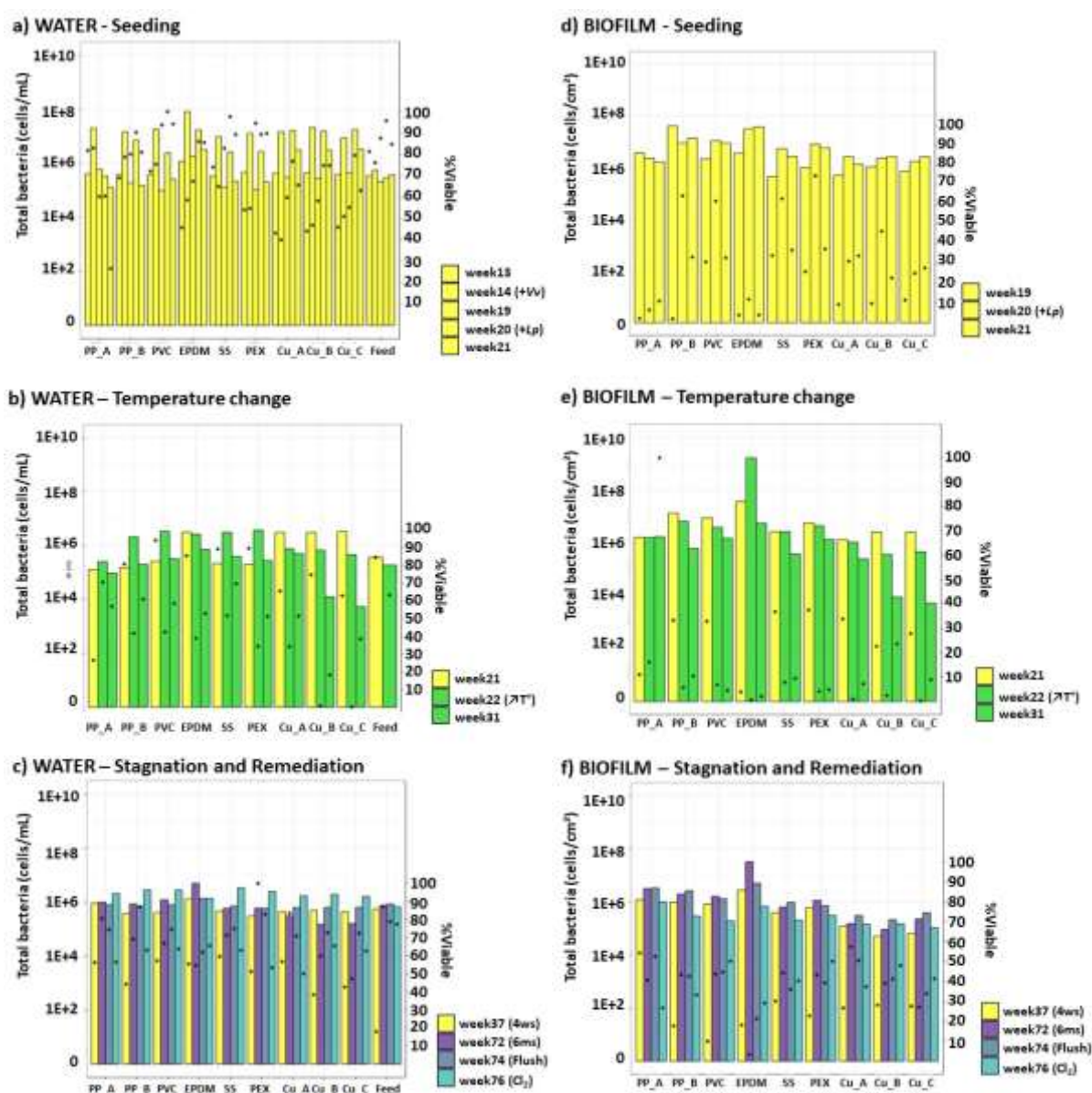


Figure 5.5 Temporal evolutions of the concentrations of total cells (bars) and the percentage of viable cells (black dots) measured by flow cytometry in water (a, b and c) and biofilms (d, e and f)

of all the reactors. Yellow is for water at room temperature, green (b and e) for when water temperature is increased, purple for after the 6-month stagnation (c and f). Blue and turquoise are for samples taken 2 weeks after flushing and 2 weeks after chlorination respectively. Cu_A reactors were heated at 40°C, Cu_B at 55°C and Cu_C at 60°C during the heating phase.

Overall, the percentage of intact cells was lower in biofilms than in water. The highest concentrations of total cells in water were measured after the addition of *V. vermiformis* (1.5×10^3 cells/mL) and the addition of *L. pneumophila* (1.5×10^7 and 2×10^7 cells/mL) in all reactors. Also, total bacterial populations in biofilms increased by at least 0.5-log over the seeding period, except in the PP_A and PP_B reactors.

Interestingly, during the first week of heating, a 1-log increase in total bacterial densities was noted in water of all reactors except the EPDM and Cu ones (Figure 5.5b). However, after 2 months of heating, a 1-log decrease of total bacterial populations was observed in all reactors except in copper reactors set at 55°C and above, where a larger decrease (2-log) was noted (Figure 5.5b). In biofilms, bacteria decreased by at least 0.5-log after 2 months of heating (Figure 5.5e). The greatest decrease (2.5-log) was noticed in copper reactors set at 55°C and 60°C. A drop of the proportion of intact cells was noticed in water and biofilm samples of all the reactors on the first week of heating, especially in copper reactors, but this percentage increased after several weeks of heating (Figure 5.5b&e).

After the 6-month stagnation, the total cell densities decreased by 50% in water from copper reactors while they increased by 2-fold in average in the other reactors (Figure 5.5c). A slight increase of the total cells was also noted in biofilms after the 6-month stagnation (Figure 5.5f). The 4-week and 6-month stagnation periods led to an increase of at least 30% of the percentage of intact cells in water of all the reactors except EPDM (Figure 5.5c). The same trend was noticed in biofilms except on the PP_A and EPDM coupons where the percentage decreased of 15% (Figure 5.5f).

A 0.5-log increase of the total bacterial population in water was noted 2 weeks after the shock chlorination and was related to a leaching of bacteria from biofilms as the total cells densities slightly decreased in biofilms (Figure 5.5c & f). After the shock chlorination, a decrease of the percentage of intact bacteria was noticed in all the water samples except the one with EPDM coupons. Interestingly, 2 weeks after the shock chlorination an increase of the fraction of intact

cells was observed in all the biofilms except the ones on PP coupons and on copper coupons set at 40°C.

5.4 General discussion

5.4.1 Biofilm formation potential

DWDS are complex systems with a large diversity of materials which directly impact the quality of water. Once established for 21 weeks, fixed bacterial densities varied within ± 0.5 -log (Figure 5.5d), even after spiking of *L. pneumophila*. Spiking led to increased variability in the bulk concentrations in the following weeks (Figure 5.5a). Other studies also reported different total bacterial populations in water (Spencer et al., 2020) and biofilms (Proctor et al., 2016) onto copper, iron, PVC, PEX and silicone, with differences tending to diminish along time for periods ranging from several weeks to months. The studied materials were ranked according to their biofilm density at week 21 (Figure 5.5d) and considering SEM observations (Figure 5.4) : EPDM > PP > PVC > PEX > Cu – SS. Plastic and metallic materials were colonized, with a biofilm density and extent dependent on the material type, as previously reported (Waines et al., 2011). The observed dense biofilm on EPDM agrees with the rapid full colonization (indicated by ATP) and complex biofilm structure of EPDM plumbing previously observed after 28 and 84 days (Waines et al., 2011). SEM observations (Figure 5.4) highlight differences of biofilm structure on colonized materials. For PVC, the presence of crevices observed (Figure 5.4) suggests a surface favourable for biofilm adherence compared to PEX and SS surfaces. The colonization observed corresponds to the 10^7 cells/cm² reported by Neu et al. (2019) for 12-month old biofilms on PVC-P shower hose fed by tap water. The denser biofilms reported by Biedroń et al. (2017) on PVC may be due to the seeding protocol used using a limited number of bacterial strains (11) added to their system (Biedroń et al., 2017). Although a similar potential was observed for PVC and PEX in this study, major differences have been reported for a same material depending on the manufacturer, especially for PVC (Proctor et al., 2016), which could lead to a change in ranking order between those two materials. As observed on our coupons, the development of a thin EPS layer was described for 84-day old biofilms on PEX (Waines et al., 2011). For metallic materials, our observations of scattered biofilms on SS and copper coupons (Figure 5.4) concur with previous findings (Waines et al., 2011). Observations made in the present study are typical of young biofilms composed of individual cells and empty areas, except on EPDM and PP (Martiny et al., 2003).

Because the nature and availability of nutrients influence bacterial growth, understanding their source is crucial to evaluate the risk of bacterial amplification in the water system according to the nature and the sequence of materials used (Prest et al., 2016a). When considering total and biodegradable dissolved organic carbon present in the bulk, two sources should be considered: 1) initial and sustained release from the material itself and 2) release from accumulated organic matter and biofilm. Materials used in drinking water systems can leach organic and inorganic components in water, especially new plastic pipes, mainly because of the stabilizers, plasticizers and antioxidants they contain (Stern & Lagos, 2008). This release, either short term (14 days, (Neu et al., 2020)) or long term (8 months, (Proctor et al., 2016)), leads to higher concentrations of biodegradable carbon and increased bulk and biofilm cell densities. PEX pipes are particularly prone to leaching TOC, DOC, and AOC in the early days of utilization (Connell et al., 2016; Lund et al., 2011).

In this study, similar DOC and BDOC concentrations were measured in all reactors after 12 weeks compared to feed water (Figure 5.3). The minimal leaching observed is attributed to the low surface to volume ratio (0.1cm^{-1}) per coupon material in the CDC reactors and the progressive elimination of the initial migration of DOC/BDOC as observed by Neu et al. (2020). On the other hand, the release from accumulated organic matter and biofilm is believed to be the primary source of the major increases in DOC and BDOC concentrations observed for all materials after heating. This hypothesis is confirmed by the highest concentrations released in copper reactors, where the bulk of the DOC and BDOC does not originate from the material. Interestingly, the largest DOC migration corresponds to the largest biofilm loss (Figure 5.5). The decrease in DOC and BDOC observed after the addition of high concentrations of microorganisms on weeks 14 and 20 (Figure 5.3) is likely due to the carbon consumption by microorganisms for cellular multiplication.

5.4.2 Concentrations of metals

Varying dynamics in lead and copper concentrations between water and biofilm were observed reflecting the sequence of precipitation/accumulation and release driven by temperature and stagnation (Figure 5.2).

First, mean Pb concentrations (average of $1.5\mu\text{g/L}$) in the bulk were slightly lower than found in the feed water (average of $2.2\mu\text{g/L}$) in the PEX reactor after six weeks. In contrast, higher releases were noted with peak concentrations ($4.7 - 34.7\mu\text{g/L}$) during the first weeks (4-20 weeks) in a PEX

building system; this release was attributed to potential leaching from brass and PEX (Inkinen et al., 2014). Secondly, an important release of Pb was noticed in the EPDM reactor, exceeding Canadian drinking water guidance level of 5µg/L (Health Canada, 2019). This suggests the presence of Pb in this brand of EPDM, possibly as a stabilizer as used in old uPVC pipes (Al-Malack, 2001).

On the opposite, lower Pb concentrations were measured in water with copper reactors, while significant accumulation of Pb concentrations were measured on copper coupons biofilms (Figure 5.2). Increasing temperature above 55°C decreased bulk water concentrations (55°C (-43%) and 60°C (-80%)) with a reverse trend on the copper coupons. We hypothesize that precipitation, co-precipitation and biofilm entrapment of Pb occur on copper coupons (Belcher & Zhang, 2018). It raises the possible issues of subsequent increases of Pb in water resulting from: (1) detachment from the biofilm and surface into the water during transient events, such as a flowrate increase (flushing) causing a resuspension of the biofilm or particulate; (2) desorption from the surface/biofilm resulting from reduced concentrations in water. Indeed, flushing resulted in small increases in Pb concentrations in almost all the reactors after two weeks, for example the SS reactor exceeded incoming water concentrations by a factor 2.8X. Pb concentrations remained stable despite prolonged stagnation, in contrasts with other studies where stagnation increased Pb leaching (Inkinen et al., 2014).

In copper reactors, bulk concentrations of copper were not significantly different ($p>0.05$) in the three copper reactors during operations at room temperature. Figure 5.2 reveals wide ranges of bulk Cu concentrations and coupons biofilm accumulations. To better assess the factors explaining these changes, Figure 5.6 shows the evolution of the dynamics on partitioning of Cu between the bulk and solid/biofilm phases. Heating reactors to 40°C, 55°C and 60°C decreased copper concentrations in water by factors of 1.6X to 5X as expected by solubility but resulted in major increases (between 1.8X and 3.8X as compared as during stagnation at room temperature) of accumulated Cu onto the biofilm coupons in the Cu reactors. Moreover, visible precipitation was noted on the glass reactor outer vessels at 55°C and 60°C and the surface of the coupons changed to blackish colour typical of tenorite which is formed at the pH, alkalinity and temperatures found in this experiment, unpublished data. A decrease of copper concentrations was noticed in water from all reactors after the 6-month stagnation, except for copper reactors previously heated to 55°C and 60°C (Cu_B and Cu_C). This concurs with the previously reported high copper leaching during

the first 10h of stagnation and the subsequent decrease of Cu concentrations (Merkel et al., 2002). Among all the non-copper reactors, the highest average copper concentrations were measured in EPDM biofilms, possibly due the accumulation of copper on the plastic (Salehi et al., 2017) and the binding and sequestering of copper ions by extracellular polymeric substances (Beech & Sunner, 2004). Overall, copper concentrations in water remained under the maximum acceptable value of 2mg/L for all reactors, but sometimes exceeded the aesthetic objective of 1mg/L as defined by Health Canada (Health Canada, 2018).

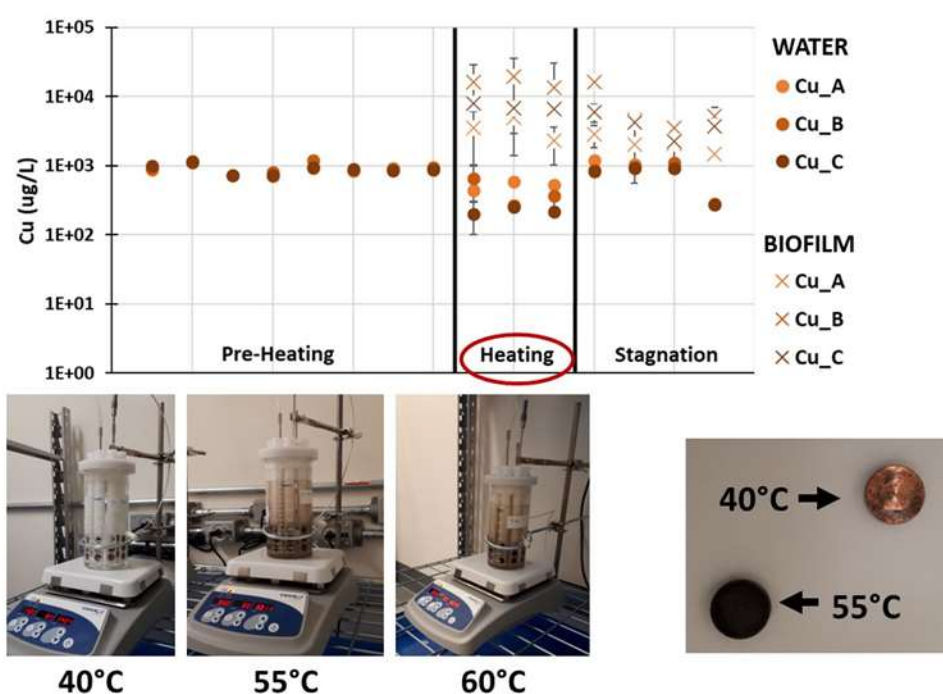


Figure 5.6 Temporal evolution of copper concentrations in water and biofilms of copper reactors and pictures of the reactors and coupons after heating. Cu_A (light orange): mean value of the 2 reactors heated at 40°C, Cu_B (orange): mean value of the 2 reactors heated at 55°C, Cu_C (brown): mean value of the 2 reactors heated at 60°C.

Trends in other metals measurements (Fe, Cr and Zn) reflect specificities of the coupon material (Figure D.2). Small concentrations of Cr were measured in bulk water of all reactors, with higher values measured in the biofilm of the SS reactor. After shock chlorination, bulk Cr concentrations in the SS reactor reached 1.82µg/L compared to a mean value of 1µg/L before, raising concerns about material damage with shock chlorination. Zinc concentration in bulk closely followed incoming water concentrations except for the EPDM reactor. Important early release, presumably from the material, resulted into Zn concentrations of 902µg/L and 197µg/cm² after 1 month and 21

weeks respectively. The mean Zn concentration in EPDM biofilms was 106µg/cm² compared to a mean value of 12µg/cm² for all the other reactors. Fe concentrations increased in water of all non-copper reactors once set at 40°C (between 1.3X and 2.2X) except the EPDM reactor. In copper reactors set at 55°C and 60°C bulk Fe concentrations decreased by 10% and 35% respectively.

5.4.3 Impact of stagnation on microbial densities

Stagnation impacted the release and use of DOC/BDOC affecting intact and total cells in the bulk and solid phases. Substrate utilization can be estimated by considering the changes of DOC in reference to the initial BDOC concentrations. Utilization of substrate will depend on both the lability of the available carbon and the ability of the intact bacteria in the bulk and fixed phases to use it. As an example, the EPDM, Cu_B and Cu_C reactors displayed a consumption of DOC after the 4-week stagnation, suggesting high carbon lability. This is supported by the high concentration of BDOC measured in those reactors, above 2.5 mg/L at the onset of stagnation. Partial utilization of the available carbon resulted in the highest increases in intact cells in those reactors (Figures 5.3 & 5.5). Fixed and suspended biomass were exposed to significant levels of organic carbon during the following 6-month stagnation. Indeed, concentrations of DOC and BDOC measured in all reactors except PP_A after 6 months of stagnation suggest depletion of the available carbon substrates during the extended stagnation (Figure 5.3). Differences in kinetics of carbon utilization may reflect differences in the nature of the labile carbon sources; in copper reactors, the main source is most likely the dead biomass, while in the EPDM, the material is a source of undefined carbon of unknown lability after heating. As a result, viable percentages remained relatively constant in water for this reactor despite an observed increase in total cells. The peak of carbon may have occurred early on during stagnation, leading to depletion of nutrients before the end of stagnation and limited growth (Proctor et al., 2020). This is not observed for short-term stagnation, as total bacteria increase was observed in the water after the 4-week stagnation at room temperature.

Carbon usage also led to an increase in total bacterial populations and the percentage of intact cells in the water of other plastic reactors. BDOC concentrations elevated by heating providing an initial stock of BDOC sufficient for bacteria to remain viable during a prolonged 6-month stagnation. These findings acknowledge a deterioration of the microbial water quality after extended periods

of stagnation (4 weeks and 6 months) and agree with other studies correlating stagnation times with increased total cells densities and intact cells proportions (Lipphaus et al., 2014).

5.4.4 Impact of flushing and chlorination on microbial densities

Differences were noted between reactors two weeks after the simulated flushing. In general, DOC and BDOC concentrations decreased directly reflecting feed water levels (Figure 5.3). Concentrations of total cells increased or decreased slightly depending on the material (Figure 5.5c). Flushing sometimes promotes the washout of high concentrations of chemical components or microbes that were present in the stagnant water (Lehtola et al., 2006). Renewal in nutrients and dissolved oxygen could explain the slight increase in percentage of intact cells that was measured in almost all the reactors (water and biofilm) two weeks after flushing. A decrease in percent intact cells was reported immediately after a partial flush of up to 40L of cold water taps (Lipphaus et al., 2014). The delay between flushing and water sampling can explain these differences. Flushing can lead to a short-term reduction in microbial densities, but over time, the renewed nutrients and oxygen brought by the flush may promote cell recovery after a few days of stagnation (Grimard-Conea et al., 2022). In addition, small changes in total bacterial densities were also noted in biofilm 2 weeks after flushing for all reactors except PEX and EPDM (Figure 5.5f). These results suggest that flushing is not efficient to eliminate biofilm as previously mentioned (Proctor et al., 2020), and should not be followed by further stagnation.

During shock chlorination, chlorine decay measured after 24h in the EPDM reactor was much faster than in other reactors, suggesting that EPDM is reactive to chlorine. Within 24 hours, copper coupons consumed chlorine faster compared to plastics except from EPDM, which concurs with previous studies (Raetz, 2010). Also, copper concentrations in copper biofilms were reduced by 50% after the shock chlorination, which is expected as chlorine will react with copper scales and copper accumulated in the extracellular polymers of the biofilm. Most importantly, even after rinsing the reactors after disinfection, BDOC concentrations measured in all reactors 2 weeks after the shock chlorination increased by 2- to 10-fold. Larger releases of DOC were observed for the PVC, EPDM and Cu_C reactors, which is consistent with a previous report of significantly higher TOC migrations in chlorinated EPDM and PEX compared to non-chlorinated ones (Mao et al., 2018). The large increase of BDOC concentrations after chlorination in all reactors may be linked to the release of dead oxidized cells from the surface biofilms. This highlights the need to rinse

thoroughly a system after chlorination to evacuate all the dead cells that can serve as nutrients and could promote microbial growth. After chlorination, total cells concentrations clearly increased in bulk of all reactors (except EPDM) while they decreased in all biofilms, showing a transfer of the bacteria from biofilms to bulk water. The decrease of intact cells percentages in water after chlorination also agrees with the previous report of a rapid decrease of intact cells after a shock chlorination (Besmer et al., 2017). In contrary, intact cells percentages increased in all biofilms except on EPDM coupons, suggesting that viable cells preferably remain in the biofilm after injured and dead cells are released and evacuated in bulk water.

5.4.5 Impact of temperature on microbial densities

Temperature greatly influences the potential of growth of bacteria in water systems (Prest et al., 2016a). After a week of heating at 40°C, densities of total cells increased in water of all non-copper and EPDM reactors while the percentage of intact cells decreased, suggesting the release of dead cells in the water. In the copper and EPDM reactors, bulk densities of total cells decreased. In biofilm, all cell densities and viability decreased, again except for the EPDM reactor. This concurs with a previous study reporting a decrease in culturability and ATP viability in the biofilms of a hot water system compared to a cold water one (Inkinen et al., 2014). A most striking observation is the marked and sustained DOC and BDOC increases in all the reactors set at 40°C by a factor of up to 2.6X and 27.8X respectively during the 10 weeks of heating, and even more in copper reactors at 55°C and 60°C (factor of min 2.5X and max 9.5X).

These results agree with previous studies showing an increase of assimilable organic carbon (AOC) and total organic carbon (TOC) concentrations with higher temperatures of 55-60°C (Hametner, 1999; Inkinen et al., 2014). DOC and BDOC concentrations intensified with increasing temperatures from 40°C to 60°C with fold changes of up to 7.5X in BDOC and 2.7X in DOC in heated copper reactors. High releases of TOC have been reported in copper-PVC convectively mixed pipe reactors at 48°C as compared to PVC ones (Spencer et al., 2020). The high release of DOC from copper pipes and coupons in hot water suggest that heating periodically and at suboptimal temperature can generate substantial amounts of nutrients that supply surviving microorganisms or the microbiomes downstream. Indeed, minerals and microbes released from copper strongly impacted the development of biofilm and the presence of *L. pneumophila* on

downstream non-copper materials (Lu et al., 2014). The nutrients produced during heating can also be consumed by microorganisms if temperature decreases or during stagnation periods.

