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OPERATIONAL FACTORS INFLUENCING OCCURRENCE AND RISK EXPOSURE TO *PSEUDOMONAS AERUGINOSA* AND *LEGIONELLA PNEUMOPHILA* FROM HOSPITAL WATER SYSTEMS

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OPERATIONAL FACTORS INFLUENCING OCCURRENCE AND RISK EXPOSURE TO *PSEUDOMONAS AERUGINOSA* AND *LEGIONELLA PNEUMOPHILA* FROM HOSPITAL WATER SYSTEMS

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RÉSUMÉ

La qualité microbienne de l'eau potable peut être altérée dans la plomberie interne de grands bâtiments. Les bactéries opportunistes telles que *Legionella pneumophila* et *Pseudomonas aeruginosa* sont présentes et même amplifiées dans les systèmes internes de distribution d'eau et peuvent représenter un risque pour la santé aux points d'utilisation (robinets, pomme de douche, etc.). La formation du biofilm, la stagnation périodique, les rapports surface-volume élevés ainsi que des plages de températures variables sont des facteurs caractéristiques des réseaux de plomberie interne pouvant favoriser la croissance bactérienne. *L. pneumophila* est une bactérie souvent associée aux systèmes de distribution d'eau chaude. Dans les établissements de santé, plusieurs pays prônent une bonne gestion de la température de l'eau afin de prévenir la prolifération des légionelles dans les réseaux de distribution d'eau. *P. aeruginosa* est à l'origine de plusieurs épidémies en milieu hospitalier, souvent directement ou indirectement liées au système d'eau. *P. aeruginosa* est principalement détecté dans les échantillons d'eau froide au robinet et dans le biofilm de l'aérateur, du robinet ou du drain. Ces deux bactéries opportunistes peuvent demeurer dans le système d'eau pour de longues périodes et sont difficiles à éradiquer une fois la contamination établie.

L'objectif principal de ce projet de recherche est de comprendre les facteurs qui influencent la présence de bactéries opportunistes dans les réseaux de distribution d'eau des hôpitaux et d'identifier des mesures permettant de diminuer le risque de prolifération. L'accent a été mis sur *L. pneumophila* dans les systèmes de distribution d'eau chaude et sur *P. aeruginosa* dans les systèmes de distribution d'eau froide. De manière plus détaillée, ce projet vise à: (1) établir le profil de la charge bactérienne dans les systèmes d'eau froide et d'eau chaude en fonction de la durée de stagnation et du volume d'eau recueilli au point d'utilisation, (2) établir la contribution du biofilm sur la charge bactérienne mesurée dans l'eau recueillie au point d'utilisation, (3) évaluer les effets du cuivre et du chlore présents dans l'eau sur la détection de *P. aeruginosa*, (4) optimiser une méthode adaptée aux conditions environnementales des réseaux internes de distribution d'eau pour la détection *P. aeruginosa* et *L. pneumophila* dans de l'eau du robinet, y compris la fraction viable, (5) évaluer la présence de *P. aeruginosa* et de *L. pneumophila* dans les systèmes de distribution d'eau des grands bâtiments, (6) fournir une méthode de diagnostic des réseaux d'eau chaude dans les grands bâtiments basée sur les profils de température et permettant

d'identifier les zones à risque de prolifération de *L. pneumophila* (7) déterminer les facteurs environnementaux et opérationnels favorables à l'établissement et à la persistance de *P. aeruginosa* au robinet, et (8) développer un outil de classement de risque pour les systèmes d'eau chaude basé sur les résultats de suivi de températures aux points de contrôle afin de prioriser les secteurs à risque et l'implantation de mesures correctives.

La première phase du projet de recherche était de localiser à quel endroit se retrouvent les bactéries dans les systèmes de distribution d'eau et comment la présence de facteurs tels le chlore et le cuivre dans les échantillons d'eau potable peuvent affecter les résultats de détection. Des profils de charge bactérienne ont été générés par échantillonnage de volumes successifs au robinet, pour des périodes de stagnation contrôlées. Les premiers 15 mL représentaient plus de 50% des bactéries hétérotrophes aérobies (BHA) mesurées dans le premier litre d'eau froide recueilli à l'ouverture du robinet Ce volume correspond au volume d'eau qui demeure dans le robinet entre deux utilisations. Malgré une baisse importante des BHAs suite au rinçage du robinet, une stagnation d'une heure a suffi pour observer une augmentation des BHA de 1,7 log dans le premier volume de 15 mL prélevé à l'ouverture du robinet. Cette hausse rapide a été attribuée à l'importante contribution du biofilm, mise en évidence par une corrélation exponentielle entre les concentrations en BHA dans l'eau et le rapport surface/volume des conduites. Des périodes de stagnation plus longues ont contribuées à une hausse des BHAs dans le premier litre d'eau prélevé mais l'impact n'était pas aussi prononcé pour les profils de bactéries viables en eau froide et en eau chaude, de même que pour les BHAs en eau chaude. L'impact du chlore résiduel et du cuivre présents à des concentrations retrouvées dans l'eau potable a été étudié pour *P. aeruginosa*. Des concentrations en chlore résiduel entre 0,3 et 2 mg Cl_2/L étaient suffisantes pour supprimer la cultivabilité des bactéries. Cependant, la viabilité n'a été que partiellement affectée et une récupération immédiate a été observée suite à la disparition du résiduel de chlore. La présence de cuivre à des concentrations de 250 µg/L a entraîné une baisse de la cultivabilité de 6 log en moins de deux heures sans toutefois affecter la viabilité. Les cellules ont retrouvé graduellement leur cultivabilité peu de temps après la chélation du cuivre.

La deuxième phase du projet a permis d'identifier les facteurs opérationnels et environnementaux favorisant la présence et la prolifération des bactéries dans l'eau chaude et dans l'eau froide au moyen de campagnes d'échantillonnage des systèmes de plomberie interne d'hôpitaux. Pour les systèmes d'eau chaude, l'étude a porté sur la caractérisation du système, le suivi des températures

aux points de contrôle (chauffe-eau et retour de la recirculation), les profils de températures aux points d'utilisation et la détection de *L. pneumophila* dans l'eau par culture et qPCR (réaction en chaîne de la polymérase quantitative). Les résultats on démontrés que les systèmes pour lesquels la température de l'eau à la sortie du chauffe-eau était maintenue au-dessus de 60°C en tous temps et qui maintenaient une température supérieure à 55°C en tous points du réseau n'avaient pas ou très peu d'échantillons positifs pour *L. pneumophila*. Pour les systèmes ne répondant pas à ces critères de température, les zones à risque ont pu être identifiées grâce à l'interprétation des résultats de profils de température et de la caractérisation détaillée du système. Le risque plus élevé a été confirmé par la détection microbiologique plus fréquente de *L. pneumophila* par les méthodes de culture et de qPCR dans les secteurs identifiés.

Dans les systèmes de distribution d'eau froide, un échantillonnage a été effectué afin d'évaluer le pourcentage de robinets positifs pour *P. aeruginosa* et d'évaluer l'influence de facteurs opérationnels et environnementaux. Des échantillons d'eau et de biofilm (aérateur et drain) ont été prélevés au robinet puis analysés par culture et qPCR. Seulement 7% des échantillons d'eau étaient positifs lorsqu'évalués par culture, comparativement à plus de 50% lorsqu'évalués par qPCR. *P. aeruginosa* a été retrouvé aux trois sites d'échantillonnage d'un même robinet (eau, aérateur et drain) pour 21% des points échantillonnés. L'alignement entre le robinet et le drain, le mode d'activation du robinet et la qualité microbienne de l'eau étaient des facteurs significatifs pour la positivité de l'eau. De plus, la présence d'un aérateur positif était une variable importante pour prédire la concentration de *P. aeruginosa* évaluée par qPCR. Les robinets dont l'eau était positive avaient des concentrations 100-fois plus élevées si l'aérateur était aussi positif. Les méthodes de culture traditionnelles ne permettent pas toujours de détecter *P. aeruginosa* dans l'eau, mais sa présence importante est suggérée par les résultats qPCR. En milieu hospitalier, des perturbations au niveau de l'opération du système d'eau engendrée par des travaux de rénovation ou un manquement dans l'application des bonnes pratiques peuvent agir comme des éléments déclencheurs conduisant à une concentration et un risque accrus d'exposition.

Finalement, l'interprétation des résultats obtenus a mené à des recommandations concernant les mesures d'atténuation de risques pouvant être mises en place. Dans les systèmes de distribution d'eau chaude, une méthode de diagnostic combinant la surveillance des températures aux points de contrôle et l'utilisation de profils de températures aux points d'utilisation est proposée afin de déterminer la vulnérabilité du système et d'identifier les secteurs à risque de prolifération de

Legionella. Le suivi des températures contribue à mieux comprendre l'hydraulique du réseau, à quantifier les pertes thermiques du système dans son ensemble et à identifier les colonnes de distribution qui nécessitent un équilibrage. Une classification du niveau de risque associé à un système d'eau chaude est également proposée. En plus d'une température minimale à la sortie du chauffe-eau et au retour de la boucle de recirculation, des critères concernant le pourcentage du temps où la température en sortie de chauffe-eau est atteinte ainsi que les pertes de chaleur dans le système ont été ajoutés. Ces critères permettent d'évaluer le temps de contact et les températures dans l'ensemble du système, à l'exception du volume distal non-recirculé.

Dans l'ensemble, ce projet de recherche a permis de mettre en évidence certains des facteurs influençant la présence de *L. pneumophila* et *P. aeruginosa* dans les réseaux d'eau des hôpitaux et d'identifier des mesures de gestion des risques. La stagnation périodique, le rapport surface-volume des conduites et les températures du réseau d'eau chaude ont été identifiés comme des facteurs importants affectant la concentration et la fraction cultivable de bactéries viables à laquelle le consommateur est exposé. Les résultats montrent que la charge bactérienne est significativement plus élevée au premier jet, phénomène qui est amplifié après une période de stagnation. Dans les systèmes de distribution d'eau chaude, le maintien de températures suffisamment élevées supprime la cultivabilité de *L. pneumophila* mais un risque demeure, tel que suggéré par la persistance de *L. pneumophila* détectée par qPCR. Les outils de diagnostic et de classification de risque proposés aideront les établissements de soins de santé à interpréter les données de surveillance de température et à prioriser l'implantation de mesures correctives selon le niveau de risque. Au robinet, un choix approprié du robinet ainsi que de la disposition de l'évier contribueront à minimiser la contamination de l'eau par *P. aeruginosa* et le risque d'exposition pour les patients.

ABSTRACT

Microbial quality of drinking water can be altered in the premise plumbing of large buildings. Opportunistic pathogens such as *Legionella pneumophila* and *Pseudomonas aeruginosa* are present and even amplified in the water distribution system of large buildings and represent a health risk at points-of-use (taps, shower head, etc). Biofilm formation, periodical stagnation, high surface-to-volume ratios, and favorable temperatures are characteristics of the premise plumbing system that can promote bacterial growth. *Legionella pneumophila* is frequently detected in hot water distribution systems and thermal control is a common measure implemented by healthcare facilities. *Pseudomonas aeruginosa* is a source of outbreaks, especially in intensive care units (ICU) and several of these outbreaks have been directly or indirectly linked to water systems. *P. aeruginosa* is primarily detected in cold water and faucet biofilm samples, specifically at the distal end of the water system. Both of these opportunistic pathogens can persist for prolonged periods of time once contamination is established.

The main objective of this research project is to understand factors influencing occurrence of opportunistic pathogenic bacteria in hospital premise plumbing and identify risk mitigating measures. The emphasis was put on *Legionella pneumophila* in hot water distribution systems and on *Pseudomonas aeruginosa* in cold water distribution systems. On a more detailed level, this project sought to: (1) establish the bacterial load profile in cold and hot water systems according to the stagnation time and the volume of water collected at the point-of-use in order to define the optimum sampling protocol and interpret sampling results, (2) establish the biofilm detachment contribution to the bacterial load in the water collected at the point-of-use, (3) evaluate the effect of copper and chlorine present in premise plumbing on the detection of *P. aeruginosa* in water, (4) optimize a selective method for environmental detection of *P. aeruginosa* and *L. pneumophila* in tap water, including the viable fraction, (5) assess the occurrence of *P. aeruginosa* and *L. pneumophila* in the premise plumbing of large buildings, (6) provide a diagnostic methodology for health care facilities and other large buildings based on temperature profiling of the hot water distribution systems to identify *L. pneumophila* risk areas (7) determine the environmental and operational factors favorable to the establishment and persistence of *P. aeruginosa* at the tap and in the premise plumbing, and (8) develop a risk

classification tool for hot water distribution systems based on temperature measurements and monitoring at key control points to prioritize corrective actions.

Initially, preliminary investigation was conducted to understand where to find bacteria within the premise plumbing and the impact of common drinking water stressors on sampling results. Bacterial profiles were generated through sampling successive volumes at the tap after controlled stagnation periods. More than 50% of heterotrophic plate counts (HPCs) from the liter collected immediately upon opening the cold water were recovered from the first 15 mL, corresponding to the volume contained within the faucet body. After a short stagnation of one hour after flushing the tap, HPCs were up by 1.7 log in the 15 mL volume from the faucet. The dominant contribution of the biofilm was evidenced by an exponential correlation between HPCs in water and the pipe surface-to-volume ratio after one hour stagnation. The effect of stagnation on HPCs and viable cell counts was not as pronounced for hot water. The impact of residual chlorine and copper at concentrations present in drinking water was demonstrated for *P. aeruginosa*. A chlorine residual between 0.3 and 2 ppm was sufficient to suppress cell culturability. However, viability was only partially affected and rapid recovery was observed once residual chlorine disappeared. For copper concentrations of 250 μg/L, culturability decreased by 6 log in the span of 2 hours but copper ions did not depress viability; viable cells started to regain culturability shortly after copper ion chelation.

Once these aspects were established, field investigations of hospital water systems were conducted to identify key operational and environmental factors promoting bacteria in water. In hot water distribution systems, temperature and heat loss at control points (water heater, recirculation, and representative points-of-use) were monitored for five hot water distribution systems. Systems in which water temperature at water heater outlet was consistently above 60°C and maintained above 55°C across the network were negative for *Legionella* by culture or quantitative polymerase chain reaction (qPCR). For systems not meeting these temperature criteria, risk areas for *L. pneumophila* were identified using temperature profiling and system's characterization. In cold water distribution systems, sampling was conducted to evaluate faucet positivity for *P. aeruginosa* and to assess influencing operational and environmental factors. Water, aerator and drain swab samples were collected from faucets and analyzed by culture and qPCR. *P. aeruginosa* contamination in various components of the sink environment was detected several years after the resolution of an outbreak, illustrating the difficulty to eradicate *P.*

aeruginosa from the plumbing components once established. A low *P. aeruginosa* contamination of water was detected by culture (7%) while qPCR detection revealed a 50% positivity rate. *P. aeruginosa* was recovered in all three sampling site (water, aerator and the drain biofilms) for 21% of the sinks. Drain alignment, the type of device and water microbial quality were significant factors for water positivity, and aerator positivity was a significant variable to predict the load of *P. aeruginosa*. Faucets with a positive aerator had average *P. aeruginosa* concentrations 100-fold higher than positive faucets with a negative aerator. Although the bacteria may not be detectable in the water by traditional culture methods, *P. aeruginosa* is present and can recover its culturability under favorable conditions. The importance to have clear maintenance protocol of the water systems, including the drainage component of the system, is highlighted.

Based on these results and current literature, the last step was to transfer these findings into applicable risk mitigation measures. In hot water distribution systems, a diagnostic flowchart combining temperature monitoring of the main hot water system components and temperature profiling at points-of-use is proposed to determine the overall susceptibility of the system and to identify specific areas at risk for *Legionella* proliferation. Temperature monitoring will help understand the hydraulics, quantify the thermal losses of the recirculating system and identify the distribution columns that need balancing. A system risk classification is also proposed, with selection criteria developed based on results from this research and reported in the literature. In addition to a minimum temperature at the water heater outlet and at the end of the recirculation loop, the percentage of time the temperature is met together with the total heat loss in the system were included. These criteria provide conditions to maximise the contact time at temperatures above 55°C throughout the system, except for the non-recirculating distal volume.

Overall, this research project evidenced factors influencing the occurrence of *L. pneumophila* and *P. aeruginosa* in hospital premise plumbing and identified risk mitigating measures. Periodical stagnation, pipe surface-to-volume ratio and water temperatures at the tap were identified as major factors affecting the concentration and culturable fraction of viable bacteria to which the consumer is exposed. Findings show significantly higher bacterial loads in the tap's first volume after stagnation. In hot water distribution systems, results confirmed that maintaining sufficiently high temperatures within hot water distribution systems suppressed *L. pneumophila* culturability. However, the persistence of *L. pneumophila* in the system was observed by qPCR. The proposed

risk diagnostic and risk classification are a step forward to help healthcare facilities interpret temperature monitoring data and prioritize areas where corrective measures should be implemented. At the tap, an appropriate choice of device and sink layout will contribute to minimizing the contamination of taps by *P. aeruginosa* and the risk of exposure for patients.

CONDENSÉ EN FRANÇAIS

La contamination des réseaux de distribution d'eau potable dans les grands bâtiments est de plus en plus préoccupante. Certaines bactéries pathogènes opportunistes tels *Legionella pneumophila* et *Pseudomonas aeruginosa* sont présentes et même amplifiées dans ces réseaux et représentent un risque sanitaire aux points d'utilisation (robinets, pommeau de douche, etc). Elles se développent dans le biofilm formé naturellement à l'intérieur du réseau de distribution et y persistent grâce à la stabilité et la résistance du biofilm. *L. pneumophila* colonise les réseaux de distribution d'eau chaude et froide entre 20°C et 50°C (température optimale de 35°C) et a été identifié comme la principale cause des épidémies reliées à l'eau aux États-Unis depuis 2005 (> 50%). *P. aeruginosa* peut se développer à des températures allant jusqu'à 42°C bien que sa température optimale de croissance se situe entre 30°C et 37°C. Il est responsable d'environ 10% des infections nosocomiales aux États-Unis et représente la plus fréquente cause d'infection chez les grands brûlés et la deuxième plus grande cause de pneumonies nosocomiales.

Bien que certaines directives d'exploitation aient été avancées, aucun consensus n'existe sur la détection, le suivi et le contrôle de ces bactéries opportunistes dans les réseaux internes de distribution d'eau complexes. Les facteurs influençant l'établissement et la persistance de *L. pneumophila et P. aeruginosa* ont été définis par la recherche effectuée au cours des dernières années : les matériaux, la présence préalable d'un biofilm hétérotrophe, la température, le régime hydraulique, la concentration de carbone organique assimilable, la concentration et le type de désinfectant ainsi que la présence d'amibes en sont les principaux. Toutefois l'importance relative de ces facteurs est mal définie, particulièrement dans les réseaux internes réels. De plus, l'étendue de la présence de *L. pneumophila et P. aeruginosa* ainsi que le type d'équipement contaminé sont mal connus.

La plupart des études en milieu hospitalier ne permettent pas d'identifier les lieux et causes de l'amplification des bactéries pathogènes opportunistes à l'intérieur du bâtiment. Pour préciser si cette amplification est généralisée ou plutôt localisée, il est nécessaire de mesurer à différents temps de séjour de manière à mettre en évidence le lieu et les causes de l'amplification. De plus, peu d'études incluent les drains, qui peuvent pourtant être une source importante de contamination du point d'utilisation et du matériel de soin à proximité.

L’expérience européenne a clairement mis en évidence l’impact prédominant du temps de séjour sur la prolifération de pathogènes opportunistes dans les réseaux internes. La maîtrise de l’hydraulique de ces réseaux y est maintenant réglementée par l’émission de critères de conception et d’exploitation des réseaux. Elle consiste en une modification de la configuration et/ou de l’exploitation des réseaux pour éliminer les zones à faibles écoulement (temps de séjour élevé), à haut potentiel de déposition et les antennes (culs-de sac). La maîtrise de l’hydraulique permet de maintenir un contrôle thermique adéquat en tous points du réseau afin de limiter la présence et la prolifération de *L. pneumophila*. Cette approche est appliquée avec succès, et est préférée à l’implantation d’un traitement d’appoint au point d’entrée du bâtiment. Toutefois, l’application de ces directives et la démonstration de leurs impacts directs sur l’occurrence des bactéries opportunistes sont peu documentées.

Le principal objectif de cette thèse est de comprendre les facteurs qui influencent la présence de bactéries opportunistes dans les réseaux de distribution d’eau des hôpitaux et d'identifier des mesures pour diminuer le risque de prolifération. L'accent a été mis sur *L. pneumophila* dans les systèmes de distribution d'eau chaude et sur *P. aeruginosa* dans les systèmes de distribution d'eau froide. De manière plus détaillée, ce projet vise à: (1) établir le profil de la charge bactérienne dans les systèmes d'eau froide et d’eau chaude en fonction de la durée de stagnation et du volume d'eau recueilli au point d'utilisation, (2) établir la contribution du biofilm sur la charge bactérienne mesurée dans l'eau recueillie au point d'utilisation, (3) évaluer les effets du cuivre et du chlore présents dans l’eau sur la détection de *P. aeruginosa*, (4) optimiser une méthode adaptée aux conditions environnementales des réseaux internes de distribution d’eau pour la détection *P. aeruginosa* et *L. pneumophila* dans de l'eau du robinet, y compris la fraction viable, (5) évaluer la présence de *P. aeruginosa* et de *L. pneumophila* dans les systèmes de distribution d’eau des grands bâtiments, (6) fournir une méthode de diagnostic des réseaux d’eau chaude dans les grands bâtiments basée sur les profils de température et permettant d’identifier les zones à risque de prolifération de *L. pneumophila* (7) déterminer les facteurs environnementaux et opérationnels favorables à l'établissement et à la persistance de *P. aeruginosa* au robinet, et (8) développer un outil de classement de risque pour les systèmes d'eau chaude basé sur les résultats de suivi de températures aux points de contrôle afin de prioriser les secteurs à risque et l’implantation de mesures correctives.

La première phase du projet de recherche visait à comprendre à quel endroit se trouvent les bactéries dans le système d'eau chaude et d'eau froide (Chapitre 3) et comment la présence de facteurs tels le chlore et le cuivre dans les échantillons d'eau potable peuvent affecter les résultats de détection (Chapitre 4). Dans un premier temps, des profils de charge bactérienne en fonction du volume écoulé au robinet ont été établis pour les 10 litres recueillis immédiatement à l'ouverture du robinet (Chapitre 3). L'échantillonnage de volumes successifs a été effectué séparément pour l'eau froide et pour l'eau chaude, après des périodes de stagnation contrôlées allant d'une heure à 10 jours. Les résultats ont révélé des profils décroissant des concentrations en bactéries hétérotrophes aérobies (BHA) dans le premier litre échantillonné pour l'eau chaude et l'eau froide. Les premiers 15 mL représentaient en moyenne 53% (eau froide) et 35% (eau chaude) de la charge bactérienne cultivable (BHA) du premier litre recueilli après stagnation. Ce volume correspond au volume estimé d'eau qui demeure dans le robinet entre deux utilisations. Malgré une baisse importante des BHA suite au rinçage du robinet, une stagnation d'une heure a suffi pour observer une augmentation de 1,7 log dans ce même volume de 15 mL. Cette hausse rapide a été attribuée à l'importante contribution du biofilm, mise en évidence par une corrélation exponentielle entre les concentrations en BHA dans l'eau et le rapport surface/volume des conduites et du robinet. Des périodes de stagnation plus longues ont contribuées à une hausse des concentrations en BHA dans le premier litre d'eau prélevé mais l'impact n'était pas aussi prononcé pour les profils de bactéries viables en eau froide et en eau chaude, de même que pour les profils de concentration de BHA en eau chaude. Les profils de bactéries viables étaient comparables en eau chaude et en eau froide, mais contrairement aux BHA, la diminution a été observée seulement après 500 mL d'écoulement. En moyenne, le pourcentage de cellules cultivables est passé de 1% dans le premier 15 mL prélevé à 0,005% après 10L d'écoulement, et ce pour les deux systèmes étudiés. La stagnation périodique et le rapport surface/volume élevé ont été identifiés comme des facteurs importants qui affectent la concentration en bactéries et la fraction cultivable à laquelle l'utilisateur est exposé. Les résultats montrent que la charge bactérienne est significativement plus élevée dans le premier volume du robinet, ce phénomène étant amplifié après une période de stagnation.

Dans un deuxième temps, l'effet d'une exposition de la bactérie *P. aeruginosa* à des concentrations de chlore résiduel (2 mg Cl_2/L) et d'ions cuivre (250 µg/L) représentatives des réseaux internes de distribution d'eau des grands bâtiments a été étudié (Chapitre 4). Ces facteurs

peuvent représenter un stress pour la cultivabilité des bactéries, sans pour autant en affecter la viabilité. Des concentrations en chlore résiduel entre 0,3 et 2 mg Cl_2/L étaient suffisantes pour supprimer la cultivabilité des bactéries. En dépit d'une réduction de la viabilité de 3.5 log, une récupération immédiate a été observée suite à la disparition du résiduel de chlore et une reprise partielle de la cultivabilité a été observée après 24 heures. La présence de cuivre a entraîné une baisse de la cultivabilité de 6 log en moins de deux heures sans toutefois affecter la viabilité des cellules. Ces dernières ont retrouvé graduellement leur cultivabilité, peu de temps après la chélation du cuivre. Les résultats indiquent que *P. aeruginosa* peut non seulement survivre dans les réseaux d'eau potable mais qu'un potentiel de récupération de la cultivabilité et de multiplication est possible suite à une exposition au cuivre ou au chlore pour les concentrations étudiées.

La deuxième phase du projet a été amorcée par des campagnes d'échantillonnage de systèmes de distribution interne d'eau dans les hôpitaux, qui ont permis d'identifier les facteurs opérationnels et environnementaux favorisant la présence et la prolifération des bactéries. Tout d'abord, cinq systèmes d'eau chaude ont été évalués (Chapitre 5). Après une caractérisation de chacun des systèmes, un suivi des températures a été effectué aux points de contrôle (chauffe-eau et retour de la boucle de recirculation), des profils de température ont été établis en des points d'utilisation représentatifs et la présence de *L. pneumophila* dans l'eau a été évaluée par deux méthodes : culture et qPCR (réaction en chaîne de la polymérase quantitative). Les résultats ont démontré que les systèmes pour lesquels la température de l'eau à la sortie du chauffe-eau était maintenue au-dessus de 60°C en tous temps et qui maintenaient une température supérieure à 55°C en tous points du réseau n'avaient pas ou très peu d'échantillons positifs pour *L. pneumophila* ni par culture, ni par qPCR. Pour les systèmes ne répondant pas à ces critères de température, les zones à risque ont pu être identifiées grâce à l'interprétation des résultats de profils de température et de la caractérisation détaillée du système. L'identification d'un risque plus élevé par le biais des critères de température a été confirmée par la détection microbiologique plus fréquente de *L. pneumophila* par les méthodes de culture et de qPCR dans ces secteurs. Afin de comprendre l'incapacité de certains secteurs à maintenir les températures aux niveaux désirés, une investigation détaillée a été menée dans un secteur. Les résultats ont montré que la présence de clapets anti-retour défectueux dans les robinets pouvait causer des baisses de température importantes dans les colonnes de distribution d'eau chaude du secteur, suggérant un apport d'eau

froide. Cet exemple laisse entrevoir les multiples raisons pouvant affecter le maintien des températures dans des secteurs définis d'un système.