Regardless of elevated BDOC in Cu reactors heated to 55-60°C, maintaining the temperature over time prevents bacterial increases and depresses viability (Figures 5.3, 5.5b and D.3) in agreement with the demonstrated efficacy of thermal control as shown in large buildings by Bédard et al. (2015b) and Bédard et al. (2016a).

If heating is not constant, as it is the case when applying thermal shocks, it is critical to thoroughly rinse a system, to flush all the nutrients released by the materials and biofilms during heating. Stagnation at lower temperature after a heat treatment without efficient flushing may lead to microbial regrowth eliminating the benefits of shock thermal treatment, as these conditions promote the growth of opportunistic pathogens. Indeed, necrotrophic growth of *Legionella pneumophila* has been shown following a heat shock treatment followed by a stagnation period at a lower temperature (Temmerman et al., 2006).

5.5 Conclusions

- All materials (PP, PVC, EPDM, PEX, stainless steel, copper) were colonized by bacteria after 14 weeks at 25°C, but the type of material impacted the density and the structure of biofilms, leading to the following ranking : EPDM > PP > PVC > PEX > Cu – SS
- EPDM leached up to 14µg/L of Pb in water while EPDM biofilms accumulated up to 256 and 197µg/cm² of Cu and Zn respectively.
- Precipitation, co-precipitation, and biofilm entrapment of Pb occurred on copper coupons with Pb concentrations as high as 9.8µg/cm².
- All materials leached substantial concentrations of DOC and BDOC when heated at 40°C; increasing temperature to 55°C and 60°C increased this release by up to 4.2X and 7.2X-fold respectively. It highlights the importance to thoroughly flush a system after periodic curative heating to prevent microbial regrowth.
- The high availability of nutrients after heating combined with a 6-month stagnation at room temperature led to an increase of the total bacterial densities and viability.

- Shock chlorination led to a transfer of dead and injured cells from biofilms to the liquid phase and BDOC concentrations increased by up to 10X-fold. Again, thorough flushing must be carried after to evacuate nutrients and cells released to prevent microbial growth.

Declaration of competing interests

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

Acknowledgements

The authors acknowledge Margot Doberva and Tristan Marri  for their help on the pilot. They also thank the Centre for characterization and microscopy of materials (CM)² of Polytechnique Montr al for the SEM pictures. The authors thank the team of Madjid Hadioui at the University of Montr al for the analyses of the concentrations of metals. The authors thank the CREDEAU, Industrial Chair in Drinking Water (Polytechnique Montreal) and its technical staff which benefit from the financial support of NSERC grants, the City of Montreal, Veolia Water Technologies Canada, the City of Laval, the City of Longueuil, the City of Repentigny, the City of Joliette and the City of L'Assomption.

Funding

This research was funded by Natural Sciences and Engineering Research Council of Canada (NSERC) under the Industrial Chair in Drinking Water to M.P [Alliance grant number ALLRP 545363-19]. M.C. was funded by a B2X Ph. D. scholarship from Fonds de Recherche du Qu bec–Nature et Technologies (FRQNT) [grant number 302102].

**CHAPTER 6 ARTICLE 3: FACTORS AFFECTING THE DYNAMICS
OF *LEGIONELLA PNEUMOPHILA* AND NONTUBERCULOUS
MYCOBACTERIA AND THEIR HOST *VERMAMOEBIA*
VERMIFORMIS IN PREMISE PLUMBING**

Opportunistic pathogens are ubiquitous of premise plumbing systems, especially in biofilms. However, our understanding of the impact of several parameters such as temperature, materials, stagnation and chlorination on their occurrence and survival in both bulk water and biofilms is still limited. In this chapter, the interplay of materials, temperature, stagnation, and chlorination on the concentrations of *L. pneumophila*, NTM and *V. vermiformis* was observed through a pilot-scale study. This paper was submitted on May 4th 2023 in *Environmental Science and Technology*, then transferred on June 3rd 2023 in *ES&T Water*. Supplementary information is provided in Appendix E.

**Factors affecting the dynamics of *Legionella pneumophila* and nontuberculous
mycobacteria and their host *Vermamoeba vermiformis* in premise plumbing**

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Keywords: *Legionella pneumophila*, *Mycobacterium* spp., amoeba, premise plumbing, materials, temperature, stagnation, hot water

Abstract

Premise plumbing are complex systems with diverse materials, variable temperature and stagnation time. Opportunistic pathogens are commonly found in premise plumbing, but little is known on the impact of design and operational characteristics of building water systems on their occurrence and survival in water and biofilms. The main objective of this study was to investigate the interplay between material type, temperature and stagnation on the occurrence and survival of *Legionella pneumophila*, nontuberculous mycobacteria (NTM), and their host *Vermamoeba vermiformis*. Twelve CDC biofilm reactors were used to compare concentrations of these microorganisms measured by qPCR, in water and biofilms in contact with coupons of 6 materials: polypropylene (PP), polyvinyl chloride (PVC), EPDM, cross-linked polyethylene (PEX), stainless steel (SS) and copper (Cu). The addition of *V. vermiformis* induced an average 1.4-log increase of NTM concentrations, while the spiking of two *L. pneumophila* strains caused a 3-log decrease of NTM in PP and PEX biofilms. Heating at 55°C and 60°C in copper reactors led to the decline of *V. vermiformis* and *L. pneumophila* below qPCR quantification limit. A rebound of culturable *L. pneumophila* was noted after a 6-month stagnation period at room temperature in water with PVC and stainless steel coupons.

Short synopsis

Pilot study revealed that the addition of *V. vermiformis* led to an increase of NTM while spiking *Legionella pneumophila* decreased NTM.

Abstract Graphics

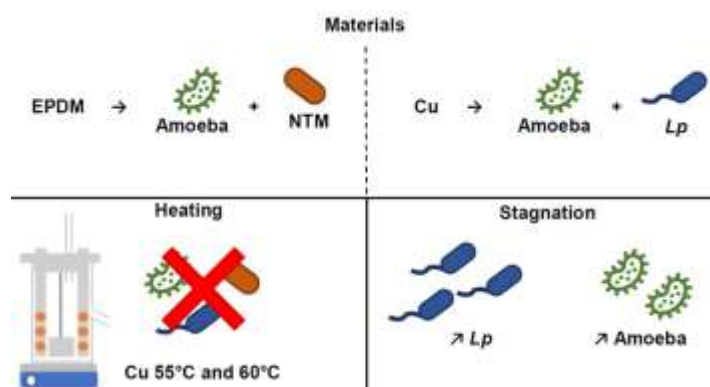


Figure 6.1 Abstract Graphics

6.1 Introduction

Opportunistic pathogens (OPs) are microorganisms naturally present in premise plumbing that can infect people with predisposing conditions such as reduced immune competence or age (>70 year old) (Falkinham et al., 2015a). These microorganisms share some characteristics like their ability to integrate and form biofilms, to grow in free-living amoebae, and their resistance to disinfection and high temperature (Falkinham et al., 2015b). *Legionella pneumophila* and nontuberculous mycobacteria (NTM) are the OPs associated with most of hospitalizations with more than 108,000 cases and a total direct healthcare cost of more than 1,900 millions US\$ in 2014 (Collier et al., 2021). Intracellular proliferation of OPs in hosts like amoebae or ciliates plays a crucial role in their survival and proliferation in water distribution systems. The amoeba *V. vermiformis* has been described as a host supporting the multiplication of both *L. pneumophila* (Kuiper et al., 2004) and some NTM species like *M. chimaera* (Sarink et al., 2023). The intracellular growth in amoebae enhances the virulence of *L. pneumophila* and *M. avium* (Cirillo et al., 1999; Cirillo et al., 1997). Amoebae also protect intracellular bacteria from disinfection (He et al., 2021).

Most microorganisms present in water distribution systems are in fixed phase: either in deposits or as fixed communities on pipe surfaces called biofilms (Wingender, 2011; Zacheus et al., 2001). Biofilms are mostly composed of bacteria, protozoans, and extracellular polymeric substances (EPS), and provide numerous advantages to microorganisms such as nutrient supply and a protection against disinfection (Wingender & Flemming, 2011). Biofilms can be a threat for the health of the consumers as they are recognized niches of opportunistic pathogens (Wingender &

Flemming, 2011). There is currently no disinfection strategy that can completely remove biofilms in water systems, and research efforts are mostly focused on finding the best way to manage them and to avoid the occurrence of OPs.

Many different materials are found in premise plumbing. Among them, polymeric materials can leach carbon and nutrients used by the microorganisms to proliferate (Mao et al., 2018) while copper pipes less support biofilm formation (Waines et al., 2011). Materials also influence the composition and amount of biofilm formed in the distribution system by their surface structure, and because they affect the effectiveness of several disinfection strategies (Lehtola et al., 2005; Waines et al., 2011).

Temperature is an important parameter in the prevention of OPs occurrence, especially *L. pneumophila* which grows preferably between 25 and 42°C (Falkinham et al., 2015b). Heat shock is also used as a remedial tool in water system colonized by OPs, with temporal limited efficiency and adaptation of the microorganisms over time (Allegra et al., 2011). Hosts of OPs also show good resistance to temperatures up to 60°C and are often isolated from hot water systems (Rohr et al., 1998). In most countries, temperatures in hot water distribution systems of buildings are regulated to prevent the risk of *L. pneumophila* contamination. In Quebec (Canada), water must be at least at 60°C at the water heater outlet and at least at 55°C in the recirculation loop (Régie du bâtiment du Québec (RBQ), 2014). In long-term and healthcare facilities, hot water should not exceed 43°C at the tap to prevent scalding (Régie du bâtiment du Québec (RBQ), 2014).

Unlike other microorganisms, OPs are able to survive and multiply under low oxygen levels usually found during stagnation periods (Falkinham et al., 2015b). After the COVID-19 shutdown, as contamination of systems by *L. pneumophila* was a main preoccupation, flushing and shock chlorination were the most recommended actions for the recommissioning of building water distribution systems (Grimard-Conea et al., 2022; Proctor et al., 2020).

Knowledge is still incomplete on the interplay between materials, temperature, and flow conditions, and the occurrence and survival of planktonic and fixed OPs. The present study aims to (1) investigate the impact of materials on the occurrence and survival of *L. pneumophila*, NTM, and the amoeba *V. vermiformis* in pilot reactors with coupons of 6 different materials, (2) assess the survival of these microorganisms in temperatures typical of hot water systems and, (3) evaluate the impact of intermediate (4 weeks) and long (6 months) stagnation periods on the survival of

these microorganisms, as well as the impact of recommended recommissioning methods (flushing and shock chlorination).

6.2 Materials and methods

Pilot operation

A pilot consisting of 12 CDC Biofilm reactors (BioSurface Technologies Corporation, USA) was operated for 76 weeks to compare 6 different materials : polypropylene (PP), polyvinyl chloride (PVC), EPDM rubber, cross-linked polyethylene (PEX), stainless steel (SS) and copper (Cu). Details were presented in a previous work (Cazals et al., 2023) and pilot's timeline is provided in Figure E. 1. Briefly, reactors were filled with naturally dechlorinated tap water and operated for three months to form biofilm. *Vermamoeba vermiformis* (ATCC 50237) was then added (final concentration of 1.5×10^3 cells/mL), followed by *Legionella pneumophila* serogroup 1 (final concentration of 2×10^7 cells/mL) and serogroup 5 (final concentration of 1.5×10^7 cells/mL) 2 weeks later. Only one reactor with PP coupons (called PP_A) was inoculated with only *L. pneumophila* serogroup 5. Set point temperature of all reactors except PP_A was increased 2 weeks after the addition of *L. pneumophila*. After 9 more weeks of operation at set temperature, a 4-week stagnation at room temperature was then simulated in the pilot, which was then followed by a 6-month stagnation period because of the COVID-19 shutdown. Reactors were then flushed, and a shock chlorination was performed using a 20ppm chlorine solution for 24h.

Filtration and DNA extraction

DNA extraction was performed following the bead beating method described in Bédard et al. (2015b) and adapted from Yu and Mohn (1999). Briefly, 250mL of water samples were filtered on 0.2µm filters on the day of sampling and frozen until DNA extraction. Filters (water samples) or 500µL (biofilm samples) were then added into an extraction tube (Lysing Matrix A, MP Biomedicals, USA). Prior to bead beating, 1mL of lysing buffer supplemented with RNase was added to each tube. Bead beating was performed on a FastPrep-24 (MP Biomedicals, USA), followed by ammonium acetate precipitation and successive ethanol washes.

Quantification of target microorganisms

Culturable *L. pneumophila* concentrations were estimated using the culture-based enzymatic Legiortest* test (IDEXX Laboratories, USA) following the 100 mL potable water protocol for water

samples, and the 1mL non-potable water protocol for biofilm samples. qPCR quantification of *L. pneumophila* was performed using the iQ-Check® Quanti *L. pneumophila* Real-Time PCR Quantification Kit (Bio-Rad, USA). All qPCR analyses were performed on a CFX Opus 96 Real-Time PCR System (Bio-Rad, USA).

NTM were quantified using a qPCR protocol adapted from Haig et al. (2018) and Radomski et al. (2013). Each 20µL reaction mixture was composed of 0.5µL of bovine serum albumin (Thermo Fisher Scientific, Canada), 1µL of each primer, 10µL of SsoFast EvaGreen Supermix (Bio-Rad, USA), and 2µL of DNA. qPCR conditions were as follows : 5min initial denaturation at 95°C followed by 40 cycles with denaturation at 95°C for 20s, annealing at 57°C for 30s, and elongation at 72°C for 30s.

V. vermiformis were quantified by qPCR following the protocol of Kuiper et al. (2006). Each 20µL reaction mixture contained 1µL of each primer, 10µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), and 1µL of DNA. Concentrations in cell equivalents were calculated based on the estimation of $1,330 \pm 127$ copies of the targeted 18S rRNA gene per *V. vermiformis* cell made by Kuiper et al. (2006).

Illumina amplicon sequencing

Sequencing of microbial 16S (V4 region, (Caporaso et al., 2012)) and 18S (V9 region, (Paranjape et al., 2020)) rRNA genes was performed on all water samples with an Illumina MiSeq Sequencer (2×250 bp) at McGill Genome Centre (Montreal, QC, Canada). Sequences were processed using the Mothur pipeline (Schloss et al., 2009) as described in Paranjape et al. (2020) (Paranjape et al., 2020). The MicrobiomeAnalyst web-based platform was then used to create the taxonomic abundance profiles and for alpha- and beta-diversity analyses (Chong et al., 2020).

6.3 Results

Quantification of *Legionella pneumophila*

L. pneumophila (*Lp*) concentrations were estimated by culture-based enzymatic method and qPCR, in both water and biofilm samples (Figures 6.2&6.3).

WATER		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(MPN/mL)		wk13	wk14 (+Vv)	wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4w5)	wk72 (6m5)	wk74 (F)	wk76 (C12)
Feed water	<-2	<-2	<-2	<-2	<-2	<-2	<-2	<-2	<-2	<-2	<-2	<-2
PP_A	<-2	<-2	<-2	<-2	>1.36	3.34	2.68	1.14	2.35	0.78	0.53	<-2
PP_B	<-2	<-2	<-2	<-2	>1.36	3.76	3.81	<-2	<-2	<-2	<-2	<-2
PVC	<-2	<-2	<-2	<-2	>1.36	4.68	3.94	0.86	-0.51	1.28	0.90	-1.96
EPDM	<-2	<-2	<-2	<-2	>1.36	3.51	1.82	<-2	<-2	<-2	<-2	<-2
SS	<-2	<-2	<-2	<-2	>1.36	4.19	3.93	<-2	<-2	0.61	0.88	<-2
PEX	<-2	<-2	<-2	<-2	>1.36	4.27	6.01	<-2	<-2	<-2	<-2	<-2
Cu_A	<-2	<-2	<-2	<-2	>1.36	3.46	3.00	<-2	<-2	<-2	<-2	<-2
Cu_B	<-2	<-2	<-2	<-2	>1.36	3.33	-1.83	<-2	<-2	<-2	<-2	<-2
Cu_C	<-2	<-2	<-2	<-2	>1.36	3.47	<-2	<-2	<-2	-1.00	0.05	<-2
BIOFILM		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(MPN/cm ²)				wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4w5)	wk72 (6m5)	wk74 (F)	wk76 (C12)
PP_A	/	/	/	<-0.1	2.60	2.35	2.43	2.55	2.76	2.44	2.19	<-0.1
PP_B	/	/	/	<-0.1	>3.25	3.44	3.92	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1
PVC	/	/	/	<-0.1	>3.25	2.94	3.62	0.24	<-0.1	2.26	2.02	<-0.1
EPDM	/	/	/	<-0.1	>3.25	2.35	<2.90*	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1
SS	/	/	/	<-0.1	>3.25	>3.25	2.61	<-0.1	<-0.1	0.26	<-0.1	<-0.1
PEX	/	/	/	<-0.1	>3.25	3.57	<3.90*	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1
Cu_A	/	/	/	<-0.1	1.68	1.55	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1
Cu_B	/	/	/	<-0.1	1.05	1.60	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1
Cu_C	/	/	/	<-0.1	0.32	2.96	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1	<-0.1

Figure 6.2 Heatmap of log-transformed *L. pneumophila* concentrations estimated by culture-based enzymatic method in water (upper, MPN/mL) and biofilms (lower, MPN/cm²). Colour gradient goes from blue (lowest values) to red (highest values). Grey boxes indicate concentrations below the detection limit of the method in water and biofilms. >1.36 (water) and >3.25 (biofilm) indicate concentrations higher than the upper quantification limit of the method. (* sample below the detection limit was diluted before analysis)

WATER		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(gc/mL)		wk13	wk14 (+Vv)	wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4w5)	wk72 (6m5)	wk74 (F)	wk76 (C12)
Feed water	<-1.1	<-0.7	<-1.7	<-1.7	<-1.1	1.96	1.39	<-1.1	<-1.7	<-1.1	<-1.7	<-1.7
PP_A	<-1.1	<-0.7*	<-1.7	<-1.7	3.71	2.72	2.07	0.52	1.34	0.44	<-1.1	<-1.1
PP_B	0.51	<-0.7*	<-1.7	<-1.7	3.38	3.84	4.55	1.81	1.24	<-1.1	<-1.1	<-1.7
PVC	0.47	<-0.7*	<-1.7	<-1.7	3.36	4.22	3.43	1.39	0.93	1.05	0.34	<-1.1
EPDM	<-1.1	0.95	<-1.7	<-1.7	6.26	5.72	4.31	1.29	1.02	<-1.1	<-1.1	<-1.1
SS	<-1.7	<-0.7*	<-1.7	<-1.7	4.92	>3.39	5.21	1.27	1.50	<-1.1	0.36	<-1.1
PEX	<-1.7	<-0.7*	<-1.7	<-1.7	5.24	>3.39	5.21	1.46	1.21	<-1.1	<-1.1	<-1.1
Cu_A	<-1.1	<-0.7*	0.57	<-1.7	5.39	5.04	4.37	0.81	0.80	<-1.1	<-1.1	<-1.1
Cu_B	<-1.1	<-0.7*	<-1.7	<-1.7	6.08	4.93	3.55	<-1.1	0.21	<-1.1	<-1.1	<-1.1
Cu_C	0.51	<0.9*	0.76	<-1.7	6.01	5.26	2.94	<-1.1	<-1.7	<-1.1	<-1.1	<-1.1
BIOFILM		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(gc/cm ²)				wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4w5)	wk72 (6m5)	wk74 (F)	wk76 (C12)
PP_A	/	/	/	<0.90	<1.50	2.88	<0.90	3.12	<0.90	<0.90	<0.90	<0.90
PP_B	/	/	/	<0.90	4.05	5.09	4.40	3.01	<1.50	<0.90	<0.90	<0.90
PVC	/	/	/	<0.90	5.44	5.15	4.11	<1.50	<0.90	<0.90	<0.90	<0.90
EPDM	/	/	/	<0.90	4.43	4.22	3.61	<1.50	<1.50	<0.90	<0.90	<0.90
SS	/	/	/	<0.90	4.41	4.17	4.10	<0.90	<0.90	<0.90	<0.90	<0.90
PEX	/	/	/	<0.90	5.06	4.96	4.57	<0.90	<0.90	<1.50	<1.50	<1.50
Cu_A	/	/	/	<1.50	3.44	2.94	3.01	<1.50	<1.50	<1.50	<1.50	<1.50
Cu_B	/	/	/	<0.90	2.76	3.37	<1.50	<1.50	<1.50	<1.50	<1.50	<1.50
Cu_C	/	/	/	<0.90	<1.50	3.07	<1.50	<0.90	<0.90	<1.50	<1.50	<1.50

Figure 6.3 Heatmap of log-transformed *L. pneumophila* concentrations measured by qPCR in water (upper, gene copies/mL) and biofilms (lower, gene copies/cm²). Colour gradient goes from blue (lowest values) to red (highest values). Light grey boxes indicate concentrations below the lower quantification limit of the method, and dark grey boxes indicate concentrations below the detection limit of the method in water and biofilms. (* sample was diluted due to inhibition)

Heating initially increased both *Lp* culturable concentrations and qPCR signals in water of PP_B, PVC, SS and PEX reactors by at least 1.2-log, while they decreased in EPDM and Cu reactors (Figures 6.2&6.3). Especially in Cu reactors heated at 60°C, culturable *Lp* was no longer detected after the first week of heating. After 10 weeks at 40°C and higher, bulk culturable and qPCR *Lp* concentrations dropped in all the reactors, especially in Cu reactors heated at 55°C and 60°C where no *Lp* could be quantified by both culture and qPCR (Figures 6.2&6.3). Despite a reduction of at least 3-log of *Lp* qPCR signals in water, a rebound of culturable *Lp* was detected in the water of PVC, SS and one Cu reactors after the 6-month stagnation. Flushing did not affect bulk *Lp* culturable concentrations but qPCR signal in water of SS reactor increased while it decreased in PP_A and PVC reactors to the point that PP_A fell under the quantification limit. After chlorination no qPCR signal could be quantified for *Lp* in water of all reactors, except for PVC where culturable *Lp* was still detected.

The absence of *Lp* by qPCR in biofilms before spiking confirmed successful integration of inoculated *Lp* in all biofilms as detected a week after spiking. After the first week of heating, a drop of at least 1.5-log of culturable *Lp* concentrations was observed in all biofilms except PP_B and PVC, and after several weeks, PVC coupons were the only heated coupons on which culturable *Lp* were still detected (Figure 6.2). Simultaneously, a decrease of qPCR signals was noted in all biofilms the first week of heating except in metallic (Cu and SS) reactors heated at 40°C where the signals remained stable. Like culturable *Lp* concentrations, qPCR signal decreased after several weeks of heating to the point that the only quantifiable signal was measured in PP_B biofilm (Figure 6.3). Following the 4-week stagnation, no *L. pneumophila* could be quantified by qPCR in biofilms samples. However, rebounds of culturable *Lp* of 2.3-log and 0.3-log were noted in biofilms from PVC and SS reactors respectively after the 6-month stagnation at room temperature. Flushing did not affect the culturable *Lp* densities on PP_A and PVC coupons, but no culturable *Lp* could be recovered from any biofilm after chlorination.