Par la suite, une campagne d'échantillonnage a été effectuée dans le système d'eau froide d'un hôpital où une épidémie de *P. aeruginosa* était survenue en 2004-2005, dans une unité néonatale de soins intensifs. Au moment de l'épidémie, les résultats de génotypage des souches cliniques et environnementales isolées avaient identifié les drains et les aérateurs comme étant à la source de l'épidémie. L'échantillonnage mené dans le cadre du projet de recherche actuel a ciblé 28 éviers de cet hôpital afin d'évaluer le pourcentage de robinets positifs pour *P. aeruginosa* et d'évaluer l'influence de facteurs opérationnels et environnementaux (Chapitre 6). Une caractérisation détaillée a d'abord été effectuée, incluant entre autre le type de robinet, le matériau de la conduite de connexion, le diamètre interne du robinet, l'alignement entre le drain et le robinet, ainsi que l'efficacité de drainage. Les éviers ont ensuite été échantillonnés comme suit: 1) un frottis de l'intérieur du drain; 2) un litre d'eau prélevé à l'ouverture de l'eau froide; 3) un frottis de l'extérieur de l'aérateur. Les analyses microbiologiques ont révélé que seulement 7% des échantillons d'eau étaient positifs lorsqu'évalués par méthode de culture, comparativement à plus de 50% lorsqu'évalués par méthode qPCR. Un important pourcentage des aérateurs (64%) et des drains (89%) étaient aussi positifs en qPCR. Au total, 21% des éviers étaient positifs simultanément aux trois sites d'échantillonnage (eau, aérateur et drain). La position du drain par rapport au jet d'eau, le mode d'activation du robinet et la qualité microbienne de l'eau étaient des facteurs significatifs pour la positivité de l'eau. De plus, la présence d'un aérateur positif était une variable importante pour prédire la concentration de *P. aeruginosa* mesurée par qPCR. Les concentrations en *P. aeruginosa* mesurées dans l'eau étaient en moyenne 2 log plus élevées pour les éviers ayant un aérateur positif. Ces résultats soulignent l'importance de mieux comprendre le rôle de l'aérateur dans la contamination du robinet et de l'eau. Bien que les bactéries ne soient pas toujours détectées dans l'eau par des méthodes de culture traditionnelles, *P. aeruginosa* est présent et peut retrouver sa cultivabilité dans des conditions favorables. En milieu hospitalier, des événements perturbateurs du système d'eau comme les travaux de rénovation ou un manquement dans l'application des bonnes pratiques peuvent agir comme des éléments déclencheurs conduisant à une concentration et un risque d'exposition accrus. Le rôle du drain dans la contamination rétrograde de l'aérateur suggère l'importance d'établir un protocole de maintenance détaillé des systèmes d'eau incluant le système de drainage.

Finalement, l'interprétation des résultats obtenus a mené à l'élaboration de mesures d'atténuation de risques pouvant être mises en place. Dans les systèmes de distribution d'eau chaude, une méthode de diagnostic combinant la surveillance des températures aux points de contrôle et l'utilisation de profils de températures aux points d'utilisation est proposée afin de déterminer la vulnérabilité du système et d'identifier les secteurs à risque de prolifération de *Legionella* (Chapitre 5). Un diagnostic systématique est nécessaire pour identifier les zones les plus à risque dans les systèmes où la circulation n'est pas équilibrée, particulièrement pour les bâtiments existants dont les plans datent et qui n'ont pas été mis à jour au fil des rénovations et de réaménagements du système. Lorsque le système comprend plusieurs boucles connectées à un collecteur principal, la température doit être surveillée à chacune des boucles de retour. Le suivi des températures en continu contribue à mieux comprendre l'hydraulique du réseau, à quantifier les pertes thermiques du système dans son ensemble et à identifier les colonnes de distribution qui nécessitent un équilibrage. Une classification du niveau de risque associé au système d'eau chaude est également proposée. En plus d'une température minimale à la sortie du chauffe-eau et au retour de la boucle de recirculation, des critères concernant le pourcentage du temps où la température en sortie de chauffe-eau est atteinte, le temps requis pour atteindre la température d'équilibre au robinet ainsi que les pertes de chaleur dans le système ont été ajoutés. Ces critères permettent d'évaluer le temps de contact et les températures dans l'ensemble du système, à l'exception du volume distal non-recirculé. L'approche de diagnostic et de classification de risque des systèmes d'eau chaude proposée est peu coûteuse et peut facilement être mise en œuvre afin d'optimiser les ressources disponibles vers les zones à risque plus élevé.

Ce projet de recherche visait à améliorer notre capacité à limiter la prolifération des bactéries opportunistes dans les systèmes de distribution d'eau chaude et d'eau froide des hôpitaux grâce à une meilleure maîtrise des facteurs opérationnels et environnementaux. Les principales conclusions sont les suivantes:

- Le biofilm joue un rôle important sur la concentration en bactéries libres dans l'eau au point d'utilisation en raison du rapport surface-volume élevé dans les antennes terminales des systèmes de distribution d'eau.
- L'exposition de *P. aeruginosa* au chlore et au cuivre présents dans l'eau potable peut résulter en une sous-estimation des concentrations telles qu'évaluées par des méthodes de culture

standard. Ce résultat souligne l'importance de définir un protocole de suivi approprié, incluant le volume d'échantillonnage, le traitement de l'échantillon et la méthode d'analyse pour évaluer les risques directs et indirects d'exposition à *P. aeruginosa* aux points d'utilisation.

- La méthode de détection par qPCR est un outil de surveillance intéressant permettant de détecter rapidement les variations de concentration en *L. pneumophila* ou *P. aeruginosa* dans l'eau des systèmes de distribution par rapport aux valeurs de base établies par un suivi périodique et ce, malgré la présence de chlore et de cuivre dans l'eau. Une augmentation du signal qPCR indique une multiplication des cellules et permet de mettre en place des mesures correctives rapidement afin de minimiser le risque.
- L'approche de diagnostic progressif du réseau interne de distribution d'eau chaude proposé permet de déterminer les secteurs les plus à risque d'un système de distribution d'eau chaude pour la prolifération de *L. pneumophila.* Le diagnostic peut être utilisé pour définir un plan d'action, incluant les actions préventives à mettre en place et la sélection des points de prélèvement pour le suivi de *L. pneumophila*.

De plus, des recommandations à plusieurs niveaux ont été formulées :

- Les éviers :
  - La sélection de robinets de conception simple est à privilégier, visant à minimiser : la surface en contact avec l'eau, le volume d'eau froide et chaude mélangé et la présence de matériaux plastiques et élastomères.
  - L'utilisation d'aérateurs ou de régulateurs de débit devrait être évitée, tel que recommandé en Angleterre (Department of Health (DH), Estates & Facilities, & Government of Great Britain, 2013).
  - L'installation de valves thermostatiques ne devrait être permise que si le risque de brûlure est plus important que le risque d'infection. La valve thermostatique devrait être située le plus près possible du robinet.
  - La longueur des tuyaux de connexion sous l'évier (souvent en tressé flexible) devrait être minimale de façon à réduire le volume d'eau exposé à des rapports surface/volume plus élevés.

- Un programme d'entretien des drains devrait être mis en place afin d'éviter un mauvais drainage de l'eau lors de l'utilisation du robinet.
- L'utilisateur devrait éviter de mettre les mains sous le premier jet d'eau en raison des concentrations plus élevées présentes dans le premier 250 mL.

➢ Le système d'eau chaude :

- La circulation de l'eau chaude ne devrait pas être interrompue périodiquement pour des considérations énergétiques. Il est primordial de maintenir des températures adéquates en tout temps dans l'ensemble du système d'eau chaude afin de prévenir des plages de températures favorisant la prolifération des légionelles. Le maintien de températures élevées permet de contrôler *L. pneumophila* sans toutefois l'éradiquer.
- Le volume stagnant de l'antenne terminale (robinet et connexion) devrait être minimisé en connectant le système de recirculation le plus près possible du point d'utilisation.
- La température des boucles de recirculation secondaires devraient être suivie en continu, avant le collecteur et la boucle de recirculation principale.
- Les profils de température devraient être appliqués au plus grand nombre de points possible de façon à mieux comprendre la nature (distale ou systémique) et l'étendue (volume) des volumes d'eau en présence de températures favorables à *L. pneumophila*.

➢ Évaluation du risque et échantillonnage:

- Un plan de sécurité de l'eau devrait être implanté pour chaque établissement de santé. En plus de l'évaluation technique du risque requise lors de l'implantation, le risque devrait être réévalué lorsqu'une modification doit être faite aux installations existantes (plomberie ou point d'utilisation).
- Lorsque qu'un traitement de désinfection en continu est présent à l'entrée du bâtiment, le traitement doit être appliqué de façon assidue et faire l'objet d'un suivi serré de manière à éviter les périodes prolongées où le traitement n'est pas optimal. Pour ces systèmes, une détection par qPCR est souhaitable en complément de la culture afin de détecter plus rapidement des changements microbiologiques qui pourraient ne pas être détectés par culture en raison de la présence d'un désinfectant résiduel.

- L’échantillonnage de l’eau devrait être fait en période de faible utilisation ou après stagnation afin de maximiser la détection des bactéries libres présentes.
- Idéalement, un échantillon du premier jet et un échantillon après écoulement devraient être prélevés afin de déterminer si la contamination est distale (point d’utilisation) ou systémique. Pour les échantillons récoltés au premier jet, un trop grand volume échantillonné ne sera pas représentatif du point d’utilisation.

➢ Nouvelles constructions:

- Une procédure de mise en service des systèmes d’eau des nouveaux bâtiments ou des secteurs rénovés devrait être en place afin d’évaluer la présence de bactéries opportunistes, les traitements requis et les mesures correctives à mettre en œuvre avant le début de l’utilisation du système.
- La conception spatiale des chambres et de l’évier devrait inclure les éléments suivants : 1) minimiser le nombre d’éviers afin d’éviter les robinets peu ou pas utilisés; 2) choisir des éviers dont le drain est positionné de façon à ce que le jet d’eau ne puisse pas générer des éclaboussures en entrant en contact avec le drain; 3) la position du lit et du matériel de soin destiné aux patients devraient être situé à l’extérieur du rayon sujet aux éclaboussures provenant de l’utilisation de l’évier (i.e. lavage de mains).

Le rôle de l'environnement dans la transmission d'infections nosocomiales liées aux soins de santé est de plus en plus reconnu**.** Des efforts de recherche soutenus permettront une compréhension accrue des systèmes de distribution d’eau des hôpitaux, où de multiples variables peuvent influencer la dynamique de prolifération des bactéries opportunistes. Une approche multidisciplinaire et une analyse des causes fondamentales sont nécessaires à la mise en œuvre réussie d’un plan de gestion du risque.

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LIST OF ABBREVIATIONS

AFNOR	Association française de normalisation
APHA	American Public Health Association
APIC	Association for Professionals in Infection Control and Epidemiology
ASHE	American Society for Healthcare Engineering
ASHRAE	American Society of Heating, Refrigerating and Air-Conditioning Engineers
ASPE	American Society of Plumbing Engineering
ATP	Adenosine Triphosphate
AWWA	American Water Works Association
BAP	Blood Agar Plate
BCYE	Buffered Charcoal Yeast Extract
BSI	British Standard Institution
CDC	Center for Disease Control
Cl_2	Chlorine
CSA	Canadian Standard Organization
CFU	Colony forming unit
CMMTQ	Corporation des maîtres mécaniciens en tuyauterie du Québec
CSTB	Centre Scientifique et Technique du Bâtiment
CT	Concentration x Time
Cu	Copper ions
DDTC	diethyldithiocarbamate
DH	Department of Health (Great Britain)
DNA	deoxyribonucleic acid

ECDC	European Center of disease prevention and control
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EPDM	Ethylene propylene diene monomer
Fe	Iron
FEMS	Federation of European Microbiological Societies
GU	Genomic Unit
GVPC	Glycine, Vancomycin, Polymixin, Cycloheximide
HCF	Health care facility
HCN	Cyanide
HPC	Heterotrophic Plate Counts
HQNO	2-heptyl-4-hydroxyquinoline *N*-oxide
HSE	Health and Safety Executive
HWDS	Hot Water Distribution System
ICU	Intensive Care Unit
ISO	International Organization for Standardization
INSPQ	Institut national de santé publique du Québec
Lp	*Legionella pneumophila*
LSPQ	Laboratoire de Santé Publique du Québec
MacAP	MacConkey Agar Plate
min	Minutes
MPN	Most Probable Number
MQ	MilliQ Ultrapure Water
N	Final cell count

N_0	Initial cell count
Na_2SO_3	Sodium Thiosulfate
NICU	Neonatal Intensive Care Unit
NSERC	Natural Sciences and Engineering Research Council
POU	Point-Of-Use
ppm	Parts Per Million
PVC	Polyvinyl Chloride
Qc	Québec
qPCR	Quantitative Polymerase Chain Reaction
RAISIN	Réseau d'alerte, d'investigation et de surveillance des infections nosocomiales
RBQ	Régie du Bâtiment du Québec
RNAse	Ribonuclease
SDS	Sodium dodecyl sulfate
sg.	Serogroup
Spp.	Species
S/V	Surface-to-Volume Ratio
TSB	Tryptic Soy Broth
UNG	Uracil-N glycoslyase
VBNC	Viable But Non Culturable
WEF	Water Environment Federation
WHO	World Health Organization
Wks	Weeks

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INTRODUCTION

Background

Contamination of drinking water systems in large buildings is an increasing concern and raises questions regarding the role of drinking water distributors to prevent it. Indeed, following the recent improvements of disinfection processes, contamination of water during distribution is now responsible for the largest proportion of waterborne outbreaks in North America (Brunkard et al., 2011; Craun, 2012; Reynolds, Mena, & Gerba, 2008). Opportunistic pathogens such as *Legionella pneumophila* and *Pseudomonas aeruginosa* are present and even amplified in the water distribution system of large buildings and represent a health risk at points-of-use (taps, shower head, etc). They thrive in the biofilm naturally formed within the distribution network and their persistence has been attributed to the biofilm resistance and stability (Wingender, 2011). Several of these microorganisms have been identified as important sources of health care associated infections. *Pseudomonas*, specifically *P. aeruginosa*, causes 4-17% of nosocomial infections related to water, making it the largest reported cause (Hidron et al., 2008; Institut national de santé publique du Québec (INSPQ), 2011; The RAISIN Working Group, 2009). *Legionella* in plumbing systems was identified as the main cause of outbreaks related to water in the United States in 2009-2010 (> 50%) (Centers for Disease Control and Prevention (CDC) & Morbidity and Mortality Weekly Report (MMWR), 2013) and is on the EPA Contaminant Candidate list (United States Environmental Protection Agency (USEPA), 2009a). In France, it accounted for 7% of nosocomial infections between 2001 and 2006 (The RAISIN Working Group, 2009).

L. pneumophila is a Gram-negative water associated opportunistic pathogen responsible for two forms of respiratory disease: severe pneumonia (or legionellosis) and Pontiac fever. Pontiac fever is milder and has flu-like symptoms. It is estimated that between 8,000 and 18,000 people are hospitalized each year in the United States because of legionellosis (Marston, Plouffe, File, Jr, & et al., 1997). The mortality rate can be very high, reaching up to 50% for people with predisposing factors (Bartram, Chartier, Lee, Pond, & Surman-Lee, 2007; Morbidity and Mortality Weekly Report (MMWR), 1997). Other species of the genus *Legionella* can cause Legionnaire's disease, but *L. pneumophila* alone is responsible for over 90% of cases worldwide

(Benin, Benson, & Besser, 2002; Yu, V. L. et al., 2002). The principal mode of transmission is by inhalation of fine water droplets (1 to 10 μm) containing the bacteria at a dose sufficient to be infectious (Buse, Schoen, & Ashbolt, 2012; Schoen & Ashbolt, 2011). A minimum infectious dose has not been clearly established in the literature. However, concentrations of 1,000 to 100,000 CFU/mL were measured in water samples suspected of being the source of contamination during infection (Giglio, Monis, & Saint, 2005). In hospital settings, a dose as low as 10 CFU/mL may be sufficient to cause an infection (Torii et al., 2003). Control of *Legionella* risks in health care facilities is addressed through regulations and guidelines (Bartram et al., 2007; BSR/ASHRAE, 2013; Centers for Disease Control and Prevention (CDC), 2003; Health and Safety Executive (HSE), 2013; République Française, 2010a). Approaches to control *L. pneumophila* in hot water distribution systems vary considerably, but all guides include objectives or obligations for optimal operating temperatures at critical points in the distribution systems. System characterization and environmental monitoring are among the first steps to establish a water safety plan or to evaluate the operational risk in hot water distribution systems, especially in health care facilities (BSR/ASHRAE, 2013; Department of Health (DH) & Estates and Facilities Division, 2006b; République Française, 2010b; World Health Organization (WHO), 2011). Recent guidelines stress the need to properly manage hydraulics to ensure homogeneous temperature and biocidal control in all areas of the hot water distribution system (Centre scientifique et technique du bâtiment (CSTB), 2012), and system balancing under varying demand should be verified.

L. pneumophila has been typically recovered from cooling towers, hot and cold water premise plumbing, humidifiers and whirlpools (Arvand, Jungkind, & Hack, 2011; Brousseau et al., 2012; Walser et al., 2014; Yiallouros et al., 2013). It was isolated in systems with temperatures ranging between 0 and 60°C (Carvalho, Nastasi, Gamba, Foronda, & Pellizari, 2008; Martinelli et al., 2000) but its optimum growth temperature is 35°C (Katz & Hammel, 1987). *Legionella* grows in the biofilm naturally established in water distribution networks (Murga et al., 2001). Many studies have shown the presence of *L. pneumophila* in large buildings water systems such as hospitals (Bargellini et al., 2011; Marchesi et al., 2011; Martinelli et al., 2000; Moore et al., 2006), hotels (Borella et al., 2005; Leoni et al., 2005), in dentistry lines (Barbeau, Gauthier, & Payment, 1998; Petti, Iannazzo, & Tarsitani, 2004), in spas (Guillemet et al., 2010) and in

residential showers (Stout et al., 1992). Most cases are associated with water systems at temperatures higher than room temperature.

Hospital studies show that the prevalence estimated by percent positivity varies from 10 to 50% of hot water samples taken from taps and showers (Bargellini et al., 2011; Bonadonna et al., 2009; Borella et al., 2005; Flannery et al., 2006; Leoni et al., 2005; Martinelli et al., 2000; Mathys, Stanke, Harmuth, & Junge-Mathys, 2008; Stout et al., 1992). In Germany, a large-scale sampling of private residences hot water revealed that 9% of systems were positive for *L. pneumophila* (Mathys et al., 2008) whereas 15% were positive in Italy (Borella et al., 2005). However, the potential contribution of cold water systems as a source of *L. pneumophila* should not be overlooked. In the Netherlands, the proportion of positive taps reached 3.9% while 4.2% of cold water samples were positive in the network of a dental clinic in Italy (Borella et al., 2005). Some factors have been identified in recent years as being favorable to the growth of *L. pneumophila* in water systems. Temperature, presence of amoeba, use of disinfectants, pipe materials and other plumbing components, stagnation and the presence of biofilm are the main factors to consider (Buse et al., 2012).

Ideally, systems should be maintained in control through optimized hydraulics and system temperatures (Health and Safety Executive (HSE), 2013; République Française, 2010b). However, if sampling results indicate microbial contamination of the system or if the system cannot be maintained under control, disinfection should be applied. There are several water disinfection methods that are used to control *Legionella* in hot water distribution systems (Lin, Stout, & Yu, 2011). No disinfection method has demonstrated an absolute efficiency, especially on the biofilm established within the pipes. Some solutions such as chlorine dioxide and monochloramine proved effective in controlling the concentrations of *L. pneumophila* in the liquid phase under the thresholds of 10^2 CFU/mL when applied continuously in premise plumbing (Marchesi et al., 2012; Moore et al., 2006; Srinivasan et al., 2003). However, episodes of periodic interruption of disinfection promote system contamination and monitoring of the microbiological quality of water is needed to ensure effective treatment. Indeed, the vast majority of studies have evaluated the effectiveness of treatment through a culture method. However, *L. pneumophila* is known to enter VBNC or intra-amoeba state when in the presence of a stress, and would be undetected by culture. It is possible that the disinfectant induces a VBNC state or intra-amoeba state without creating cell death. Thus, when the disinfectant concentration falls or is

stopped, the cells can proliferate in the system. This would explain the rapid regrowth observed after cessation of treatment or when there was a temporary failure of the system (Saby et al. 2003, Thomas et al. 2004). Furthermore, disinfectants were not effective against tested amoeba, especially for amoebae in the biofilm (Cervero-Arago, Rodriguez-Martinez, Canals, Salvado, & Araujo, 2013; Thomas et al. 2004). The implementation of a disinfection regime should also assess the impact on other opportunistic pathogenic bacteria also present in the water or the biofilm. For example, in a study comparing disinfection with chlorine and chlorine dioxide, improved efficiency of monochloramine for *L. pneumophila* was noted, but no effect on *Pseudomonas* spp. while the chlorine dioxide had a marked effect on reducing the number of sites positive for *Pseudomonas* spp. (Marchesi et al., 2011).

Pseudomonas aeruginosa is an opportunistic pathogen ubiquitous in the environment and recognized for its capacity to form or integrate biofilms. Although infrequently recovered in municipal water, *P. aeruginosa* can occur at higher frequency within large building water distribution systems where it can persist and multiply (Loveday et al., 2014; Trautmann, M., Lepper, & Haller, 2005). Environmental reservoirs are numerous, including water and biofilm recovered from faucets, aerators and drains (Hota et al., 2009; Lavenir et al., 2008; Walker, J. T. et al., 2014). *P. aeruginosa* can be transmitted by a number of routes, including healthcare workers' hands (Jones, 2011), patient-to-patient (Bergmans et al., 1998) and environmental contamination (Jefferies, Cooper, Yam, & Clarke, 2012). It is responsible for about 10% of nosocomial infections in the US and it is the most common cause of infection in burn patients and the second leading cause of nosocomial pneumonia (Maier et al. 2000). A detailed review of *P. aeruginosa* ecology in drinking water is presented in Chapter 1.

L. pneumophila and *P. aeruginosa* can colonize and proliferate in water from premise plumbing and its existing biofilm. *L. pneumophila* is most often associated to the hot water distribution system and can be systemic or distal whereas *P. aeruginosa* is primarily detected in cold water samples, specifically at the distal end of the water system (Cristina et al., 2014). In both cases, once contamination is established, it can persist for prolonged periods of time. Several disinfection methods have been tested, but have mitigated success once applied in a premise plumbing environment, with all additional factors. In light of this, it appears that a better understanding of the environmental factors associated with the presence of these bacteria in the water system is needed to have a preventive rather than curative approach.

Structure of dissertation

This thesis is subdivided in eight chapters. Following a detailed review of *P. aeruginosa* in drinking water (Chapter 1, submitted to *Water Research*), the research objectives along with the methodology are presented (Chapter 2). Chapters 3 through 6 present research results in the form of four submitted or published scientific publications. The first article aims at better understanding the localization of bacterial amplification within premise plumbing and the impact of water stagnation on the bacterial load at the faucet (Chapter 3, submitted to *Environmental Science and Technology*). The next chapter investigates the effect of chlorine and copper ions, commonly found in water from premise plumbing, on the detection of *P. aeruginosa* (Chapter 4, published in *FEMS Microbiology Letters*). The following 2 chapters summarize findings from extensive sampling campaigns in large buildings targeting the occurrence of *L. pneumophila* and *P. aeruginosa*. Chapter 5 proposes a new diagnostic method to identify risk areas for *L. pneumophila* in a large building using temperature profiles and control points (published in *Water Research*). Chapter 6 reports the results of a field investigation of factors associated to the occurrence of *P. aeruginosa* within hospital sink environments (submitted to *Infection Control and Hospital Epidemiology*). Finally, a general discussion is provided in Chapter 7 followed by conclusions and recommendations.

CHAPTER 1. ARTICLE 1 – *PSEUDOMONAS AERUGINOSA* IN DRINKING WATER SYSTEMS: A REVIEW

Pseudomonas aeruginosa is one of the leading causes of waterborne infections in healthcare facilities. This chapter proposes a critical overview of the ecology of *P. aeruginosa* in drinking water systems, the key factors promoting its growth, amplification and persistence and a summary of current regulations and guidelines to control *P. aeruginosa.* This review brings an engineering outlook on this microbial contaminant, looking into the effects of the operating conditions prevailing within the distribution system (disinfection, temperature and hydraulic regime) and recommendations with regards to proactive control measures that can be implemented in light of the information reviewed. This paper is under review by *Water Research*.

PSEUDOMONAS AERUGINOSA IN DRINKING WATER SYSTEMS: A REVIEW

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is ubiquitous in the environment and is recognized for its capacity to form or join biofilms. Although infrequently recovered from municipal water, *P. aeruginosa* can occur at a higher frequency under certain conditions within drinking water distribution systems, where it can persist and multiply. The present review focuses on the ecology and key factors that promote the growth and persistence of *P. aeruginosa* within drinking water networks. The role of the premise plumbing system, including the materials and the devices that are at, or within, close proximity to the point-of-use (faucet, aerator, drains), is summarized. Furthermore, the effects of the operating conditions that prevail within the distribution system (disinfection, temperature and hydraulic regime) are

reviewed. Limited guidelines and recommendations to control water contamination by *P. aeruginosa* are available. A better understanding of its ecology and key influencing factors will help to identify culprit areas and implement control measures.

KEYWORDS

Pseudomonas aeruginosa, premise plumbing, environmental factors, disinfection, faucets, healthcare facilities

1.1 Introduction

Pseudomonas aeruginosa is a versatile bacterial pathogen that is associated with an ever-widening spectrum of infections in humans that can be associated with significant morbidity and mortality (Kerr, K. G. & Snelling, 2009). This opportunistic pathogen mainly affects immune-suppressed patients and is found in 50-60% of hospitalized patients (especially on burns and scabs), as opposed to 1.2-6% in healthy individuals (Botzenhart & Döring, 1993; Shooter, 1971). One of the most common causes of healthcare-associated infections, *P. aeruginosa* represented 8-11% of reported nosocomial infections in Europe and in the United States between 2001 and 2010 (Hidron et al., 2008; The RAISIN Working Group, 2009; Zarb et al., 2012). It is the most frequently recovered Gram-negative bacteria from patients with healthcare-acquired pneumonia over the last two decades in the United States (Weinstein, Gaynes, Edwards, & System, 2005). For individuals who suffer from cystic fibrosis, it is the most important cause of morbidity (Pier, 2012) and a major predictor of mortality (Emerson, Rosenfeld, McNamara, Ramsey, & Gibson, 2002).