Quantification of nontuberculous mycobacteria (NTM)

Concentrations of NTM in water and biofilm samples were measured by qPCR throughout the entire experiment (Figure 6.4).

WATER		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(gc/mL)		wk13	wk14 (+Vv)	wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4wS)	wk72 (6mS)	wk74 (F)	wk76 (C12)
Feed water		3.89	4.67	3.44	2.70		2.85	4.01	4.11	4.11	3.93	3.80
PP_A		3.87	5.38	4.02	3.10	3.47	3.87	2.16	4.25	3.93	3.31	4.09
PP_B		3.79	5.17	3.41	<1.90*	<0.90*	<1.90*	3.81	3.95	4.05	4.03	4.35
PVC		3.49	5.23	2.19	2.91	<1.90*	3.84	3.84	4.05	4.49	3.53	3.86
EPDM		4.65	5.31	5.48	<2.90*	4.96	<1.90*	4.15	4.38	4.55	4.24	4.00
SS		3.69	4.95	2.98	2.79	3.20	3.67	3.86	4.09	3.78	3.69	4.32
PEX		4.00	5.40	2.38	2.77	2.98	<1.90*	4.00	3.79	3.81	3.98	4.01
Cu_A		3.68	5.16	3.48	<2.90*	3.28	<0.90*	3.53	4.00	3.37	3.83	4.12
Cu_B		3.51	5.40	3.07	4.18	3.48	<-0.10*	0.89		2.52		3.97
Cu_C		3.99	4.83	3.15	4.25	4.29	1.52	<-1.10	3.73	2.77	4.05	4.00

BIOFILM		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(gc/cm ²)				wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4wS)	wk72 (6mS)	wk74 (F)	wk76 (C12)
PP_A	/	/		<1.50	<1.50	<1.50	<1.50	<1.50	<0.50	<1.50	<1.50	<1.50
PP_B	/	/		3.22	<1.50	<2.50*	<1.50	<0.50	<0.50	<1.50	<1.50	<1.50
PVC	/	/		3.45	3.73	<2.50*	<1.50*	<1.50	<1.50	<1.50	<0.50	<0.50
EPDM	/	/		5.85	5.31	5.86	3.07	4.23	<1.50	<1.50	<1.50	<0.50
SS	/	/		<1.50	<1.50	3.83	<1.50	<1.50	<0.50	<0.50	<0.50	<0.50
PEX	/	/		3.67	<1.50	4.08	<1.50	<1.50	<0.50	<1.50	<1.50	<0.50
Cu_A	/	/		<1.50	<1.50	<1.50	<1.50	<1.50	<1.50	<0.50	<0.50	<0.50
Cu_B	/	/		<1.50	<1.50	<1.50	<1.50	<0.50	<1.50	<0.50	<0.50	<0.50
Cu_C	/	/		<1.50	<1.50	<1.50	<1.50	<1.50	<1.50	<0.50	<0.50	<0.50

Figure 6.4 Heatmap of log-transformed concentrations of NTM estimated by qPCR in water (upper, gene copies/mL) and biofilms (lower, gene copies/cm²). Colour gradient goes from blue (lowest values) to red (highest values). Light grey boxes indicate concentrations below the lower quantification limit of the method, and dark grey boxes indicate concentrations below the detection limit of the method in water and biofilms. (* sample was diluted due to inhibition)

During the first week of heating, NTM concentrations decreased in water of EPDM and Cu reactors while they increased in the others (except PEX reactor where no conclusion can be made because of the dilution of the sample) (Figure 6.4). After several weeks of heating, NTM concentrations in water of Cu reactors heated at 60°C decreased by 3-log (Figure 6.4). The 6-month stagnation at room temperature led to a decrease of the NTM qPCR signal in water of Cu reactors of at least 0.6-log while it remained stable in other reactors. During heating, NTM were quantifiable in EPDM biofilms only, but their density declined by 1.8-log after 10 weeks of heating. The density dropped after the 4-week stagnation at room temperature (Figure 6.4). On week 19, biofilm samples from plastic coupons had at least 10³ gc/cm² of NTM while biofilms developed on metallic coupons (SS and Cu) were positive but unquantifiable. PP_A coupons were the only ones with non-quantifiable concentrations of NTM.

Quantification of *V. vermiformis*

Concentrations of the amoeba *V. vermiformis*, host of both *L. pneumophila* and NTM, were assessed using qPCR (Figure 6.5).

WATER		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(cells eq/ml)		wk13	wk14 (+Vv)	wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4wS)	wk72 (6mS)	wk74 (F)	wk76 (C12)
Feed water		0.20	0.72	<0.21	<0.37	<0.31	<0.30	<0.03	0.20	0.81	0.12	0.04
PP_A		0.65	2.27	0.43	1.26	<1.26*	0.61	<0.24	0.99	1.05	0.27	1.07
PP_B		0.66	1.90	0.00	<0.74*	<2.82*	<0.26*	0.53	0.97	1.23	0.77	1.05
PVC		0.40	2.58	<0.79	1.18	<0.26*	<0.26*	0.80	0.62	1.38	0.42	0.97
EPDM		0.74	0.98	0.86	<0.74*	<0.26*	<0.26*	0.19	1.22	1.45	1.08	0.80
SS		0.12	1.85	<0.09	1.15	<0.42	1.88	<0.18	0.62	0.70	0.34	0.80
PEX		0.65	1.96	<0.72	1.17	<0.73	<0.26*	<0.01	0.42	1.02	0.29	0.40
Cu_A		0.59	2.55	0.24	<0.74*	<0.26*	<1.26*	0.29	0.29	0.71	0.15	0.65
Cu_B		0.63	2.75	<0.10	<0.74*	<0.26*	<1.26*	<3.26*	0.19	0.07	0.18	0.69
Cu_C		0.77	2.10	0.09	<1.82*	<0.26*	<3.26*	<3.26*	0.51	0.14	0.59	0.66

BIOFILM		Seeding					Increase of Temperature		Stagnation and Remediation			
Log(cells eq/cm ²)		wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4wS)	wk72 (6mS)	wk74 (F)	wk76 (C12)		
PP_A	/	<-3.23	<-0.67	<-0.67	<-0.67	<-3.23	<-3.23	<-0.67	<-0.67	<-3.23		
PP_B	/	<-0.67	<-0.67	<-1.23*	<-3.23	<-3.23	<-3.23	<-0.67	<-0.67	<-3.23		
PVC	/	<-0.67	<-0.67*	<-1.23*	<-0.33*	<-3.23	<-0.67	<-0.67	<-3.23	<-0.67		
EPDM	/	2.47	2.34	<-0.67	1.97	<-0.67	<-0.67	<-0.67	<-0.67	<-3.23		
SS	/	<-0.67	<-0.67	<-0.67	<-0.67	<-0.67	<-0.67	<-3.23	<-0.67	<-3.23		
PEX	/	<-0.67	<-0.67	<-0.67	<-0.67	<-3.23	<-3.23	<-0.67	<-0.67	<-3.23		
Cu_A	/	<-3.23	<-0.67	<-0.67	<-0.67	<-0.67	<-3.23	<-0.67	<-0.67	<-0.67		
Cu_B	/	<-3.23	<-0.67	<-0.67	<-0.67	<-0.67	<-3.23	<-0.67	<-0.67	<-3.23		
Cu_C	/	<-3.23	<-3.23	<-0.67	<-0.67	<-0.67	<-3.23	<-0.67	<-0.67	<-3.23		

Figure 6.5 Heatmap of log-transformed *V. vermiformis* concentrations in cell equivalents in water (upper) and biofilms (lower) determined by qPCR. Colour gradient goes from blue (lowest values) to red (highest values). Light grey boxes indicate concentrations below the lower quantification limit of the method, and dark grey boxes indicate concentrations below the detection limit of the method in water and biofilms. (* sample was diluted to prevent inhibition)

V. vermiformis was still quantified in water of all reactors set at 40°C while it could not be quantified in Cu reactors heated at 55°C and 60°C (Figure 6.5). Also, a 2-log decrease of *V. vermiformis* concentration was noted in SS reactor after 10 weeks of heating at 40°C. Stagnation at room temperature induced increased *V. vermiformis* concentrations (between 0.1 and 0.7-log) in water of all reactors except in Cu_B and Cu_C reactors. Finally, *V. vermiformis* concentrations were not really impacted by flushing as measured 2 weeks after the intervention. On the other hand, *V. vermiformis* DNA concentrations increased by at least 0.5-log 2 weeks after shock chlorination in PP_A, PVC, SS, Cu_A and Cu_B reactors (Figure 6.5).

V. vermiformis only colonized EPDM biofilm, but several weeks of heating at 40°C induced the drop of their population without recovery after 6-month stagnation at room temperature (Figure 6.5).

Eukaryotic diversity

Vermamoeba represented 2 out of the ten most abundant ASVs obtained in water samples analysed by 18S rRNA amplicon sequencing. Many ASVs were classified as “Eukaryota_unclassified” because of the lack of reference sequences for the V9 region of the 18S rRNA gene in the SILVA SSU 138 taxonomic database. No other potential host of *Lp* was found in the taxonomy list, and

the other most dominant eukaryotes were typical of aquatic environment (*Rhodotorula* – an environmental yeast found in water, and *Chrysophyceae* – algae mostly found in freshwater).

The relative abundance of sequences identified as *Vermamoeba* relative to the total number of sequences was calculated for each water sample (Figure 6.6).

WATER	Seeding				Increase of Temperature			Stagnation and Remediation			
%abundance	wk13	wk14 (+Vv)	wk19	wk 20 (+Lp)	wk21	wk22 (ΔT)	wk31	wk37 (4wS)	wk72 (6mS)	wk74 (F)	wk76 (C12)
Feed water	NA	1.86	NA	NA	0.20	1.08	0.10	NA	0.10	2.06	0.00
PP_A	0.00	1.76	0.00	0.00	0.00	0.59	0.00	0.00	3.72	0.00	1.57
PP_B	0.00	0.98	0.10	NA	0.00	3.33	NA	35.55	4.21	5.68	NA
PVC	0.00	6.07	NA	NA	0.49	NA	NA	0.00	0.00	0.00	64.84
EPDM	NA	NA	95.98	NA	NA	NA	NA	NA	97.85	50.64	NA
SS	0.00	10.58	7.25	7.74	NA	NA	NA	0.00	0.00	0.78	2.06
PEX	0.00	0.00	NA	0.39	0.88	0.49	0.00	0.00	0.10	1.76	0.00
Cu_A	0.00	57.99	NA	NA	NA	NA	NA	0.00	0.00	0.00	0.00
Cu_B	NA	96.23	NA	NA	NA	NA	NA	84.82	0.00	0.05	0.73
Cu_C	NA	89.37	NA	NA	NA	NA	NA	94.32	1.47	0.00	NA

Figure 6.6 Heatmap of the relative abundance (%) of *Vermamoeba* sequences over the total number of rarefied sequences obtained by 18S sequencing (V9 region) in water samples. Colour gradient goes from green (lowest values) to red (highest values). Blue cases indicate samples with a null abundance of *Vermamoeba*. NA indicate samples for which data was not available after rarefaction.

Generally, the relative abundance of *Vermamoeba* was similar to the qPCR results. When detected in water, *Vermamoeba* had the highest abundance in EPDM reactor. After its spiking in reactors, highest abundance of *Vermamoeba* was measured in copper reactors. Heating led to the absence of *Vermamoeba* sequences in all reactors after 10 weeks. The percentage of *Vermamoeba* sequences increased in PP_B, Cu_B and Cu_C after 4-week stagnation at room temperature but dropped in copper reactors after 6-month stagnation. A relative abundance for *Vermamoeba* was quantified in almost all reactors after flushing.

6.4 Discussion

Feed water microbial diversity

No culturable *L. pneumophila* was detected in the feed water nor in the reactors before spiking, but positive qPCR signals were measured in feed water at weeks 21 and 22, suggesting a low presence of *Lp* in municipal water. Several strains of *L. pneumophila* were previously isolated from hospitals water systems in Montreal (Bédard et al., 2019). This also highlights the capacity of the qPCR method to detect all the *L. pneumophila* cells present in the sample, including dead and viable but non culturable (VBNC) cells. Even if the sanitary significance of VBNC *Legionella* remains unclear, they can still produce virulence proteins and infect amoebae and human macrophages

(Alleron et al., 2013; Dietersdorfer et al., 2018), especially VBNC *L. pneumophila* induced after exposures at temperatures between 55°C and 70°C (Cervero-Aragó et al., 2019).

V. vermiformis and NTM were both detected repeatedly in feed water and the reactors from the onset of the pilot. The NTM and *V. vermiformis* strains detected before any inoculation were therefore well adapted to the feed water used in our pilot. Indeed, NTM are ubiquitous in hydric environments, especially premise plumbing (Falkinham et al., 2015a). In our study, NTM were also the only monitored microorganisms that were not spiked in the reactors but were naturally present in high concentrations in the dechlorinated tap water used as feed water. *V. vermiformis* was also naturally present in feed water and in the pilot, but in low concentrations prior to spiking (Figure 6.5). *V. vermiformis* has already been isolated from water samples from dental unit waterlines and water taps in Montreal (Barbeau & Buhler, 2001).

Impact of materials on occurrence and survival of opportunistic pathogens and hosts

Different dynamics were observed according to the materials, especially with EPDM. *V. vermiformis* was quantified only in biofilms developed on EPDM coupons where the highest densities of NTM (at least 1.6-log higher than the other biofilms) were measured (Figures 6.4&6.5). Throughout our experiment, *Vermamoeba* also had the highest abundance in eukaryotic populations of EPDM reactors (Figure 6.6). In a previous study comparing biofilms formed on 4 different materials by scanning electron microscopy, protozoa were only found in EPDM biofilms (Waines et al., 2011). Here, NTM concentrations in water in contact with EPDM coupons were on average 1-log higher compared to feed water. Interestingly, *Lp* concentrations in EPDM biofilms were the lowest (by at least 0.5-log) among all the biofilms developed on polymeric materials heated at 40°C. This may be explained by a form of competition between NTM and *Lp*, or by the fact that the dense biofilm developed on EPDM coupons provides for other bacterial species preferred by *V. vermiformis* over *Lp* for grazing, preventing its intracellular multiplication (Cazals et al., 2023; Shaheen & Ashbolt, 2021).

In our study, NTM seemed less resistant to copper than *Lp* for biofilm colonization of the coupons. Indeed, no quantifiable colonization of the Cu coupons was noted by NTM while an average quantifiable density of 1×10^3 gc/cm² of *Lp* was measured on these coupons before heating (Figures 6.3&6.4). This concurs with the absence of active *Mycobacterium* spp. in biofilm samples from a copper pipeline by Inkinen et al. (2016), and the detection of *Lp* in mature copper biofilms when

cocultured with *V. vermiformis* (Buse et al., 2017). On the other hand, concentrations of fixed *Lp* estimated by qPCR were on average 1.5-log lower in Cu reactors compared to the non-copper reactors. This is similar to the low culturable numbers of *Lp* found in copper biofilms by Moritz et al. (2010) compared to PEX and EPDM biofilms (Moritz et al., 2010). Interestingly, a few days after its addition in the reactors, *Vermamoeba* was the most abundant eukaryote in copper reactors but then its percentage decreased (Figure 6.6). PP_A and PVC reactors supported best the presence of *Lp* in water and remained positive for bulk *Lp* throughout the entire experiment (Figures 6.2&6.3).

Impact of heating on the survival of opportunistic pathogens and hosts

The decrease of *Lp* concentrations observed in the biofilm within the first week of increased temperatures corresponds to an increased concentration in bulk water for all materials except copper and EPDM (Figure 6.3). It suggests a transfer of *Lp* from biofilms to the bulk phase in all reactors, except in EPDM and Cu reactors where *Lp* initially was less detected. For copper, a decrease in *Lp* concentrations was observed in water (culturable and qPCR) and biofilm after the first week of heating (Figures 6.2&6.3), except for copper biofilm at 40°C measured by qPCR (Figure 6.3). The decrease in bulk water of *Lp* concentrations can be attributed to the high concentrations of Cu (10mg/L) measured in the copper reactors during the heating phase at 55°C and 60°C (Cazals et al., 2023). Even if the two *Lp* strains added in our pilot presented a resistance to copper concentrations up to 5mg/L (Bédard et al., 2021), concentrations of 10mg/L may have been fatal. In EPDM, the decline of *Lp* in water and biofilm may be linked to the degradation of the material, as reported for EPDM seal used for supplying water systems for 3 years at 20°C to 45°C (Nakamura et al., 2011).

After 10 weeks of heating, culturable *Lp* persisted only in the PVC reactor and the PP_A reactor maintained at room temperature. The prolonged colonization of this reactor 56 weeks after its inoculation is a reminder that *Lp* is not only a hot-water issue but an overall water distribution system issue. It also concurs with a previous study reporting survival of *L. pneumophila* in tap water at 25°C for more than 3 months (Schwake et al., 2015).

V. vermiformis resisted well the 2 months of heating in water of all reactors set at 40°C with an average concentration of 1,856 cell equivalents/L, except in copper reactors heated at 55°C and 60°C where it was no longer quantifiable (Figure 6.5). A study reported fewer than 0.05% of *V.*

vermiformis survivors after 30min at 55°C and no survivors at all at 60°C (Kuchta et al., 1993). Fixed *V. vermiformis* densities on EPDM coupons also dropped under the quantification limit after 10 weeks of heating at 40°C. This concurs with previous tests reporting a persistence of the same strain of *V. vermiformis* in autoclaved tap water to temperature up to 40°C but a decline of the population measured by flow cytometry at 43°C (Cazals et al., 2022).

Impact of stagnation on survival of opportunistic pathogens and hosts

The ability of *Lp* to survive prolonged stagnation might be linked to the water quality prior to the start of stagnation. Here, the heating phase before the 4-week and 6-month stagnations led to important leaching of dissolved organic carbon (up to 13.2mg/L) and biodegradable dissolved organic carbon (up to 3.4mg/L) in water of all the reactors (Cazals et al., 2023). This large reservoir of nutrients allowed microorganisms to survive and multiply in water (Cazals et al., 2023). This was also observed for *Lp* after the 6-month stagnation, where a rebound of culturable *Lp* was noted in water of PVC, SS, and one Cu reactor, as well as in the biofilms developed on PVC and SS coupons. Some previous studies notified increases of *L. pneumophila* of up to 4-log after a winter break (Rhoads et al., 2022) or mean concentrations of more than 4,400 MPN/L in first draw water after a 16-week stagnation due to COVID-19 shutdown (Grimard-Conea et al., 2022). An increase of *V. vermiformis* was also observed in water of all the reactors after prolonged stagnation, except in Cu reactors that were previously heated at 55°C and 60°C. These reactors were also the only ones in which *V. vermiformis* could not be quantified by qPCR after 10 weeks of heating, suggesting that if present, the remaining population of amoebae was too low to recover.

Impact of flushing and shock chlorination on opportunistic pathogens and hosts concentrations

As NTM and *V. vermiformis* were naturally present in the feed water used to flush the reactors, the impact of flushing on the concentrations of opportunistic pathogens can only be assessed based on *Lp* concentrations.

Despite flushing of the reactors, culturable *Lp* concentrations in water of PP_A, PVC, SS and Cu_C reactors and qPCR concentrations in PVC reactor remained stable 2 weeks after flushing (Figures 6.2&6.3). In SS reactor, concentrations increased from <80gc/L before flushing to 2290gc/L after. Flushing after stagnation only had a temporary effect to control *Lp* concentrations in reactors, as

previously reported during recommissioning flushing in colonized water systems of buildings (Grimard-Conea et al., 2022).

Shock chlorination was effective against *Lp*, as all culturable concentrations dropped below the detection limit (except PVC reactor) and qPCR signals fell below the quantification limit, both in water and in biofilms (Figures 6.2&6.3). Indeed, *Lp* is susceptible to high concentrations of chlorine (or lower concentrations with long contact times) (Kuchta et al., 1983).

On the other hand, similar concentrations of NTM and *V. vermiformis* were measured in water of reactors 2 weeks after the shock chlorination compared to before chlorination. Although, it must be noted that both microorganisms were present in feed water used to refill the reactors after the shock chlorination (Figures 6.4&6.5), so no conclusion can be drawn on the efficiency of shock chlorination on NTM and *V. vermiformis* with our data. However, previous studies mentioned a resistance to chlorine by NTM like *M. avium* as their membrane acts as a barrier to disinfectants like chlorine, enhanced for slower growing species and NTM in biofilms (Falkinham, 2003; Steed & Falkinham, 2006; Taylor et al., 2000). Treatment with solutions of 10ppm of chlorine was lethal to both trophozoites and cysts of *V. vermiformis* (<0.01% survival) but amoebae supported growth of *Lp* even after 30min in a 4ppm chlorine solution (Kuchta et al., 1993). Similarly, the adapted strain of *V. vermiformis* found in our system may be able to protect intracellular NTM during chlorine treatment as previously reported (Rhoads et al., 2022).

Microorganisms and their relationships

In our study, it appears that *Lp* survived better in the liquid phase of the reactors than in the biofilms. It concurs with the results of Buse et al. (2017) who reported a steady decrease of *Lp* concentrations in 610 day-old biofilms after their addition in reactors set at room temperature. As *Lp* is a secondary colonizer of biofilms, it depends on the bacterial species already present in the biofilms to form associations, which may not happen because of competition or antagonism (Buse et al., 2017). Also, *V. vermiformis* appears to be more present in water than in biofilms for all the reactors. The presence of hosts in the liquid phase may provide more favourable conditions for the survival of *Lp* than in biofilms, as formerly observed in a lab-scale cooling tower (Paniagua et al., 2020).

NTM concentrations increased in water of all reactors by 1.4-log on average a few days after the addition of *V. vermiformis*. Indeed, *V. vermiformis* is a potential host for numerous opportunistic pathogens like NTM and can protect them once under the cyst form. For instance, *V. vermiformis*

contributes to a better survival of *M. chimaera* after exposure to 1000ppm available chlorine solution for up to 5min (Sarink et al., 2023).

Opposite patterns observed for *Lp* and NTM suggest a form of competition for *V. vermiformis* between *Lp* and NTM, and the importance of *V. vermiformis* for the attachment of NTM in biofilms. The spiking of two strains of *L. pneumophila* in reactors led to a 0.5-log decrease of NTM densities in EPDM biofilm and a decrease of more than 2-log in PP_B and PEX biofilms. NTM concentrations also decreased by almost 1-log in the water of PP_A while only one *Lp* strain was added. NTM were also more present in EPDM biofilms where *V. vermiformis* were also highly present and fewer *Lp* were measured. However, the spiking of *Lp* led to the increase of bulk NTM concentrations in PVC (0.7-log), PEX (0.4-log) and Cu reactors (1-log). Interestingly, PVC and PEX biofilms were the ones with highest *Lp* densities a few days after the spiking (Figure 6.3). A decrease of NTM in PEX biofilm after the addition of *Lp* suggests a migration of NTM from biofilm to water, maybe to avoid *Lp*. These observations also assent that manipulating the premise plumbing microbiome by controlling the presence and concentrations of hosts could prevent the proliferation of some opportunistic pathogens (Wang et al., 2013).

The spiking of *Lp* in reactors induced an increase of *V. vermiformis* concentrations in all reactors after a few days, even PP_A where only one strain was added. However, ATCC *V. vermiformis* as measured by qPCR did not even survive one week after the spiking of *Lp*, and without host *Lp* did not multiply or remain long.

The ratio of *Lp* over *V. vermiformis* at time of inoculation of *Lp* might have been too high, explaining our observations. Indeed, infection experiments of amoebae by *Lp* are usually conducted with multiplicity of infection lower than 1 (Holden et al., 1984) and only a fraction of amoebae are infected. If too many *Lp* are present compared to amoebae, amoebae are attacked by too many bacteria at the same time and die before being infected, preventing intracellular replication of *Lp*. For instance, high multiplicity of infection led to the destruction of the monolayer in macrophage model (Caparon & Johnson, 1988). Also, cell-concentration dependent intracellular growth of *Lp* in amoebae and the equilibrium between *Lp* and amoebae in biofilms have previously been described (Shaheen et al., 2019). In our study the ratio between NTM and *V. vermiformis* when the amoeba was added to the reactors probably allowed the intracellular multiplication of NTM, while

the ratio between *Lp* and *V. vermiformis* when bacteria were added led to the death of amoebae and the limited replication of *Lp*.

Interventions studies aiming to show differences between materials, thermal conditions, oxidants, and stagnation typically use some spiking procedure in order to have a measurable response. Indeed, previous studies have used spiking of amoebae and OPs like *Lp* to assess the impact of different environmental and operational parameters on their survival (Buse et al., 2017; Proctor et al., 2017). Spiking of high concentrations of microorganisms in pilots or replica of water distribution systems is needed for microorganisms to integrate the system, and for researchers to be able to measure differences between the different conditions applied. However, the addition of such concentrations may result in the dominance of the spiked species and a change in the microbial diversity which is usually not representative of the natural ecology in water systems. Conclusions drawn from studies with spiking of high concentrations of microorganisms may have limited representativeness of the natural diversity of premise plumbing systems but provide information to buildings managers on actions to take in case of a contamination of a system with risk for the health of the consumers.

The interplay between opportunistic pathogens is complex and the impact of environmental factors such as materials, temperature, periods of stagnation and adaptation to the environment will influence how one OP will dominate over another. In the present study NTM and *Lp* displayed a form of competition for their common host the amoeba *V. vermiformis*. Dynamics between microorganisms varied between materials, with enhanced NTM-*V. vermiformis* interactions with EPDM while the interaction *Lp*-*V. vermiformis* seemed more decisive in copper reactors. Heating for 10 weeks at 55°C and 60°C in copper reactors led to the absence of quantifiable qPCR signal for *V. vermiformis* and *Lp*. A 6-month stagnation period led to a rebound of *Lp* in PVC, SS and one copper Cu_C reactor, but no more culturable *Lp* was measured after shock chlorination (20ppm, 24h).

Acknowledgements

The authors thank Tristan Marrié and Margot Doberva for their contribution on the pilot and lab assays, and Elliston Vallarino Reyes for support on the analysis of the sequencing data. The authors acknowledge the CREDEAU, Industrial Chair in Drinking Water (Polytechnique Montreal) and its technical staff which benefit from the financial support of NSERC grants, the City of Montreal, Veolia Water Technologies Canada, the City of Laval, the City of Longueuil, the City of Repentigny, the City of Joliette and the City of L'Assomption.

Funding

This research was funded by NSERC under the Industrial Chair in Drinking Water to M. Prévost [Alliance grant number ALLRP 545363-19]. M. Cazals was funded by a B2X Ph. D. scholarship from FRQNT [grant number 302102].

CHAPTER 7 ARTICLE 4: HOW CLEAN IS YOUR ICE MACHINE? REVEALING MICROBIAL AMPLIFICATION AND PRESENCE OF OPPORTUNISTIC PATHOGENS IN HOSPITAL ICE-WATER MACHINES

Ice machines in healthcare facilities have been associated with outbreaks and pseudo-outbreaks but guidelines regarding their maintenance and disinfection are still lacking. This chapter presents the results of a monitoring survey of 36 ice-water machines in a hospital and the results of additional profiling samplings performed on a subset of machines. This paper was submitted on May 6th 2023 in *Journal of Hospital Infection* and supplementary information is provided Appendix F.

How clean is your ice machine ? Revealing microbial amplification and presence of opportunistic pathogens in hospital ice-water machines

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Keywords: ice machines, nontuberculous mycobacteria, *Candida*, microbial amplification, cleaning, disinfection

Structured summary

Background: Ice machines in healthcare facilities have been suspected and even linked to outbreaks and pseudo-outbreaks. Guidelines exist for maintenance of these devices but there is no clear independent infection control standard, and little is known on their microbial contamination.

Aim: To evaluate the microbial contamination, amplification, and presence of opportunistic pathogens in ice-water machines in a healthcare facility.

Methods: Concentrations of general microbial indicators (heterotrophic plate counts (HPC), total and intact cells), faecal indicators (enterococci) and opportunistic pathogens (*Pseudomonas aeruginosa*, nontuberculous mycobacteria (NTM), *Candida* spp.) were measured in 36 ice-water machines on patient wards of a 772-bed hospital. Profiling sampling was performed on 5 ice-water machines and adjacent faucets to identify sites of microbial proliferation.

Findings: *Candida* spp. were found in half of ice-water samples while enterococci and *P. aeruginosa* were present in 6 and 11 drain inlets respectively. NTM were measured in all ice-water samples and 35/36 biofilms. Pre-filters and ice machines are sites for additional amplification: NTM densities were on average 1.3 log higher in water of ice machine flushed 5 min compared to flushed adjacent tap water.

Conclusion: Ice machines design needs to be adapted to reduce microbial proliferation. The absence of correlation between HPC densities (current microbial indicators) and NTM concentrations suggests a need for cleaning efficiency indicators better correlated with opportunistic pathogens. Cleaning and disinfection guidelines of ice machines in healthcare facilities need to be improved, especially when ice is given to the most vulnerable patients, and NTM may be an efficiency indicator.

7.1 Introduction

Ice is commonly used in healthcare facilities for consumption by the staff and patients, and for treatment (Millar & Moore, 2020). Outbreaks and pseudo-outbreaks related to ice-water machines in healthcare facilities have been reported for opportunistic pathogens such as *Legionella pneumophila* (Bencini et al., 2005), nontuberculous mycobacteria (NTM) (Guspiel et al., 2017; LaBombardi et al., 2002; Paul et al., 2020) and yeasts like *Candida* spp. (Kanwar et al., 2018). NTM are opportunistic pathogens ubiquitous of water systems, especially in biofilms. In 2014, 97,000 cases of domestically acquired NTM infections were estimated in the USA for a total direct healthcare cost of 1,530 million US dollars (Collier et al., 2021), and NTM-associated hospitalisations in the USA are increasing (Billinger et al., 2009). NTM were previously isolated from hospital tap water, ice-water, or ice machines (Millar & Moore, 2020; Shin et al., 2007). *Candida* species are a major cause of nosocomial infections, especially in severely ill or immunocompromised patients, and exogenous acquisition mostly happens via the hands of the healthcare workers or contaminated environment (Pfaller & Diekema, 2007).

Maintenance and disinfection of ice-water machines in healthcare facilities are not regulated. Some guidelines have been published by provincial authorities and national institutions, but mostly refer to manufacturer's guidelines regarding the cleaning, disinfection and maintenance procedures and products to use. Among the most frequently found recommendations are daily external check and cleaning of the machines and thorough internal disinfection at least every 6 months (Public Health Ontario & Provincial Infectious Diseases Advisory Committee (PIDAC), 2018). Guidelines also insist that long stagnation should be avoided in the machines by regularly draining the ice reservoirs (Centers for Disease Control and Prevention (CDC), 2019; Department of Health (DH) et al., 2016a; Public Health Ontario & Provincial Infectious Diseases Advisory Committee (PIDAC), 2018) and avoiding over-dimensioned machines. Microbial sampling is currently only indicated during epidemiological investigations (Centers for Disease Control and Prevention (CDC), 2019).

Based on observations made by the Infection Prevention and Control (IPC) team, this study aims to assess the microbial contamination of ice-water machines in healthcare facilities by sampling 36 ice-water machines in a hospital following the hospital approved procedure for these devices. We further investigated the origin of the microbial contamination of ice-water machines by evaluating the microbial proliferation from the building's water inlet to the machines.

7.2 Methods

Step 1 – Monitoring survey of ice-water machines

The study was performed early 2020 in a building of a 772-bed teaching hospital in Montreal, Canada. Two ice-water machine models were present in the building: Symphony Plus 12 (Follett, USA) (n=4) and Meridian HID312 (Scotsman, USA) (n=32). All machines were in small kitchens or corridors, and adjacent to a sink. Two water filters were placed upstream of all ice-water machines : (1) a 5 µm membrane, and (2) a 1 µm membrane containing phosphate beads. Machines exterior was cleaned daily, and an anti-scaling agent was applied weekly, while internal anti-scaling and disinfection were performed every 6 months (Text F.1). Ice-making system included a condenser.

The first step of the study consisted in the sampling of 36 ice-water machines in the same hospitalisation building (Table F.1), with 2 samples taken per machine: a combined sample of ice(1.5 L) and water(0.5 L) simultaneously taken in a sterile container, and a swab of 19,24 cm² of biofilm taken at the drain inlet was resuspended in 2 mL of saline solution (0.85 w/v%). Temperature, pH, total and free chlorine concentrations were measured on site. When free chlorine residual was above 0.1 mg/L, 2 mL of sodium thiosulfate 10% were added in the ice-water sample. HPC concentrations were estimated by culture, and total and intact cells concentrations were measured by flow cytometry with SYBRGreen and SYBRGreen-Propidium Iodide staining (Gatza et al., 2013) (Figure F.1). Enterococci and *Pseudomonas aeruginosa* concentrations were estimated using the EnterolertTM and PseudalertTM culture-based enzymatic tests (IDEXX, USA). For each machine, 1 L of ice-water was filtered on 0.2µm polyethersulfone membranes (Pall Corporation, USA), while 500 µL of biofilm resuspension were frozen before extraction. DNA extraction was performed using a phenol-chloroform protocol (Haig et al., 2018). Concentrations of NTM were estimated by qPCR with the protocol developed by Radomski et al. (Radomski et al., 2013) using a Rotor-Gene Q thermocycler (Qiagen, USA). Briefly, *atpE* gene was amplified and standards were made of 10-fold dilutions of *M. avium subsp. paratuberculosis* (ATCC® BAA968DTM) for quantification. *Candida* spp. presence was verified by culture on CHROMagar Candida (CHROMagar, France) and incubation for 24 h and 48 h at 35°C. Colonies from 4 positive samples were cryoconserved and confirmed using the API Candida test (BioMerieux, USA).

Statistical analyses and Wilcoxon tests were conducted using R version 4.2.2.

Step 2 – Identification of source of microbial amplification

Profiling sampling, consisting of collecting volumes of water corresponding to different sections of plumbing and devices to locate microbial niches with elevated concentrations, was performed in June 2022 on 5 ice-water machines (selection criteria in Table F.2). First, 3 consecutive samples were taken at the cold water of the adjacent faucet of each machine: (1) 1 L the first draw (F1J), (2) 1 L after a 30 s flushing (F30S), and (3) 1 L after 30 s stable water temperature (F) (Figure 7.1). Then, water present in the filters placed upstream of the machine (both pre- and post-filtration) was collected (WF). Feed water to the machine was sampled (W) as well as the water in the internal water reservoir of each machine (WR). The water in the reservoir is pumped by the machine to make ice and is never directly used by the consumer. Finally, 1 L of ice (I) was collected from the ice chute and a 1 L sample was collected at the water chute after a 5 min flush (W5m) (Figure 7.1). A 10 L sample was collected at the building's water inlet, and 4-month-old filters were collected. Swabs were taken on inner and outer surfaces of both membranes and analysed by ion chromatography. Precipitates observed in some water samples (Figure F.2) were filtered and analysed by scanning electron microscopy (SEM) and X-ray elementary analyse. Temperature, pH, total and free chlorine concentrations were measured on site in the three samples taken at the faucet while HPC concentrations, total and intact cells densities, and NTM concentrations were measured in all samples. For NTM qPCR, DNA extraction was performed using the FastDNATM SPIN kit (MP Biomedicals, USA) and a CFX96 BioRad Thermocycler (BioRad, USA) was used (Haig et al., 2018). No ethical approval was required for this study.

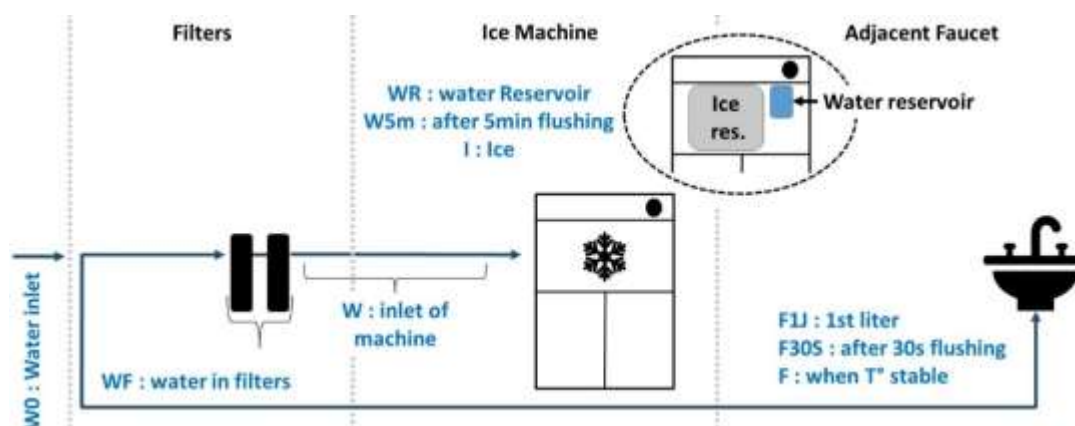


Figure 7.1 Schematic of sampling sites and samples taken during the profiling sampling

7.3 Results

Monitoring survey of ice-water machines

Among the 36 machines sampled, temperatures range from 10.2 to 23.4°C and residual chlorine concentrations varied from non-detected to 0.69 mg/L (Table F.1). First draw temperatures of water exceeded 20°C in 6/36 machines and very low free chlorine residuals <0.2 mg/L were detected in 8/36 water samples. HPC concentrations exceeded 500 CFU/mL in 18/36 of the ice-water samples while HPC densities higher than 3×10^6 cells/cm² were obtained in 19 drain biofilms (Figure 7.2). On average, intact cells constituted 55% of total cells in biofilms compared to 41% in ice-water samples.

Machine	ICE-WATER				BIOFILM			
	HPC (CFU/ml)	Total cells (cells/ml)	Intact cells (cells/ml)	NTM (gc/ml)	HPC (CFU/cm ²)	Total cells (cells/cm ²)	Intact cells (cells/cm ²)	NTM (gc/cm ²)
2	1.80x10 ⁴	4.90x10 ⁴	4.80x10 ⁴	3.5x10 ⁴	1.68x10 ⁵	8.30x10 ⁷	4.51x10 ⁷	8.87x10 ⁶
30	1.30x10 ⁴	1.40x10 ⁵	1.30x10 ⁵	7.5x10 ²	1.67x10 ⁵	3.50x10 ⁶	1.04x10 ⁶	1.22x10 ⁴
6	9.6x10 ³	7.10x10 ⁴	5.20x10 ⁴	2.47x10 ³	1.10x10 ⁶	1.12x10 ⁷	6.26x10 ⁶	1.70x10 ⁵
12	7.30x10 ³	7.40x10 ⁴	4.07x10 ⁴	6.19x10 ⁴	1.10x10 ⁶	5.67x10 ⁶	3.12x10 ⁶	6.66x10 ⁵
9	5.20x10 ³	5.20x10 ⁴	3.00x10 ⁴	5.63x10 ²	1.10x10 ⁶	5.86x10 ⁶	1.37x10 ⁶	2.33x10 ⁵
25	3.85x10 ³	6.22x10 ⁴	2.64x10 ⁴	3.57x10 ²	1.10x10 ⁶	1.26x10 ⁷	8.61x10 ⁶	4.70x10 ⁴
17	3.10x10 ³	2.36x10 ⁴	1.78x10 ⁴	5.72x10 ²	1.76x10 ⁵	6.50x10 ⁵	3.67x10 ⁵	4.38x10 ²
1	3.05x10 ³	1.08x10 ⁴	3.05x10 ³	9.27x10 ²	1.10x10 ⁶	6.16x10 ⁶	1.21x10 ⁶	8.66x10 ³
28	2.95x10 ³	4.59x10 ⁴	2.89x10 ⁴	7.42x10 ³	1.10x10 ⁶	3.92x10 ⁶	2.98x10 ⁶	1.47x10 ⁴
22	2.40x10 ³	2.31x10 ⁴	1.15x10 ⁴	4.24x10 ³	1.10x10 ⁶	1.20x10 ⁷	7.34x10 ⁶	9.51x10 ⁴
35	2.40x10 ³	8.45x10 ⁴	1.33x10 ⁴	2.26x10 ³	1.27x10 ³	1.82x10 ⁴	4.78x10 ³	3.12
8	1.43x10 ³	1.11x10 ⁴	2.67x10 ⁴	2.92x10 ³	1.10x10 ⁶	2.91x10 ⁶	1.72x10 ⁶	3.41x10 ⁴
18	1.10x10 ³	3.89x10 ⁴	2.46x10 ⁴	4.63x10 ²	1.10x10 ⁶	2.21x10 ⁵	1.85x10 ⁵	0.81
5	8.15x10 ²	4.66x10 ⁴	1.51x10 ⁴	5.44x10 ³	1.10x10 ⁶	8.74x10 ⁷	4.29x10 ⁷	5.74x10 ⁶
24	8.15x10 ²	3.91x10 ⁴	2.50x10 ³	1.03x10 ³	6.09x10 ⁴	1.87x10 ⁵	1.02x10 ⁵	1.95x10 ⁴
27	6.80x10 ²	1.15x10 ⁴	3.84x10 ³	8.11x10 ²	1.58x10 ⁴	1.72x10 ⁵	5.48x10 ⁴	3.12
33	5.80x10 ²	1.82x10 ⁴	4.47x10 ³	1.82x10 ²	1.86x10 ⁵	2.64x10 ⁶	1.45x10 ⁶	8.25x10 ³
32	5.43x10 ²	2.19x10 ⁴	1.11x10 ⁴	2.97x10 ³	1.10x10 ⁶	3.34x10 ⁶	2.04x10 ⁶	5.51x10 ⁴
10	4.90x10 ²	4.71x10 ⁴	2.23x10 ⁴	1.40x10 ²	1.10x10 ⁶	1.44x10 ⁷	7.19x10 ⁶	3.42x10 ⁴
4	3.95x10 ²	3.82x10 ⁴	1.79x10 ⁴	1.27x10 ³	3.59x10 ⁴	7.77x10 ⁵	4.19x10 ⁵	3.24x10 ³
31	3.90x10 ²	1.95x10 ⁴	1.08x10 ⁴	4.16x10 ³	1.10x10 ⁶	4.59x10 ⁶	2.56x10 ⁶	1.86x10 ⁴
3	3.15x10 ²	2.68x10 ⁴	1.35x10 ⁴	1.77x10 ³	1.10x10 ⁶	4.20x10 ⁶	1.89x10 ⁶	3.59x10 ³
11	3.15x10 ²	3.09x10 ⁴	8.44x10 ³	4.91x10 ²	0.83	8.50x10 ⁷	7.19x10 ⁷	1.30x10 ⁷
14	2.70x10 ²	1.79x10 ⁴	3.02x10 ⁴	1.82x10 ³	5.42x10 ⁴	9.69x10 ⁵	6.83x10 ⁴	6.74x10 ⁴
20	2.55x10 ²	2.71x10 ⁴	4.17x10 ³	9.83x10 ³	1.10x10 ⁶	9.01x10 ⁶	3.90x10 ⁶	2.41x10 ⁴
7	2.20x10 ²	9.52x10 ⁴	1.12x10 ⁴	9.31x10 ³	1.10x10 ⁶	1.01x10 ⁴	1.51x10 ⁴	6.28x10 ⁶
29	7.00x10 ¹	3.30x10 ⁴	1.81x10 ³	1.60x10 ²	1.10x10 ⁶	1.53x10 ⁶	1.23x10 ⁶	2.65x10 ²
26	4.20x10 ¹	1.13x10 ⁴	1.56x10 ³	3.02x10 ²	4.04x10 ⁵	1.12x10 ⁶	7.54x10 ⁵	3.15x10 ²
34	3.05x10 ¹	2.68x10 ⁴	3.02x10 ³	1.99x10 ³	1.10x10 ⁶	2.58x10 ⁶	1.96x10 ⁶	8.00x10 ²
36	2.25x10 ¹	1.50x10 ⁴	6.23x10 ³	4.50x10 ³	7.28x10 ⁴	3.26x10 ⁵	1.09x10 ⁵	3.26x10 ²
21	1.50x10 ¹	1.20x10 ³	5.10x10 ²	1.72	6.58x10 ³	2.40x10 ⁴	1.44x10 ⁴	3.12
23	1.20x10 ¹	6.28x10 ³	1.13x10 ³	1.59x10 ²	1.10x10 ⁶	2.87x10 ⁶	1.63x10 ⁶	3.91x10 ³
19	1.00x10 ¹	2.80x10 ⁴	4.08x10 ³	6.65x10 ³	1.10x10 ⁶	2.40x10 ⁶	1.41x10 ⁶	2.62x10 ⁴
13	0.50	2.79x10 ³	3.79x10 ²	1.01x10 ³	9.13x10 ⁴	9.42x10 ⁵	3.73x10 ⁵	3.95x10 ²
15	0.50	1.20x10 ³	1.30x10 ²	1.58x10 ³	3.24x10 ⁵	5.63x10 ⁶	4.71x10 ⁶	3.58x10 ⁵
16	0.00	4.87x10 ³	1.30x10 ³	7.89x10 ²	1.94x10 ⁴	3.74x10 ⁵	2.07x10 ⁵	7.07x10 ²

Figure 7.2 Heatmap of the concentrations of HPC, Total cells, Intact cells and NTM (gene copies (gc)) in ice-water and biofilm samples of 36 ice-water machines. In ice-water samples, green colour indicates lower concentrations (HPC from 0 CFU/mL, Total and Intact cells from 10³ cells/mL, NTM from 0 gc/mL) and red colour higher concentrations (HPC from 10³ CFU/mL, Total and Intact cells from 10⁵ cells/mL, NTM from 10³ gc/mL). In biofilm samples colour gradient comes from white (lowest concentrations) to dark blue (highest concentrations). Bold indicates samples too numerous to count for HPC (3.12x10⁶ CFU/cm²) or below quantification (3.12 gc/mL) or detection limit (3.1x10⁻¹ gc/mL) for NTM.

Enterococci were found in one ice-water sample and 6 drain inlet biofilms, while *P. aeruginosa* was present in one ice-water sample and 11 drain inlet biofilms. *Candida* spp. were recovered from 15 ice-water samples and 26 drain inlet biofilms (Table F.3). Among the 4 colonies/strains obtained at 35°C and tested, one was identified as *C. parapsilosis*, two were *C. guilliermondii* and one was a *Trichosporon* spp. 1. NTM were detected in all ice-water (mean of 4.75x10³ gc/mL) and in 35/36 biofilm samples (mean of 1.55x10⁶ gc/cm²) (Figure 7.2).