Infections can be acquired from community settings (hot tubs, Jacuzzis, swimming pools) but occur mainly in healthcare settings, especially in critical care units and following procedures that involve physical breaches in the hosts' defenses, such as surgical incisions and the use of invasive devices (Jefferies et al., 2012; Kerr, K. G. & Snelling, 2009). At risk populations include neonates, patients with deep neutropenia, severely burned patients, patients subject to foreign device installation (e.g., vascular and urinary catheters, endotracheal tube, ventilator) and patients who have underlying pulmonary disease such as bronchiectasis and cystic fibrosis (Jefferies et al., 2012; Kerr, K. G. & Snelling, 2009; Leclerc, Schwartzbrod, & Dei-Cas, 2002). *P. aeruginosa*

can cause a long list of infections, including pneumonia, bacteremia, urosepsis and wound infections (Berthelot et al., 2001; de Victorica & Galvan, 2001; Kerr, K. G. & Snelling, 2009; Leclerc et al., 2002).

P. aeruginosa is a ubiquitous environmental bacterium that is capable of colonizing a number of wet and moist sites in plants and soils and a wide variety of aquatic environments (Hardalo & Edberg, 1997). Confirmed environmental reservoirs of *P. aeruginosa* in hospitals are numerous and include aerosols, potable water, wastewater piping, milk preparations, mineral water, milk pasteurizers, faucets/taps, sinks, sink traps, sink and shower drains, disinfectants and soaps, respiratory equipment, humidifiers, ice makers, flower vases, shaving and toothbrushes, endoscopes and endoscope washers, urodynamic pressure transducers, medication, water baths and hydrotherapy pools, infant feeding basins, bathing basins, bath toys and cleaning equipment (Aumeran et al., 2007; Bert, Maubec, Bruneau, Berry, & Lambert-Zechovsky, 1998; Bilavsky et al., 2013; Breathnach, Cubbon, Karunaharan, Pope, & Planche, 2012; Jefferies et al., 2012; Kerr, K. G. & Snelling, 2009; Molina-Cabrillana et al., 2013; Muscarella, 2004; Yapicioglu et al., 2011).

P. aeruginosa can be transmitted by a number of routes, including healthcare workers' hands (Jones, 2011), patient-to-patient (Bergmans et al., 1998) and environmental contamination (Jefferies et al., 2012), while ingestion is not considered to be a significant mode of transmission (Buck & Cooke, 1969). Although a varying proportion of infections can be attributed to cross-transmission (Bertrand et al., 2001; Cuttelod et al., 2011), water is believed to be a major contributor to the amplification and transmission of *P. aeruginosa* in hospital environments. Indeed, due to its ability to form biofilm on most inanimate surfaces, *P. aeruginosa* widely colonizes water distribution systems, which results in amplified bulk concentrations, especially in large buildings (Lavenir et al., 2008; Petignat et al., 2006; Trautmann, M. et al., 2005), dental unit lines (Barbeau et al., 1996; Zanetti et al., 2000) and spa installations (Brousseau et al., 2012; Germinario et al., 2012). It must also be noted that in most of the outbreaks that were investigated, water was implicated either directly or indirectly. For example, devices that were previously in contact with contaminated tap water (Blanc, Parret, Janin, Raselli, & Francioli, 1997; Molina-Cabrillana et al., 2013) and hand washing with contaminated tap water (Bert et al., 1998; Ferroni et al., 1998) have been traced back as the source of contamination in hospital settings. In some cases, up to 42% of the strains that cause invasive infections in intensive care

units (ICUs) originated from water (Blanc et al., 2004; Reuter, Sigge, Wiedeck, & Trautmann, 2002) and were the principal source of colonization in patients, with over 60% of tap water samples positive for *P. aeruginosa* (Vallés et al., 2004). Trautmann et al. (2001) reported that up to 68% of tap water samples that were taken in an ICU were positive for *P. aeruginosa*, while 29% of the infections originated from strains that were recovered from those samples (Trautmann, M. , Michalsky, Wiedeck, Radosavljevic, & Ruhnke, 2001). A recent study that was conducted in 10 ICUs reported that *P. aeruginosa*-contaminated faucets were an important risk factor for acquisition, where 32% of the patients with *P. aeruginosa* had previously been exposed to positive water in their room (Venier et al., 2014). In the intensive care unit, 30-50% of the *P. aeruginosa* infections were associated with water (Exner, 2012).

The true importance of *P. aeruginosa* from water systems has been debated because it could be argued that patients contaminate their environment directly or indirectly rather than vice versa. However, recent prospective studies on ICUs, including isolate typing, confirm that contaminated water systems can be the source of infection; this finding was demonstrated by the fact that corrective action on water systems led to a significant decrease in the infections that were caused by water isolates (Petignat et al., 2006; Rogues et al., 2007; Romano et al., 2013; Vallés et al., 2004). This is further supported by observations of a correlation between having a high prevalence of faucet contamination and the number of cases of patients who harbor a phenotype that is identical to one isolated from the water (Cuttelod et al., 2011). Overall, recent evidence points toward waterborne *P. aeruginosa* as being a significant cause of primary and secondary infections in healthcare settings. Furthermore, the emergence of multidrug resistant *P. aeruginosa* is becoming a concern in healthcare units; this emergence stresses the need for efficient control strategies to be identified.

The objective of this review is to provide a critical overview of the ecology of *P. aeruginosa* in drinking water systems, the key factors that promote its growth, amplification and persistence and a summary of current regulations and guidelines to control *P. aeruginosa.*

1.2 Ecology of *P. aeruginosa* in natural and engineered water systems

P. aeruginosa is a rod-shaped heterotrophic Gram-negative aerobic bacterium with a single polar flagellum. Measuring 0.5-1.0 μm in diameter and 1.5-5.0 μm in length, this organism has minimal requirements for survival and can adapt to its environment (Leclerc et al., 2002). Although it prefers organic and fatty acids as sources of carbon, it can use a wide range of other carbon sources (over 75 organic compounds), even at very low concentrations (<100 μg/L) (van der Kooij, Oranje, & Hijnen, 1982; van der Kooij, Visser, & Oranje, 1982), and can survive for months in deionized or distilled water (Warburton, Bowen, & Konkle, 1994). Similarly, nitrogen can be obtained from multiple sources, but amino acids, organic acids and DNA are the preferred sources. *P. aeruginosa* can also be found in low nutrient or oligotrophic environments (saline solutions) as well as high nutrient (copiotrophic) environments. Adaptability to low nutrient concentrations makes its growth in water not directly linked to the level of organic matter content.

Although its optimum growth temperature is 37°C, *P. aeruginosa* can grow between 10 and 42°C (Brown, 1957). Its adaptability to various environments and ability to thrive in biofilm conditions might be linked to its capacity to use nitrate as an electron acceptor instead of oxygen. This capacity allows for growth to take place under anaerobic as well as microaerophilic conditions, where oxygen is present in lower concentrations than in the environment (which creates denitrifying conditions). The range of pH through which *P. aeruginosa* can survive and grow has not been clearly defined, but information points toward better resistance at a lower pH (5.5-6.5) vs higher pH (7.5-8.5), with an optimal growth observed at pH 7.2 for suspended cells in rich media (Beyenal, Chen, & Lewandowski, 2003). Similar information is not available for minimal media or a drinking water environment.

1.2.1 Antagonism to other bacteria

P. aeruginosa produces several substances to compete against other bacteria and fungi within its environment. Its production of pyoverdine and pyocyanin is characteristic and often used for identification purposes. Pyoverdine is a siderophore that is secreted to compete against other bacteria for iron present in the environment (Harrison, F., Paul, Massey, & Buckling, 2008).

Pyocyanin is one of the phenazines produced by *P. aeruginosa;* these compounds have antimicrobial and antifungal activities. *Candida albicans, Aspergillus fumigatus* and several other yeasts and fungi are inhibited by pyocyanin (Kerr, C. J., Osborn, Robson, & Handley, 1999; Kerr, J. R., 1994). The antimicrobial activity of pyocyanin is linked to the toxicity of oxygen reduction products (an increased production of O_2^- and H_2O_2) (Hassan & Fridovich, 1980). Hassan et al. also showed the resistance of *P. aeruginosa* to these by-products, which could have an impact on their resistance to ozone and hydrogen peroxide disinfection.

Production of other antimicrobial substances against Gram-positive bacteria and fungi are specific to *P. aeruginosa.* Rhamnolipids show good to high antimicrobial activity against several microorganisms (Haba et al., 2003; McClure & Schiller, 1996; Vatsa, Sanchez, Clement, Baillieul, & Dorey, 2010). Cyanide (HCN) is produced by *P. aeruginosa* as a secondary metabolite and is responsible for the inhibition of fungi (Voisard, Keel, Haas, & Defago, 1989). , Another secondary metabolite that is unique to *P. aeruginosa*, HQNO (2-heptyl-4-hydroxyquinoline *N*-oxide), inhibits up to 94% of *Helicobacter pylori* strains (Krausse, Piening, & Ullmann, 2005) and is active against a variety of Gram-positive bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis* (Déziel et al., 2004). Pyocins are bacteriocins that act mainly against other *Pseudomonas* of the same or other species and on other Gram-negative bacteria. More than 90% of *P. aeruginosa* strains produce pyocin, and most strains of *P. aeruginosa* are pyocinogenic (Michel-Briand & Baysse, 2002). The presence of *P. aeruginosa* in premise plumbing could impact the behavior of other bacteria, such as *S. aureus*, for which the presence of HQNO produced by *P. aeruginosa* selected for small resistant colonies, which leads to the development of antibiotic-resistant variants (Hoffman et al., 2006) and favors biofilm formation (Fugere et al., 2014; Mitchell et al., 2010). Most antagonistic effects of *P. aeruginosa* against other microorganisms have been observed in medical or laboratory environments, using suspended bacteria that were grown in nutrient-rich conditions. Only scarce data on the occurrence and relative importance of these effects in nutrient-poor biofilm structures is available.

1.2.2 Interaction with amoebae

In potable water, *P. aeruginosa* often share its habitat with free-living amoebae. Although protist independent growth is clearly established, different types of interactions with amoebae are

present depending on the environment. In most cases, *P. aeruginosa* will survive and even reproduce following phagocytosis by amoebae (Greub & Raoult, 2004). Intracellular multiplication of *P. aeruginosa* was observed within *Acanthamoeba polyphaga* in synthetic drinking water (Hwang, Katayama, & Ohgaki, 2006) and within *Acanthamoeba* and *Echinamoeba* after isolation from a hospital drinking water system (Michel, Burghardt, & Bergmann, 1995). The cell count within the amoebae was estimated to be $4x10^4$ CFU/amoeba (Hwang et al., 2006). In another study, 97% of *Acanthamoeba castellanii* were readily colonized by *P. aeruginosa* within 24 hours (Matz et al., 2008). In cases where the ratio of bacteria/amoeba was high, *P. aeruginosa* was even inhibitory to *A. castellanii* (Matz et al., 2008; Wang, X. & Ahearn, 1997). Other authors observed no toxicity toward *Hartmannella vermiformis* and *A. castellanii,* but ingestion of *P. aeruginosa* still slowed the movements and ingestion process of the amoebae (Pickup, Pickup, & Parry, 2007). On the other hand, amoebae can also be a predator to *P. aeruginosa*. Depending on the biofilm formation stage, a succession of amoebae species will evolve as the biofilm matures (Weitere, Bergfeld, Rice, Matz, & Kjelleberg, 2005). Early colonizers are grazers, feeding on suspended bacteria, and the resistance of *P. aeruginosa* to grazing will depend on its strain (with environmental strains being more resistant than a mucoid lab strain) and the type of amoebae present (Weitere et al., 2005). Within the drinking water distribution system, this interaction can be beneficial to *P. aeruginosa* because the amoebae might serve as a protection against chemical disinfection or high temperatures, with some amoeba able to survive in the premise plumbing at temperatures above 55°C, especially if they are in the cyst form (Cervero-Arago et al., 2013; Thomas et al., 2004). Amoebae can also play a role in the final structure of the biofilm, favoring more resistant biofilms. The grazing of the amoebae will trigger antipredatory mechanisms such as microcolony formation and the production of toxins (Thomas, McDonnell, Denyer, & Maillard, 2010).

1.2.3 Role and interaction in biofilm

P. aeruginosa has the ability to form highly structured biofilm with distinct architectural/chemical properties (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995) and is often used as a model organism to study biofilm development. The polymer network represents 73–98 % of the biofilm volume, leaving a small volume to be occupied by microbial cells (United States Environmental Protection Agency (USEPA), 1983). *P. aeruginosa* flagellar

and twitching motilities are among the essential factors for its ability to develop into a biofilm (O'Toole & Kolter, 1998).

In drinking water distribution systems, studies report the predominance of fixed bacteria over suspended bacteria (Prévost, Besner, Laurent, & Servais, 2014). Because drinking water is an oligotrophic environment, microorganisms tend to colonize surfaces and form a biofilm as a survival strategy (Costerton et al., 1987). *P. aeruginosa* can colonize new surfaces or integrate into existing biofilms (Revetta et al., 2013; Wingender, 2011). The biofilm plays a protective role for the bacteria, providing increased resistance to disinfectants, antibiotics and other environmental stresses compared to planktonic bacteria (Wingender & Flemming, 2011). For all of these reasons, the potential for biofilm development and its control are important considerations in engineered water systems, especially in premise plumbing. A recent review refers to the plumbing material, water temperature, use and resistance to disinfectant and water velocity as key factors that promote biofilm formation within premise plumbing (Eboigbodin, Seth, & Biggs, 2008).

Iron and phosphorus concentrations in drinking water also play a role in biofilm formation (Banin, Vasil, & Greenberg, 2005; Critchley, Cromar, McClure, & Fallowfield, 2001; Lehtola, Markku J., Miettinen, & Martikainen, 2002). Some authors report an observed correlation between an increase in soluble phosphate concentrations and a higher biofilm total biomass in water in which low phosphorus is the limiting factor (Critchley et al., 2001; Lehtola, Markku J. et al., 2002). However, in systems where carbon is the limiting factor, the addition of phosphorus does not impact the biofilm. Furthermore, as summarized by Prévost et al., recent studies observed a weaker biofilm when increased concentrations of phosphorus where present (Prévost et al., 2014). In this context, the use of orthophosphate as a corrosion control strategy in a distribution system might have various effects on the biofilm, depending on the water quality.

Despite *P. aeruginosa* preference for the biofilm lifestyle (Schleheck et al., 2009) and the established presence of biofilms in engineered water systems, *P. aeruginosa* is not always identified within such systems and is seldom detected in drinking water distribution systems (Emtiazi, Schwartz, Marten, Krolla-Sidenstein, & Obst, 2004; Kilb, Lange, Schaule, Flemming, & Wingender, 2003; Lee, D.-G. & Kim, 2003; September, Els, Venter, & Brozel, 2007; Wingender & Flemming, 2004). However, it colonizes existing biofilms in plumbing fixtures,

especially within the sink systems of hospital premise plumbing (Blanc et al., 2004; Hota et al., 2009; Lavenir et al., 2008; Vianelli et al., 2006; Walker, J. T. et al., 2014). In fact, the presence of *P. aeruginosa* in tap water appears to be strongly related to point-of-use biofilm colonization (faucets, drain, sink, showerhead) rather than to the water distribution system (Mena & Gerba, 2009). Although *P. aeruginosa* is usually a minor fraction of the microbial community in the mature biofilms of water networks (Wingender, 2011), it will integrate, survive and proliferate within this environment (Ghadakpour et al., 2014) and become a threat for immune-compromised patients when given favorable conditions, such as stagnation, warm water temperature or materials that promote biofilm growth. In addition, *P. aeruginosa* can enter a viable but non-culturable (VBNC) state when it is present in drinking water biofilms and become undetectable by standard methods (Bédard, Charron, Lalancette, Déziel, & Prévost, 2014; Moritz, M.M., Flemming, & Wingender, 2010). Cells in the VBNC state are still alive and are capable of metabolic activity but fail to multiply and grow on routine media on which they would normally grow (Oliver, J.D., 2005). To better understand its occurrence, the next section focuses on the factors that influence colonization and the persistence of *P. aeruginosa* within hospital premise plumbing biofilms and water.

1.3 Key factors that promote growth, amplification and persistence of *P. aeruginosa* in engineered water systems

Although naturally present in moist environments, *P. aeruginosa* is not frequently detected in treated water distribution systems, and there is little documentation of the treatment impact on the *P. aeruginosa* population. Early findings by Van der Kooij showed no detection in water distribution systems of *P. aeruginosa*, either before or after the treatment for both surface and ground water (van der Kooij, 1977). In another study, only 3% of 700 samples from drinking water systems, mostly groundwater sources, were positive for *P. aeruginosa* (Allen, M. J. & Geldreich, 1975), which is supported by results from a chloraminated distribution system (Wang, H., Edwards, Falkinham III, & Pruden, 2012). Similarly, *P. aeruginosa* was not detected in biofilm that was sampled from 18 pipes in different distribution systems made of various materials over an 18-month study with non-chlorinated groundwater (Wingender & Flemming, 2004). The authors suggested that biofilm formation in a public water distribution system during normal operations might not be a common habitat for *P. aeruginosa*, although it will easily

survive traditional physical and chemical treatment (Emtiazi et al., 2004). A potential impact of the treatment process would be the elimination of some other microbial genera that are more susceptible to disinfection, leaving a niche opportunity for *P. aeruginosa*. Still, survival breakthroughs of *P. aeruginosa* were reported as less frequent than *Legionella* and even less than *Mycobacterium* in two chloraminated distribution systems (Wang, H. et al., 2012). Furthermore, a direct link between the detection of *P. aeruginosa* in treated water and colonization observed in large building premise plumbing has not been established. For example, studies in which 15-58% of the taps were positive for *P. aeruginosa* had negative results for all of the water main samples (Ferroni et al., 1998; Reuter et al., 2002). These results point toward a local amplification within the premise plumbing or directly at the point-of-use rather than from the main water distribution system.

1.3.1 Materials

Types of surface materials can have a significant impact on biofilm formation and attachment, either by substances they release (organic compounds or inhibitors) or by their surface characteristics. Materials that are generally used in larger water distribution systems have not been documented to promote *P. aeruginosa* growth. In cast iron pipes, 1 month was sufficient for a biofilm to develop (Donlan, Pipes, & Yohe, 1994), but there was no specific measurement of *P. aeruginosa*. Lee et al. ran a semi-pilot made of galvanized iron for a 3-month period and measured bacterial species that were present in the influent, biofilm and effluent (Lee, D.-G. & Kim, 2003). Out of 12 sampling events, *P. aeruginosa* was isolated only once in the biofilm and once in the effluent. Although limited, this information supports the low contamination that is observed within distribution systems. In premise plumbing, copper, plastic and elastomeric materials are commonly used. Although copper is not typically installed in new constructions, it is predominant within older premises (Rahman, Encarnacion, & Camper, 2011).

Several studies have been conducted in recent years to better understand the impact of the material choice on the colonization and amplification of *P. aeruginosa* (Prévost et al., 2014). Initial studies showed that there was a low colonization rate of copper piping by *P. aeruginosa*. In a lab study, biofilm growth was observed on copper piping, but it was consistently less than for plastic surfaces (Rogers, J., Dowsett, A. B., Dennis, P.J., Lee, J.V., & Keevil, C.W., 1994b). The lower microbial diversity was dominated by *pseudomonads*; however, no *P. aeruginosa* were

detected. The analysis of used copper plumbing pipes (>12 months) that received either filtered or unfiltered water confirmed the presence of a biofilm, but still there was no detection of *P. aeruginosa* (Critchley et al., 2001). More recently, the absence of *P. aeruginosa* in a biofilm grown on copper piping was attributed to the toxicity of the copper ions that were preventing the surface colonization (Moritz, M.M. et al., 2010). However, recent studies demonstrated the loss of culturability of planktonic *P. aeruginosa* in the presence of copper after 24 hours at concentrations that are typically found in drinking water, but there was an unchanged viable cell count, which indicates the induction of a VBNC state for *P. aeruginosa* (Bédard et al., 2014; Dwidjosiswojo et al., 2011). Once the copper stress was removed, *P. aeruginosa* could fully recover its culturability and cytotoxicity (Dwidjosiswojo et al., 2011). In biofilm, *P. aeruginosa* demonstrated a higher resistance to copper, with an estimated 600-fold compared to planktonic cells (Teitzel & Parsek, 2003). Additional tests with planktonic cells revealed that there was an increased lag phase with increasing copper concentrations, but growth was observed until the minimum inhibitory concentration of 127 mg/L was reached, a concentration more than 100-fold the regulated maximum concentration in drinking water between 0.3 and 2 mg/L (California Environmental Protection Agency, Pesticide and Environmental Toxicology Branch, & Office of Environmental Health Hazard Assessment, 2008; World Health Organization (WHO), 2008).

Plastic and elastomeric materials such as polypropylene, polyethylene, ethylene propylene diene monomer (EPDM), PVC, nitrile butadiene rubber, silicone and latex all support a much denser biofilm than materials such as glass, copper or stainless steel (Tsvetanova & Hoekstra, 2010). Plastic and elastomeric materials contain organic substances that can enhance microbial growth. The release of organic contaminants such as plasticizers from elastomers generates a source of carbon that can be readily available for bacteria, as observed by Rogers et al., who saw the total carbon increase by an average 100X for polyethylene, ethylene-propylene and latex over a 3-day period (Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V., & Keevil, C.W., 1994a). Phosphorus is also released in significant amounts into the water from plastic material, increasing the microbial available phosphorus by 10X (Lehtola, M.J. et al., 2004). More specifically, plastic and elastomeric materials will support the growth of *P. aeruginosa*, especially when integrating the preformed biofilm (Colbourne, 1985; Kilb et al., 2003; Moritz, M.M. et al., 2010; Rogers et al., 1994a). Inoculated *P. aeruginosa* was incorporated into the preformed biofilm within 24 hours on polyethylene and polypropylene surfaces, compared to 21 days for mild steel (Rogers et al.,

1994a). *P. aeruginosa* incorporated quickly into biofilm that was grown on EPDM, and a release of bacteria into the water phase was observed (Bressler, Balzer, Dannehl, Flemming, & Wingender, 2009). In dental line units that were made of polyurethane, *P. aeruginosa* was repeatedly isolated from 24% of the dental units, where it represented 75-100% of the microflora (Barbeau et al., 1996). Another study concluded that there was a direct influence of the plumbing material type on the integration of *P. aeruginosa* into an established biofilm (Moritz, M.M. et al., 2010). Incorporation into the existing biofilm was observed within one day and persisted over the course of the experiment (4 wks) for the elastomeric materials, as opposed to the biofilm that was grown on copper, where *P. aeruginosa* could not incorporate.

1.3.2 Devices

Over the past decade, electronic faucets (non-touch, metered, hand-free, sensored) have been installed in buildings to reduce the water consumption and the risk of contamination during hand washing. Although it is expected that electronic faucets would eliminate hand touching by staff and thus prevent recontamination, the impact of such devices on improving hand hygiene and reducing infections has not been documented. However, these devices appear to favor the proliferation of heterotrophic bacteria, especially *P. aeruginosa*. An early warning of the potential for bacterial proliferation in electronic faucets was issued in 2001 based on the observation that a higher proportion of faucets showed elevated heterotrophic plate count (HPC >500 UFC/mL) levels for a brand of electronic faucets compared to another brand and compared to conventional faucets (Hargreaves et al., 2001).

The evaluation of the impact of electronic faucet devices on the colonization and amplification of *P. aeruginosa* has been studied mostly in hospital settings, both during normal operations and in outbreak situations. Table 1-1 lists the percent positivity by *P. aeruginosa* that was reported for various manual and electronic faucet devices as well as sink drains.

1.3.2.1 Conventional faucet devices

Most studies on electronic faucet contamination are comparisons with manual faucets within a similar environment. In addition, over the past 20 years, several authors have reported the level of contamination by *P. aeruginosa* for manual faucets. The first section of Table 1-1 shows the range of prevalence of *P. aeruginosa* contamination that is associated with manual faucets. The

percentage of positive faucets varies greatly from one study to another (0 to 100%). Looking more closely at the methodology behind these results, some of that variation can be attributed to the differences in the type of sample (swab vs water), the volume sampled (50 to 250 ml), the number of taps sampled, the number of samples per tap and the context (prospective study vs outbreak situation). In several cases, the contamination was identified as distal because there was no detection of *P. aeruginosa* in the main water samples (Ferroni et al., 1998; Reuter et al., 2002) or in water samples from outside the rooms (Lavenir et al., 2008). However, once the contamination was present at a faucet, it persisted over time. Trautmann et al. observed the same clones within individual faucets over prolonged periods of time (7 months), and Lavenir et al. concluded that close to 50% of the rooms that were sampled were permanently colonized over the 18-month study period (Lavenir et al., 2008; Trautmann, M. et al., 2001). The latter was observed even though the taps were routinely disinfected by the staff throughout the study. Similarly, Reuter et al. measured a high prevalence of faucet contamination despite the ongoing practice of removing and autoclaving aerators every 2 weeks (Reuter et al., 2002). Ferroni et al. were able to reduce the percent contamination by disinfecting the devices and chlorinating the whole water system, but had to change the taps to completely eradicate the contamination (Ferroni et al., 1998). Table 1-1 also shows that larger-scale studies (number of faucets >25) had a lower percentage of contamination (0 to 18 % vs 58 to 100%).

Table 1-1 : Reported faucets and drains contamination by *P. aeruginosa* in healthcare facilities

	Location	No Sites	No Samples	Type of Device	% Samples Positives	Sample Volume (mL)	Context (Duration)	Notes	Reference
Manual Faucets	Surgical ICU (16 beds)	6	72	Faucets	68	100	Prospective study (30 wks)	Every 2 weeks over 7 months, individual faucets harbored their clones over prolonged periods of time, despite cleaning & autoclaving aerator.	Trautmann et al. (2001)
	Surgical ICU (17 beds) + 12 peripheral wards	n.s.[1] 5	127 132	Faucets	58	100	Prospective study (40 wks)	Tap aerators were removed and autoclaved every 2 weeks prior to start of study. Hot and cold water samples from the central system were negative	Reuter et al. (2002)
	ICUs (870 beds hospital)	16	216 64	Faucets and mixing valve Faucets	9.7 0	Swabs 100	Prospective study (52 wks)	Hot-cold water mixing chamber was swabbed at end of study. Percent positivity ranged from 1.6-18.8	Blanc et al. (2004)
	Surgical and medical ICU (30 beds)	28	224	Faucets	4.5	150	Prospective study (8 weeks)	Weekly sampling	Cholley et al. (2008)
	Medical-surgical ICU (400 beds)	n.s.	53	Sink faucets and shower heads	3.8	n.s.	Outbreak - 36 patients, new building	No detection in source water (n=39) or on equipment tested (n=27)	Hota et al. (2009)
	Surgical pediatric unit (59 beds)	118 98	214 98	Faucets Showers & faucet nozzle	15 7	50 Swab	Outbreak, 14 urinary tract infections, 10 year old taps	Water sampled after a flush of few seconds. None found in 4 samples from main water pipes. 18% positivity in surgical ICU. Resolution through replacement of taps and hygiene measures.	Ferroni et al. (1998)
	Long stay care unit (22 beds)	18 18	91 53	Faucets Faucet nozzle	68 74	100 Swab	Long-term study (2 years)	Water sampled after 1 min flush. 6 out of 14 rooms permanently colonized despite descaling and aerators changed 8 months before end of study. Outdoor tap water never positive.	Lavenir et al. (2008)
	Hospital care unit	8 23	8 23	Faucets Faucets, shower heads	12.5 48	n.d. Swab	Higher *P. aeruginosa* bacteriemias than usual	Corrective measures : 5 min flush before use and POU filtration	Vianelli et al. (2006)
	ICU (16 beds)	39	484 189	Faucets in patient's room Faucets outside rooms	11.4 5.3	250 + swab	Prospective study (26 wks)	After 11 weeks into the study, aerators removed and disinfected every 2 weeks, taps disinfected with chlorine. Samples still positive after.	Rogues et al. (2007)

Table 1-1 : Reported faucets and drains contamination by *P. aeruginosa* in healthcare facilities (continued)

	Location	No Sites	No Samples	Type of Device	% Samples Positives	Sample Volume (mL)	Context (Duration)	Notes	Reference
Electronic and Manual Faucets	Hospital (450 beds)	10	10	Manual faucets	0	500	Monitoring study after replacement	Aerators not removed before sampling. Central pipe system negative. No contamination detected prior to magnetic valve for electronic faucet without temperature control.	Halabi et al. (2001)
		23	23	efaucets[2] without T° control	74				
		15	15	efaucets with T° control	7				
	Neonatal ICU (25 beds in 1200 beds hospital)	9	9	efaucets	100	swab + water	Outbreak (12 patients) after taps replacement	Samples from faucet filter (swab) and from faucet water. None of the manual faucets sampled were contaminated.	Yapicioglu et al. (2011)
	Hospital Kitchen (1333 beds hospital)	27	144	efaucets	7.6	500	Observation after renovations (26 wks)	No *P. aeruginosa* detected after chlorination; total bacterial still too high despite changing the aerator.	Chaberny and Gastmeier (2004)
	Hospital (90 rooms)	n.s.	31	efaucets	100	100	Control before opening new department	All faucets and central pipes positive for *P. aeruginosa* on reopening. No detection in central system and manual faucets after chlorination, efaucets still positive.	Van der Mee-Marquet et al. (2005)
			33	central pipe/manual faucets	0				
	Hematology ward	3	21	efaucets	90	n.s.	Control before reopening after renovations	Manual faucets negative. Chlorination 15 min, 6 times not effective.	Leprat et al. (2003)
	ICU (15 beds)	n.s.	10	Taps, water outlets, water suply	100	n.s.	Outbreak, 10 patients after renovations	Resolution through replacement of new sensor mixer tap systems with conventional mixer taps. No further detection of *P. aeruginosa* or cases.	Durojaiye et al. (2011)
	Hematology and ICU wards (900 & 500 beds)	n.s.	92	efaucets with T° control	39	500	Study	Aerator removed, faucet nose disinfected with alcohol and flushed for 1 min prior to sampling . No contamination of incoming water to e-faucets	Merrer et al. (2005)
			135	Manual faucets	1				
	NICU (28 beds)	37	296	efaucets outside NICU	12.5	Swab	Outbreak (8 patients)	All swab samples were taken from the flow restrective devices.	Ehrhardt et al. (2006)
		12	12	efaucets in NICU	71				
		5	5	Manual faucets	0	Water			
	Hospital (2168 beds)	36	18	efaucets	0	250	Study	Magnetic valves installed within < 25 cm from water basin, minimizing volume at mitigated temperature.	Assadian et al. (2002)
			18	Manual faucets	2.7				
	ICU operating suite (491 beds) & Neonatalogy unit (430 beds)	19	304	Faucets	5.3	n.s.	Prospective study (52 wks)	Sampling with aerator in place. Water from the main supply was negative for *P. aeruginosa*.	Berthelot et al. (2006)
	Hospitals (405, 420, 80 & 450 beds)	90	90	Manual faucets	2	1000	Study	Sampling with aerator in place. Low positivity by culture. Enzymatic detection method had higher positivity: 14% for manual, 29% for foot operated and 16% for efaucets.	Charron et al. (2015)
		14	14	Foot operated faucets	14				
		105	105	efaucets	5				

Table 1-1 : Reported faucets and drains contamination by *P. aeruginosa* in healthcare facilities (continued)

	Location	No Sites	No Samples	Type of Device	% Samples Positives	Sample Volume (mL)	Context (Duration)	Notes	Reference
Drains	Medical-surgical ICU (12 beds)	11	66	Sink drains	100	Swab	Study (6 weeks)	56% of drains strains, high level of antibiotic resistance. For 2 of 5 infected patients, same strain as the one isolated in the drain.	Levin et al. (1984)
	Medical-surgical ICU (400 beds)	n.s.	213	Sink drains	12.2	Swab	Outbreak - 36 patients, new building	Fluorescent marker showed drain splashed at least 1 m	Hota et al. (2009)
	Surgical and medical ICU (30 beds)	28	224	Sink drains	86.2	10	Study (8 weeks)	Water sampled in the U-bend. Each room sampled every week. Drains in all rooms were colonized at least once. 5 of 28 rooms had permanent colonization	Cholley et al. (2008)
	Pediatric oncology (18 beds)	12 34	12 12	Sink drains	25 58	Swab 10	Outbreak - 3 patients	Tap design caused errant jet in the drain creating aerosols. Resolution: installation of longer neck faucet, offset from the drain and installation of self-cleaning drains. After 18 months, *P. aeruginosa* still detected in drains except for the new self-cleaning drains and no new cases reported.	Schneider et al. (2012)
	Hospitals (405, 420, 80 & 450 beds)	210	210	Sink drains	51	Swab	Study	Sampling in 4 hospitals	Charron et al. (2015)
	Mixed infectious disease unit (11 beds)	34	76 52 8	Washing basin sinks Toilet sinks Shower and bathtube	89.5 46.2 100	Swab	Study (4 weeks)	Demonstrated that aerosols from the drains were contaminating personnel's hands. Resolution through the use of a heating device on drains (70°C) to eliminate presence of *P. aeruginosa*.	Döring et al. (1991)

1.3.2.2 Electronic faucet devices

The next section in Table 1-1 reports various case studies in which the level of contamination found in electronic faucets was compared to manual devices that were installed in similar areas of hospitals. Studies were conducted mainly in intensive care units, surgical, neonatology and hematology wards.

Most studies on electronic faucet contamination were conducted in a non-outbreak setting, after renovation or device replacement, either during a control period before the start of use (Berthelot et al., 2006; Leprat, Denizot, Bertr, & Talon, 2003; Van der Mee-Marquet, Bloc, Briand, Besnier, & Quentin, 2005) or during the monitoring period following the start of use (Chaberny & Gastmeier, 2004; Halabi, Wiesholzer-Pittl, Schöberl, & Mittermayer, 2001). Water bacterial contamination in a newly built hospital wing was measured before opening (Van der Mee-Marquet et al., 2005). Both conventional and non-touch water taps were positive for *P. aeruginosa*. After intensive chlorination of the whole system, the water samples from central pipes and conventional taps were negative, whereas all of the samples that were collected from non-touch taps remained positive for *P. aeruginosa*. Following the replacement of conventional faucets by electronic non-touch faucets, Leprat et al. and Berthelot et al. also found electronic faucets to be already contaminated with *P. aeruginosa*, even before their usage was initiated, while no contamination was detected in conventional faucets (Berthelot et al., 2006; Leprat et al., 2003). Chaberny et al. documented 12% of newly installed hospital kitchen electronic faucets to be positive for *P. aeruginosa*, after 6 months of running (Chaberny & Gastmeier, 2004), and similar levels of contamination were observed in later sampling events. Similarly, Halabi et al. compared the level of contamination after 3 months of usage and observed 100% contamination of the 10 electronic faucets that were sampled compared to no contamination being detected in the 10 conventional faucets (Halabi et al., 2001). Merrer et al. also showed a systematic and significantly higher proportion of contamination in electronic compared to conventional faucets (36/92 vs 2/135) in several high-risk areas of two hospitals and concluded that electronic faucets were a major reservoir of *P. aeruginosa* (Merrer et al., 2005). Although most comparative studies on the contamination of electronic faucets point to a higher potential of contamination than manual faucets, two studies observed no differences (Assadian et al., 2002; Charron, Bédard, Lalancette, Laferrière, & Prévost, 2015).

Electronic faucets have also been identified as a probable source of outbreaks in ICUs (Durojaiye, Carbarns, Murray, & Majumdar, 2011; Ehrhardt, Terashita, & English, 2006; Walker, J. T. et al., 2014; Yapicioglu et al., 2011). Ehrhardt et al. reported an outbreak in which 8 infants in a neonatal ICU (NICU) were infected with the same *P. aeruginosa* strain, as isolated from 11 infrared sensored faucets in patient rooms (Ehrhardt et al., 2006). More recently, an outbreak in a NICU was attributed to the use of contaminated electronic faucets (Yapicioglu et al., 2011). Within six months following the replacement of conventional faucets by electronic faucets, four patients were infected with *P. aeruginosa* within a few days. Environmental sampling detected *P. aeruginosa* in one liquid hand soap as well as in water and filters from all of the electronic faucets. No detection was observed in the conventional faucets. Several additional infections by *P. aeruginosa* were observed over the subsequent months, until the electronic faucets were replaced by conventional faucets. A similar resolution was reported by Durojaiye et al. following a *P. aeruginosa* outbreak that occurred during the 5 months after the reopening of a renovated ICU (Durojaiye et al., 2011). Positive results for all of the taps, water outlets and water supplies to the electronic faucets combined with results from sampling at various points of the hospital pointed to the newly installed electronic faucets as the likely source of the outbreak. Evidence of electronic faucet colonization has also been reported for other opportunistic pathogens, including *Burkholderia cepacia* (Kotsanas, Brett, Kidd, Stuart, & Korman, 2008), *Legionella pneumophila* (Sydnor et al., 2012), and *Mycobacterium mucogenicum* (Livni et al., 2008). Once established, contamination is reported as being difficult to eradicate for electronic faucets (Berthelot et al., 2006; Durojaiye et al., 2011; Merrer et al., 2005; Van der Mee-Marquet et al., 2005) and, in some cases, for manual faucets as well (Ferroni et al., 1998; Reuter et al., 2002; Trautmann, M. et al., 2001). Despite repeated chlorination (Leprat et al., 2003; Merrer et al., 2005) or silver ion treatment (Durojaiye et al., 2011), electronic faucets retained some level of contamination for *P. aeruginosa*. In one case, *P. aeruginosa* appeared to have been eliminated after chlorine dioxide disinfection (no colony counts), but the total bacterial count was still high (Chaberny & Gastmeier, 2004). Several outbreaks and contaminations were resolved by changing all of the faucets back to conventional faucets.

The majority of studies (with or without associated infections) conducted with electronic faucets took place immediately or shortly after their installation. The higher prevalence of positive electronic faucets has been linked in many cases to newly renovated or constructed hospital

wings or units. A construction setting presents additional risk factors in the distribution systems, such as increased stagnation and pressure changes, occasionally introducing backflows and openings for contamination (Williams, Armbruster, & Arduino, 2013). The reported contamination by *P. aeruginosa* of a new electronic faucet prior to its installation was attributed to prior testing conducted on the magnetic valve by the manufacturer (Berthelot et al., 2006). These risks are exacerbated because water is often stagnant for a long period of time between the commissioning of the system and the start of use. Even when water starts flowing through the system, the reduced flow rates often associated with electronic faucets might not be sufficient to flush the equipment and clean it from bacteria that are likely established as a biofilm by then. In addition, the activation mechanism of electronic faucets requires the user to put their hands under the spout, which causes them to be exposed to the first flush of water. This circumstance might be an important factor in transmission when contamination is present and amplified in the first volume out of the faucet (Lipphaus et al., 2014). With manual faucets, a minimum volume of water will flow during the time that the hands are removed from the handles and placed under the water stream.

1.3.2.3 Faucet design

The high contamination prevalence of electronic faucets could be caused by their design features, where low flow, low pressure and water stagnation combined with a temperature of 35°C and materials such as rubber and PVC provide ideal conditions for cell adhesion and biofilm growth (Chaberny & Gastmeier, 2004; Halabi et al., 2001; Merrer et al., 2005). However, electronic faucet designs vary and could have different susceptibilities to bacterial contamination. Hargreaves et al. observed large differences between two brands of electronic faucets, with 52% contamination for brand A compared to 8% for brand B, and 9% for manual faucets (Hargreaves et al., 2001). Halabi et al. compared electronic faucets that had a manual local temperature control lever with those that did not have a manual temperature control, and their results showed a much higher proportion of faucets contaminated by *P. aeruginosa* in the absence of temperature control (74% vs 7%) (Halabi et al., 2001). In this case, the % positive that was observed on the temperature-controlled electronic faucets was comparable to the average level of contamination observed on the conventional faucets. Charron et al. compared two types of electronic faucets that were equipped with a manual local temperature lever (Charron et al., 2015). They observed

that electronic faucets with a temperature lever that was located on the side of the sink were more often positive for *P. aeruginosa* (31%) compared to electronic faucets that had a temperature lever on the faucet body (14%). Another factor that can play a role in the establishment and persistence of bacterial contamination is the presence of a thermal mixing valve, which induces an average temperature that ranges between 38 and 44°C; this temperature range is ideal for the growth of mesophilic bacteria (Health and Safety Executive (HSE), 2013). Furthermore, some conventional or pedal-activated faucets could present similar design features and have higher contamination levels (Charron et al., 2015). Makinen et al. studied two types of electronic faucets: the first type with the mixing chamber integrated in the faucet while the second type had the mixing chamber separate from the faucet (Mäkinen et al., 2013). Contamination levels for HPC and *L. pneumophila* were significantly higher in the faucets that had a separate mixing chamber. This design increases the volume of mixed hot and cold water and is designed to allow a minimal flow of cold water at all times, which prevents the flow of hot water through this volume. Interestingly, in this same study, the manual faucets presented the highest level of contamination for HPC and *L. pneumophila*, while no *P. aeruginosa* were detected in any of the samples.

In light of these observations, it appears that the type of electronic faucet plays an important role in the colonization by *P. aeruginosa*. However, most of the studies provide very limited information on the types of devices that were sampled, although this information is important to understanding the location and cause of the contamination and to clearly establish that there is a greater risk associated with electronic devices. Several features should be considered when interpreting the results, such as the following: the presence of a mixing chamber, the materials and volume of the mixing chamber/column, the temperature maintained in the mixing chamber/column, the presence and type of a flow reduction device, the materials used for the mixing valve, the complexity of the internal structure of the device (the presence of nooks and crevices), the ability to flush with hot water, and the materials used for connecting these devices. For example, the distance between the mixing valve and the tap will have an impact on the volume of stagnant water at a mitigated temperature. In their study, Assadian et al. attributed the absence of contamination in the sampled electronic faucets to the short distance of the pipe between the mixing valve and the tap (< 25 cm) (Assadian et al., 2002). Looking at different reports of electronic faucet contamination in hospitals over the last decade, it is difficult to

confirm this suggestion as most studies do not document the length of the pipe between the mixing valve and the tap. Still, a correlation between the tap positivity and the volume at the mitigated temperature was reported (Charron et al., 2015).

1.3.2.4 Flow straighteners and flow restriction devices

Flow restriction devices are used to reduce the water consumption and, therefore, limit the peak flow conditions that can be used for flushing and cleaning a tap. Although characteristic of electronic faucets, they can also be used on conventional faucets. The higher positivity and level of contamination reported when sampling the first volume at the point-of-use vs a sample representative of the system also suggests that there is a contribution from the flow restriction devices (Cristina et al., 2014). A recent study showed that complex flow straighteners were susceptible to biofilm accumulation and presented higher rates of colonization by *P. aeruginosa* compared to simple plastic and metal aerators (Walker, J. T. et al., 2014). The contribution of the restricting flow device to the higher percent contamination of electronic faucets deserves further investigation as those types of devices are used increasingly to reduce water consumption.

1.3.2.5 Drains

Shower and sink drains are also probable sources of *P. aeruginosa* infections (Table 1-1) (Breathnach et al., 2012; Hota et al., 2009; Levin, Olson, Nathan, Kabins, & Weinstein, 1984; Maltezou et al., 2012; Schneider et al., 2012). In a newly constructed hospital, an outbreak of *P. aeruginosa* was linked to a contaminated sink drain (Hota et al., 2009). The use of a fluorescent marker demonstrated that the drain content splashed at least 1 m from the sink when it was used for handwashing. The sink was directly adjacent to medical material intended for patient care, and the head of the bed was within less than 1.5 m from the sink. Similarly, an outbreak was attributed to the water flow that was directed into contaminated drains (Schneider et al., 2012). Two recent hospital outbreaks of antibiotic-resistant *P. aeruginosa* were linked to faulty shower drains and sewage backflows in showers and toilets (Breathnach et al., 2012), and another suspected the drains as the source, although the isolated strains were untypable (Maltezou et al., 2012).

Some authors suggested that tap colonization might not come from the main water but instead could be a retrograde contamination from the drain into the different tap components (Cholley,

Thouverez, Floret, Bertrand, & Talon, 2008; Döring et al., 1991; Kirschke et al., 2003; Trautmann, M. et al., 2005). In addition, reduced water flow that is associated with electronic faucets and the installation of flow restrictive devices on conventional faucets have led to an increased number of complaints regarding drain blockages, as reported by technical services personnel from four hospitals (2013), which is likely associated with the inability of the reduced water flow to prevent biofilm from accumulating over time within the drain. This information is critical when planning a renovation or construction in a hospital setting.

Overall, the reported data suggest that there is a higher potential of colonization and amplification of *P. aeruginosa* in electronic faucet devices. As a result, several authors have recommended avoiding the installation of electronic faucets in at-risk patient areas (Chaberny & Gastmeier, 2004; Halabi et al., 2001; Hargreaves et al., 2001; Merrer et al., 2005; Yapicioglu et al., 2011). However, the low number of faucets in most of the studies that involve electronic devices ($n < 40$) might influence the outcome, as observed with conventional faucet studies. In addition, differences in the types of electronic faucets (Halabi et al., 2001) or their environment (Ehrhardt et al., 2006) might lead to important variations in the observed percentage of positive faucets. Furthermore, the colonization of electronic devices is associated with multiple factors that are not unique to them, such as stagnation volumes and materials that are present in these devices. Better documentation of the connecting materials and the faucet technical details involved in prevalence studies for *P. aeruginosa* or other opportunistic pathogens is essential to help focus research efforts on reducing the risk of infections that are related to current installations and on improving future designs. Aerators and drains are also important devices to consider because they present a humid environment with increased biofilm potential compared to the wet environment within pipes. The choice and the positioning of the faucets and drains as well as the room layout could contribute to minimizing the tap colonization by *P. aeruginosa* and to reducing the risk of exposure that is related to drain contamination.

1.3.3 Disinfectants

A wide range of disinfection methods have been used or tested to control *P. aeruginosa*, and some have had more success than others, as presented in Table 1-2. *P. aeruginosa* is one of the most resistant Gram-negative bacteria toward disinfection, especially when growing in a biofilm.

The principal disinfectants that are used in premise plumbing water disinfection and their documented impact on *P. aeruginosa* are presented below.

1.3.3.1 Chlorination

Chlorination can be achieved through the application of chlorine, monochloramine or chlorine dioxide. Although resistance to chlorination will vary depending on the strain of *P. aeruginosa*, it will survive chlorination at concentrations that are applicable to drinking water (Grobe, Wingender, & Flemming, 2001). Planktonic cells are more susceptible to chlorination than biofilm cells (Behnke, Parker, Woodall, & Camper, 2011). Several mechanisms are involved in the increased biofilm cells resistance, including the difficulty for the disinfectant to penetrate the biofilm matrix, the high chlorine demand generated by EPS, DNA and other extracellular material forming the matrix, or bacterial resistance acquired through genetic transfer within the biofilm (Ortolano et al., 2005). A recent study by Xue et al. identifies EPS as the key to increased resistance (Xue & Seo, 2013). The EPS located at the surface of the cell membrane will consume disinfectant residual but will also impact the accessibility of the reactive sites on the cell surface and delay the interaction between the disinfectant and the cell membrane. In addition, EPS would reduce membrane permeabilization by disinfectants, which suggests that extensive damage might not occur and bacteria might be able to recover once the disinfectant is depleted.

There is little information on the effects of monochloramine and chlorine dioxide on *P. aeruginosa*. Because these oxidants are efficient for the control of other opportunistic waterborne pathogens (i.e., *Legionella pneumophila*) and their use in hospitals is increasing, it would be important to understand their impact on *P. aeruginosa*. Suboptimal chlorine disinfection and periodical chlorine depletion in drinking water systems potentially accelerate the development of bacteria in biofilm by reducing their susceptibility to disinfection (Codony, Morato, & Mas, 2005) and by leading to the selection of multidrug-resistant *P. aeruginosa* (Shrivastava et al., 2004).

Table 1-2: Reported efficacy of various disinfectants against *P. aeruginosa*

Disinfectant	Suspended or biofilm cells	Experimental Scale	Disinfectant Dose	Contact time (min)	Initial cell concentration (cfu/mL)	Log reduction	Additional information	References
Chlorine	Suspended	Laboratory	0.5 mg Cl_2/L	< 1	10^6	4		Xue and Seo (2013)
		Laboratory	0.5 mg Cl_2/L	30	$8x10^{-1}$	0.6		Shrivastava et al. (2004)
		Laboratory	0.1-0.6 mg Cl_2/L	5	10^6	0.4-4.3		Grobe et al. (2001)
	Biofilm	Laboratory	0.5 mg Cl_2/L	30	10^6	1.7		Kim et al. (2009)
		Laboratory	5.8 mg Cl_2/L	60	nd	2		van der Wende (1991)
Chlorine dioxide	Suspended	Laboratory	0.5 mg Cl_2/L	30	10^7	5		Behnke and Camper (2012)
			1.5 mg Cl_2/L	30	10^7	7		
	Biofilm	Laboratory	1.5 mg Cl_2/L	30	10^7	< 1		Behnke and Camper (2012)
Mono-chloramine	Suspended	Laboratory	2 mg Cl_2/L	30	10^6	5		Xue and Seo (2013)
	Biofilm	Laboratory	4 mg Cl_2/L	60	$3.8x10^{12}$ cfu/m^2	4		Chen et al. (1993)
Ozone	Suspended	Pilot	15 ppm	10	$8x10^4$	2		Liberti et al. (2000)
		Laboratory	0.2 ppm	1	10^6	5		Restaino et al. (1995)
	Biofilm	Not reported						
Silver ions	Suspended	Laboratory	5 mg/L	20	$3x10^7$	2	Resistance observed over time	Wu (2010)
		Laboratory	0.08 mg/L	720	$3x10^6$	6		Huang et al. (2008)
		Laboratory	0.1 mg/L	480	10^6	5.5		(Silvestry-Rodriguez et al. 2007)
	Biofilm	Laboratory	5 mg/L	20	$6.3x10^7$	1	Resistance observed over time	Wu (2010)
		Laboratory	10 mg/L	30	10^6	0.6		Kim et al. (2009)
Copper ions	Suspended	Laboratory	0.6 mg/L	600	10^6	6	Full recovery	Dwidjosiswojo et al. (2011)
		Laboratory	0.1 mg/L	90	$3x10^6$	6		Huang et al. (2008)
		Laboratory	2 mg/L	300	10^6	6		Teitzel and Parsek (2003)
	Biofilm	Laboratory	16 mg/L	300	$3x10^7$	3.5	Resistance to copper observed	Teitzel and Parsek (2003)
Thermal shock	Suspended	Hospital	70°C	30	Not applicable		Contamination at the tap eliminated after thermal shock treatments	Van der Mee-Marquet et al. (2005)
		Hospital	75°C	60				Bukholm et al. (2002)
	Biofilm	Laboratory	65°C	2	10^8cfu/cm^2	5		Park et al. (2011)
		Laboratory	85°C	1	$4x10^4$cfu/cm^2	2-3		Kisko and Szabo-Szabo (2011)

There are few chlorination studies for *P. aeruginos*a, and most of them have been conducted under laboratory conditions with culture methods, which do not account for the VBNC state. A recent lab study on multi-species biofilms from drinking water demonstrated the high level of resistance of *P. aeruginosa* in such environments, which require up to 600 mg Cl_2/L to reduce their survival below detectable levels (Schwering, Song, Louie, Turner, & Ceri, 2014). In premise plumbing systems, the presence of a multispecies biofilm could impact the resistance to disinfection and must be accounted for. For example, hyper-chlorination of electronic faucets was reported as not being successful in eliminating contamination (Van der Mee-Marquet et al., 2005).

1.3.3.2 Copper-silver ionization

Copper-silver ionization disinfection is increasingly used, especially for building distribution systems applications. A lab study reported the efficacy of copper (0.1-0.8 mg/L) and silver ions (0.08 mg/L) to eliminate *P. aeruginosa* from water (Huang et al., 2008). Similarly, the use of silver ions (0.1 mg/L) on planktonic *P. aeruginosa* led to a 4-6 log reduction (Silvestry-Rodriguez, Bright, Uhlmann, Slack, & Gerba, 2007). In both studies, the disinfection effectiveness was evaluated based on cultivation methods. Despite the reported efficacy, the use of silver nitrate (Durojaiye et al., 2011) and copper-silver ionization (Petignat et al., 2006) failed to eliminate contamination in recent *P. aeruginosa* outbreaks. Silver was also observed to be ineffective at preventing biofilm formation (Silvestry-Rodriguez et al., 2007). The discrepancy between the initial laboratory observations and the application to a real system can be attributed to the mode of action of silver and copper ions on bacteria. Recent work has shown that copper induces a loss of culturability without a measurable change in the viable bacteria counts (Bédard et al., 2014; Dwidjosiswojo et al., 2011). Bacteria enter a VBNC state when they are exposed to copper and fully recover culturability and cytotoxicity once the stressor is removed. The adaptation of *P. aeruginosa* biofilm to silver-ion toxicity has been observed and led to silver ions resistance after an exposure of 51 days (Wu, 2010).

Given the suggested resistance of *P. aeruginosa* toward copper and silver ions, even at high concentrations, and the recommended maximum levels of copper (2 mg/L) and silver (0.1 mg/L) in drinking water (World Health Organization (WHO), 2006), more work is required to

understand the efficacy of copper-silver ions toward *P. aeruginosa* over longer periods of time despite an initial suppression of culturability. Furthermore, the effectiveness of copper-silver ions on premise plumbing biofilm must be further investigated as an increased resistance of biofilm cells has been observed (Harrison, J. J., Turner, & Ceri, 2005; Teitzel & Parsek, 2003), and multispecies biofilm are typically more resistant to disinfection than single-species biofilm (Behnke & Camper, 2012). The evaluation of copper-silver ion disinfection through traditional culture methods could also provide misleading information if the cells convert to a VBNC state and later have the ability to recover once the ion concentration is depleted or in the presence of a susceptible host.