Identification of source of microbial amplification

Profiling sampling revealed total and intact cells densities at least 1 log higher in faucet samples compared to the water at the inlet of the building, except for one machine. Flushing the tap resulted

in variable reduction of total cells by a factor 1.5X to 4.9X, and a reduction by 1.6X to 2.6X of intact cells densities (Figure 7.3). Bacterial densities up to 3 log higher were measured in filters and machines water reservoirs compared to flushed faucet. Total and intact cells densities in water collected after 5 min flushing at the machines were equal or higher than those in the respective flushed faucets, and the ones in ice samples were 1 to 2 log higher than the water inlet, showing a bacterial amplification in the machines (Figure 7.3).

HPC concentrations at taps were 0.5 log to 3.8 log higher than in the building water inlet even after stabilisation of temperature for 30 s (Figure 7.3). Highest HPC concentrations were measured in the filters, the machine inlets, and water reservoirs. Even after a 5 min flushing HPC concentrations in the water of machines 1 and 4 remained 7X and 41X higher than in the water sample collected at the faucet after flushing until stabilisation of the temperature. HPC concentrations in all ice samples were higher than 10^3 cells/mL.

NTM concentrations were 0.9 log to 3.1 log higher in samples collected at the faucets as compared to the building's water inlet, indicating an amplification in the building's cold water distribution system (Figure 7.3). Highest NTM densities were measured in machines water inlets and internal water reservoirs (mean of 4.3×10^6 gc/mL). NTM concentrations in ice samples were 0.9 log to 2.7 log higher than in faucet first draws. Even after a 5 min flushing, NTM concentrations in the water of the ice-water dispensers remained 3 log higher than building's water inlet, revealing a colonization of the machine/water system by NTM.

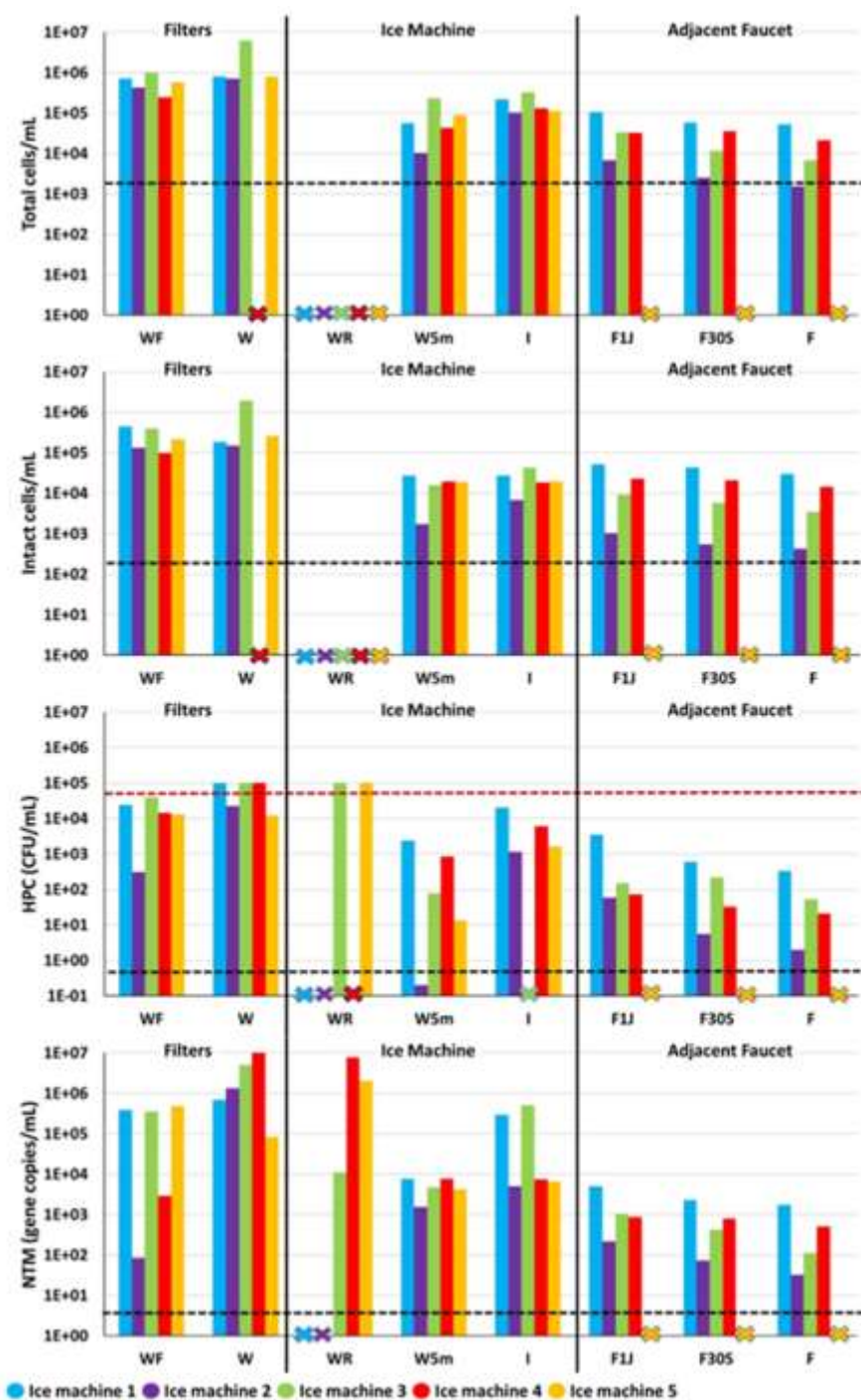


Figure 7.3 Concentrations of Total cells, Intact cells, HPC, and NTM (gene copies/mL) measured during profiling sampling. WF: water in filters, W: water coming out of filters to the machines, WR: water in machine reservoirs, W5m: water at the machine after 5min flushing, I: ice, F1J: 1st draw at faucets, F30S: 1L of water after 30s of flushing at faucets, F: 1L of water after stable temperature at faucets. Black dotted lines represent the concentrations measured at the building's

water inlet. Red dotted line on the HPC graph represents the upper quantification limit. Crosses indicate that the measurement was not performed.

Pre-treatment filters increased phosphate concentrations from a background of $<0,2$ to $1,9$ mg/L- PO_4^{3-} . Light blue precipitates were observed in 3/5 internal water reservoirs when sampling and green particles were found in the inlet water sample of a machine when filtered (Figure F.2). SEM and X-ray elementary analyses showed a dominance of P(21.7%), O(48.7%), Ca(23%), Mg(1.7%) and Cu(4.5%) in the blue precipitates, indicative of precipitation of phosphates with hardness and trace metals from the plumbing, while their inorganic nature was confirmed by microscopy. Elemental composition of the green particles suggests a dominance of copper oxide (Cu(31.6%) O(47.8%)).

7.4 Discussion

Opportunistic pathogens and faecal indicators

When considering all 36 ice-water machines surveyed, NTM were found in all ice-water samples with concentrations from 1.7 to 6.2×10^4 gc/mL. NTM are ubiquitous of water systems and have been reported in hospitals water systems (El Sahly et al., 2002; Millar & Moore, 2020). Similar concentrations between 2×10^1 and 1.3×10^4 gc/mL of NTM 16S rRNA gene were previously measured in unchlorinated water distribution systems (van der Wielen & van der Kooij, 2013). Although the building is supplied by chlorinated drinking water, chlorine residuals measured in the water dispensed by ice-water machines were less than 0.2 mg/L for more than 25% of the sampled machines, which can explain the similarity between our NTM concentrations and those measured in unchlorinated water systems. Similar levels up to 7.76×10^3 gc/mL were reported in faucets by Haig et al. (2020) although amplification of 1.5 to 3 orders of magnitude occurred more specifically in first draws, unlike results shown on Figure 7.3 (Haig et al., 2020). Although present in the municipal distribution system (DS), additional profiling sampling from this study revealed that NTM are progressively amplified in the hospital DS at the adjacent tap (up to 2.7 log after flushing), in the machine's water circuit (up to 3.3 log after 5min flushing) and ice (up to 5.1 log). Operating conditions, usage patterns and design of ice-water machines created favourable conditions (temperature, nutrients, stagnation/poor flow) for elevated NTM growth in the internal water reservoir dedicated to ice production.

The opportunistic pathogen *P. aeruginosa* and faecal indicator enterococci were detected once in water-ice samples but were more often found in biofilms (11 and 6 machines respectively). *P. aeruginosa* infections are mainly acquired in healthcare facilities, especially in intensive care units, and are often directly or indirectly linked to buildings water systems (Bédard et al., 2016c). On the other hand, enterococci are used as indicators of hand hygiene and faecal contamination in water (Boehm & Sassoubre, 2014) and a positive sample leads to a boiling advisory (Ministère du Développement durable de l'Environnement et des Parcs du Québec (MDDEP), 2014). The presence of these microorganisms in the water could be linked to retro contamination through splattering from the drains to the staff hands, water/ice chutes and patient glasses (Kanwar et al., 2018).

Here, *Candida* spp. were present in 42% and 72% of ice-water and biofilm samples respectively. It concurs with previous works reporting *Candida* spp. from drains and chutes of 64 ice machines in 7 healthcare facilities and from hospital water samples (Arvanitidou et al., 1999; Kanwar et al., 2018). Although no clinical cases were reported in the present facility, *C. parapsilosis* was identified among the strains isolated and is a recognized opportunistic pathogen (Trofa et al., 2008). Some *Trichosporon* spp. were also identified here. They are related to human diseases, from superficial infections to invasive infections with high mortality rates (Montoya Mendoza & González González, 2014).

The high prevalence of pathogens detected in the drains and the suspected retro contamination of the water highlight the need for enhanced and validated cleaning procedures and rethinking of the design of the water chute and drain to eliminate retro contamination.

Microbial indicators

As observed for NTM, mean increases of 2.4 log of HPC and 1.1 log of total cells were observed in the flushed samples at the faucets in reference to incoming municipal water at the building inlet. This is higher than the maximal acceptable amplification factor of 1 log for HPC in French healthcare facilities and indicative of growth or renewed culturability in the cold water building system (Castex & Houssin, 2005). Amplification of general microbial indicators (HPC, total and viable cells) was also noted in filters and ice-water machines during profiling sampling and attributed to water stagnation in filters, internal water reservoirs of the machines, and the possible presence of biofilm in the piping of the machines. The larger increase in HPC than in total cells is

attributed to the impact of chlorine on culturability as supported by the correlation between chlorine residual and HPC in water samples collected at the faucets (Figure F.3, $R^2=0.5$). The corresponding larger increase in NTM suggests that the cold water distribution system selects for NTM even in flushed samples. As opposed to trends observed for NTM, total and intact cells densities in ice-water samples were comparable to stagnant faucet cold water levels observed in this study and previously reported (3.91×10^4 and 1.83×10^4 cells/mL) (Bédard et al., 2018). As expected, high mean concentrations of 1.05×10^7 and 6.26×10^6 cells/cm² of total and intact cells were measured in drains, in agreement with mean total HPC detected in sink drain biofilms (between 10^4 and 10^8 CFU/cm²) (El-Liethy et al., 2020).

Critical components and factors causing amplification

Profiling sampling identified three components causing microbial amplification, including NTM, in the ice-water machine circuit (Figure 7.3): the water tubing after prefilters, the internal reservoir and condenser for ice-making, and the ice storage bin. Passage of water through the water circuit of the ice machine (5 min flushing) increased densities by 0.8 log of HPC and 0.6 log of total bacteria, suggesting continuous release from the biofilm in the tubing up flow of the dispenser. Figure 7.3 clearly shows the largest amplification of all microbial indicators and NTM occurring in the tubing connecting the outlet of the pre-filters to the ice-water machine. Conditions in the connecting tubing is especially favourable to biofilm and suspended bacteria growth because of the high surface to volume ratio (Bédard et al., 2015a) and regular stagnation between uses. Additionally, some flexible tubing materials have been shown to be highly favourable to biofilm growth (Proctor et al., 2016).

Chemical water quality was also altered in ice-water machines leading to precipitation of phosphates complexes in the machines and possibly enhancing bacterial growth. The second prefilter is used to minimize scaling through the release of slowly soluble solid polyphosphates beads. Reservoir precipitates can be washed out to the ice making circuit. Their presence is most likely related to the low use frequency of some of these machines (observations by staff). Phosphate addition has been shown to promote biofilm growth and reduces diversity in favour of bacterial pathogens and fungi (Batté et al., 2003b; Douterelo et al., 2020). Concentrations of 3.6 and 39.5 mgPO₄/L have been linked to in vitro stimulated growth of *L. pneumophila* (Jereb et al., 2022).

Pre-filters, especially activated carbon filters, have been related to microbial contamination of water and ice machines as they reduce chlorine concentrations (Klompas et al., 2023).

While ice machines are considered as cold environments, our initial monitoring showed that water temperatures exceeded 20°C in 17% of the machines mainly because of the condenser emanating heat while producing ice. Also, there is no refrigeration of the water in the machine, and these temperatures accelerate the potential microbial growth in its various components as compared to a chilled system.

Implications for risk management

The results of this study demonstrate the high potential of microbial amplification in ice-water machines and their potential to become a source of exposure to opportunistic pathogens, especially NTM, *P. aeruginosa*, *Candida* spp., and faecal indicators (enterococci). Li et al. (2017) showed that adequate disinfection is essential for the continuous safe use of devices and the prevention of healthcare-associated NTM infections (Li et al., 2017).

Several approaches can be explored to prevent this amplification and reduce risks to users. Firstly, it appears necessary to revise the design of ice-water machines to eliminate components and conditions favourable to microbial amplification and deposits accumulation. For example, ice-water machines combine two circuits that are prone to the development of biofilms and subsequent release of opportunistic pathogens into water and ice. For the water circuit, as for faucets in healthcare facilities, avoiding stagnation and minimizing temperature are key to minimizing concentrations of metals and microbes (Bédard et al., 2018; Inkinen et al., 2014). The most simple and efficient approach for cold water supply would be to replace ice-water machines by refrigerated water dispensers with automated flushing, whenever ice is not needed. Pre-filters that may stimulate the growth of biofilm should be avoided and the implementation of point-of-use disinfection considered (e.g. UV at water/ice chute). In the case of ice production, the use of pre-filters, the redesign of internal reservoirs and the presence of an open drain at the point of use clearly need to be reviewed as the level of colonisation by bacteria, opportunistic pathogens and faecal indicators is significant in water, ice, and drain. Phosphates based pre-filters are used to minimize scaling and sequester metals for the ice circuit. As phosphates are limiting nutrients in drinking water, the addition of polyphosphates can lead to precipitation and potentially stimulate the growth of opportunistic pathogens, especially with low usage (Jereb et al., 2022). By

decoupling the provision of ice for cooling water intended for drinking and other uses, a reduction of the number of ice machines to a minimum can be achieved. For the remaining ice machines, more stringent and efficient disinfection regimes should be implemented. Typically, disinfection criteria are based on a target level of inactivation of the most resistant microorganism, such as *Cryptosporidium* for drinking water production (Government of Québec, 2017). NTM gene copies were found to be abundant in ice-water machines and NTM are known to be highly resistant to disinfection because of their thick cellular membrane (Falkinham, 2016). Considering their frequent presence in water systems, their resistance to disinfection, their ability to grow in ice machines and the risk they may represent to vulnerable patients, NTM could be excellent cleaning indicators for ice-water machines. In contrast, culturable indicators such as HPCs are poor indicators of the cleaning efficiency as they are highly susceptible to chlorine (Figure F.3, $R^2=0.5$). In such, they provide a false sense of security as they are not correlated with the presence of opportunistic pathogens (Figure F.4). Incorporating routine monitoring of NTM concentrations as an indicator of cleaning and disinfection efficacy could help prevent risk of exposure and infections (Klompas et al., 2023).

Limitations of this study include that NTM were measured only by qPCR which include dead, non-culturable intact cells and culturable cells. However, when observable increase occurs it is most probably related to active cells. On the other hand, persistence after treatment should be considered. Significant differences in the resistance of culturable NTM across species have been shown, with species with relevant health issues like *M. avium* being more resistant (Islam et al., 2013). Only environmental samples were taken so further epidemiological studies are needed to assess the impact of microbial contamination of ice machines on patient safety. Finally, all the samples came from the same hospital, so it is difficult to generalise with current data.

In conclusion, there is clearly a need to develop international or national or both guidelines regarding the cleaning, disinfection, and maintenance of ice-water machines in healthcare facilities. As it is now recognized that water systems can be a threat to the most vulnerable patients, some hospitals apply water-free patient care protocols for immunocompromised or critically ill patients (Klompas et al., 2023). The same could be done for ice, by using ice made from sterile water or by avoiding ice machines in intensive care units as recommended in the UK (Department of Health (DH) et al., 2016a).

7.5 Conclusions

- Bacterial amplification and the presence of opportunistic pathogens were observed in various components of ice-water dispensing machines.
- HPC are not appropriate indicators of microbial contamination and cleaning efficiency of ice-water machines.
- Careful selection of pre-treatment should be made to avoid precipitation in the ice machine components and the addition of nutrients that can foster microbial growth.
- Cleaning procedures of ice-water machines should include the elimination of all deposits and the thorough cleaning of the water reservoir
- Use of ice machine should be evaluated considering the need for ice, the risks to patients, and the availability of iceless options for cold water provision
- Further studies are required to determine the extent to which this contamination is a risk to patients and the impact of improved cleaning procedures on nosocomial infection rates.

Acknowledgements

The authors acknowledge the support of the CHUM's IPC team, hygiene and sanitation team, and Patrick Galipault from the team of technical services, and the plumbers from Veolia. SEM pictures were performed at the Centre for characterization and microscopy of materials (CM)² of Polytechnique Montréal.

Conflict of interest

None declared.

Funding

This research was funded by a research contract with CHUM and by the NSERC Discovery grant of M. Prévost (RGPIN-2021-04341). M. Cazals was funded by a B2X Ph.D. scholarship from FRQNT [grant number 302102].

CHAPTER 8 GENERAL DISCUSSION

The main findings of this research are highlighted in this chapter. The overall objective was to identify design, operation, and maintenance parameters preventing the occurrence and persistence of opportunistic pathogens in premise plumbing. The different sections and outcomes of the research are summarized in Figure 8.1. The first part aimed to evaluate the survival of *L. pneumophila* in water heaters depending on the phase (water vs loose deposits), the heating regime, and the presence of hosts. A pilot-scale study was then conducted for the second part of the research to identify environmental and operational factors preventing the persistence of *L. pneumophila*, NTM, and *V. vermiformis* in both bulk water and biofilms of cold and hot water distribution systems. Finally, an investigation was conducted on hospital water-ice machines to assess their microbial contamination and to identify better indicators and practices for the maintenance, cleaning, and disinfection of these devices in healthcare facilities.

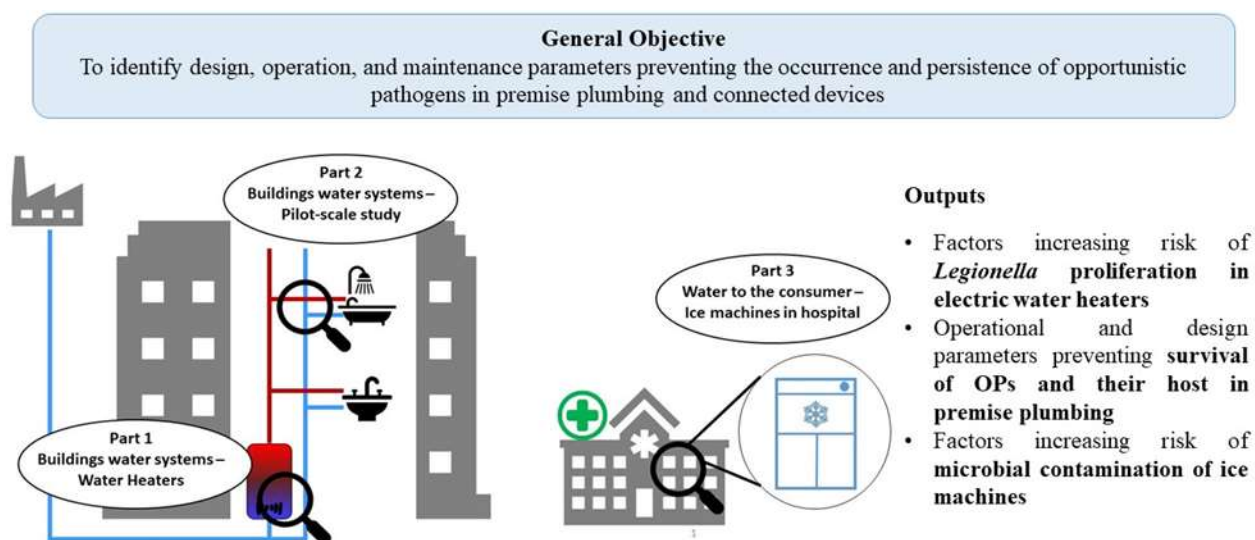


Figure 8.1 Summary of the research conducted

The implementation of a water safety plan (WSP) is now strongly recommended for the prevention and control of infections in healthcare facilities. Throughout the present research, the driving objective was to provide answers and key information for building water systems managers on how to best prevent OP proliferation in their systems, and then to translate this information in a water safety plan. The results will therefore be discussed according to the system assessment and monitoring modules of a WSP, as illustrated on Figure 8.2.

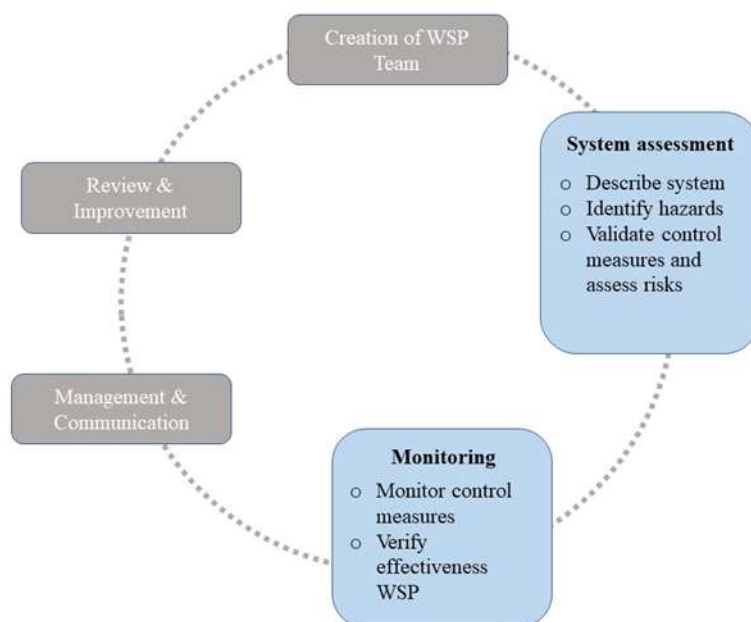


Figure 8.2 Schematic of the modules of a water safety plan (adapted from (World Health Organization (WHO), 2023))

8.1 Building water system description and identification of hazards

The first step of a successful WSP is the creation of a multidisciplinary team to initiate the definition and the implementation of a WSP. First, the assessment of the entire water system should be performed as per the 4 modules constituting system assessment step. Results from this research directly impact the first two modules which are the description of the system and the identification of hazards (World Health Organization (WHO), 2023).