1.3.3.3 Ozonation

The inactivation of bacteria by ozone is accomplished through an oxidation reaction that leads to the degradation of the membrane, which results in cell lysis (Liberti, Notarnicola, & Lopez, 2000). Inactivation of up to 5 log of *P. aeruginosa* has been observed for a short contact time at the high concentration of 20 ppm O_3 (Restaino, Frampton, Hemphill, & Palnikar, 1995,), but another study observed only a 2 log reduction with a smaller but still high concentration of 15 ppm (Liberti et al., 2000). At lower concentrations, closer to what could be used in drinking water ozonation, inactivation was achieved within 5 and 35 minutes for concentrations of 1.34 ppm and 0.39 ppm, respectively (Lezcano, Pérez Rey, Baluja, & Sánchez, 1999). Still, data on the efficacy of ozonation in killing *P. aeruginosa* should be interpreted with caution: 1) all of the results were obtained through laboratory studies over short periods of time; 2) the inactivation was evaluated through the culturability of *P. aeruginosa* without assessing if the VBNC cells were still present following the ozonation; and 3) the potential for resistance development over time is unknown. The production of pyocyanin by *P. aeruginosa* increases its production of oxygen reduction products (O_2^- and H_2O_2), and *P. aeruginosa* is resistant to these by-products, which could have an impact on the resistance to disinfection by ozone and hydrogen peroxide (Hassan & Fridovich, 1980).

1.3.3.4 Thermal Disinfection

Thermal disinfection is achieved by raising the water temperature to a level where the bacteria will not survive for a prolonged period of time. Mostly reported for the control of *Legionella pneumophila* in premise plumbing (Health and Safety Executive (HSE), 2013), thermal

disinfection has also been successfully used to eradicate *P. aeruginosa* from faucets in a few studies. In one case study, a continuous flow of water at 70°C for a period of 30 minutes was sufficient to eliminate *P. aeruginosa* from 85 non-touch water taps in a newly built hospital, with no further isolation in the following 6 months of the study (Van der Mee-Marquet et al., 2005). In another study, Buckholm et al. reported that weekly thermal treatment of taps at 75°C for 60 min was effective in eliminating *P. aeruginosa* (Bukholm, Tannæs, Kjelsberg, & Smith-Erichsen, 2002). Despite its effectiveness at reducing the bacterial load, this disinfection method can be time consuming and costly.

1.3.3.5 Premise plumbing point-of-use treatment

Installation of point-of-use (POU) filtration devices is increasing to help reduce chlorine residual, lead or bacteria that could be present in tap water. However, some types of POU could amplify the presence of *P. aeruginosa* by promoting biofilm formation. Chaidez and Gerba sampled 10 houses and observed the presence of *P. aeruginosa* in 38.6% of the activated charcoal POU-treated water samples vs 16.6% of the tap water samples (Chaidez & Gerba, 2004). A similar amplification was measured for both HPC and total coliforms. For taps with POU filters, samples were also taken through a bypass valve, thus avoiding the filter. *P. aeruginosa* was present in 33.3% of those samples, which shows potential retrograde colonization of the upflow piping due to the POU device, even in the presence of 0.3 mg Cl_2/L residual chlorine. Another study showed household commercial faucet filter contamination with *P. aeruginosa* due to improper use (de Victorica & Galvan, 2001). In contrast, the installation of 0.2-µm disposable filters at point-of-use has been reported to effectively reduce *P. aeruginosa* and other waterborne pathogen infections (Cervia, Ortolano, & Canonica, 2008). The elevated cost that is associated with the use of these filters and the potential for retrograde contamination from the drain are limitations to consider.

The reported efficacy of disinfectants on *P. aeruginosa* varies between lab experiments and actual water system environments. The presence of established biofilm in premise plumbing increases the resistance to disinfection. Furthermore, the efficiency of the disinfection will depend on good hydraulic conditions, which ensure that the disinfectant reaches the farthest point in the system.

1.3.4 Hydraulics and temperature regime

Water stagnation, average residence time and flow regime are factors that affect the establishment of biofilm and the risk of amplification of opportunistic pathogens. A recent document on the water safety in buildings published by the World Health Organization highlights low flow, stagnation and warm water temperatures as bacterial growth-promoting conditions (World Health Organization (WHO), 2011). Accordingly, the results from different studies point toward higher flow and turbulence to reduce the biofilm formation (Critchley et al., 2001; Donlan et al., 1994; Kirisits et al., 2007). A lower residence time, erosion of cells on the surface due to higher shear force and better diffusion of disinfectant with a thinner boundary layer are factors that were suggested to explain the effect of the flow dynamics on biofilm formation (Donlan et al., 1994). Another key parameter is the surface-to-volume ratio (S/V). In a recent study, the impact of S/V on the biomass production potential for pipes was investigated (Tsvetanova & Hoekstra, 2010). The authors observed a significant effect of S/V on the planktonic biomass concentration, with concentrations that were 4 to 14 times higher with higher S/V ratios. Premise plumbing piping usually has a small diameter and, therefore, has a larger S/V ratio than the distribution system. Many laboratory studies are performed in reactors or equipment that poorly represent the premise plumbing S/V ratio. Given the impact that it might have on the concentrations of planktonic bacteria, the S/V ratio should be a key design parameter when setting up laboratory or pilot experiments.

Very few studies have examined the impact of hydraulics and flow regime on *P. aeruginosa* specifically, as most of the work has been performed with respect to biofilms in general. The effect of the flow regime on cell-to-cell signaling was evaluated for *P. aeruginosa* (Kirisits et al., 2007). The authors observed that a larger amount of biofilm was required to reach full cell signaling within the biofilm community with an increased flow rate. *P. aeruginosa* cell attachment was also investigated through different shear forces and was found to increase with the shear force under low flow conditions, with its maximum attachment reached between 3.5 and 5 mN/m^2. When shear was >5mN/m^2, the attachment decreased while the shear continued to increase (Raya et al., 2010). The impact of a dead leg and stagnation has not been reported for *P. aeruginosa* specifically. However, recent studies have shown the impact of water stagnation on the microbial quality of drinking water in premise plumbing. Lautenschlager et al. showed deterioration of microbial water quality after overnight stagnation in household premise

plumbing fed by non-chlorinated water (Lautenschlager, Boon, Wang, Egli, & Hammes, 2010). They observed an increase of 1-2 log in the HPC concentration along with an increase in the concentration of intact cells of 0.1-0.3 log and an increase in the cells' biovolume. Another study that was conducted on taps fed by chlorinated water also reported an increase in the number of cells and % intact associated with periods of stagnation (Lipphaus et al., 2014).

A study in which biofilms were first established under laminar or turbulent flow looked at the effect of unsteady hydraulic conditions on the biological quality of the drinking water (Manuel, Nunes, & Melo, 2010). Periods of stagnation once the biofilm was established promoted bacterial accumulation for both the planktonic and biofilm bacteria. These cells were carried away once the flow was resumed, which increased the concentration in drinking water. This finding should be investigated further for waterborne opportunistic pathogens, to understand their response in premises that have variable demands and periodic stagnation.

In Europe, the residence time has clearly been linked with the amplification of opportunistic pathogens in premise plumbing. Recommendations have been emitted for the design and operation of premise plumbing water networks (Centre scientifique et technique du bâtiment (CSTB), 2012): they should be designed to eliminate low flow and dead leg areas. This approach is part of the strategy in France to reduce their nosocomial infections (République Française, 2010b). The importance of the water flow and hydraulics in the amplification of opportunistic pathogens within premise plumbing is emphasized. However, the application of these principles often involves high costs either in infrastructure modifications or in labor, to perform purges and periodical microbiological controls with defined action limits.

Maintaining a temperature above 60°C in hot water distribution systems is a control strategy that is recommended by the World Health Organization and has been adopted by several countries (World Health Organization (WHO), 2011). In other countries such as the USA, the risk of scalding prevails, and a temperature of 48°C is recommended. A new recommendation from ASHRAE suggests a water temperature of above 60°C at the heater outlet and 51°C at all points of the distribution system in the building (BSR/ASHRAE, 2011). However, there is little data to confirm the effectiveness of this measure to control the establishment of *P. aeruginosa* or to eradicate it once present. Increasing the temperature of the hot water network from 50 to 60°C in an ICU was believed to be the major contributor to the observed decrease in faucet contamination

over the two years that followed the temperature regime change (Cuttelod et al., 2011). However, a rise of the hot water temperature from 50 to 58°C at the tap in another ICU did not significantly decrease the rate of faucet colonization or the concentrations of *P. aeruginosa* recovered from faucet swab specimens (Petignat et al., 2006). Overall, temperature control in the hot water network could be an efficient control measure to prevent the establishment and amplification of *P. aeruginosa*, but it might not be effective in the short term to eradicate an already established contamination.

1.4 Guidelines and recommendations for the control of *P. aeruginosa* in engineered systems

P. aeruginosa is not regulated for municipal drinking water because there is no evidence that it can be a source of infection for the general population (Cunliffe et al., 2011). However, its presence in water from healthcare facilities can be significant for at risk populations, and some countries have recommended target and action levels in healthcare settings. In France, *P. aeruginosa* should be below 1 CFU/100 mL in water that is used for patient care and other specific uses within the healthcare facility (Castex & Houssin, 2005). Similarly, in United Kingdom, there is no mandatory routine monitoring of drinking water for *P. aeruginosa*, but it is expected to remain undetected in premise plumbing water from healthcare facilities (Department of Health (DH) et al., 2013).

Several control measures have been reported to limit the presence of *P. aeruginosa* in water distribution systems of healthcare facilities, but the available studies make it difficult to draw conclusions about their effectiveness (Loveday et al., 2014). Examples of reported measures to control *P. aeruginosa* were the use of disinfectants, an increase in the water temperature, replacement of devices and installation of filters. It is critical to consider that these interventions were in outbreak contexts as corrective measures, rather than preventive measures. In light of the key factors that promote the growth of *P. aeruginosa* within water distribution systems, the following recommendations can be proposed as proactive control measures for healthcare facilities:

- Thermostatic mixing valves should be installed only if a risk assessment has evaluated that its use by vulnerable patients causes them to be at risk of scalding. If a thermostatic

valve is to be installed, then it should be integral to the body of the device (faucet or shower) (Department of Health (DH) et al., 2013).

- Flow straighteners and aerators should be avoided as much as possible, as recommended by the Department of Health in UK (Department of Health (DH) et al., 2013).
- For new buildings, the room design should ensure that the bed head is not within the radius of the sink splashing.
- A drain cleaning program should be implemented to avoid plugging due to low usage or low flow.
- The number of taps should be minimized to avoid underused water outlets and low throughput.
- Promoting factors, such as flexible hoses, stagnant water, poor temperature control, and dead legs, should be avoided.
- A water safety group and a water safety plan should be implemented. The water safety plan will drive an engineering risk assessment of the water system.
- To maximize the recovery of planktonic bacteria, sampling should be performed during periods of no use or low use. Collecting pre-flush and post-flush samples will help to assess whether the source of *P. aeruginosa* is distal (a device) or systemic.
- Putting hands under the first flush of water should be avoided.

1.5 Concluding remarks

The opportunistic pathogen *P. aeruginosa* is an adaptable bacteria that readily develops resistance to antibiotics and disinfectants, which makes it difficult to control once it is established in premise plumbing biofilms. Different solutions have been applied to reduce the incidence of contaminated points of utilization, especially in hospitals. The use of 0.22-μm filters at the point of use is an effective but costly solution. Temperature control of the hot water network could be an efficient control measure to prevent the establishment and amplification of *P. aeruginosa*, but it might not be effective in the short term to eradicate an already established contamination. Furthermore, in the context of a large building distribution system, with long residence times,

periods of prolonged stagnation and the presence of a biofilm, disinfectant residual is unlikely to reach dead legs and biofilm at remote points of utilization. If disinfection is applied, it should be performed diligently and monitored closely to avoid resistance development during periods of disinfectant depletion. An appropriate choice of material and device as well as room layout will contribute to minimizing the colonization of taps by *P. aeruginosa* and the risk of exposure for patients. Electronic faucets provide ideal conditions for the amplification of *P. aeruginosa*, which then becomes extremely difficult to eradicate. Furthermore, construction or renovation settings present additional risks because water is often stagnant for long periods of time and the risk of contamination is increased through construction activities. A thorough commissioning of the water network, including a survey for *P. aeruginosa* and other opportunistic pathogen contamination, should therefore always be performed prior to building occupation.

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CHAPTER 2. RESEARCH OBJECTIVES, HYPOTHESES AND METHODOLOGY

2.1 Critical review of previous research

There is a wide body of literature reporting research conducted to better understand how to control *L. pneumophila* and *P. aeruginosa* in premise plumbing of large buildings, particularly in hospitals. Prior research provides insight on the ecology of these bacteria and their resistance to disinfection under certain conditions. However, a large portion of these observations were obtained at the laboratory scale under conditions (rich media and controlled environments) that are not representative of environmental conditions in distribution systems. A multitude of factors may interact in the establishment and amplification of these bacteria in real water distribution systems, making the transfer from laboratory scale to premise plumbing challenging. Furthermore, few studies include the sink environment, which may be a significant source of contamination of the point-of-use and care equipment (Cholley et al., 2008; Hota et al., 2009; Schneider et al., 2012). Several *P. aeruginosa* outbreaks have been attributed to the use of water in large buildings without being able to establish a direct link (Petignat et al., 2006; Rogues et al., 2007; Romano et al., 2013; Vallés et al., 2004) whereas the causal link between clinical cases and contamination of hot water systems and cooling towers is clearly established for *L. pneumophila* (Colville, Crowley, Dearden, Slack, & Lee, 1993; Walser et al., 2014).

The majority of hospital-based studies report the number of positive points-of-use by culture detection and are not always quantitative. Although some studies have been conducted across the building premise plumbing, they seldom (1) identify the locations and causes of amplification of opportunistic bacteria in the water system; (2) relate the monitoring results to risk assessment. Reported levels of bacterial contamination were obtained using a wide range of sampling protocols that preclude their comparison because of methodological differences. In existing studies, sampling protocols, including water sampling volumes and pre stagnation times vary significantly. To ensure efficient monitoring and allow inter site comparison, there is a need to specify the sampling protocol, namely: the analytical method used, the sample volume, pre stagnation duration, first flush vs post flush sample, sample pre-treatment and storage, etc. To clarify whether an amplification is widespread or localized in a large building, adapted sampling

protocols should be used. Greater contamination near the tap as compared to levels in the main premise distribution system has been evidenced (Cristina et al., 2014; Serrano-Suarez et al., 2013), but few studies provide information on the precise location and factors promoting this amplification (Lautenschlager et al., 2010; Lipphaus et al., 2014). To determine the location of bacterial proliferation, sequential sampling that target various levels of the distribution system and that consider residence time (stagnation) should be used. Finally, to relate sampling results to risk assessment, the sampling protocol should provide a basis to evaluate acute and chronic exposure. No study defines an optimum sampling protocol providing better risk assessment and interpretation at the point-of-use.

It is well established that most of the bacterial biomass in a distribution network is established in the biofilm (Flemming, H.-C., Percival, & Walker, 2002). However, the conditions governing the biofilm contribution to the suspended bacteria in a dynamically operated system are poorly known. It is not yet possible to predict the relative importance of the biofilm contributions by natural detachment of bacteria in the water or by mechanical detachment of biofilm by shear forces. The role and importance of biofilm detachment in premise plumbing water contamination at the point of use needs to be better understood to determine the control strategy: either to target biofilm control or control suspended bacteria released from the biofilm.

The detection of opportunistic pathogens in drinking water presents several challenges including a preference for biofilm vs planktonic state as well as its presence in low and variable concentrations. Moreover, environmental factors such as a nutrient-poor environment and the presence of inhibitors and stressors can lead to a dominance of the viable but not culturable (VBNC) state. When monitoring for *P. aeruginosa* at the tap, two main environmental stresses can be present: disinfectant residual and metals, namely, copper, zinc and lead. Depending on prior stagnation and residual chlorine at time of sampling, these stressors can impact culturability and traditional culture methods may fail to reveal the presence of bacteria. These undetected bacteria should not be neglected in an environment with vulnerable patients. VBNC bacteria lose their culturability when confronted with stress but to have the ability to regain viability and infectivity once stressors disappear. This situation happens frequently in premise plumbing, as chlorine residual will be lost during stagnation and preferred materials near the tap are often elastomeric or plastic materials, limiting the exposure to copper. At a larger scale, the effectiveness of various disinfectants reported in the literature to control *P. aeruginosa* and *L.*

pneumophila may be misleading if evaluated based on culturability, not measuring the undetected cells able to recover (Allegra et al., 2011; Chang, Hwang, Cheng, & Chang, 2007; Dwidjosiswojo et al., 2011; Xue, Hessler, Panmanee, Hassett, & Seo, 2013). In addition, most of these studies were performed in conditions non-representative of those typical to premise plumbing: variable flow regimes, frequent and variable stagnation periods, the presence of mixed heterotrophic biofilm, increased surface-to-volume ratio, etc. Some hospital case studies using disinfection of premise plumbing have evidenced the inefficacy of treatment, the emergence of resistance or even the selection of strains with greater pathogenic potential (Allegra et al., 2011; Blanc, Carrara, Zanetti, & Francioli, 2005; Shrivastava et al., 2004). It appears more productive to define an operating regime that efficiently controls viable bacterial proliferation in the biofilm to prevent contamination of the water, rather than relying on secondary disinfection or periodical microbiological monitoring results as a first line of defense.

The European experience has clearly demonstrated the predominant impact of residence time and thermal control on the proliferation of opportunistic pathogens in premise plumbing hot water systems. Reported corrective action and recent guidelines stress the need to properly manage hydraulics to ensure homogeneous temperature and biocidal control in all areas of the hot water distribution system (Centre scientifique et technique du bâtiment (CSTB), 2012). Also commonly specified are construction and operational standards to minimize stagnation and reduce heat loss: recirculation loops, elimination of hydraulic and physical dead ends. This approach can be successful and is considered as essential before the addition of a secondary disinfection at the building point of entry. However, despite the growing number of regulations and guidelines to control *Legionella* in hot water distribution systems, the application of these guidelines and the demonstration of their impact on the occurrence of opportunistic pathogens are poorly documented. Furthermore, the implementation of these guidelines in existing buildings often requires important modifications to the system to ensure hydraulic circulation or extensive resources to implement flushing and monitoring.

2.2 Objectives

The main objective of this project is to understand factors influencing occurrence of opportunistic pathogenic bacteria in hospital premise plumbing and identify risk mitigating measures.

On a more detailed level, this project will seek to:

1. Establish the bacterial load profile in cold and hot water systems according to the stagnation time and the volume of water collected at the point-of-use in order to define the optimum sampling protocol and interpret sampling results;
2. Establish the biofilm detachment contribution to the bacterial load in the water collected at the point-of-use;
3. Evaluate the effect of copper and chlorine present in premise plumbing on the detection of *P. aeruginosa* in water;
4. Optimize a selective method for environmental detection of *P. aeruginosa* and *L. pneumophila* in tap water, including the viable fraction;
5. Assess the occurrence of *P. aeruginosa* and *L. pneumophila* in the premise plumbing of large buildings;
6. Provide a diagnostic methodology for health care facilities and other large buildings based on temperature profiling of the hot water distribution systems to identify *L. pneumophila* risk areas;
7. Determine the environmental and operational factors favorable to the establishment and persistence of *P. aeruginosa* at the tap and in the premise plumbing;
8. Develop a risk classification tool for hot water distribution systems based on temperature measurements and monitoring at key control points to prioritize corrective actions.

Achieving these objectives will allow us to answer fundamental questions with regards to risk management related premise plumbing microbial water quality:

- Where are the bacteria located in the premise plumbing?
- Do short stagnation periods impact bacterial load in the water?
- Is the presence of common drinking water inhibitors masking the presence of bacteria when measured by traditional culture methods?
- Which factors lead to the amplification of *P. aeruginosa* at the point-of-use?
- How can *L. pneumophila* be controlled to reduce the risk of health-care related infections?

The project objectives are derived from the following research hypotheses:

1. The deterioration of the microbiological and physico-chemical quality of water in premise plumbing occurs after stagnation, mainly at the point-of-use (faucet, shower, etc.) and in its immediate connecting pipes. *Existing data on microbiological quality of water at the point-of-use has been established from samples collected with or without prior flush, after varying stagnation time and with variable sampling volumes, including water from various sectors of the distribution system (tap, connecting pipes, secondary and principal distribution pipes). A sequential sampling approach will help identify the sectors contributing most to the deterioration of water quality. Moreover, this will define the optimum sampling protocol for further research. Indeed, the volume required to reduce the bacterial load and the time required to return to the initial values are not documented.*

2. The presence of stressors commonly present in drinking water premise plumbing will affect the detection of bacteria in water and may lead to an underestimation of the potential risk in the system. *P. aeruginosa prevalence studies are generally conducted using the culture detection method. Several studies were not able to isolate P. aeruginosa from the water sampled at the tap. One reason might be the presence of environmental stressors that may impact the culturability of P. aeruginosa without eradicating it.*

3. It is possible to optimize a reproducible method to detect *L. pneumophila* and *P. aeruginosa* in water samples from the premise plumbing. *The methods reported in the literature lead to losses of microorganisms or rely solely on culturability, leading to an underestimation of the population. Culture methods do not detect viable but non-culturable bacteria (VBNC) or environmentally stressed bacteria. The use of qPCR is increasingly reported, but the DNA extraction has not been optimized for drinking water specific inhibitors such as chlorine, metals (copper, lead, iron, etc.) and low concentrations. A method optimization is required to maximize cell recovery and eliminate PCR inhibitory substances present in premise plumbing water samples. There is no data on the reproducibility of the DNA extraction method.*

4. The assessment of water temperatures at the point-of-use and at key control points throughout the hot water distribution system of a building is a good predictor of sectors and points-of-use at risk for *L. pneumophila* growth. *Many studies report the use of*

temperature regime as a control measure for L. pneumophila. However, assessment of the risk is commonly achieved through costly microbial detection of L. pneumophila at selected points-of-use. The work proposed will demonstrate the potential of detailed temperature profiling to identify areas at risk of L. pneumophila in the hot water distribution systems. This work will provide a scientific basis to identify effective monitoring strategies and guidance to conduct temperature profiling and interpret monitoring results.

5. The sink environment including parameters such as the type of device, the volume of mixed hot and cold water, drain and aerator contamination affects the establishment and persistence of *P. aeruginosa* in the network. *Existing studies have focused on positivity of faucets, relating mainly the observed differences to the type of faucets. However, other influencing parameters such as volume of mixed hot and cold water, piping material, water temperature, drain and aerator positivity, chlorine and copper concentrations have not been systematically investigated. Such data will help to identify the device characteristics promoting the contamination and to generate information for hospitals to better choose the equipment and installation design.*
6. It is possible to define a risk assessment and control approach for *L. pneumophila* based on temperature regime and hydraulic optimization in domestic drinking water network. *Propose a risk characterization approach based on temperature diagnostic at critical control points.*

2.3 Methodology

The experimental approach was conducted in five main steps:

1) Define the bacterial load profiling and the impact of stagnation in cold and hot water systems of large buildings (Obj. 1 & 2);
2) Evaluate the impact of chlorine and copper present in drinking water on detection of *P. aeruginosa* (Obj. 3);
3) Establish temperature profiling and monitoring at critical control points in five hot water systems (Obj. 6 & 8);

4) Measure the prevalence of *L. pneumophila* in five hot water systems (Obj. 5 & 8);

5) Measure the prevalence of *P. aeruginosa* in the cold water system of a large hospital (Obj. 5 & 7).

The experimental protocol is described for each part, with reference to the specific microbiological analysis performed. The microbiological methods used throughout this research project are summarized in section 3.3.6, including a description of the work done to optimize the DNA extraction from premise plumbing water samples and the qPCR detection method for *P. aeruginosa* in environmental samples, which will address objective #4.

2.3.1 Bacterial load profiling and impact of stagnation

This first part of the research is to determine the total bacterial load profile as a function of the volume of water sampled from a tap and of the stagnation time. It is aimed at understanding the contribution of each section of the water distribution system within a premise plumbing to the level of contamination measured in a sample. The impact of stagnation is studied by repeating the bacterial load profiling for various stagnation times. This first step addresses objectives #1 and #2.

Sampling was conducted in a ten-story 450 bed children's hospital fed by surface filtered chlorinated water and a hot water recirculating distribution system. The sampling was conducted separately for cold and hot water systems, on two designated taps. The cold water system was sampled in July 2012 with an incoming municipal water temperature averaging 26.2°C and a measured residual chlorine of 0.41 mg Cl_2/L. The hot water system was sampled between November 14th and December 11th 2012, with a hot water temperature coming out of the heater averaging 61.6°C and residual chlorine below 0.1 mg Cl_2/L.

Bacterial load sampling was performed immediately upon opening the water after the designated stagnation time. For each sampling event at a tap, successive volumes of the first liter were sampled in sterile 50 mL tubes or propylene bottles with sodium thiosulfate (final concentration 1.1 mg L^{-1}) : 1st volume of 15 mL, 2nd volume of 35 mL, 3rd volume of 200 mL, 4th volume of 250mL, 5th volume of 500mL (Figure 2-1). These sampled volumes cumulated the first liter contained within the tap and connecting pipes at the start of sampling. Three additional samples of 250 mL were collected after 2L, 5L and 10L of flow (Figure 2-1). For each sampled volume

(1 to 8), heterotrophic plate counts (HPC), viable and total bacterial counts were assessed as described in section 2.3.6. Sampling events were conducted after 1, 24, 48, 72, 120 & 240 hours of controlled stagnation. Hot and cold water systems were sampled in separate events to ensure prior stagnation of the first liter. Temperature and residual chlorine were measured immediately following the first liter for each sampling event. Residual chlorine concentrations were measured with a Pocket Colorimeter II (HACH, USA).