To be able to accurately identify risk areas, hazards, and effective control measures, it is crucial to adequately describe the water system, from the water supplied to the building to the point of use (World Health Organization (WHO), 2011b). As building plumbing systems are complex and to some degree site specific, it is important to describe the structure of the water systems using plans and on-site investigations. Then a thorough description of the devices and materials present in the system and their compliance for safe use in drinking water systems should be documented in the WSP (World Health Organization (WHO), 2011b). Indeed, both plastic and metallic materials are present in water distribution systems, whether in pipes or fixtures (Julien et al., 2020). In Chapter 5, clear structural differences were observed between biofilms formed on coupons of

polypropylene (PP), polyvinyl chloride (PVC), ethylene propylene diene monomer (EPDM), cross-linked polyethylene (PEX), stainless steel (SS) and copper (Cu). Particularly, biofilms developed on EPDM coupons were denser and total bacterial concentrations were higher in water and biofilms sampled in the EPDM reactor as estimated by flow cytometry (FCM) (Chapter 5). Highest concentrations of *V. vermiformis* and NTM by qPCR measurements were found on biofilms developed on EPDM (Chapter 6). Previous studies have already signalled the potential of EPDM biofilms to be reservoirs for OPs and possible sources of microbial contamination of water (Bressler et al., 2009; Moritz et al., 2010). A previous study reported higher HPC and total cells counts in biofilms developed on EPDM than in biofilms developed on PEX and Cu (Moritz et al., 2010). EPDM also consumes chlorine and increased release of organic carbon are measured when in contact with chlorinated water, resulting in enhanced biofilm growth potential (Mao et al., 2018; Neu et al., 2020). As EPDM is mainly used as a sealing material, it can easily be overseen when listing materials present in water systems while it can also have considerable impacts on microbial water quality. EPDM is also approved for other drinking water applications such as flexible connecting hoses. It is present in the solenoid valves on electronic faucets and has been repeatably proven to be susceptible to colonization by *P. aeruginosa* (Hutchins et al., 2020). Considering the high potential of EPDM for OPs and its impact on water quality, special attention should be put toward sectors of the plumbing system that contain any EPDM. The use of EPDM should also be minimised in the system, by avoiding EPDM flexible hoses in the last meter before the faucet for example.

The impact of copper on water quality has been widely investigated. In this work, only microcolonies were observed after immersing copper coupons in water for 3 months (Chapter 5) which concurs with bactericidal properties and slower biofilm formation previously associated with copper (Gomes et al., 2019; Proctor et al., 2017). Copper is mainly used in hot water distribution systems, especially in healthcare facilities (Waines et al., 2011). Overall bacterial concentrations as well as concentrations of *Lp*, NTM and *V. vermiformis* dropped both in water and biofilms of copper reactors heated at 55°C and 60°C (Chapters 5&6). The targeted microorganisms remained unquantifiable by qPCR after 10 weeks of heating (Chapter 6). However, some *Lp* strains isolated from biofilms which had been in contact with copper piping or copper alloys for a long time can develop a high tolerance to copper (Bédard et al., 2021).

For the system description to accurately represent the water system in its entirety, it is important to list all the fixtures and devices connected to the system (both cold and hot water). For hot water systems, a mapping of the components used for heating, their capacity and temperature set points should be considered. Temperature and temperature shifts will impact both chemical (metals, carbon release) and microbial (total bacterial concentrations, OPs) water quality (Chapters 5&6). In Chapter 4, temperature assays performed on *V. vermiformis* showed a shift from a live to dead state when increasing temperature from 40°C to 43°C (Chapter 4, Figure 4.3). A decrease of more than 2-log of *Lp* concentrations was measured after 1h exposures at 55°C and 60°C while a 1h exposure at 70°C led to a 4-log decrease in *Lp* concentrations (Chapter 4, Figure 4.1). Proctor et al. (2017) also defined temperature as the most influential parameter on both microbial composition and *Lp* concentrations in hot water systems. It is therefore important to monitor water temperature in all parts of the hot water system and to be particularly attentive to areas with lower temperatures. Also, all point-of-use devices and their frequency of use should be identified in the WSP (World Health Organization (WHO), 2011b). Although water-ice machines are part of the equipment that must be evaluated for risks, there is no specific regulation for water-ice machines in healthcare facilities. Moreover, it remains unclear whether the quality of the ice and water produced by these machines falls under food or drinking water standards. Results presented in Chapter 7 demonstrate that significant microbial amplification takes place in these devices. Microbial contamination by Gram-negative bacilli and *Candida* spp. of different external elements of ice machines in healthcare facilities and nursing homes was reported by Kanwar et al. (2018). Ice machines have also been linked to infections and pseudo-infections by *Lp* and NTM (Bencini et al., 2005; Guspiel et al., 2017). Point-of-use treatments such as membrane filters should also be considered in WSP as they can alter the quality of water and be sites of microbial amplification (Chapter 7) (World Health Organization (WHO), 2011b). Indeed, profiling sampling performed on 5 water-ice machines in this research revealed that pre-filters leaching nutrients such as phosphates can contribute to the amplification of total bacteria, HPC and NTM (Chapter 7). These results illustrate the importance of identifying and characterizing the risks associated with all devices.

Once all the elements of a water system have been listed, hazards and hazardous events must be identified by the WSP team (World Health Organization (WHO), 2011b). WHO defines a hazard as “a biological, chemical, physical or radiological agent that has the potential to cause harm”

(World Health Organization (WHO), 2011b), while a hazardous event is a situation leading to the existence of a hazard (World Health Organization (WHO), 2011b).

The different materials used in plumbing systems may create biological and chemical hazards depending on their specificities. For instance, accumulation of Pb by biofilms has been previously described (Belcher & Zhang, 2018). In this work, we showed the potential for biofilms to accumulate metals and the potential of leaching of metals and organic carbon from materials. Biofilms developed on Cu coupons accumulated Pb (up to 10µg/cm²) with a possible release of Pb in water in case of desorption or biofilm detachment (Chapter 5). In addition to harbouring a dense biofilm, EPDM also released Pb in water at concentrations sometimes exceeding Canadian drinking water guidance value (Chapter 5) suggesting the use of Pb in the manufacturing of this brand of EPDM and representing a chemical hazard for consumers (Health Canada, 2019). Biofilms developed on EPDM can also harbour high concentrations of OPs and their hosts, with the highest concentrations of both NTM and *V. vermiformis* being measured in EPDM biofilms throughout 76 weeks of pilot-scale study (Chapter 6). Integration of *P. aeruginosa* and *L. pneumophila* in EPDM biofilms has already been described (Bressler et al., 2009; Moritz et al., 2010). Another characteristic of materials is their propensity to release nutrients and carbon assimilable by microorganisms. Higher potential of biofilm formation and carbon leaching have been reported for plastic materials (Neu & Hammes, 2020). In this work, we showed the interplay between materials and temperature on the leaching of organics. Heating at 40°C and above resulted in substantial leaching of DOC and BDOC (up to 13.2mg/L and 3.40mg/L respectively) in water for all 6 studied materials (Chapter 5). Part of this carbon release can be attributed to the detachment of dead biomass from biofilms. The release of such amounts of nutrients in water can support important microbial growth locally or downstream in the system, leading to a decrease of water quality and hazards for the consumers, especially the most vulnerable ones.

A hazard condition to consider in hot water systems, is the presence of temperatures optimal for the growth of OPs especially *Lp* because of poor thermal distribution across the building hot water system (National Academies of Sciences, 2019). While regulated temperatures are set at the point of heating and in the return loops to avoid OP growth temperature ranges in hot water systems, poor balancing and heat loss can lead to insufficient temperatures in some areas of the system. In Chapter 4, 4-log reductions of *Lp* concentrations in water and water heater loose deposits were obtained after 1h exposure at 70°C and 60°C respectively while exposures at lower temperature

led to much lower reductions (2-log reductions and less). The strain of *V. vermiformis* used during this study also showed good resistance to temperatures up to 40°C with all materials, while it could not be quantified in Cu reactors heated at 55°C and 60°C, which concurs with the previous observation of a shift from a live to dead state at 43°C (Chapters 4&6).

Another situation often encountered in water systems of large buildings is the presence of stagnant water. Indeed, stagnation of water in distribution systems has been linked to release of metals in water and enhanced microbial growth, threatening consumer health (Inkinen et al., 2014; Lipphaus et al., 2014; Proctor et al., 2020; Tian et al., 2021). Disinfectant maintenance is not possible in areas of low flow stagnation caused by inadequate hydraulic balancing or because of dead legs in the system (Thomas et al., 2004). Stagnation is also a recognized factor contributing to *Legionella* growth and biofilm formation in plumbing systems (Liu et al., 2006; Raetz, 2010). In this work, we have shown that stagnation in internal reservoirs of water-ice machines because of infrequent use was suspected to cause precipitation of phosphates favouring bacterial amplification and NTM growth (Chapter 7).

In addition to the periodic stagnation that occurs in large building plumbing systems, extended periods of stagnation can occur. Recently, the prolonged closure of many large buildings worldwide because of the COVID-19 pandemic focused the attention on the potential hazards related to these extended periods of low or no water use (Proctor et al., 2020). Indeed, a 6-month stagnation at room temperature in reactors previously heated at 40°C or more led to a rebound of *V. vermiformis* (up to 3-log in Cu, measured by qPCR) in all reactors, and a rebound of culturable *Lp* was measured in PVC, SS and Cu (up to 2-log in PVC) (Chapter 6). Hozalski et al. (2020) also reported widespread detection of NTM and *Legionella* spp. in showers of 5 university buildings after 2 months of stagnation. Intermittent use of point-of-use devices is also a factor associated with biofilm formation and microbial growth in drinking water distribution systems (World Health Organization (WHO), 2011b).

8.2 Monitoring and effectiveness of control measures

After system and hazard assessment, the next component of a WSP is the implementation of control measures and the validation of their efficacy by monitoring. The objective here is to define and implement an operational monitoring plan to ensure that control measures are operating properly

(World Health Organization (WHO), 2023). During this phase, operational limits and corrective actions must also be defined (World Health Organization (WHO), 2011b).

8.2.1 Definition of a monitoring plan

The monitoring plan incorporated in a WSP should specify what will be monitored, where, how, when and by whom (World Health Organization (WHO), 2011b).

What should be monitored ?

Operational monitoring may consist of biological and physico-chemical parameters testing which do not necessarily need to be time-consuming or complex (World Health Organization (WHO), 2011b). For microbial parameters, there are still discussions on the pros and cons of measuring general microbial indicators instead of measuring concentrations of OPs. Currently, primary indicators of drinking water quality are mostly faecal indicators (coliforms or *E. coli*) but OPs are more resistant to disinfection than these indicators and are therefore not well-correlated (Falkinham et al., 2015a; Lu et al., 2015; Wen et al., 2020). In buildings, heterotrophic plate counts (HPC) have been used to monitor general microbial quality of drinking water, although they are highly variable in buildings and are not associated with any known health effects (Bartram et al., 2003; Inkinen et al., 2014; Siebel et al., 2008). However, this method is time-consuming, detects only culturable bacteria, does not detect some of the changes (increase of total cells, community changes) happening in water distribution systems (Lautenschlager et al., 2013; Siebel et al., 2008). In ice machines, we showed frequent exceedances of the often-used reference level of HPC<500CFU/mL in some ice-water samples. More importantly, lower HPC levels were observed in samples with high concentrations of NTM or the presence of *Candida* spp., showing the poor predictive capacity of this indicator for these OPs (Chapter 7). Furthermore, the only ice-water sample positive for *P. aeruginosa* had a HPC result below 500CFU/mL.

A key element of this work is the definition of indicator organisms to suit the objective of monitoring, such as establishing prevalence or measuring the efficacy of disinfection. In those cases, an indicator organism should be abundant and more resistant to disinfection. In the case of ice-water machines, NTM appears to be a better indicator of the presence of other opportunistic pathogens such as *Candida* spp. in ice-water. Considering their ubiquity in water systems and ice machines, the hazard that some of the species of NTM represent to vulnerable patients, and their resistance to disinfection, NTM appear as an excellent indicator of cleaning and disinfection

efficacy of ice machines, and their routine monitoring in healthcare facilities could prevent risk of infections (Klompas et al., 2023). Traditional methods used for the detection and quantification of NTM are culture-based and were developed for clinical microbiology. Recent developments are adapted for the detection and enumeration of NTM in water samples (Ditommaso et al., 2022; Hammer-Dedet et al., 2021; Radomski et al., 2010). However, NTM detection by culture is quite laborious as it typically requires treatment for the suppression of rapidly growing microorganisms, and a long incubation time in rich media; therefore qPCR appears as a good alternative, and protocols are still being improved (Haig et al., 2018; Radomski et al., 2010).

Once the indicator is selected, the next step is to define critical limits which delimit range values of acceptability for the performance of control measures (World Health Organization (WHO), 2023). When these limits are not respected, corrective actions must be implemented to bring the system under control (World Health Organization (WHO), 2011b). Traditionally, an approach consisting of establishing the absence or the limited amplification of an indicator is implemented. As an example, an increase of 1-log of HPC is considered to be acceptable in drinking water in healthcare facilities in France (Castex & Houssin, 2005). A change in levels of a pathogen is an indication of amplification in a device and can be used to measure the need for preventative action. However, it is not a health or risk base standard, only concentrations can be indicative of risk. Further studies are needed to define acceptable concentrations through exposure and epidemiological studies to support the assessment of the risk for patients.

Although most control measures target specific OPs, free-living amoebae (FLA) have been proposed as an indicator of the risk and a better target for control measures for *Lp* and NTM (NASEM, 2019). Because of their common existence in water systems, and their essential role in the growth of *Lp* and NTM, some researchers propose controlling their concentrations to control OP concentrations and proliferation, especially in healthcare facilities (Delafont et al., 2019; Falkinham et al., 2015a). This is particularly important as OPs multiplied in FLA displayed enhanced virulence and resistance to disinfection (Buse et al., 2013; Cirillo et al., 1997; Sarink et al., 2023). In this work, spiking of *V. vermiformis* in drinking water led to the increase of NTM concentrations (by 0.9-log to 1.9-log) within less than a week for all tested materials (Chapter 6). Also, after 4h of exposure at 55°C, the addition of *V. vermiformis* allowed a 6-log increase of *Lp* concentrations in water heater loose deposits in 4 days compared to a 1-log increase without amoeba (Chapter 4). These findings are in line with previous studies reporting increased

concentrations of OPs in presence of amoebae and reinforce the value of controlling FLA concentrations to control OP concentrations in premise plumbing and the hazard they represent for patients.

Some physico-chemical parameters such as temperature can also be used as indicators of risks of OPs (Wang et al., 2017). That is why guidance and regulations call for minimum temperatures in hot water systems and maximum temperatures of 20°C in cold water. Temperature monitoring (preferably continuous) at control points of hot and cold water systems is already required in healthcare facilities in France as an indicator of the risk of proliferation of *Lp* in the systems (Castex & Houssin, 2005). For *Legionella* control in healthcare facilities in the UK, minimal monthly temperature monitoring is required at control points, with a continuous temperature monitoring being recommended at water heater outlets and return loops (Department of Health (DH) & Estates and Facilities Division, 2006; Health and Safety Executive (HSE), 2013). A tool based on temperature profiling and temperature distribution within a water system was also developed to identify areas with high risk of *Lp* proliferation (Bédard et al., 2015b). Temperature <15°C in ice machines and $\geq 55^{\circ}\text{C}$ in reactors were associated with HPCs <500CFU/mL and lower total cell levels below 1×10^5 cells/mL respectively (Chapters 5&7). In Chapter 7, a temperature >20°C or free chlorine residual <0.2mg/L or both in water were well-correlated with HPCs >500CFU/mL in ice-water samples taken at the 36 ice machines of a hospital, but the correlation was weaker with *Candida* spp. presence/absence.

Where should we monitor ?

There is currently no standard on the selection of sampling sites for the monitoring of OPs (Wang et al., 2017). Typically, sampling locations will reflect the priorities of infection control teams considering the main exposure routes of the target microorganisms, the vulnerability of the population, and the environmental preferences of the microorganisms.

Lp is the main OP for which sampling sites for routine monitoring plans have been defined in regulations and guidelines. Sampling sites are selected based on the higher risk of *Legionella* proliferation (infrequent use, dead legs...) and the vulnerability of occupants (intensive care units, burn units...) (BSR/ASHRAE, 2015; Castex & Houssin, 2005; Department of Health (DH) & Estates and Facilities Division, 2006; Wang et al., 2017). More recently, mandatory and

prospective surveillance of NTM in hospital heater cooler units have been implemented and WSP are mandatory in European hospitals.

Another aspect of sampling is whether water or biofilm is collected. To this discussion, we propose to add the sampling of loose deposits to the sites for sampling. In Chapter 4, temperature inactivation assays of an environmental strain of *Lp* showed different inactivation curves when heating was performed in water or in water heater loose deposits. Indeed, accumulation of loose deposits at the bottom of electric water heaters and the thermal stratification occurring in the water tank of electric water heaters can increase the risk for *Legionella* proliferation (Alary & Joly, 1991; Dewailly & Joly, 1991). Thus, sampling and analysing loose deposits from electric water heaters should be considered when sampling for *Legionella*.

Selecting sampling sites and sample type selection should be adjusted to meet the sampling objectives. For instance, sampling biofilm at the drain inlets of ice machines provides information on the efficiency of cleaning procedures as well as the hazard of retro-contamination from drain to the hands of users (medical personnel, patients, or visitors) through splattering (Chapter 7) (Kanwar et al., 2018). On the other hand, sampling ice and water dispensed by ice machines provides relevant information about the hazard of infection or colonization of patients by ingestion or inhalation of droplets and aerosols when drinking or sucking on ice chips (Chapter 7) (Graman et al., 1997). Monitoring for the presence of *Candida* spp. and NTM in both these types of samples is relevant as both groups of pathogens cause superficial (skin) and systemic or pulmonary infections, and both direct contact and ingestion are transmission routes reported for them (Dowdell et al., 2019; Papon et al., 2013).

Results from profiling sampling is more exhaustive but needed to identify more precisely the source of contamination and amplification. Profiling sampling performed on 5 ice machines in a hospital revealed a progressive amplification of NTM in the drinking water distribution system of the hospital, from the building's water inlet to the faucets, even after flushing. Profiling sampling also showed that pre-filters and ice machines were sites of additional amplification of total and culturable bacteria and of NTM (Figure 7.3). Profiling sampling protocols have been proposed for both chemical (especially Pb release) and microbial water quality assessment (Bédard et al., 2018; Deshommes et al., 2013). Sampling the first draw after stagnation informs WSP teams on the hazard for consumers as people usually do not flush water before filling their glass for instance. It

is also in the first draw that the probability to find OPs is the highest (Wang et al., 2017). Thus, sampling after at least 2h of stagnation is recommended in the UK for *P. aeruginosa* detection in augmented care units (Department of Health (DH) et al., 2013). On the other hand, samples taken from subsequent volumes of water at the same point of use or after flushing provide information on water quality at different locations of the building water system. For instance, bacterial profiles obtained after sampling of successive volumes in cold and hot water systems revealed that most of bacterial decline occurred after the first 15mL (Bédard et al., 2018). This study also showed that biofilm, which formation was favoured by the high surface-to-volume ratio in point-of-use devices, contributed the most to the increase in suspended culturable cells in bulk water after 1h stagnation (Bédard et al., 2018).

How should we monitor ?

To finalise the monitoring plan, it is important to define the methods and frequencies for the monitoring of the different control measures identified. The variability of a parameter as well as its significance for public health should be considered when choosing the monitoring frequency (World Health Organization (WHO), 2023).

Regarding methods used for microbial analyses, regulations are still based on culture-based methods, but other easier and less time-consuming methods have been widely used and optimized in research (see 2.2.5). A balance must be found between speed and ease of testing, and the significance of the results provided. For instance, during the pilot-scale study *Lp* were detected and quantified by qPCR in the feed water and some reactors while no *Lp* were measured by culture (Chapter 6). Indeed, qPCR detects and measures all target DNA present in the sample once extracted and does not differentiate between live and dead bacteria (Delgado-Viscogliosi et al., 2009). Higher rates of detection of *Legionella* spp. by qPCR compared to culture have been reported (Whiley & Taylor, 2016). On the other hand, some of our biofilm samples were not quantifiable by qPCR while positive and quantifiable by culture (Chapter 6). This is probably due to the differences of volumes analysed with each method and the different quantification limits: 1mL directly analysed by culture compared to 5µL of DNA extracted from 500µL of sample analysed by qPCR. During our monitoring survey of ice machines in a hospital, a better equivalence was found between HPC and intact cells as compared to HPC and total cells (Chapter 7, Figure 7.2). While HPC usually represent less than 1% of all bacteria in water, total and intact cell densities

measured by FCM reliably quantify the entire bacterial community present (Van Nevel et al., 2017). Finally, quantification of NTM by qPCR in samples from ice machines provided information on the contamination levels and presence of opportunistic pathogens (Chapter 7). However, this method does not allow the identification and quantification of NTM pathogenic species specifically, limiting its relevance during post-outbreak investigations for instance.

8.2.2 Verify the effectiveness of WSP

Once the monitoring plan has been defined and implemented, the objective is to verify that the WSP is effective and that water system operates safely (World Health Organization (WHO), 2011b). This step aims to verify the effectiveness of the control measures and monitoring implemented. Once corrective or remediation measures are implemented, it is also necessary to verify their effectiveness and to ensure safe water supply.

This verification step was the initiating point of the research project on ice machines. Indeed, the whole project started when routine monitoring performed on ice and water samples from 6 ice machines revealed high HPC levels in all samples. The monitoring survey then conducted on 36 ice machines confirmed high HPC levels and the presence of OPs in most of the machines (Chapter 7). The second part of the project aiming to assess the temporal efficiency of the disinfection procedure is currently in progress. The impact of the replacement of some components of the pre-filters and machines as well as the effect of a higher dose of disinfectant will also be assessed. At this point, the work presented in this thesis provides the baseline contamination and informed on the source of OP contamination and amplification. Our preliminary results on cleaning efficacy show that standard cleaning and optimized cleaning procedures do not lower indicators and OPs levels on the long term, showing the need to verify procedures and control methods in a WSP.

In the pilot study part of this thesis, the impact of extended stagnation and the efficacy of remediation measures implemented for the recommissioning of a water system after an extended closure were investigated. Guidelines published during the COVID-19 pandemic recommended two main remediation measures for the safe reopening of large buildings: (1) flushing, and (2) shock chlorination (Proctor et al., 2020). Simulations in 12 CDC biofilm reactors showed that flushing did not impact concentrations of OPs. In particular, culturable *Lp* were still measured after flushing was performed (Chapter 6). Flushing was not efficient either to eliminate biofilms (Chapter 5). Limited temporal efficiency of recommissioning flushing on *Lp* concentration after an

extended period of stagnation was also reported by Grimard-Conea et al. (2022). On the other hand, shock chlorination (20 mg/L Cl_2 for 24h) performed in the same 12 CDC biofilm reactors was effective against *Lp* (Chapter 6). A transfer of dead cells from biofilms to bulk water was also noted in all reactors with a decrease of intact cells percentage in bulk water but an increase of this same percentage in biofilms (Chapter 5). The application of remedial measures should also take into consideration any secondary impact such as the massive release of nutrients. Shock chlorination caused an increase of BDOC concentrations of at least a factor 1.9X in water of all reactors (Chapter 5). These results highlight the need of thorough and repeated flushing of the system after shock chlorination to evacuate the nutrients that might otherwise lead to significant microbial growth (Chapter 5). Finally, the impact of such remediation measures on the integrity of the system should also be assessed. Indeed, high concentrations of chlorine can react with the different materials present in the system and damage them (Proctor et al., 2020). During the shock chlorination performed in reactors at concentrations meeting guidelines, chlorine reacted with both EPDM and Cu coupons (Chapter 5). Chlorine decay was much faster in EPDM and Cu compared to the 4 other materials tested. Cu concentrations were also reduced by a factor 2X, probably because of the reaction of chlorine with Cu accumulated in biofilms and Cu scales (Raetz, 2010).