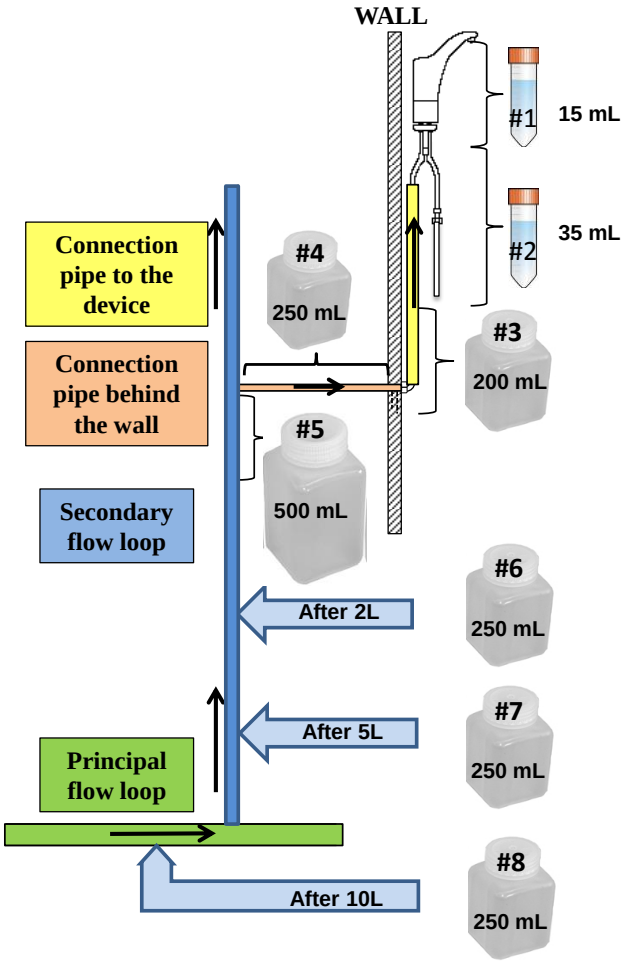

Figure 2-1: Sampling sequence and approximate corresponding volume within the plumbing

2.3.2 Impact of chlorine and copper on the detection of *P. aeruginosa*

When monitoring for *P. aeruginosa* at the tap, two main environmental stressors can be present: disinfectant residual and metal ions from plumbing (copper, zinc and lead). The second part of the research was conducted in the laboratory, to evaluate the impact of chlorine residual and copper concentrations present in drinking water at the time of sampling with four different

detection methods. Depending on prior stagnation and residual chlorine concentration at the time of sampling, traditional culture methods may fail to reveal the presence of VBNC *P. aeruginosa* because of the impact of these factors on culturability. This part of the research addresses objective #3.

The experiments were performed with *P. aeruginosa* strain PA14 (Lee, D. G. et al., 2006) prepared in Tryptic Soy Broth (TSB; Difco) and grown overnight at 37 °C. Cells were harvested by centrifugation (3000 x g for 30 min) and washed twice in sterile 2 mM phosphate buffer for a final estimated cell density of 5×10^9 cells/mL.

Sterile polypropylene bottles containing 500 mL buffered water (2 mM phosphate buffer, pH 7.3±0.1) were inoculated (final concentration 10^7 cells/mL) and monitored throughout the experiment. A control cell suspension was not exposed to chlorine or copper. For the first part of the experiment, chlorine was added to inoculated water for an initial chlorine residual of 2 mg Cl_2/L. Table 2-1 summarizes the sampling plan for each studied parameters.

Table 2-1: Sampling plan to evaluate the impact of chlorine and copper on recovery of *P. aeruginosa*

	Day 1						Day 2		Day 3	Day 5	Day 9
	0	0,5h	1h	2h	4h	6h	24h	24+2h	48h	4d	8d
Control	x										x
Cl_2		x	x		x		x		x	x	x
Cu				x		x	x	x	x	x	x

For each sampling points, residual chlorine was measured (Hach pocket colorimeter) and 1% sodium thiosulfate was added in order to neutralize chlorine prior to conducting microbiological analysis. For the second part of the study, copper stress was induced by adding copper sulphate to the water prior to inoculation ($CuSO_4 \bullet 5H_2O$, final copper concentration 4 µM). Copper stress was stopped after 24 hours by the addition of the chelator diethyldithiocarbamate (DDTC) (Moritz, M. M., 2011). All samples were mixed thoroughly and divided to perform the following microbiological analysis: culture, enzymatic assay, Live/Dead BacLight and qPCR (see section 2.3.6).

2.3.3 Temperature profiling and monitoring

Monitoring *L. pneumophila* can be costly and only provides a snap shot at a point in time. Using temperature profiles at points-of-use and monitoring of temperature in key control points of the system can provide an initial assessment of a complex hot water distribution system and help identify problematic areas. In this third step of the research project, five hot water distribution systems were analyzed. Results obtained will address objectives # 6 and #8.

Temperature profiling and monitoring was studied in five different hot water distribution systems. Systems 1 to 4 are smaller systems within a 7-story general hospital facility of 255 beds using conventional electric water heater being fed chlorinated ground water. System 5 has a larger flash system feeding a ten-story 450 bed children's hospital fed by surface filtered chlorinated water. Characterization of the different systems and connected units was completed between July 2012 and October 2013. The principal flow and return loop of each system was sampled at the water heater outlet, in the principal return loop and prior to the return point into the water heater. Water samples were collected through sampling ports which were flushed prior to sampling to ensure no stagnant water from the sampling port would be collected. After flushing, the sampling port was cleaned with ethanol and sterilized MilliQ water. A volume of 250 mL was collected for pH, temperature, chlorine and conductivity measurements. Municipal water feeding the hot water systems was sampled following the same protocol. To better identify problematic sectors, continuous temperature monitoring was conducted on 3 subordinate return loops for system 5. A Datalogger (RDXL4SD 4-Channel, Omega, Qc, Canada) using a surface probe was installed on the selected piping. Sentinel taps where sampling was performed were selected based on the following criteria: representative of different building levels, some at the far end and preferably in areas serving vulnerable patients such as intensive care units, surgical ward, transplant, infectious diseases.

Following systems characterization, sampling was conducted to establish temperature profiles at each selected sentinel point-of-use in hot water, across all 5 systems between July 2012 and March 2013. A temperature probe was inserted into the water to measure the temperature over a 20 minute period of continuous flow. Each system had a number of taps sampled proportional to the size of the system. Systems 1 to 5 had respectively 3, 6, 3, 7 and 36 taps sampled. The temperature profiles could not be generated for 1 mitigated tap in system 1, 3 in system 2 and 1 in

system 3. In addition, three taps from system 5 were selected for a repeat temperature profile sampling. Residual chlorine was measured onsite (Pocket ColorimeterTM II, Hach, USA) for all samples.

2.3.4 Prevalence of *L. pneumophila* in the hot water system

In order to validate temperature profiles as predictor of *L. pneumophila* risk, the forth step of the project was to conduct a targeted sampling campaign for *L. pneumophila* across the 5 studied systems. The sampling was conducted to evaluate the presence of *L. pneumophila* at the point-of-use in order to address objectives #5 and #8.

All five hot water systems were sampled for *L. pneumophila*. The principal flow and return loop of each system was sampled at the water heater outlet, in the principal return loop and prior to the return point into the water heater. The samples were collected at the time of the systems characterization, as described in section 2.3.3. A sample of 2L was collected in sterile polypropylene bottles with sodium thiosulfate (final concentration of 1.1 mg/L), with 1 L for qPCR and 1 L for culture.

L. pneumophila sampling was also conducted for all sentinel points of systems 1 to 4 and 8 selected sentinel points from system 5. Sentinel points from system 5 were selected based on temperature profile results, targeting areas with poor temperature results. For each sampling point, 3L of hot water were collected without prior flush into sterile polypropylene bottles containing sodium thiosulfate (final concentration of 1.1 mg/L). Of the 3 liters collected, 1L was used for culture, 1L for qPCR and 1L was collected as extra. Analyses were performed as described in section 2.3.6.2.

In order to evaluate the repeatability of *L. pneumophila* levels at a given sampling point collected on different days and times, multiple sampling were performed on a few selected points. Two systems were selected for the repeat sampling: a system with no positive sites for *L. pneumophila* (system 1) and a system with a high positivity rate (system 4). Both systems were fed by the same source water for easier comparison. In each system, 3 sampling points were selected as representative control points: the water heater outlet, one representative tap and the principal return loop. This sampling was repeated at total of 5 times at each selected point, following the same protocol as described for the initial evaluation.

2.3.5 Prevalence of *P. aeruginosa* in the cold water system

In 2004-2005, an outbreak of *P. aeruginosa* in the neonatal intensive care unit of a Montreal hospital was reported. Environmental investigation led to the water system, especially faucet aerator and drain biofilm, as the most probable source of the outbreak. Corrective measures were put in place and the outbreak was resolved. At the time of the investigation, none of the water samples were positive for *P. aeruginosa* by culture. To better understand the source of the water system contamination and evaluate the occurrence of *P. aeruginosa* in a non-outbreak situation, an investigation of the cold water system of this hospital was performed as part of this research project. A new sampling protocol combined with additional detection methods were put in place. Results obtained will address objectives #5 and #7.

A total of 28 conventional faucets were sampled as follow: 1) a swab from the drain, 2) 1L of first flush cold water in sterile propylene bottle with 1% sodium thiosulfate and 3) a swab of the aerator. Three additional faucets were sampled for water only by culture and qPCR. Culture, heterotrophic plate counts (HPC), qPCR and viable and total cell counts were performed on the water. Culture and qPCR were performed on swabs. In addition, each faucet and its environment was characterized in detail, including the type of activating device, the connecting pipe material, the faucet internal diameter, the faucet alignment to drain and the drainage efficiency.

2.3.6 Microbiological analysis

Several microbiological detection methods were used throughout the different steps of the project. Heterotrophic plate counts (HPC) were evaluated on R2A agar at 22°C, after 7 days of incubation according to method 9215-D (American Public Health Association (APHA), American Water Works Association (AWWA), & Water Environment Federation (WEF), 2012). Viable and total cell counts were determined using LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes) (Boulos, Prévost, Barbeau, Coallier, & Desjardins, 1999). This kit differentiates viable from dead cells using membrane integrity criteria. Briefly, 1 mL of sample or dilution in 0.85% sterile saline solution was mixed with 3 µl of stain (propidium iodide and SYTO9), incubated in the dark for 15 min and filtered on black 0.2 µm polycarbonate filter (Millipore). Enumeration was done at 1000-fold magnification, with an epifluorescence microscope (Olympus). Microbiological methods specific to the detection of *P. aeruginosa* and

L. pneumophila are described in more details in the following sections. In addition, the work done to optimize the DNA extraction and the qPCR for drinking water samples is described, addressing objective #4.

2.3.6.1 *P. aeruginosa* culture and enzymatic detection

Culture was performed according to International Organization for Standardization (ISO) method 16266:2006 for *P. aeruginosa* detection and enumeration in water (ISO, 2006). Briefly, three dilutions were done in duplicate for each sample, filtered on a 0.45 µm mixed cellulose ester membrane. The filters were deposited on cetrimide-nalidixic acid agar plates (45.3 g L^{-1} of Cetrimide Selective Agar (Remel), 10 mL L^{-1} glycerol (Fisher), 0.015 g L^{-1} nalidixic acid (Sigma-Aldrich)) and incubated at 37.5°C for 24 h before enumeration. Detection was also performed using the Pseudalert®/QuantiTray2000 (IDEXX) according to the manufacturer's instructions.

2.3.6.2 *Legionella spp.* and *L. pneumophila* culture

Quantification of *Legionella spp*. and *L. pneumophila* by culture were performed by the Centre d'expertise environnementale du Québec, under the supervision of Dr. Manuela Villion. *Legionella* spp. were cultured according to the standard AFNOR NF T90-431 procedure (AFNOR 2003, 2006). For each sample, 1 liter was filtered on sterile 0.4 µm polycarbonate membranes (0.47 mm diameter; Maine Manufacturing, LLC). Membrane filters were then placed in 5 ml sterile water and ultrasonicated at 47 kHz for 1 min (Bransonic, Danbury, USA). In order to decrease the interfering flora, 1 ml of the resulting concentrate was heat-treated (50°C, 30 min), 1 ml was treated by acidic solution (pH=2; 5 min), and another milliliter was first heat-treated, then treated with acidic solution. For each sample, six GVPC selective agar (Innovation Diagnostics Inc.) were surface inoculated with 100–200 µl of (i) sample before filtration, (ii) of concentrate, (iii) of concentrate diluted to 1/10, (iv) of heat-treated concentrate, (v) of acid-treated concentrate, and finally (vi) of combined treated concentrate. The agars were incubated at 36°C for 10 days and colonies counted at three different times during the 10 days incubation period. Typical colonies were subcultured on buffered charcoal yeast extract (BCYE) agar without cysteine, on blood agar and on BCYE agar (Innovation Diagnostics Inc.), and then incubated for 4 days at 36°C. Resulting colonies that developed on BCYE agar, but neither on blood agar nor on BCYE without cysteine were considered as *Legionella* spp. Identification to

Legionella pneumophila species was further verified using the *Legionella* latex test (OXOID Limited).

2.3.6.3 DNA Extraction

Environmental samples are often characterized by very low bacterial concentrations and high levels of inhibitors. In potable water, samples will often contain substances such as metal ions (Fe and Cu) and humic and fulvic acids, which are known to introduce PCR inhibition (Tsai & Olson, 1992; Yeates, Gillings, Davison, Altavilla, & Veal, 1997). It is therefore important to ensure an optimized DNA extraction procedure, which will maximize the recovery rate and removal of potential inhibitors for subsequent PCR assays. A recent study compared eight different combinations of DNA extraction and purification methods on complex biofilm samples (Ferrera et al., 2010). The authors recommend a purification step when working with complex samples with high levels of extracellular substances and cell concentration. The bead beating method combined to enzymatic lysis and phenol extraction was amongst the highest recovery rates while preserving the bacterial diversity of the real community. Results also suggest the addition of enzyme during the extraction process might greatly improve recovery rates. Another review showed that ammonium acetate precipitation led to a higher purity DNA and lower degradation level than with phenol-chloroform purification (Lemarchand et al., 2005). In this comparative study, bead beating method combined to ammonium acetate was suggested as the most effective method and has successfully been used by other authors working with environmental samples (Maynard et al., 2005; Yu, Z. & Mohn, 1999). The bead beating method followed by precipitation with ammonium acetate was selected over other methods available. The bead beating method uses the FastPrep-24 offering a unique and optimized technology to break the cell samples. To maximize the efficiency and eliminate the presence of inhibitors, the method has been combined with an ammonium acetate precipitation. Briefly, the water sample was filtered on 0.45 µm mixed cellulose ester membrane and the filter was inserted into an extraction tube containing a garnet matrix and one 1/4-inch ceramic sphere (Lysing Matrix A, MP Biomedicals). A garnet and ceramic matrix was selected to promote the extraction of the DNA directly on filters, simultaneously breaking the filter and the cells to release the DNA. A volume of 1mL of lysing buffer (Tris HCL pH8 50mM, EDTA-2Na pH8 5mM, SDS 3%) with RNase (20 µg/ml) was then added to each tube prior to the bead beating step performed on a FastPrep

MPBio-24. Ammonium acetate (2M) was added followed by cold 70% isopropanol and glycogen (concentration) to the supernatant. DNA was washed twice with cold ethanol (70%) and resuspended in 100 µl of sterile DNA-free water.

Several factors may influence DNA recovery and PCR inhibitors removal through the extraction process. In drinking water from premise plumbing, the presence of dissolved and particulate metals may result in PCR inhibition. The addition of sodium thiosulfate to neutralize residual chlorine may also impact the effectiveness of the lysing buffer by complexing with EDTA. Finally, it was elected to extract DNA directly from the filtering membrane by introducing the membrane into the extraction tube to avoid considerable DNA losses through elution or sonication of the filter. However, the presence of a filtering membrane may impact the extraction process either through adding inhibitors or generating DNA losses. In order to confirm the applicability of the selected DNA extraction process to the drinking water samples handled in this project, different conditions were tested and results are presented in Figure 2-2. All tests were conducted with a pure culture of *P. aeruginosa* PA14 spiked into sterile MilliQ water for final concentration of $2x10^6$ cells/mL. The addition of an equivalent quantity of pure culture directly into the extraction tube was used as the positive control to evaluate the rate of recovery and presence of inhibitors for all tested conditions. Low concentration condition was tested with an initial concentration of $2x10^3$ cells/mL. All conditions were tested in triplicate except for the impact of metals, which was tested in duplicate. Furthermore, each sample was evaluated in triplicate by qPCR. Resulting in n=9 for all conditions except metals, with n=6. Error bars represent standard deviation between all replicates.

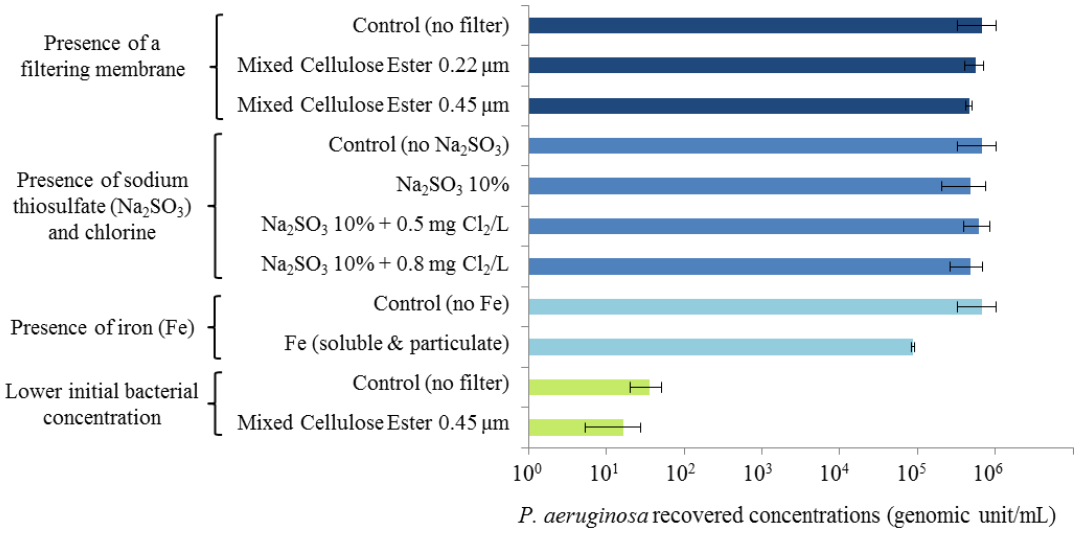

Figure 2-2 : Drinking water sample conditions tested for DNA extraction method performance.

Results confirmed good DNA recovery and removal of inhibitors for all drinking water conditions tested, with the exception of the presence of dissolved and particulate iron. In this case, the high concentration of iron added was too elevated for the complete elimination of inhibitors. However, DNA samples were re-evaluated by qPCR with a 100-fold dilution and resulting recovery was 100%. This stresses the importance of using an internal positive control in the qPCR method, as described in the next paragraph. At lower initial concentrations, the impact of the filter was more significant, but still within less than 1 log. However, recovery of the positive control was lower than the theoretical value of $2x10^3$ cells/mL. This loss might be attributed to the intrinsic losses associated with DNA extractions, which are negligible at high concentrations, but significantly impact the results for lower concentration samples.

Quantitative polymerase chain reaction analyses were performed for *L. pneumophila* and *P. aeruginosa* specifically, on a Corbett Rotorgene 6000. For *L. pneumophila*, qPCR was conducted using the iQ-Check Quanti *L. pneumophila* kit (Bio-Rad, Mississauga, Canada) with the following protocol: 15 min initial denaturation at 95°C followed by 50 cycles with denaturation at 95°C for 15 s, annealing at 57°C for 30 s, elongation at 72°C for 30s and final elongation for 15 min at 72°C (Bonetta et al. 2010). An internal control and four DNA standards ranging between 19 and $3.9x10^4$ genomic units (GU) were supplied with the kit. Sterilized water was used as negative control.

For *P. aeruginosa*, there was no available kit using a standard curve and an internal control. Furthermore, given the nature of the samples to be analyzed, it was important to select a target gene that would be highly specific and sensitive. The optimization of a qPCR method meeting these requirements was therefore undertaken as part of the research project.

Several target genes for *P. aeruginosa*, are reported in the literature: 16S rRNA, *algD, exoA, exoT, oprI, oprL, toxA, gyrB* and *ecfX*. However, some of those genes have generated false-positive (16S rRNA and *oprI*) or false-negative (*algD* and *toxA*), have been described as non-100% specific (*oprL* and *oprI*) or not always present in the genome (*exoT*) (Cattoir et al., 2010; Kaszab et al., 2011; Lavenir, Jocktane, Laurent, Nazaret, & Cournoyer, 2007; Qin et al., 2003). The *gyrB* and *ecfX* genes came out as the most specific and sensitive genes for *P. aeruginosa* (Lavenir et al., 2007) and have been used by several authors (Anuj et al., 2009; Fothergill et al., 2013; Lee, C. S., Wetzel, Buckley, Wozniak, & Lee, 2011; Motoshima et al., 2007; Qin et al., 2003). After testing of primer sets for those two genes, the primers and probe for *gyrB* from Lee et al. were selected for the qPCR method optimization (Lee, C. S. et al., 2011).

An internal amplification control was added in order to detect the presence of PCR inhibitors which may lead to false negative results (Hoorfar, 2003). An internal amplification control is another DNA sequence that is not part of the targeted microorganism gene and is coamplified simultaneously with target sequence through the PCR reaction. The internal amplification control amplification will insure the reaction was not inhibited either due to presence of inhibitory substances, malfunction of the thermal cycler, deficient polymerase activity or polymerase mixture (Hoorfar, 2003). The internal positive control kit from Life was used. It is a synthetic DNA that comes out in the yellow. Optimization of the internal was required due to the low concentration of *P. aeruginosa* in drinking water samples. The internal control DNA as therefore added to the qPCR mix in a 1/10 concentration.

The qPCR reaction volume was 20 µl, including 5 µl of DNA, 10 µl TaqMan® Universal Master Mix II, no UNG (Life Technologies), 1 µl primers and probe (Custom TaqMan® Gene Expression Assay) and internal control reagents (2 µl Internal Control Positivie (ICP) Master Mix and 0.04 µl ICP DNA, TaqMan® Exogenous Internal Positive Control Reagents, Life technologies). The qPCR conditions where the same as Lee et al. : initial cycle at 95°C for 10 min, 50 cycles consisting of denaturation step at 95°C for 30s and a combined annealing and

extension step at 60°C for 90 s. The number of cycles was increased to 50 compared to Lee et al. Sterile water was used as negative control. For each qPCR run, a standard curves from 10^6 to 10^1 genomic unit/reaction was generated using a stock of isolated *gyrB* 356 pb fragment containing the gyrB section targeted by the qPCR primers. For the combined qPCR and DNA extraction, the detection limit was 10 GU/reaction or 200 GU/L, and the quantification limit was 1000 GU/reaction or 20000 GU/L.

Table 2-2 presents a summary of the experimental approach for each hypothesis together with expected results and the corresponding scientific paper presenting these results.

Table 2-2 : Experimental approach developed to validate (or invalidate) the research hypotheses and corresponding published articles

	Hypothesis	Scale	Experimental approach	Expected results	Article
1	The deterioration of the microbiological and physico-chemical quality of water in premise plumbing occurs after stagnation, mainly at the point-of-use (faucet, shower, fountain, etc.) and its immediate connecting pipes	Hospital hot and cold water distribution systems	Sequential sampling of volumes between 15 ml and 10 L of cold and hot water from taps Repeat sampling for stagnation periods of 1h to 10 days Assessment of total and viable bacterial load and heterotrophic plate counts for all samples.	Bacterial load profile depending on the volume collected Stagnation time required to restore the initial bacterial load	2
2	The presence of stressors commonly present in drinking water premise plumbing will affect the detection of bacteria in water and may lead to an underestimation of the potential risk in the system.	Laboratory	Expose *P. aeruginosa* spiked water samples to copper and chlorine for 24 hours Monitor *P. aeruginosa* during and after exposition to stressors by culture, enzymatic detection, Live/Dead and qPCR	Impact of copper and chlorine on the culturability and viability of *P. aeruginosa* Possible recovery of *P. aeruginosa* culturability after stress removal	3
3	It is possible to optimize a reproducible method to detect *L. pneumophila* and *P. aeruginosa* in water samples from the premise plumbing.	Laboratory	Optimization of DNA extraction to remove PCR inhibitors and maximize recovery Development of a *P. aeruginosa* specific qPCR including standard curve and internal control	Quantification of the recovery of *P. aeruginosa* after DNA extraction and qPCR	

Table 2-2: Experimental approach developed to validate (or invalidate) the research hypotheses and corresponding published articles (continued)

	Hypothesis	Scale	Experimental approach	Expected results	Article
4	The assessment of water temperatures at the point-of-use and at key control points throughout the hot water distribution system of a building is a good predictor of sectors and points-of-use at risk for *L. pneumophila* growth.	Hospital hot water distribution systems	Hot water systems characterization Temperature profiling at selected points-of-use in different hospital hot water systems Quantification of *L. pneumophila* in multiple control points in different hot water systems	Prevalence of *L. pneumophila* in hot water Relation between temperature profiles and detection of *L. pneumophila*	4
5	The sink environment including parameters such as the type of device, the volume of mixed hot and cold water, drain and aerator contamination affects the establishment and persistence of *P. aeruginosa* in the network.	Hospital cold water distribution systems	Sampling of cold water, aerator and drain biofilm at selected conventional taps in a hospital Quantification of *P. aeruginosa* by culture and qPCR Assessment of total and viable bacterial load and heterotrophic plate counts for all samples. Characterization of equipment and connection Measurement of physico-chemical characteristics of water	Definition of the variables affecting the prevalence *of P. aeruginosa* at the tap Mapping of the positive sites within a hospital system in a non-outbreak situation Comparison of positive results obtained by culture and by qPCR	5
6	It is possible to define a risk assessment and control approach for *L. pneumophila* based on temperature regime and hydraulic optimization in domestic drinking water network.	Hospital hot water distribution systems	In depth review of the existing guidelines and regulations in place for thermal control of *L. pneumophila* Analysis of results gathered from 5 hot water distribution systems from hospital	Step by step approach to prioritize risk areas within a system Guidance to interpret temperature monitoring results	4

CHAPTER 3. ARTICLE 2 - IMPACT OF STAGNATION AND BIOFILM ON BACTERIAL LOAD PROFILES IN WATER FROM PREMISE PLUMBING OF A LARGE BUILDING

In premise plumbing, especially in large buildings, a better control of bacterial amplification requires an understanding of its localization and the impact of water stagnation on the bacterial load at the faucet. This chapter compares culturable and viable cell profiles within the first ten liters of cold and hot tap water for stagnation periods of various durations. The contribution of the biofilm to the culturable counts in the water bulk phase is highlighted, especially in cold water after one hour stagnation. The results presented were generated through a sampling campaign in cold and hot water distribution systems from a hospital. This paper was submitted to *Environmental Science and Technology*. Supplementary information is presented in Appendix 1.

IMPACT OF STAGNATION AND BIOFILM ON BACTERIAL LOAD PROFILES IN WATER FROM PREMISE PLUMBING OF A LARGE BUILDING

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ABSTRACT

Microbial quality of drinking water can be altered in the premise plumbing of large buildings. To understand the location and the causes of the bacterial amplification, successive volumes of water were sampled from taps after controlled stagnation periods. Bacterial profiles revealed a steep decline in heterotrophic plate counts (HPCs) in the first 250 mL sampled from the hot and cold

water systems. The first 15 mL accounted for 53% (cold water) and 35% (hot water) of the HPC load in the first liter after stagnation. HPCs increased again rapidly (1.7 log) after a short stagnation of one hour. The dominant contribution of the biofilm was evidenced by an exponential correlation between HPCs in water and the pipe surface-to-volume ratio. Direct viable cell count profiles were comparable in hot and cold water, decreasing only after 500 mL. The proportion of culturable cells decreased rapidly after the first flush volume from 0.9±1% to 0.005% in both systems. Periodical stagnation, small surface-to-volume ratio and water temperatures at the tap were identified as major factors affecting the concentration and culturable fraction of viable bacteria to which the consumer is exposed. Findings show significantly higher bacterial loads in the tap's first volume after stagnation.

3.1 Introduction

Drinking water quality is regulated and monitored prior to and throughout municipal distribution systems, ensuring microbial quality water is delivered to the consumer's premise plumbing (World Health Organization (WHO), 2006). However, monitoring of microbial quality after stagnation in premise plumbing is generally not required, despite the significant alterations that can occur, especially within large buildings (Bagh, Albrechtsen, Arvin, & Ovesen, 2004; Inkinen et al., 2014; Lipphaus et al., 2014). Biofilm formation, periodical stagnation, high surface-to-volume ratios, favorable temperatures are factors that can promote bacterial growth in premise plumbing. Heterotrophic plate counts (HPCs) have been used as an indicator of the general microbial quality of the water in main distribution systems. Several countries such as the USA, Australia, Netherlands, Japan, France and Canada have established recommended HPC levels for drinking water between 100 and 500 CFU/mL (Bartram, Cotruvo, Exner, Fricker, & Glasmacher, 2003). HPCs are also used to monitor quality variations within health care facilities premise plumbing in France, where a change of more than 1 log between the cold water infeed and the point-of-use is considered an abnormal variation (Castex & Houssin, 2005). However, HPCs are only representative of the readily culturable bacteria and do not provide indications of the presence of viable but non culturable (VBNC) cells in full scale distribution systems and premise plumbing (Coallier, Prévost, Rompré, & Duchesne, 1994; Prévost et al., 1998). Moreover, VBNC cells may present a health risk as they have been shown to retain and are able to regain

virulence together with their culturability under suitable conditions (Li, Mendis, Trigui, Oliver, & Faucher, 2014).

Recent studies reported distal amplification in large building premise plumbing, with 5 to 30 fold increases observed in HPC concentrations between the plumbing system and the point-of-use (Cristina et al., 2014; Flemming, H. C. & Bendinger, 2014; Serrano-Suarez et al., 2013). Comparisons are typically done between a first flush sample of 1 liter at a point-of-use and a sample from the principal water system taken after some flushing (1-5 minutes). Bacterial profiling in full scale buildings has revealed that bacterial loads as estimated by HPCs, total and viable counts, decrease rapidly from the first liter and then progressively with flushing in the cold and hot water premise systems (Gatel et al., 1994; Lautenschlager et al., 2010; Lipphaus et al., 2014). These observations raise important questions with regards to the choice of sampling strategy, especially the use of flushing and the sample volume, which can greatly affect results.

Recent investigations on the role of stagnation in the distal amplification of bacterial loads in premise plumbing have revealed the importance of overnight inactivity (Lautenschlager et al., 2010; Lipphaus et al., 2014; Pepper et al., 2004). They hypothesize that this amplification is caused by a combination of bacterial growth, bacterial cell detachment and sloughing from the biofilm during stagnation and flow. The risk of exposure can be reduced by implementing a practice of flushing taps for 1 to 5 min after stagnation, a procedure recommended in areas inactive for prolonged periods of time (Health and Safety Executive (HSE), 2013). However, systematic flushing of all faucets after overnight stagnation is impractical and time-consuming, especially in large buildings with multiple points-of-use such as in healthcare settings. In addition, the impact of shorter stagnation periods frequently occurring throughout the day is not well documented. Furthermore, to the best of our knowledge, this is the first report on the impact of stagnation on the microbial quality profiles in hot water.

In this study, HPC, direct viable and total cell count profiles in hot and cold water systems from a large building were systematically performed within the first 10 L at the tap. Variable controlled stagnation time periods were induced to understand the impact on water microbial quality for both cold and hot water systems in a large building. The objectives of this study were to: 1) compare culturable and direct viable cells profiles within the first 10 L of cold and hot tap water;

2) assess the impact of stagnation time on those profiles for both water systems, including short stagnation periods during the day.

3.2 Materials and Methods

3.2.1 Description of the study site

The study was performed in a ten-story 450 bed children's hospital fed by chlorinated surface filtered drinking water. The cold water system was sampled in July 2012 with an incoming municipal water temperature averaging 26.2°C and a measured residual chlorine of 0.41 mg Cl_2/L. The hot water system was sampled between November 14th and December 11th 2012, with a water temperature coming out of the heater averaging 61.6°C and a residual chlorine concentration below 0.1 mg Cl_2/L.

3.2.2 Sampling protocol

Bacterial load sampling was performed immediately upon opening the water after the designated stagnation time. The sampling was conducted separately for cold and hot water systems, on two designated manual taps. For each sampling event at a tap, successive separate volumes composing the first liter were sampled in sterile 50 mL tubes or propylene bottles containing sodium thiosulfate (final concentration 1.1 mg/L): 1st volume of 15 mL, 2nd volume of 35 mL, 3rd volume of 200 mL, 4th volume of 250 mL, 5th volume of 500mL (Figure 3-1). Three additional samples of 250 mL were collected after 2L, 5L and 10L of flow. The last sampled volume after 10 liters of flow was equivalent to a two minute flush. For each sampled volume, heterotrophic plate counts (HPCs), direct viable and total bacterial counts were assessed as described in section 2.3. Sampling events were conducted after 1, 24, 48, 72, 120 and 240 hours of controlled stagnation. Hot and cold water systems were sampled in separate events to ensure controlled prior stagnation of the first liter. Temperature and residual chlorine were measured immediately following the first liter for each sampling event. Residual chlorine concentrations were measured with a Pocket Colorimeter II (HACH, USA).

3.2.3 Microbiological analysis

Heterotrophic plate counts (HPCs) were performed on R2A agar at 22°C, after 7 days of incubation according to method 9215-D (American Public Health Association (APHA) et al., 2012) . Viable and total cell counts were determined using LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, USA) (Boulos et al., 1999) . This kit differentiates viable from dead cells using membrane integrity criteria. Briefly, 1 mL of sample or dilution in 0.85% sterile saline solution was mixed with 3 µl of stain (propidium iodide and SYTO9), incubated in the dark for 15 min and filtered on a black 0.2 µm pore diameter, 25 mm diameter polycarbonate filter (Millipore, Bedford, USA). Enumeration was done at 1000-fold magnification, with an epifluorescence microscope (Olympus). Total bacterial cells are defined as the sum of viable (green) and dead (red) cells.

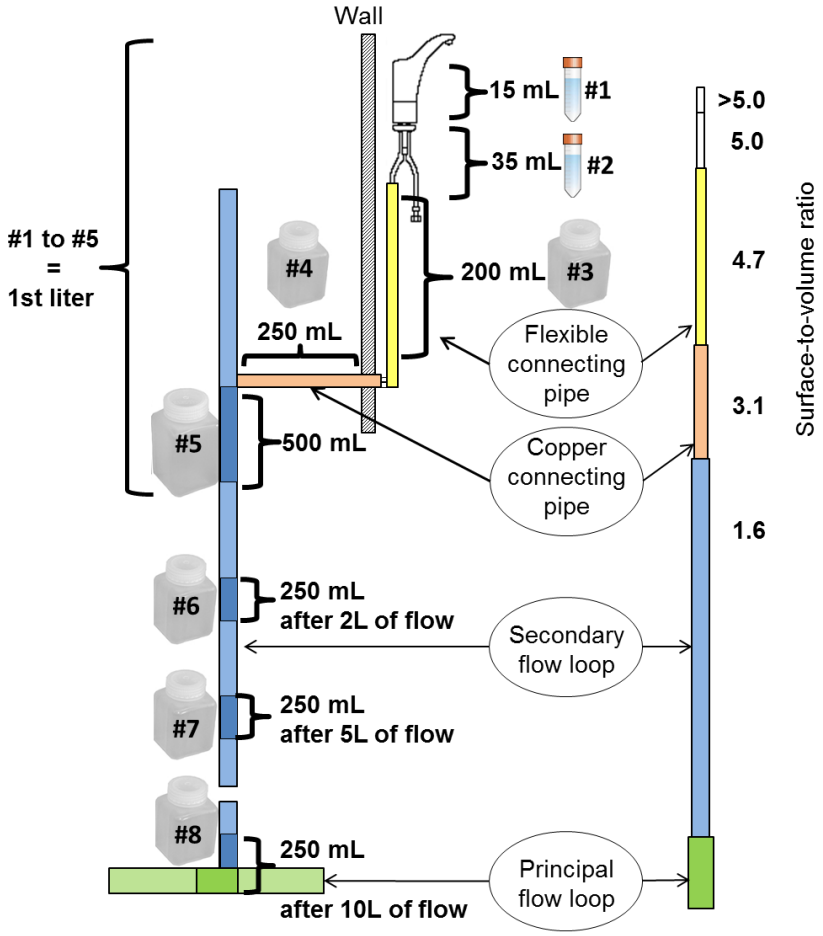

Figure 3-1 : Sampling sequence illustrating water origin within the premise plumbing and estimated surface-to-volume ratio for each section

3.3 Results & Discussion

3.3.1 Bacterial load profiles at the faucet

Sequential volume monitoring revealed a rapid decline in HPCs concentration in the first 250 mL for hot and cold water (Figure 3-2 a,c). Concentrations continued to decline in cold water whereas they stabilized after one liter in hot water. The HPC profiles can be interpreted as a function of the section of the premise plumbing from which the water originates. The first 50 mL corresponds to the faucet volume (10-30 mL) and potentially includes some water from the flexible connecting pipes between the faucet and the wall, made of elastomeric material (Bédard et al., 2015). The higher HPC concentrations observed in the initial 50 mL correspond to the presence of biofilm growth promoting factors such as stagnation and the very high surface to volume ratio. During stagnation, the small disinfectant residual dissipates and temperatures reach room temperature creating conditions favorable for bacterial growth. The capacity for biofilm growth of materials used in piping and faucets is determined both by the nature of the material and the surface available to colonization (Donlan, 2002; Laurent et al., 2005; Moritz, M.M. et al., 2010). The presence of various plastic and elastomeric materials in contact with water may be favorable to biofilm growth (Moritz, M.M. et al., 2010; Tsvetanova & Hoekstra, 2010). A higher surface-to-volume ratio will provide more attachment sites and results in greater bacterial concentrations in the bulk liquid. Faucets contain recesses and crevices and a large number of internal parts (ball, cylinder or cartridge assembly) required for flow control; these elements add up to large projected surfaces. Figure 3-3 presents the internal parts of a simple faucet showing the variety of materials and the large surface area in the mixing chamber of a simple monolever manual faucet. Increasing the surface-to-volume ratio in 10 piping materials from 0.7 cm^{-1} to 1.7 cm^{-1} increased bulk concentrations of HPCs by 4 to 14 fold, but did not significantly alter fixed biomass as estimated by ATP production potential (Tsvetanova & Hoekstra, 2010).

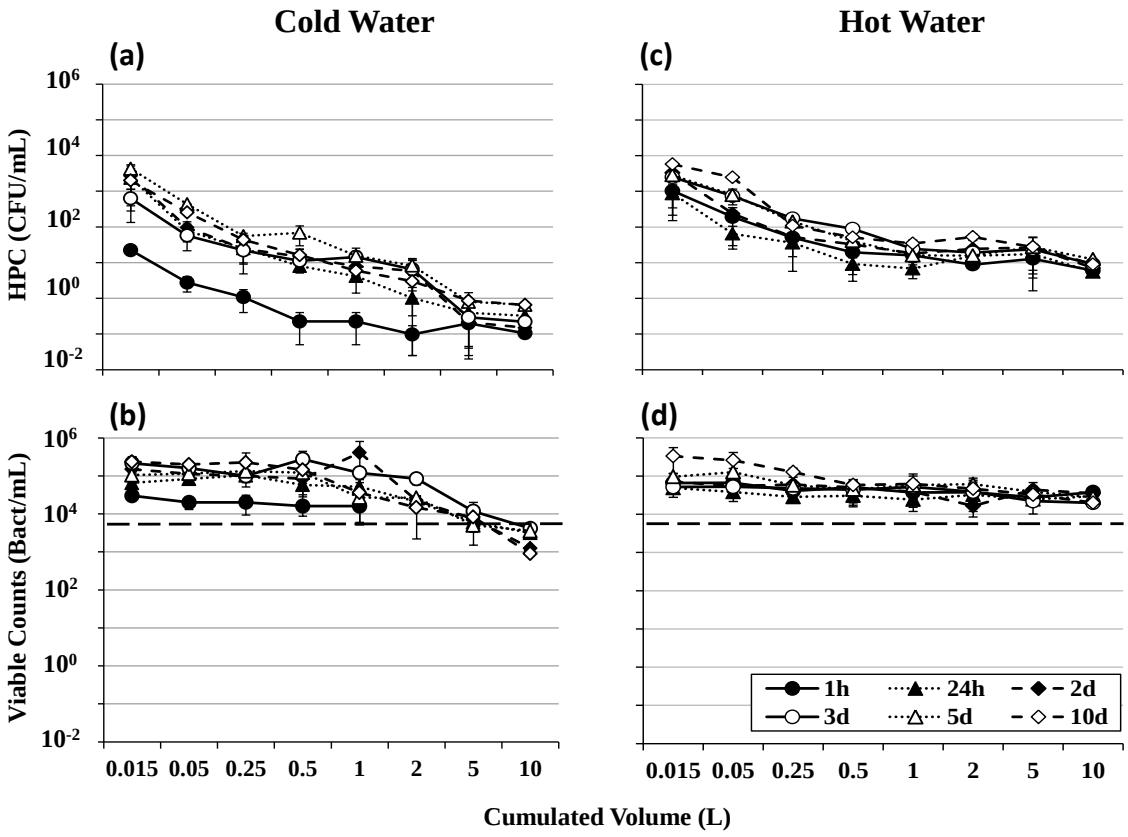

Figure 3-2 : Mean HPC and direct viable counts profiles in cold (a, b) and hot (c, d) water from 2 taps for different stagnation times. Incoming water total counts are indicated on (b) and (d) by a dotted line.

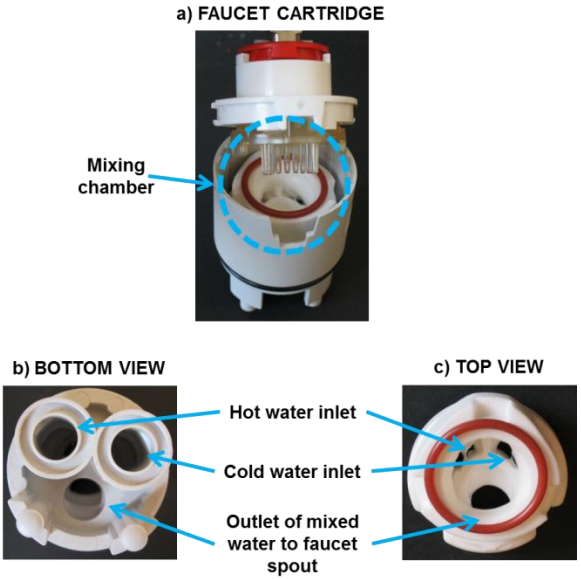

Figure 3-3 : Images of a cartridge inside a monolever manual faucet.

A large reduction of the HPC concentrations is observed in next volumes sampled (>50 mL up to a liter). This volume originates in part from the flexible connecting pipe and mostly from the connecting piping from the wall to the secondary distribution pipes. The water in this section, also referred to as the terminal piping, is also stagnant between faucet usages, a growth promoting factor (Lautenschlager et al., 2010). On the other hand, the pipe material for this section was copper, a material reported to repress the culturability of bacteria (Bédard et al., 2014; Dwidjosiswojo et al., 2011; Moritz, M.M. et al., 2010). Concentrations of copper in this building have been shown to be in the range capable of inducing the loss of culturability with a mean copper concentration of 547 µg/L (n=59). Stagnation and copper injury may offset each other in copper piping, resulting in an apparent levelling of culturable bacteria in this volume.

The last section of the distribution system sampled (>1L) corresponds to the volume of water from the secondary and primary sections of the distribution system (Figure 3-1). The origin of the water between 1 and 10 L will vary depending on the building configuration. Depending on the distance between the main distributing pipe and the sampling site, the proportion of water from the secondary distribution piping and the primary distribution piping reflects the distance between the principal flow pipe and the faucet. In the building investigated, the faucets were located on the 6^{th} and the 7^{th} floor and the water after 10L was still coming from the secondary flow pipe.

Figure 3-2a shows that a steady decline of HPCs persisted after the first liter in cold water. The presence of other devices connected to the secondary distribution pipe forces water circulation despite the absence of systematic recirculation. Despite the significant decrease (3.9±1.1 log), bacterial concentrations after 2 min flushing remained 2 log higher than those in the incoming municipal water ($5x10^{-3}$ CFU/mL not shown). A similar decrease of 1.6±0.3 log in viable counts is also observed in this volume but the levels after 10L reach those found in the incoming cold water source (as shown on Figure 3-2 b). A similar effect of long term flushing was also observed by Lautenschlager et al in cold water, between the first liter and 5 min flushed samples (Lautenschlager et al., 2010).

In hot water, a distinctly slower decrease in HPC concentrations was observed in the last section. This can be attributed to the fact that the secondary distribution pipe is maintained in circulation at all times, with water quality expected to be uniform throughout the system. In hot water

systems from large buildings, the water has to be recirculating in order to maintain control temperatures throughout (World Health Organization (WHO), 2011). Different trends were observed between culturable and viable cells from hot and cold water systems. HPC results were higher in hot water, except for the first 15 mL where levels were comparable (Figure 3-2 a,c). The first 15 mL sampled corresponds to the volume within the tap, which can be a mix of hot and cold water, depending on the previous user settings. It is therefore not surprising to observe comparable values between the hot and cold water for this volume. The following samples (50 mL to 1 L) came from the cold and hot water connecting pipes. In this section, the mean HPC levels were 0.3 to 1.3 log higher in hot water. An earlier study reported HPC level in hot water 1 log above cold water (Bagh et al., 2004). In hot water, total cell counts remained constant throughout the first 10 L ($2.1\pm0.6 \times 10^5$ cells/mL) whereas viable cell counts decreased 1.26×10^5 to 0.27×10^5 cells/mL, a high residual concentration of viable bacteria. It is interesting to note that the decrease in viable cells is far less pronounced (0.67± log) than observed in cold water, with comparable counts in the first liter between hot and cold water (Figure 3-2 b,d) but higher in hot water after 1 L, remaining 0.56 log above incoming cold water levels (Figure 3-2 d). This could be partially explained by the deficient hot water recirculation that was present in the system studied. The percent viability profiles confirm different trends between the cold and hot water systems studied. Figure 3-4a shows a slow decrease in percent viability with flush time in the first liter of hot water compared to stable proportions of viable bacteria in cold water. In cold water, the mean percentage of viability remained stable throughout the first 10 L but was highly variable between sampling events. Total direct bacterial counts decreased with flushing in cold water, but the percent viability was unaffected. Cold water sampling was conducted in summer, with temperatures at the tap similar to the temperatures within the premise plumbing.

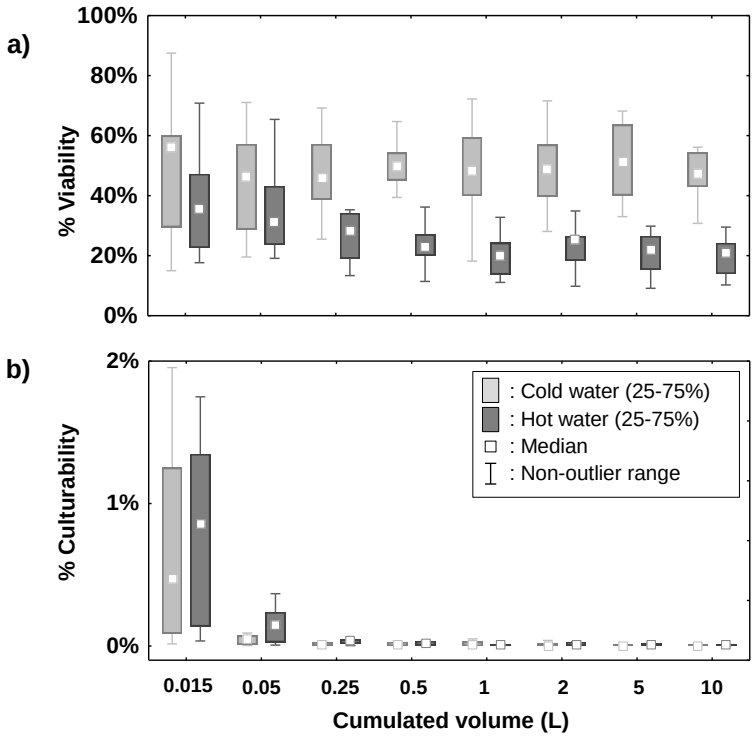

Figure 3-4 : Percent viability (a) and percent culturability (b) profiles in cold and hot water systems measured at the faucet for 10 L (n=14).

Figure 3-2 shows that, for hot and cold water systems alike, HPCs concentrations profiles vary over several orders of magnitude (0.4 to 5.2 log) while never approaching the levels found in the incoming water (Figure 3-2 b,d). On the other hand, in cold water, incoming water quality is maintained throughout the system until a gradual increase in the last meters of piping before the tap. In the case of hot water, a stable and notable increase of total viable counts is observed when compared to levels in the incoming water, reflecting the presence recirculation in the system. This increase is significant when considering the concentrations present. When applying the same three sections within the plumbing system to bacterial load profiles performed for intact and total cell counts in a recent study, similar trends were observed (Lipphaus et al., 2014). Lautenschlager et al. measured HPC concentrations in the first flush liter of cold water compared to samples after taken after 5 min flush and reported 1.98 log reductions as compared to 3.8 log in the present study (Lautenschlager et al., 2010). The apparent difference between the results can be attributed to the first flush volumes and the flushing times. Actually, if an equivalent first flush volume of one liter is used, the mean concentrations obtained in the present study were $5.5 \pm 4.0 \times 10^{1}$ CFU/mL, for a reduction of 2.17 log, closely comparable to results by Lautenschlager et al. This

shows the importance and potential impact of the selected sampling volume on HPC measurements and compliance to existing guidelines. Figure 3-5 presents the HPC concentrations for various first flush sampling volumes. In this case, a sampling volume of one liter or more would meet the recommended HPC target levels (Bartram et al., 2003). Therefore, sampling 15 mL or 1 L can make the difference between meeting recommended threshold values, especially in health care facilities. In this case, the total culturable bacteria in the first 15 mL represented more than half of the total culturable bacteria load in the first liter (3.42×10^4 vs 5.5×10^4 CFU/mL). These results illustrate the importance of standardized sampling protocols for consistent interpretation and comparison of results against targeted levels for infection prevention.

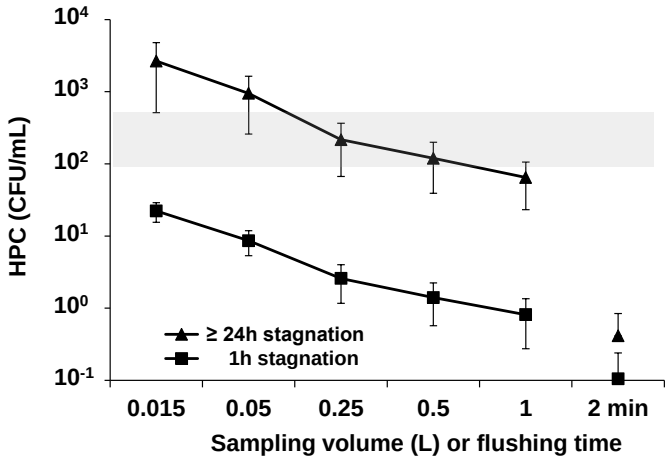

Figure 3-5 : Mean HPC concentration calculated for the cumulated sampling volume after 1 h stagnation (n = 2) and 24h or more of stagnation (n=12). Shaded area represents recommended HPC target levels.[5]

The viable load in cold and hot water was high with a small fraction of readily culturable cells. Viable cells can recover culturability partly depending on the prevailing conditions and the existing biofilm. As expected, the correlation between the viable counts and HPCs was poor when considering the first 15 mL volume sampled from the tap (Appendix 1, Figure A-1.1a). The lack of correlation is likely attributed to the inherent variability in environmental stressors such as chlorine residual, pH, copper concentrations in the volume stagnating in the tap, reflecting the local conditions and prior water usage. These parameters have been shown to influence cell culturability and determine the level of recovery of culturability (Bédard et al., 2014; Dwidjosiswojo et al., 2011). These variables will affect culturability, but are not likely to affect total cell counts. During stagnation in the tap, water quality will deteriorate with temperature

changes, residual disinfectant consumption, nutrient leaching materials and detachment from the biofilm. However, the correlation greatly improved in flushed samples (2 minutes) both for HPCs vs total counts (R^2=0.60) and HPCs vs viable counts (R^2= 0.69) (Appendix 1, Figure A-1.1b). The increased correlation observed reflects the more stable quality conditions in the circulating water system as compared to the small volume at the tap. In comparison, a correlation coefficient of 0.36 was reported between HPCs and total cell counts in cold water collected on first 15 mL flush from taps in a building (Siebel, Wang, Egli, & Hammes, 2008). First flush samples were collected from taps every hour throughout the day, with variable water usage in between sampling. With random stagnation prior to sample collection, water could either be representative of the tap (no usage between samplings) or from the system (tap used shortly before sampling equivalent to a flush).

The limited impact of flushing, the sustained high concentrations of viable bacteria and the potential recovery of culturability at the tap, raise concerns about the presence of VBNC bacteria at the tap. Bacteria such as *Pseudomonas aeruginosa, Legionella pneumophila* and *Mycobacterium tuberculosis* enter VBNC state in unfavorable conditions, and are likely to follow trends observed for HPCs and viable counts at the tap. Once water resides within the tap, the favorable conditions may trigger the capacity of these VBNC pathogens to recover virulence once resuscitation occurs (Li et al., 2014), therefore representing a risk for vulnerable users at the point of care.