CHAPTER 9 CONCLUSION AND RECOMMENDATIONS

This research project aimed to identify design, operation, and maintenance parameters preventing the occurrence and persistence of opportunistic pathogens in large building premise plumbing. Several research questions were initially raised: What conditions should be maintained in electric water heaters to prevent the proliferation of *Lp* ? Which factors limit the survival of *Lp* and *V. vermiformis*, and the occurrence of NTM in cold and hot water distribution systems ? Are current disinfection procedures and guidelines efficient regarding ice machines in healthcare facilities ?

The results from the first part of this research bring some new elements and confirmed some others regarding the conditions preventing proliferation of *Lp* in electric water heaters:

- The importance of meeting regulated temperatures in water heaters as *Lp* inactivation logs were higher at 60°C compared to 55°C in both water and loose deposits. Regulated temperatures must also be met in hot water distribution systems as a shift from live to dead state of *V. vermiformis* was observed at 43°C.
- After one week, successive daily 4h exposures at 55°C can prevent the growth of culturable *Lp* in absence of other microorganisms.
- The presence of hosts can nullify the impact of heating as a 6-log increase of *Lp* concentrations was observed 4 days after the addition of *V. vermiformis* to suspensions of heat-exposed *Lp*.

Factors impacting the survival of *L. pneumophila* and *V. vermiformis*, and the occurrence of NTM were investigated with a pilot-scale study. The following conclusions were reached regarding the materials used in premise plumbing:

- EPDM coupons leached important concentrations of Pb sometimes exceeding the Canadian guidance value of 5µg/L.
- EPDM biofilms accumulated Cu and Zn, while Pb accumulated in copper biofilms, which may present a health risk for consumers in the event of biofilm detachment.
- A ranking of the 6 studied materials was established based on the density of their biofilms: EPDM > PP > PVC > PEX > Cu – SS
- NTM and *V. vermiformis* were preferentially detected in EPDM bulk water and biofilm while *Lp* less integrated EPDM biofilms.

The increase of the temperature in the pilot led to the following conclusions regarding water quality in hot water systems:

- Heating at 40°C and above led to substantial leaching of DOC and BDOC from all materials, a fraction of these carbon concentrations being dead biomass from the biofilms. This finding highlights the importance of thoroughly flushing water systems after a thermal shock, or when all or part of a hot water system is brought down to room temperature (for maintenance or during extended closure of buildings) as these nutrients can enhance microbial growth.
- None of the target OPs and their host could be quantified in copper reactors at 55°C and 60°C. This suggests that copper systems heated at temperatures of at least 55°C effectively prevent occurrence of *Lp*, NTM and *V. vermiformis*. It would be interesting to conduct similar studies with NTM and *V. vermiformis* strains adapted to hot water systems to corroborate these observations.

The pilot was kept stagnant at room temperature during the COVID-19 shutdown, which led to the following conclusions regarding extended stagnation periods and the recommended recommissioning strategies :

- Extended stagnation (6 months) at room temperature led to the rebound of culturable *Lp* in PVC and SS reactors previously heated at 40°C, and in one Cu reactor previously heated at 60°C for more than 10 weeks. This experiment was conducted during the COVID-19 shutdown and is representative of what happened in some large buildings at the same time, with studies reporting detection of relatively high *Lp* concentrations in hot water systems upon reopening of the system.
- Shock chlorination performed on the 12 reactors led to an important increase of BDOC concentrations and a transfer of injured and dead cells from biofilms to bulk water. Again, this finding highlights the need to implement thorough and repeated flushing after shock chlorination to wash out the nutrients and cells that could promote microbial growth.
- While simulated flushing did not impact culturable *Lp* concentrations, no culturable *Lp* could be detected after shock chlorination, underlining the at least short-term efficiency of this chemical treatment in our system on *Lp*.

The final conclusions drawn from this pilot-scale study involve the interactions observed between opportunistic pathogens, their host, and their environment:

- NTM and *V. vermiformis* were naturally present in the feed water taken from the municipal drinking water system.
- The addition of *V. vermiformis* led to a substantial increase in NTM concentrations in all the reactors. This finding highlights the role of amoebae in the proliferation of NTM in premise plumbing.

The field study conducted on ice machines in a hospital led to the following conclusions:

- Ice machines and pre-filters are sites of microbial amplification and can be reservoirs of OPs.
- HPC and faecal indicators are currently used in food regulations applying to ice, but our results showed that HPC are not suitable indicators of the efficiency of disinfection of ice machines in healthcare facilities, and do not adequately represent the risk for the most vulnerable patients.
- Release of phosphates by pre-filters and stagnation in internal water reservoirs are suspected to cause precipitates in those reservoirs and to favour microbial growth.

Results from this research led to the following recommendations to mitigate the risk of OP proliferation in premise plumbing:

In hot water systems

- Water heater shutdown to accommodate peak demand shaving for instance, should be done carefully. Findings show that despite the need to reduce energy consumption, lowering the set point temperature of water heaters or changing their heating pattern may have consequences on the microbial quality of water.
- Maintaining high temperature in hot water systems allows a control of *L. pneumophila* and its hosts but does not eradicate them. Results of both pilot-scale study and temperature assays in water and loose deposits from electric water heaters highlight the need to maintain a temperature of at least 55°C in the system, and thus that the water heater should be set at a higher temperature like 60°C.

In premise plumbing

- All materials present in the system must be listed, even sealing materials like EPDM, as they can negatively impact both chemical and microbial water quality and become a vulnerable area of the system.
- Thorough flushing of the system after performing shock chlorination or heat shock as remediation or recommissioning strategies is crucial to prevent microbial growth.

In ice machines

- Design of ice machines should be modified to reduce microbial proliferation. For instance, alternatives to the internal water reservoir or means to automatically drain this reservoir on a regular basis should be considered. The benefits of the installation of UV lights in ice reservoirs could also be assessed.
- Model of ice machines and the size of the ice reservoir should be adapted to the usage made of this machine in the hospital unit.
- Pre-treatments like membrane filters should be carefully selected and adapted to the local water quality and use, as they can be sites of microbial amplification and niches of OPs.
- The need for ice machines in each unit should be evaluated, especially in units with the most vulnerable patients like ICU. Ice-free care options should also be considered as well as the use of cooled water dispensers with an auto flushing option to distribute fresh water, and the use of sterile water to produce ice.

This research project sparked ideas for future research, and it would be interesting to :

- Conduct temperature assays in bulk water and loose deposits of a real electric water heater, with its native microbiome or by filling it with non-sterile hot water, to assess the survival of *L. pneumophila* in conditions more representative of real systems.
- Analyse the microbial communities present with each material in the pilot to possibly identify species or communities favouring or inhibiting the occurrence of *L. pneumophila*, NTM, and *V. vermiformis*.
- Further study the interactions between *V. vermiformis* and NTM (host-prey relationship?), and *L. pneumophila* and NTM (competition? antagonism?).

- Conduct a similar pilot-scale study but with native communities from premise plumbing (by just using tap water) to test the impact of heating and stagnation on these communities, without spiking.
- Evaluate the temporal efficiency of current disinfection procedures for ice machines in healthcare facilities.
- Improve disinfection procedures for ice machines by assessing the efficiency of protocols previously used to disinfect water-containing equipment contaminated by NTM.
- Determine to which extent microbial and NTM contamination of ice machines is a risk to the patients in each unit of the hospital and assess the impact of improved disinfection procedures on nosocomial infection rates, with epidemiologic studies and risk assessment.
- Determine a critical amplification value or a threshold for NTM concentrations in ice machines that would ensure that the disinfection was efficient and the risk for patients is limited.
- Identify species of NTM present in ice machines to evaluate the health risk and assess the impact of phosphates and AgIon® (silver ions) on these species under lab conditions.

It is increasingly acknowledged that building plumbing systems can be sources of nosocomial infections. However, these systems remain microbial black boxes and sustained multidisciplinary research efforts are still needed to further improve our understanding of these systems, and to find and implement simple and effective barriers and solutions.

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APPENDIX A CLEANING PROTOCOL FOR COUPONS OF CDC BIOFILM REATORS®

Procedure:

1. Put metallic coupons in one 50mL sterile tube, put other coupons in the other 50mL tube (max 30 coupons per tube)
2. Add solution of 1/1000 Fisherbrand™ Versa-Clean™ (Thermo Fisher Scientific, USA) up to the 45mL graduation
3. Sonication cycle, 5min
4. Rinse each tube 3 times with autoclaved tap water filtered on 0.2µm
5. Add autoclaved tap water filtered on 0.2µm up to the 45mL graduation
6. Sonication cycle, 5min
7. Rinse each tube 3 times with autoclaved tap water filtered on 0.2µm
8. For metallic coupons: soak coupons in autoclaved tap water filtered on 0.2µm for 2h
For polymeric coupons: soak coupons in a solution of hydrogen chloride 2mol/L under the chemical hood for 2h
9. Rinse each tube 3 times with autoclaved ultrapure water filtered on 0.2µm
10. Let the coupons dry in open sterile Petri dishes under the biological hood. Use the same Petri dishes to stock clean coupons

APPENDIX B COMPARISON OF THE TWO MODELS OF ICE MACHINES

Table B.1 Comparison of the two models of ice machines

	Meridian HID312, Scotsman	Symphony Plus 12, Follett
		
Ice characteristics	Extruded ice (H² Nugget Ice). Daily production up to 260 lbs. Storage capacity of 12 lbs of ice. Continuous flow ice production if the ice tank is not full.	Chewblet® ice (compressed ice nuggets). Daily production up to 425 lbs. Storage capacity of 12 lbs of ice.
External features of the machine	Air cooled. Water and ice dispensers, non-contact sensors. Stainless steel exterior panels.	Air cooled. Water and ice dispensers, infrared non-contact SensorSAFE™ sensors. Stainless steel exterior panels. Automatic double rinse after every hour of compressor operation.

Table B.1 Comparison of the two models of ice machines (continued)

	Meridian HID312, Scotsman	Symphony Plus 12, Follett
Internal features of the machine	Stainless steel evaporator, copper clad. Stainless steel internal components: auger in evaporator, extruder head. Grease-free bearings. Key components made of AgION®, a silver-based antimicrobial protection.	Stainless steel coated evaporator. Stainless steel auger with helix profile to cut ice film from evaporator inner surface. Designed for corrosion resistance. Liners, agitation wheels, water tanks, drain lines, pan and drain screen made of AgION®, a silver-based antimicrobial protection.
Drain system	All models require a drain, sometimes with the addition of an anti-backflow system. Internal drain pan separates the drains from the various machine components.	Vacuum breaker to prevent drain water from coming into contact with water and ice distribution components if the drain is clogged.

**APPENDIX C SUPPLEMENTARY MATERIAL, ARTICLE 1:
COMPROMISED EFFECTIVENESS OF THERMAL INACTIVATION OF
LEGIONELLA PNEUMOPHILA IN WATER HEATER SEDIMENTS AND
WATER, AND INFLUENCE OF THE PRESENCE OF *VERMAMOEBIA*
*VERMIFORMIS***

Journal: Microorganisms

Title: Compromised Effectiveness of Thermal Inactivation of *Legionella pneumophila* in Water Heater Sediments and Water, and Influence of the Presence of *Vermamoeba vermiformis*

Authors: Margot Cazals, Emilie Bédard, Margot Doberva, Sébastien Faucher and Michèle Prévost

Number of figures: 1

Figure C.1

Number of tables: 3

Table C.1

Table C.2

Table C.3

Table C.1 Characteristics of the water and the deposits of the two water heaters studied

Parameters and units	Water Heater A	Water Heater B
Water		
Chlorine residual (mgCl ₂ /L) ¹	≤ DL	≤ DL
Total chlorine (mgCl ₂ /L)	≤ DL	≤ DL
pH	8.0	8.2
Alkalinity (mgCaCO ₃ /L)	27	180
Calcium (mgCaCO ₃ /L) ²	47	131
Total hardness (mgCaCO ₃ /L)	53	239
Temperature in the bottom of the water heater during sampling (°C)	33.8	37.5
Total dissolved solids (mg/L)	96	376
Dissolved O ₂ (mg/L)	10	2
Conductivity (μS/cm ²) ²	150	587
Deposits		
Turbidity (NTU)	142	342
Suspended matter (mg/L)	1344	2275
Volatile suspended solids (mg/L)	367	189
% Volatile solids	25	8
Magnesium (mg/L)	5.9	15.6
Aluminum (μg/L)	4.0	3.5
Calcium (mg/L)	13.5	30.1
Iron (μg/L)	249.9	558.7
Manganese (μg/L)	18.2	148.6
Copper (μg/L)	4.0	1196
Aerobic heterotrophic bacteria (CFU/mL)	415 000	40 000
<i>L. pneumophila</i> (MPN/mL)	ND	ND

¹ DL = 0,05 mg Cl₂/L; ² measured in municipal treated water

Table C.2 Total populations of *V. vermiformis* estimated by microscopy after 4h-exposure at temperatures from 25°C to 60°C

Temperature of the 4h exposure	Total population of <i>V. vermiformis</i> (cells/mL)
25°C	6 x 10 ⁴
30°C	8 x 10 ⁴
37°C	8 x 10 ⁴
40°C	1 x 10 ⁵
43°C	1.1 x 10 ⁵
50°C	1.3 x 10 ⁵
55°C	1.7 x 10 ⁵
60°C	2 x 10 ⁵

Table C.3 Mean values and standard deviations of the control samples for the assay on the long-term efficacy of 1h heat exposures of *Legionella pneumophila*

Time after the 1h heat exposition (hours)	Control 55°C - Mean (CFU/mL)	Control 55°C - Standard Deviation		Control 60°C and 70°C - Mean (CFU/mL)	Control 60°C and 70°C - Standard Deviation	
1	5.00E+08	1.73E+08	(34.64%)	5.67E+07	2.31E+07	(40.75%)
6	5.00E+08	1.73E+08	(34.64%)	5.67E+07	2.31E+07	(40.75%)
24	2.67E+08	5.77E+06	(2.17%)	1.77E+07	2.08E+06	(11.78%)
48	2.70E+08	5.29E+07	(19.60%)	2.07E+07	4.16E+06	(20.15%)
72	4.00E+08	1.73E+08	(43.30%)	2.03E+07	4.73E+06	(23.24%)
168	2.17E+08	4.16E+07	(19.22%)	3.67E+07	1.15E+07	(31.49%)
336	1.60E+08	2.00E+07	(12.50%)	4.67E+06	5.77E+05	(12.37%)
720	/ ¹	/	/	8.33E+06	2.52E+6	(30.20%)

¹ No value available for the control sample of the 55°C assay after 720 hours.

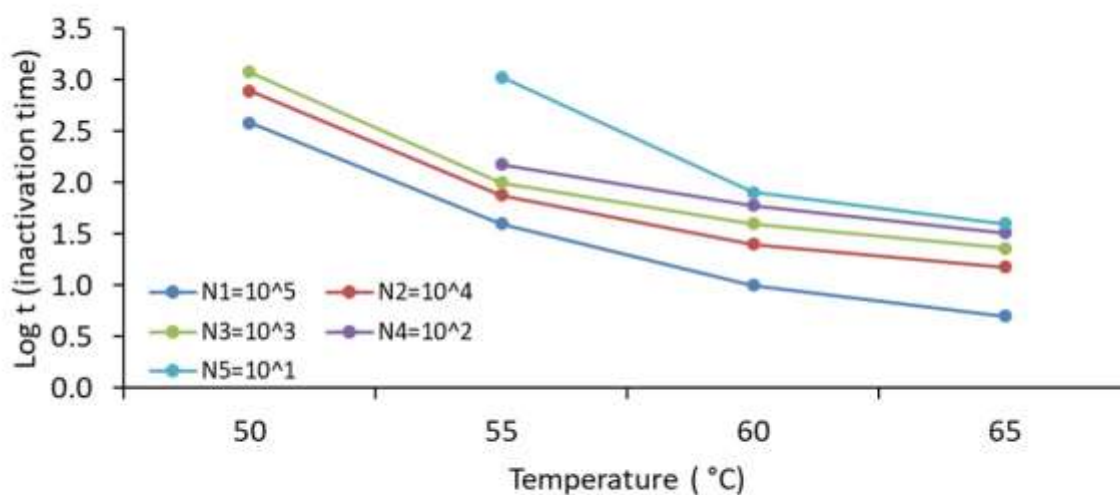


Figure C.1 Results of the Kormendy and Kormendy graphical method

**APPENDIX D SUPPLEMENTARY MATERIAL, ARTICLE 2: WHAT
HAPPENS IN YOUR WATER SYSTEM? IMPACT OF MATERIALS,
TEMPERATURE, STAGNATION AND CHLORINATION ON WATER
QUALITY AND BIOFILM FORMATION**

Journal: Water Research

Title: What happens in your water system? Impact of materials, temperature, stagnation and chlorination on water quality and biofilm formation

Authors: Margot Cazals, Emilie Bédard and Michèle Prévost

Number of figures: 3

Figure D.1

Figure D.2

Figure D.3

Number of tables: 1

Table D.1

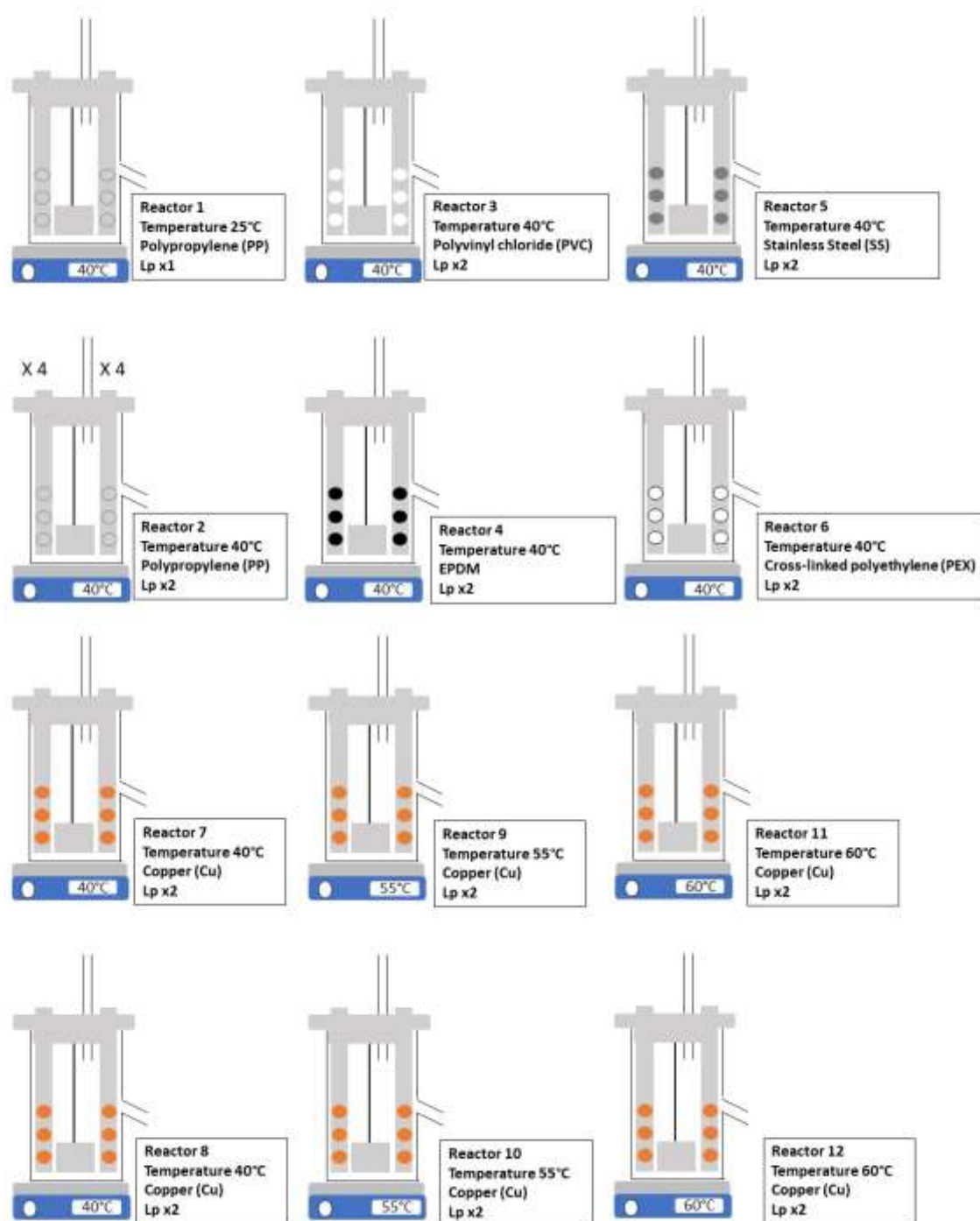


Figure D.1 Scheme of the 12 CDC Biofilm reactors used in this pilot study

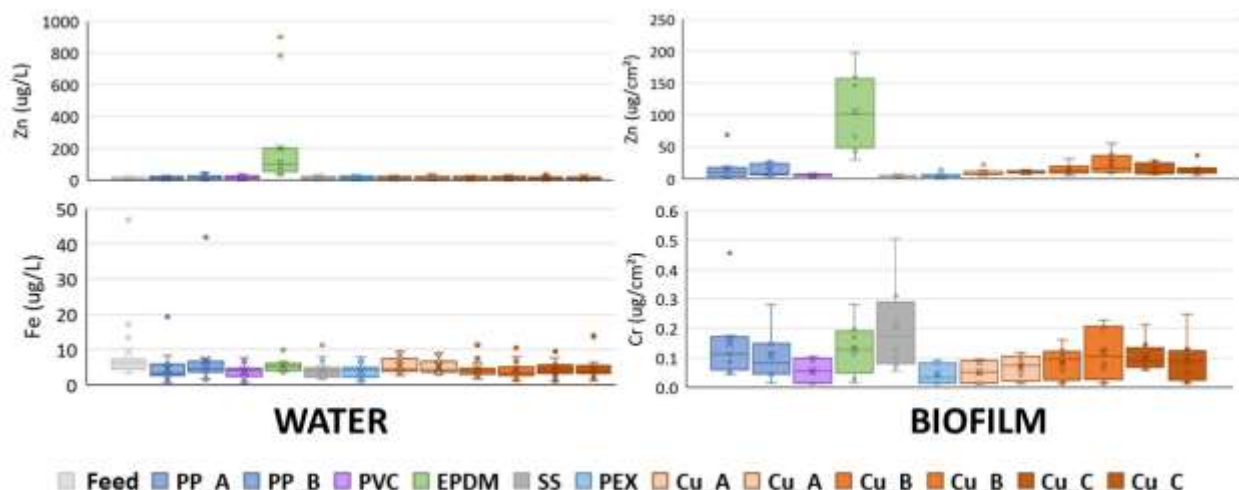


Figure D.2 Boxplots of Zn, Fe and Cr concentrations in bulk and biofilm samples. PP_A : reactor with PP coupons set at 25°C, PP_B : reactor with PP coupons set at 40°C, Cu_A : reactor with copper coupons heated at 40°C, Cu_B : reactor with copper coupons heated at 55°C, Cu_C : reactor with copper coupons heated at 60°C.

Intact Cells cells/mL	week 13	week 14 (+Vv)	week 19	week 20 (+Lp)	week 21	week 22 (ΔT)	week 31	week 37 (4wS)	week 72 (6mS)	week 74 (F)	week 76 (Cl ₂)
	Seeding					Temperature Change		Stagnation and Remediation			
Feed	2.80E+05	4.28E+05	1.83E+05	2.81E+05	3.16E+05		1.18E+05	9.77E+04	6.34E+05	6.72E+05	5.08E+05
PP_A	3.30E+05	1.66E+07	3.54E+05	2.00E+05	3.38E+04	1.75E+05	5.25E+04	4.99E+05	7.83E+05	5.91E+05	1.18E+06
PP_B	2.66E+05	1.14E+07	1.50E+05	6.33E+06	1.22E+05	8.73E+05	1.21E+05	1.67E+05	5.88E+05	6.05E+05	1.75E+06
PVC	2.72E+05	1.39E+07	9.34E+04	2.48E+06	2.46E+05	1.43E+06	1.86E+05	2.27E+05	8.08E+05	5.78E+05	1.80E+06
EPDM	5.41E+05	4.76E+07	1.19E+06	1.50E+07	2.73E+06	1.03E+06	3.81E+05	7.09E+05	2.72E+06	8.41E+05	8.91E+05
SS	2.48E+05	6.09E+06	1.08E+05	2.51E+06	1.92E+05	1.59E+06	2.72E+05	2.67E+05	4.05E+05	5.30E+05	2.08E+06
PEX	2.56E+05	7.03E+06	9.75E+04	2.39E+06	1.79E+05	1.25E+06	1.42E+05	1.53E+05	7.53E+05	4.78E+05	1.28E+06
Cu_A	1.80E+05	5.91E+06	1.74E+05	1.22E+07	2.01E+06	2.47E+05	2.63E+05	2.52E+05	2.40E+05	4.46E+05	8.26E+05
Cu_B	1.96E+05	9.89E+06	1.58E+05	1.17E+07	2.20E+06	3.85E+03	2.25E+03	1.72E+05	9.02E+04	4.59E+05	1.29E+06
Cu_C	1.80E+05	4.36E+06	2.28E+05	1.36E+07	2.06E+06	7.00E+02	2.00E+03	1.88E+05	7.75E+04	4.60E+05	1.01E+06

Figure D.3 Heatmap of intact cells densities in all the reactors and the feed water. Lowest values are in blue while higher ones are in red. Samples were collected three days after interventions (+Lp, +Vv and ΔT) and 2 weeks after flushing (F) and chlorination (Cl₂). Cu_A reactors were heated at 40°C, Cu_B at 55°C and Cu_C at 60°C during the heating phase.

Table D.1 Mean values and standard deviations of physicochemical parameters in the 12 reactors. n represent the numbers of values used for the calculations of the mean and standard deviations in each reactor. Turbidity measurements indicated in italics are the peak values measured and the week the measurements were obtained.

	Feed	PP_A	PP_B	PVC	EPDM	SS	PEX	Cu_A	Cu_B	Cu_C
pH n = 20	8.08 ± 0.39	8.34 ± 0.19	8.47 ± 0.18	8.47 ± 0.17	8.42 ± 0.19	8.48 ± 0.16	8.47 ± 0.16	8.50 ± 0.17	8.55 ± 0.25	8.54 ± 0.26
Turbidity (NTU) n = 20	0.18 ± 0.08	0.37 ± 0.39 <i>w14 = 1.25</i> <i>w37 = 1.45</i>	0.53 ± 0.51 <i>w14 = 1.21</i> <i>w24 = 2.23</i> <i>w37 = 1.21</i>	0.44 ± 0.46 <i>w14 = 1.54</i> <i>w22 = 0.76</i> <i>w37 = 1.77</i>	1.57 ± 1.46 <i>w14 = 5.88</i> <i>w22 = 4.69</i>	0.43 ± 0.29 <i>w14 = 0.79</i> <i>w27 = 1.02</i> <i>w37 = 0.96</i>	0.40 ± 0.27 <i>w14 = 1.03</i> <i>w22 = 0.86</i>	0.66 ± 0.49 <i>w14 = 1.85</i> <i>w72 = 1.72</i>	1.17 ± 1.52 <i>w14 = 2.35</i> <i>w22 = 6.53</i>	0.94 ± 1.65 <i>w14 = 1.31</i> <i>w22 = 7.88</i>
Dissolved O ₂ before ↗ T° (mg/L) n = 6	8.63 ± 0.39	8.41 ± 0.44	8.37 ± 0.40	8.41 ± 0.39	8.30 ± 0.37	8.38 ± 0.46	8.34 ± 0.44	8.40 ± 0.40	8.44 ± 0.41	8.39 ± 0.41
Dissolved O ₂ after ↗ T° (mg/L) n = 5	8.15 ± 0.52	7.81 ± 0.49	6.73 ± 0.34	6.77 ± 0.29	6.70 ± 0.25	6.65 ± 0.14	6.62 ± 0.13	6.72 ± 0.15	6.11 ± 0.27	6.16 ± 0.81
Conductivity (S/m) n = 20	391 ± 30	395 ± 36	396 ± 38	402 ± 38	402 ± 40	404 ± 39	402 ± 44	393 ± 51	396 ± 35	394 ± 36
Free Chlorine 24H after shock chlorination (mg/L)	/	15.6	15.4	15.6	3.4	13.8	13.7	11.3	10.6	12.8
DOC (mg/L) n = 11	1.89 ± 0.23	2.65 ± 0.62	3.12 ± 1.03	3.12 ± 1.09	4.14 ± 2.04	3.08 ± 1.04	3.80 ± 3.28	2.89 ± 0.98	3.93 ± 2.58	4.68 ± 3.32
BDOC (mg/L) n = 10	0.12 ± 0.05	0.29 ± 0.16	0.41 ± 0.30	0.46 ± 0.40	0.66 ± 0.91	0.46 ± 0.42	0.50 ± 0.43	0.37 ± 0.22	0.92 ± 1.06	1.23 ± 1.33

**APPENDIX E SUPPLEMENTARY MATERIAL, ARTICLE 3: FACTORS
AFFECTING THE DYNAMICS OF *LEGIONELLA PNEUMOPHILA* AND
NONTUBERCULOUS MYCOBACTERIA AND THEIR HOST
VERMAMOEBIA VERMIFORMIS IN PREMISE PLUMBING**

Journal: ES&T Water

Title: Factors affecting the dynamics of *Legionella pneumophila* and nontuberculous mycobacteria and their host *Vermamoeba vermiformis* in premise plumbing

Authors: Margot Cazals, Emilie Bédard, Sébastien P. Faucher and Michèle Prévost

Number of figures: 1

Figure E.1

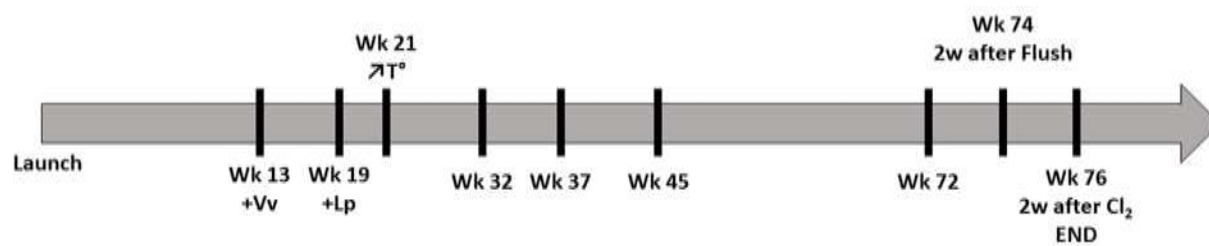


Figure E.1 Schematic of the timeline of the pilot's operation

**APPENDIX F SUPPLEMENTARY MATERIAL, ARTICLE 4: HOW
CLEAN IS YOUR ICE MACHINE? REVEALING MICROBIAL
AMPLIFICATION AND PRESENCE OF OPPORTUNISTIC PATHOGENS
IN HOSPITAL ICE-WATER MACHINES**

Journal: Journal of Hospital Infection

Title: How clean is your ice machine? Revealing microbial amplification and presence of opportunistic pathogens in hospital ice-water machines

Authors: Margot Cazals, Emilie Bédard, Chantal Soucy, Patrice Savard and Michèle Prévost

Number of figures: 4

Figure F.1

Figure F.2

Figure F.3

Figure F.4

Number of tables: 3

Table F.1

Table F.2

Table F.3

Number of texts: 1

Text F.1

Table F.1 Localisation and characteristics of sampled ice machines, and temperature and free chlorine residuals measured in water. NA indicates that the measurements could not be performed. OR = Operating Room. Concentrations of free chlorine of 0.02mg/L and below were considered as Non Detect (ND). STDV : Standard deviation.

Machine	Care Unit	Model	Specificities	Temperature (°C)	Free Chlorine (mg/L)
1	Intensive Cardiology	S	Kitchenette	22.7	ND
2	Hemodynamics	F	Open access, in the corridor	22	ND
3	Emergency Room (ER)	S	Rapid assessment room	14.5	0.42
4	Emergency Room (ER)	S	Open access, in the corridor	15.1	0.26
5	Sampling Room	F		17.8	0.05
6	Palliative Care	S	Kitchenette	22	0.05
7	Endoscopy	F	Open access	11.5	0.49
8	OR	S		21.3	0.03
9	OR	S		NA	NA
10	OR	S		14.5	0.32
11	OR	F		14.8	0.32
12	OR	S		22.5	ND
13	Neonatology	S	Kitchenette	10.2	0.57
14	Severe burns	S	Kitchenette, locked door	18.1	0.26
15	Intensive care	S	Kitchenette	12.9	0.53
16	Intensive care	S	Kitchenette	11.6	0.55
17	Addictions	S	Kitchenette	18.1	0.04
18	Psychiatry	S	Kitchenette, locked door	17.3	0.23
19		S	Kitchenette	13.4	0.53
20		S	Kitchenette	13.8	0.36
21		S	Kitchenette	18.4	0.3
22		S	Kitchenette	16.5	0.28
23		S	Kitchenette	15.5	0.41
24		S	Kitchenette	10.2	0.69
25		S	Kitchenette	15.4	0.36
26		S	Kitchenette	12	0.59
27		S	Kitchenette	14	0.51
28		S	Kitchenette	17.3	0.29
29		S	Kitchenette	12.7	0.53
30		S	Physical therapy room	23.4	ND
31		S	Kitchenette	11.5	0.56
32		S	Kitchenette	15.5	0.38
33		S	Kitchenette	14.4	0.48
34		S	Kitchenette	15.7	0.41
35		S	Kitchenette	15.5	0.52
36		S	Kitchenette	14.7	0.4
Mean +/- STDV				15.9 +/- 3.7°C	0.34mg/L +/- 0.20mg/L

Table F.2 Criteria of choices of the 5 machines for profiling sampling. OR = Operating Room

N° Figure 3	N° Figure 1	Care Unit	Model	Temperature (°C)	Free Chlorine (mg/L)	HPC (CFU/ml)	NTM (gc/ml)	Other microorganisms
1	2	Hemodynamics	F	22	0.02	1.80E+04	3.55E+04	Water: <i>Candida</i> spp. Biofilm: <i>P. aeruginosa</i> + <i>Candida</i> spp.
4	8	OR	S	21.3	0.03	1.45E+03	2.92E+03	Water: <i>Candida</i> spp. Biofilm: <i>Candida</i> spp.
5	12	OR	S	22.5	0	7.30E+03	6.19E+04	Water: <i>Candida</i> spp. Biofilm: <i>P. aeruginosa</i> + <i>Candida</i> spp.
2	25		S	15.4	0.36	3.85E+03	1.57E+02	Water: Enterococci + <i>Candida</i> spp. Biofilm: Enterococci + <i>P. aeruginosa</i> + <i>Candida</i> spp.
3	30		S	23.4	0.02	1.36E+04	7.51E+02	Water: <i>Candida</i> spp. Biofilm: <i>Candida</i> spp.

Table F.3 Number of positive samples for each microorganism measured during the monitoring survey

	Ice-water (n=36)	Drain biofilm (n=36)
HPC < 500 CFU/mL	18	35
Enterococci	1	6
<i>P. aeruginosa</i>	1	11
<i>Candida</i> spp.	15	26

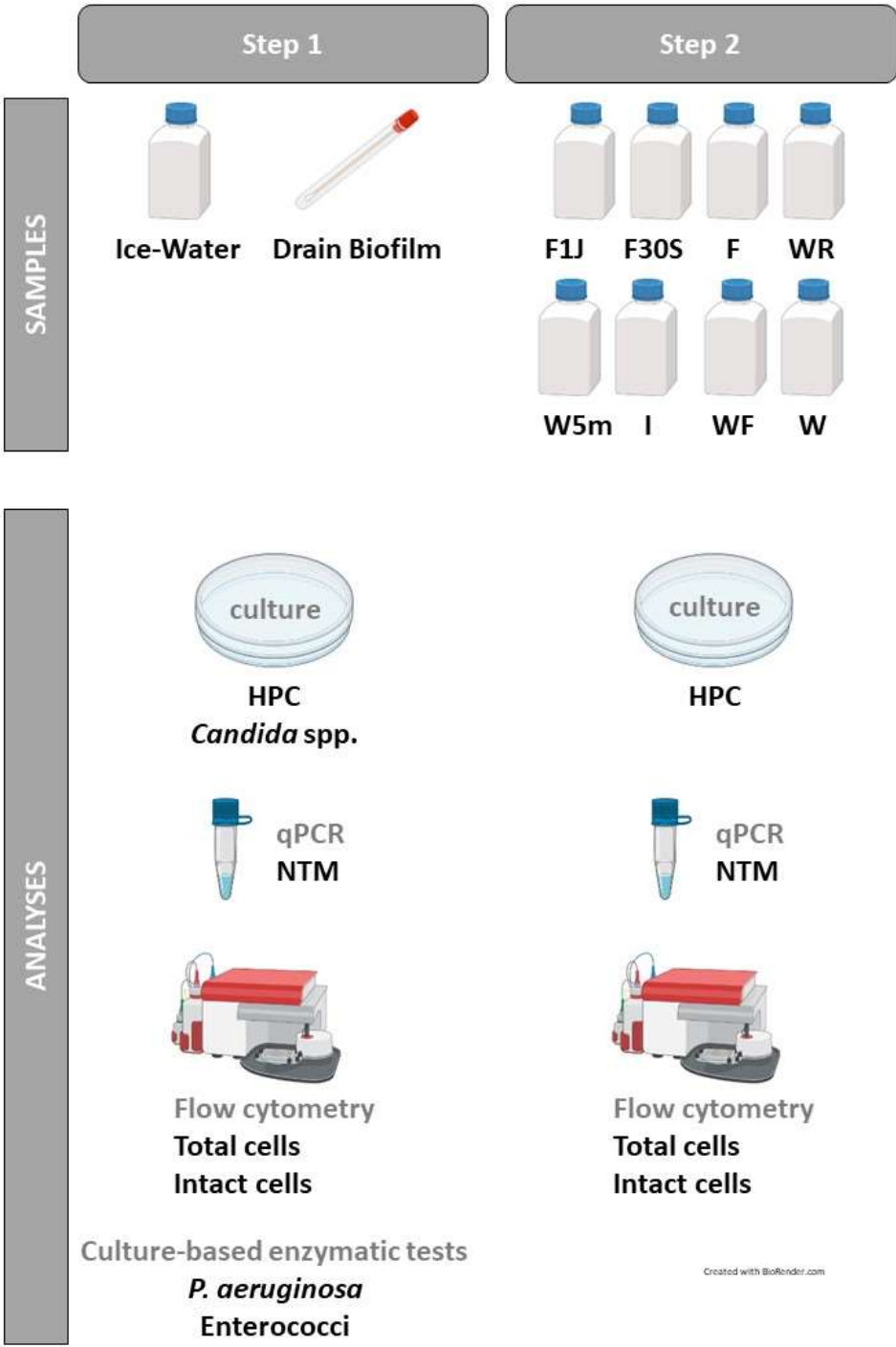


Figure F.1 Diagram of the microbiological analyses performed on all the samples collected in the study

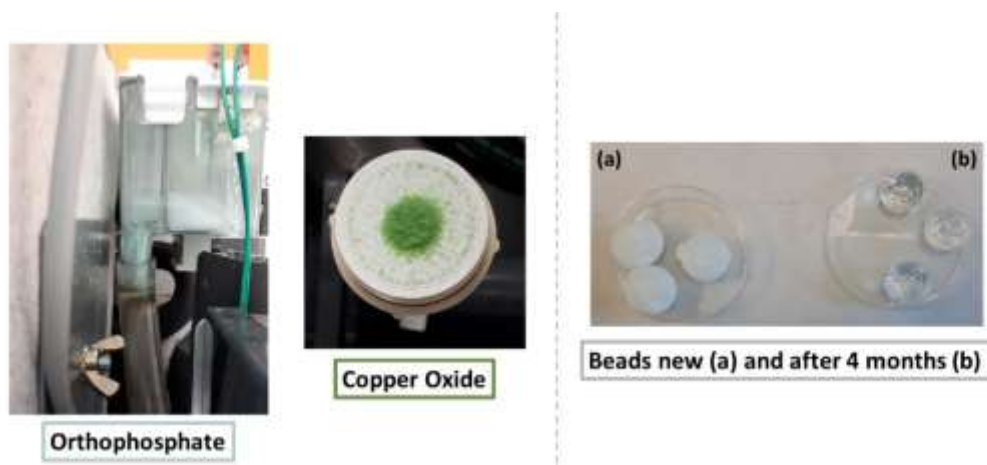


Figure F.2 Pictures of the precipitates found in the water reservoirs and phosphate beads of new filters (a) and 4-month-old filters (b).

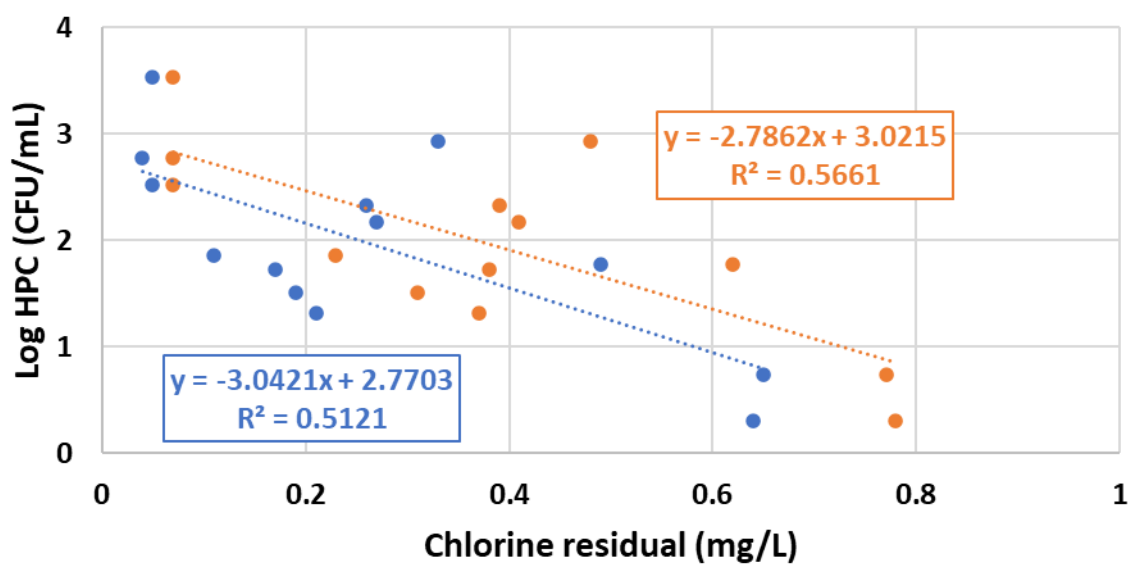


Figure F.3 Correlations between log₁₀ transformed HPC concentrations and free chlorine (in blue) and total chlorine (orange) residuals in samples taken at the faucets during profiling sampling.

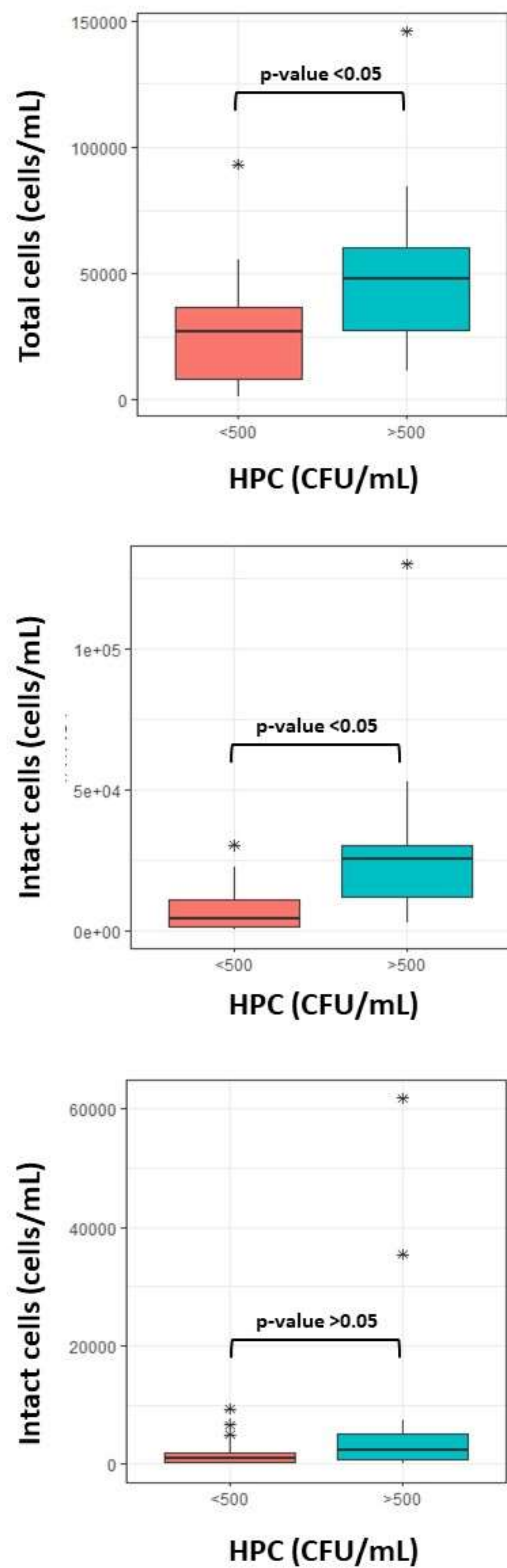


Figure F.4 Distributions of concentrations of total and intact cells and NTM depending on HPC levels (<500 CFU/mL or >500 CFU/mL) and results of Wilcoxon tests.

Text F.1

Cleaning product used on a daily basis for external parts of ice machines:

Oxivir® Plus Disinfectant Cleaner Concentrate (Diversey, USA)

Anti-scaling product used weekly for external parts of ice machines:

Diversey™ Suma® Calc D5 Descaler (Diversey, USA)

Products used every 6 months for disinfection of internal parts of ice machines:

Nickel-Safe Ice Machine Cleaner (Nu-Calgon, USA)

Stera-Sheen® Green Label Machine Sanitizer (Purdy Products Company, USA)