3.3.2 Impact of stagnation on bacterial load profile

Viable cell counts and HPC profiles were determined for stagnation times between 1h and 10 days in cold and hot water. In cold water, a stagnation time of 24 hours or more did not have a significant effect on HPCs and viable cell count profiles (Fig 2a,b). Profiles following one hour stagnation were similar in trend, but with lower initial HPC concentrations. In hot water, higher initial HPC concentrations and viable cell counts were observed in the water from the faucet and its connection piping after 10 days of stagnation (Figure 3-2 c, d). For all stagnation times, viable cell count results were comparable for the first 500 mL sampled. This volume corresponds to the volume associated to the piping serving a single device and therefore experiencing true stagnation. Low concentrations were expected after such a short stagnation since taps had been flushed prior to stagnation. However, one hour was sufficient to cause a 1.7 log increase in HPCs

as compared to the system background concentration measured after flushing. The concentration increase observed after an overnight stagnation has been attributed to cell growth (Lautenschlager et al., 2010). In the present study, the increase within a short stagnation of one hour cannot be likely attributed to cell growth. The average HPC generation rate in drinking water in absence of chlorine and at temperatures of 20°C was reported to vary between 7 and 140 hours (Servais, Billen, Laurent, Lévi, & Randon, 1992). It is also unlikely that the observed increase results from the regain of culturability of VBNC cells present in the system water and subsequently located into the first 15 mL after flushing. The favorable conditions such as no chorine residual depletion and better availability of oxygen in the faucet provide elements to improve culturability (Li et al., 2014). These injured cells are not considered as 'true' VBNC state and could recover in a short period of time (Li et al., 2014). Such recovery (3-6 log) from chlorine and copper injury has been documented for *P. aeruginosa* but over the course of several hours (Bédard et al., 2014; Dwidjosiswojo et al., 2011). The most probable source of rapid increase is the detachment of bacteria from the biofilm. In that case, biofilm release would contribute to the viable and total cell counts. The increase of 0.6 log (equivalent to 300%) was indeed observed for both viable and total cell counts in the first 15 mL after one hour stagnation, as compared to system's counts obtained after flushing. These observations point towards cells released from the biofilm as the principal contributors to the increased levels of HPC, viable and total cell after one hour of stagnation.

3.3.3 Biofilm contribution to bacterial load profile observed

To better understand the biofilm contribution to the HPC increase, the surface-to-volume ratio was calculated for the various sections of the device plumbing. In large buildings, stagnation of a given device will only reflect on the volume of water within the faucet and connecting pipes, while the rest of the system might circulate due to usage at other taps (hot and cold) or forced recirculation (hot). A plot of the HPC concentrations against the surface-to-volume ratio for samples taken up to one liter of cumulated volume revealed an excellent exponential correlation after 1 hour stagnation (Figure 3-6, $R^2 > 0.97$). These results strongly support that detachment from the biofilm causes the increased HPC concentrations observed after a short stagnation. During periods of stagnation, shear stress is reduced to zero and affects the cell adhesion strength, favoring cell release in to the water phase (Manuel et al., 2010). Similar trends were observed for

longer periods of stagnation, but with lower correlations (Appendix 1, Figure A-1.2). Other factors such as cell growth probably play a role in increasing HPC concentrations over longer periods of stagnation. A higher culturability was observed in the first 15 mL compared to water after 10L of flow for both hot and cold water (Figure 3-4b). A recent study showed an ongoing release of bacteria at very low flow (1.1 mL/h) equivalent to quasi stagnation in premise plumbing (Ghadakpour et al., 2014). They also showed that a small increase in flowrate for a short period of time was sufficient to increase cell detachment by 2 log but resumed to initial levels within 3 hours or less after the event. The two mechanisms that are likely behind the biofilm release involved during stagnation are cell dispersion and biofilm erosion (Flemming, Hans- C., Wingender, & Szewzyk, 2011). Cell dispersion may occur during stagnation or when there is a change in the biofilm environment. In a mature biofilm, such as those in premise plumbing, live cells may be released to colonize other surfaces. There is a change in the flow associated with the start of stagnation and the equilibrium might be perturbed. Cells released at that time would be mostly culturable cells, able to go colonize new surfaces. Biofilm erosion or cell sloughing may occur at the end of the stagnation period, marked by a flow of water susceptible to erode the biofilm cells that are closest to the bulk water interface are the only susceptible cells to this form of detachment (Stoodley et al., 2001). Daughter cells produced at the interface and cells not embedded in the biofilm matrix are prone to this phenomenon. These types of cells would also likely be culturable (Fux, Costerton, Stewart, & Stoodley, 2005). It could be thought that repeated cycles of short stagnation and turbulent flow would lead to depletion of cell concentrations within the biofilm, resulting in reduced release into the bulk phase after some time. However, if the fraction of bacteria released from the biofilm is minimal compared to the attached cell concentration, the effect will be negligible on the overall biofilm population. The concentration in the biofilm could not be measured as part of this study and very little information is available for premise plumbing pipes, with smaller diameter and different materials than municipal drinking water distribution system. The study from Bagh et al. measured biofilm concentrations in cold and hot water distribution systems of a large building through the use of coupons in the actual distribution system (Bagh et al., 2004). They reported HPC biofilm densities of 2.8×10^5 CFU/cm^2 in cold water and 1.29×10^6 CFU/cm^2 in the hot water system, comparable to values reported in pilot plants simulating premise plumbing (Lehtola, M.J. et al., 2006; Thomas et al., 2004). Based on these values, the total number of HPCs

present in the piping section containing the first 15 mL volume could be estimated to $2x10^7$ and $9.4x10^7$ CFU in cold and hot water, respectively. The maximum total bacteria released during stagnation can be estimated using the observed concentration increase in a determined volume. In this study, total HPCs increase by $4x10^2$ CFU in the first 15 mL. Thus, repeated releases of such small number of culturable cells from the biofilm into the water during stagnation would not significantly affect the biofilm microbial population density, even with reported steady state HPC growth rate in biofilms from drinking water systems ranging between 14 and 30 days doubling time (Boe-Hansen, Albrechtsen, Arvin, & Jorgensen, 2002). Results from this study highlight the importance of the biofilm release during variable hydraulic conditions including stagnation. The strong correlation observed between the surface-to-volume ratio of the pipes and the bacterial concentration in the bulk phase suggests a release from the biofilm as the main contributor to the increase in culturable cells concentrations after a stagnation period of at least 1 hour. Smaller diameter piping will increase surface-to-volume ratio, and bacterial load, resulting in a smaller volume of water being more contaminated. Flow restriction devices represent a much greater risk of biofilm growth and detachment. These devices are composed of complex plastic aerators that add enormous surfaces of plastic materials that favor biofilm growth and can lead to outbreaks (Walker, J. T. et al., 2014).

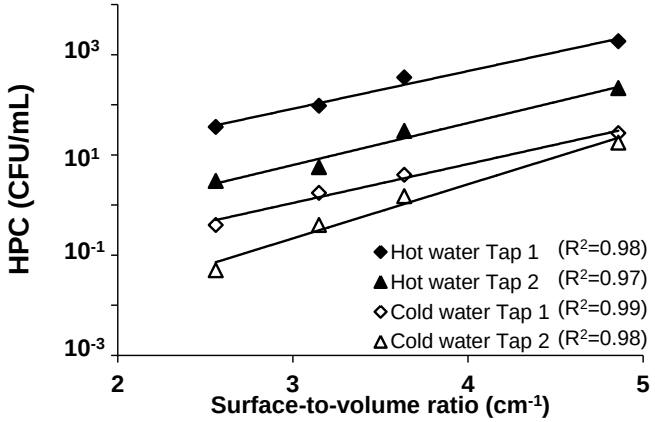

Figure 3-6 : HPC concentration after 1 hour stagnation as a function of surface-to-volume ratio in cold and hot water at 2 faucets.

As discussed previously, the volume sampled and prior flushing of the tap will have an important effect on resulting concentrations. The time of day at which the sampling is done will also greatly affect the results. Concentrations obtained after 1 hour stagnation were in average 100-fold less

than after 24 hours or more stagnation. Therefore, sampling done early in the morning would be representative of overnight stagnation whereas sampling throughout the day may represent short or no stagnation period. Furthermore, a short duration flush removing the first 250 mL before using the tap water will significantly reduce exposure to the elevated initial bacterial load observed in the bacterial profiles. It is important to point that this practice is difficult to implement with electronically activated faucets where hands will be exposed to the first volume of water while activating the faucet.

3.4 Acknowledgements

This study was supported by NSERC through the Industrial Chair on Drinking Water and the E. Bedard Vanier Canada Graduate Scholarship. The authors would like to thank the Chair staff especially Yves Fontaine, Jacinthe Mailly, Mélanie Rivard and Mireille Blais, and the participating health care facility personnel. Eric Déziel holds a Canada Research Chair in socio-microbiology.

3.5 Supporting information

Correlations are presented in supporting information for HPCs vs viable/total cell counts and for HPCs vs surface-to-volume ratio for stagnation times greater than 1 hour.

CHAPTER 4. ARTICLE 3 – RECOVERY OF *PSEUDOMONAS AERUGINOSA* CULTURABILITY FOLLOWING COPPER- AND CHLORINE-INDUCED STRESS

The previous chapter illustrates the importance of the point-of-use in the amplification of bacteria. However, the presence of environmental stressors present in drinking water at the point-of-use may impair culturability of cells and lead to an underestimated number of negative sites. This chapter reports the results from a laboratory experiment conducted to evaluate the impact of residual chlorine and copper on the detection of *P. aeruginosa* in drinking water sampled from taps. The time of recovery after stress removal is also documented, showing the masking effect of chlorine and copper on culture detection, while not killing *P. aeruginosa* which is still present in the water and able to recover once stressors are removed, a common occurrence in premise plumbing. This chapter is a paper published in the *Pseudomonas* special issue of *FEMS Microbiology Letters*. Supplementary information is presented in Appendix 2.

RECOVERY OF *PSEUDOMONAS AERUGINOSA* CULTURABILITY FOLLOWING COPPER- AND CHLORINE-INDUCED STRESS

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ABSTRACT

This study investigated how quickly cells of the opportunistic pathogen *Pseudomonas aeruginosa* recover culturability after exposure to two of the most common environmental stressors present in drinking water, free chlorine and copper ions. Viable but non-culturable (VBNC) *P. aeruginosa* undetected by direct culturing following exposure to free chlorine or copper ions can survive in drinking water systems, with potential to recover, multiply and regain infectivity. Cells

were exposed to copper sulphate (0.25 mg Cu^{2+} L^{-1}) or free chlorine (initial dose of 2 mg Cl_2 L^{-1}) for 24h. Despite total loss of culturability and a reduction in viability from $1.2x10^7$ to $4x10^3$ cells mL^{-1} (3.5 log), cells exposed to chlorine recovered viability quickly after the depletion of free chlorine, while culturability was recovered within 24 hours. Copper ions did not depress viability, but reduced culturability from $3x10^7$ to $2.3x10^2$ cells mL^{-1} (5.1 log); VBNC cells regained culturability immediately after copper ion chelation. A comparison between direct culturing and Pseudalert, a specific enzyme-based assay, was performed. Both detection methods were well correlated in the range of 10^2-10^{10} cells L^{-1}. However, correlations between the methods declined after exposure to copper ions.

KEYWORDS

VBNC - drinking water - internal plumbing – hospital - opportunistic pathogen

4.1 Introduction

Pseudomonas aeruginosa is an important source of nosocomial infections and the most frequently recovered Gram-negative bacterium from patients with nosocomial pneumonia in the USA (Weinstein et al., 2005). For cystic fibrosis patients, it is the primary cause of morbidity (Pier, 2012) and a major predictor of mortality (Emerson et al., 2002). It can also cause bacteraemia, urosepsis and secondary wound infections, including burns infection (Kerr, K. G. & Snelling, 2009). Sources of transmission are multiple, but water is believed to be an important contributor for the transmission of *P. aeruginosa* in hospitals (Trautmann, M. et al., 2005). In most intensive care unit outbreaks investigated, water has been implicated either directly or indirectly (Durojaiye et al., 2011; Ehrhardt et al., 2006; Ferroni et al., 1998; Hota et al., 2009; Schneider et al., 2012; Vianelli et al., 2006; Yapicioglu et al., 2011). According to recent information, 30 to 50% of *P. aeruginosa* infections in intensive care units are associated with water (Exner, 2012).

The detection of *P. aeruginosa* in drinking water presents several challenges, including its preference for biofilm vs planktonic state as well as its presence in low and variable concentrations. Moreover, environmental factors such as nutrient-poor conditions and the presence of inhibitors and stressors can lead to a dominance of the viable but non-culturable

(VBNC) state. Cells in the VBNC state are still alive and capable of metabolic activity but fail to multiply and grow on routine media on which they would normally grow (Oliver, J.D., 2005). A VBNC state has not been typically associated with *P. aeruginosa* and its presence in environmental samples has not fully been assessed. When monitoring for *P. aeruginosa* at drinking water taps, two main environmental stresses can be present: disinfectant residual and metals ions from plumbing (copper, zinc and lead). Depending on prior stagnation and residual chlorine concentration at the time of sampling, traditional culture methods may fail to reveal the presence of VBNC *P. aeruginosa* because of the impact of these factors on culturability. This is a well-known phenomenon for *Vibrio cholerae* in aquatic environments (Xu et al., 1982). The toxicity of copper ions to culturable *P. aeruginosa* cells has been documented at relatively high concentrations (Elguindi, Wagner, & Rensing, 2009; Harrison, J. J. et al., 2005; Teitzel et al., 2006; Teitzel & Parsek, 2003). However, the direct relevance of these results to conditions prevailing in drinking water systems is limited because of: (1) the use of growth media or mineral salt solutions in which the availability of toxic free copper ions varies with the level of chelation and binding; and (2) the systematic quenching of copper ions before enumeration. The impact of copper ions on culturable and VBNC *P. aeruginosa* was verified in drinking water containing realistic copper concentrations (63.5 μg Cu^{2+} L^{-1}) showing a greater sensitivity to copper ions and an overwhelming effect of added chelators (Moritz, M.M. et al., 2010). VBNC P. aeruginosa cells undetected by culture can survive in the system, and eventually recover and multiply when stressors are removed, e.g. free chlorine depletion during stagnation periods. Furthermore, VBNC cells can also recover cytotoxicity as shown by the reversible VBNC state of suspended P. aeruginosa induced by copper ions (Dwidjosiswojo et al., 2011).

The objectives of this study were 1) to estimate the impact of free chlorine and copper ions stresses on culturable and VBNC suspended *P. aeruginosa* cells under conditions representative of internal plumbing, 2) to test a new specific enzymatic activity-based assay for the increased recovery of stressed bacteria and 3) to quantify the recovery of *P. aeruginosa* after stress interruption for both free chlorine and copper ions.

4.2 Materials and methods

4.2.1 Bacterial strains and culture conditions

Experiments were performed with *P. aeruginosa* strain PA14 (Lee, D. G. et al., 2006) grown in Tryptic Soy Broth (Difco, Detroit, USA) overnight at 37 °C. Cells were harvested by centrifugation (3000 x *g* for 30 min), washed twice in sterile 2 mM phosphate buffer, and suspended at a final estimated cell density of 5 x 10^9 cells mL^{-1}.

4.2.2 Experimental conditions

Sterile polypropylene bottles containing 500 mL of 2 mM phosphate buffer (pH 7.3±0.1) were inoculated (final concentration 10^7 cells mL^{-1}). A control cell suspension was not exposed to free chlorine or copper ions. A chlorine solution was added to inoculated water to obtain an initial free chlorine concentration of 2 mg Cl_2 L^{-1}. Free chlorine concentrations were measured by the *N*,*N*-diethyl-*p*-phenylenediamine method using a DR5000 spectrophotometer (HACH, USA). Sodium thiosulfate (1%) was added to neutralize chlorine prior to conducting microbiological analysis. Copper sulphate was used as the source of copper ions and was added to the water prior to inoculation ($CuSO_4$, final concentration 0.25 mg Cu^{2+} L^{-1}). Copper ions were chelated after 24 hours by the addition of diethyldithiocarbamate (Moritz, M. M., 2011). All samples were mixed thoroughly and divided to perform selected analytical methods.

For the enzyme-based assay and culture methods comparison in absence of free chlorine and copper ions, serial dilutions of an early exponential phase bacterial suspension were prepared (1 to 10^6 cells mL^{-1}).

4.2.3 Microbiological analysis

Culture was performed according to International Organization for Standardization method 16266:2006 for *P. aeruginosa* detection and enumeration in water (International Organization for Standardization, 2006). Briefly, three dilutions were done in duplicate for each sample, filtered on a 0.45 µm pore size, 47 mm diameter mixed cellulose ester membrane. The filters were deposited on cetrimide-nalidixic acid agar plates (45.3 g L^{-1} of Cetrimide Selective Agar (Remel, Lenexa, USA), 10 mL L^{-1} glycerol (Fisher, Fair Lawn, USA), 0.015 g L^{-1} nalidixic acid (Sigma-Aldrich, Steinheim, Germany) and incubated at 37.5°C for 24 h before enumeration. Detection

was also performed on two sample dilutions using the Pseudalert/QuantiTray2000 (IDEXX, Chicago, USA) according to the manufacturer's instructions. Viable and total cell counts were determined using LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, USA) (Boulos et al., 1999) . This kit differentiates viable from dead cells using membrane integrity criteria. Total cells are defined as the sum of viable (green) and dead (red) cells (Figure 4-1). Briefly, 1 mL of sample or dilution in 0.85% sterile saline solution was mixed with 3 µl of stain (propidium iodide and SYTO9), incubated in the dark for 15 min and filtered on black 0.2 µm pore diameter, 25 mm diameter polycarbonate filter (Millipore, Bedford, USA). Enumeration was done at 1000-fold magnification, with an epifluorescence microscope (Olympus, Tokyo, Japan). VBNC cells were estimated as the difference between the viable and culturable cells.

4.2.4 Real-time qPCR amplification

A volume of 25 mL was filtered through a 0.45 µm pore diameter, 47 mm diameter mixed cellulose ester membrane for DNA extraction performed directly on filters using the bead beating method adapted from Yu, Z., and Mohn (1999). Briefly, the filter was inserted into an extraction tube (Lysing Matrix A, MP Biomedicals, Solon, USA). Extraction buffer containing 50 mM Tris-HCl (pH8), 5 mM EDTA (pH8), 3% sodium dodecyl sulphate and RNase (20 µg mL^{-1}, Invitrogen, Carlsbad, USA) was added to each tube prior to the bead beating step performed on a FastPrep-24 (MP Biomedicals, Solon, USA), followed by ammonium acetate precipitation (2M, Sigma-Aldrich, St.Louis, USA) and successive cold 70% ethanol washes. Quantification by quantitative polymerase chain reaction (qPCR) was performed on *gyrB* gene, using primers and probes previously described (Lee, C. S. et al., 2011). The *gyrB* gene was chosen for its specificity and sensitivity for *P. aeruginosa*. The following protocol was applied: 10 min initial denaturation at 95 °C followed by 50 cycles with denaturation at 95°C for 30s, annealing and elongation at 60°C for 90 s (Corbett Rotor-Gene 6000, San Francisco, USA). Final reaction volume (20 µl) included 5 µl of DNA, 2X Universal MasterMix (Life Technologies, Foster City, USA) and TaqMan Exogenous Internal Positive Control Reagent (Applied Biosystems, Austin, USA).

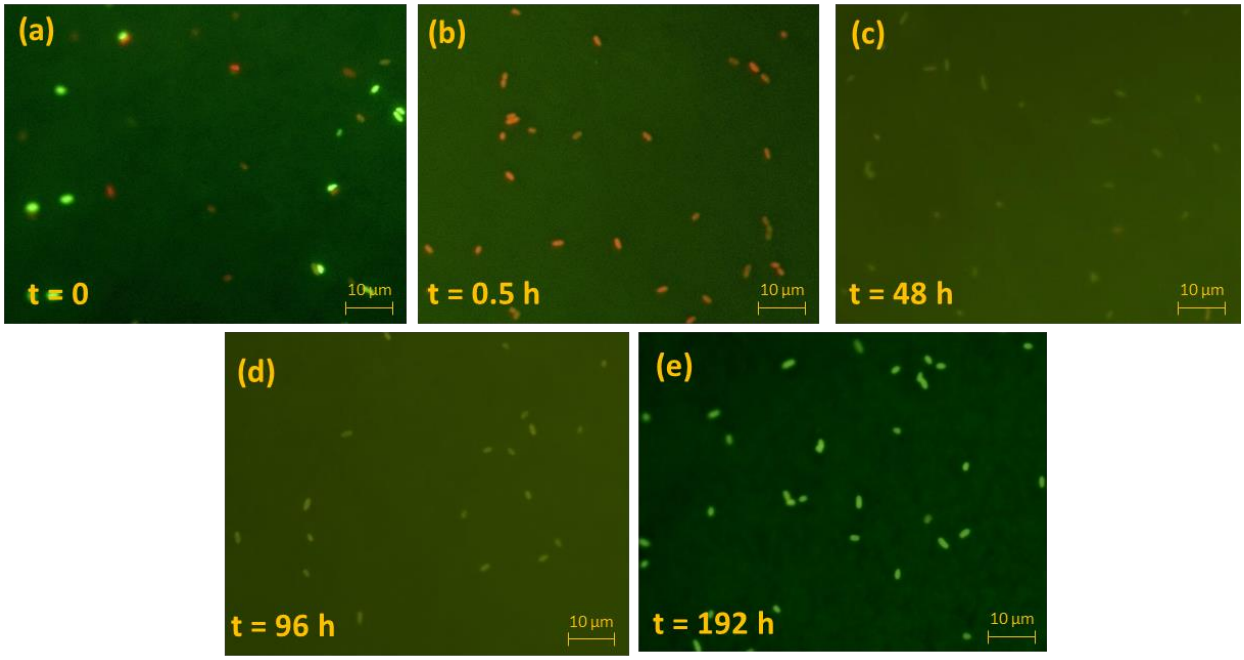

Figure 4-1: LIVE/DEAD stained *P. aeruginosa* PA14 in buffered sterile water (pH = 7.3) for various contact times after chlorination illustrating the transient cell marking observed: green prior to chlorination (a), red at t = 0.5 h (b), faded yellow at t = 48 h (c), light green t = 96 h (d) and green at t = 192 h (e). Green cells represent viable cells and red cells represent dead cells.

4.3 Results and Discussion

The objective was to measure the impact of chlorine-based disinfectant and copper-containing water on the detection of *P. aeruginosa* under environmental conditions and to document its culturability recovery after those stressors were removed. Suspensions of *P. aeruginosa* cells were monitored for up to 8 days, with free chlorine and copper ions present only during the first 24h.

4.3.1 Effect of chlorine stress on *P. aeruginosa* culturability

A representative concentration of free chlorine found in drinking water distribution systems was selected to reach a target concentration of < 0.1 mg Cl_2 L^{-1} after 24 h. Figure 4-2 shows the loss of culturability (final to initial CFU mL^{-1}, N/N_0) of *P. aeruginosa* as a function of exposure to free chlorine expressed as the product of concentration and contact time (also expressed as Ct in the literature) observed during the first 24 hours. Similar data at lower exposure to chlorine

values reported by Xue *et al.*, 2013 are also presented. Elevated initial rates of apparent inactivation of culturable *P. aeruginosa* by chlorine have been documented in batch and chemostat experiments (Behnke et al., 2011; Xue et al., 2013) showing large reductions (3.5 to 6 log) at initial free chlorine dosage of > 1 mg Cl_2 L^{-1}. Results presented in Figure 4-2 show a trend of inactivation similar to the one obtained by Xue *et al*. The loss of culturability observed in this study increased with exposure to free chlorine (product of concentration and contact time) resulting from low chlorine concentrations maintained over long periods of time (24h). These chlorination conditions are representative of those found in the internal plumbing of buildings of drinking water systems.

Cell inactivation (final to initial measured cell count ratio, N/N_0) as a function of time is shown for the various detection methods in Figure 4-3. Despite complete loss of culturability during the exposure to free chlorine and a poor nutrient environment (phosphate-buffered water), recovery of culturability after an additional delay of 24 hours following stress interruption was observed with both CFU-based and enzyme-based detection methods. A decrease in viable counts was also observed during exposure to chlorine (Figure 4-3a), indicating membrane injury for a large portion of cells. After the onset of chlorination, the majority of cells labelled with LIVE/DEAD stain (total and viable) showed poor fluorescence in the presence of free chlorine, an observation in agreement with the documented loss of DAPI fluorescence after chlorination at 5 mg Cl_2 L^{-1} (Saby, Sibille, Mathieu, Paquin, & Block, 1997). Poor fluorescence was attributed to the inability of the dye to bind to chlorine-modified DNA or to physical damage to the DNA. However, as chlorine concentrations declined over time, a transition in cell marking was observed, from typical red-stained associated with membrane permeabilization to orange and light yellow before reverting to light green (Figure 4-1). The dye combination of SYTO9 and PI can detect both inner and outer membrane permeabilization in Gram negative bacteria as detected by flow cytometry, and outer membrane damage can be reversible (Berney, Hammes, Bosshard, Weilenmann, & Egli, 2007). According to Xue *et al.* 2013, similar intermediate states of binding after chlorination are an indication of reversible cell injury (i.e. enzyme activity or functional group deformation) as opposed to lethal membrane damage. Although it is not possible to confirm the state of the cells showing intermediate staining, the observed trends in viable and cultivable cells provide evidence that most cells can survive and regrow rapidly after the dissipation of free chlorine. This is clearly observed for VBNC cells after 24 hours,

corresponding to the loss of significant concentration in free chlorine (Figure 4-3a). qPCR results show a decrease within the first hours of contact time with free chlorine, but revert to levels comparable to total cell counts obtained by LIVE/DEAD staining after 24 h.

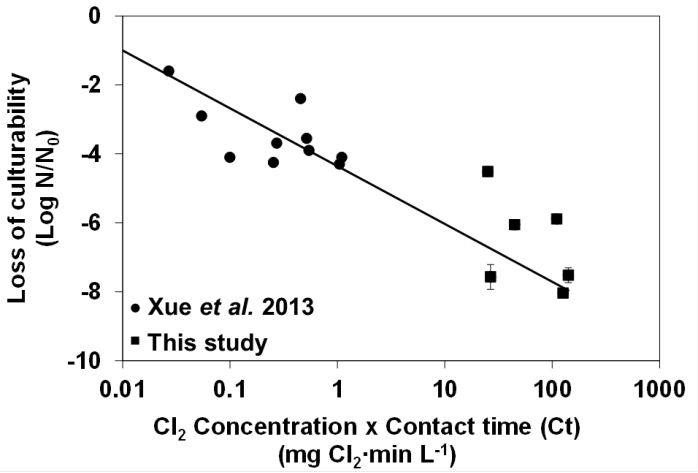

Figure 4-2: Loss of culturability for *P. aeruginosa* PAO1 (Xue *et al*. 2013) and PA14 (this study) assessed by culture method (final to initial CFU mL^{-1} ratio, N/N_0) as a function of free chlorine concentration (mg $Cl_2 \cdot L^{-1}$) and contact time (min) product expressed as Ct. Error bars for this study indicate standard deviation.

These results are highly relevant to conditions prevailing in drinking water distribution systems. The levelling off of the inactivation curves observed in disinfection studies show that a significant fraction of suspended bacteria can breakthrough primary disinfection and enter the distribution system (Behnke et al., 2011; Xue et al., 2013). Secondary disinfection conditions are not sufficient to completely inactivate suspended or clustered cells as free chlorine concentrations decline progressively with water age (Rossman, Clark, & Grayman, 1994). Internal plumbing in large buildings offer highly favorable conditions for biofilm development because of the large surface area provided by small diameter pipes and corrosion, long residence times including dead-end sections and disinfectant consumption (Prévost, Rompré, Baribeau, Coallier, & Lafrance, 1997). A significant fraction of chlorine-injured cells in the present study are still viable but unlikely to be detected by standard culture methods.

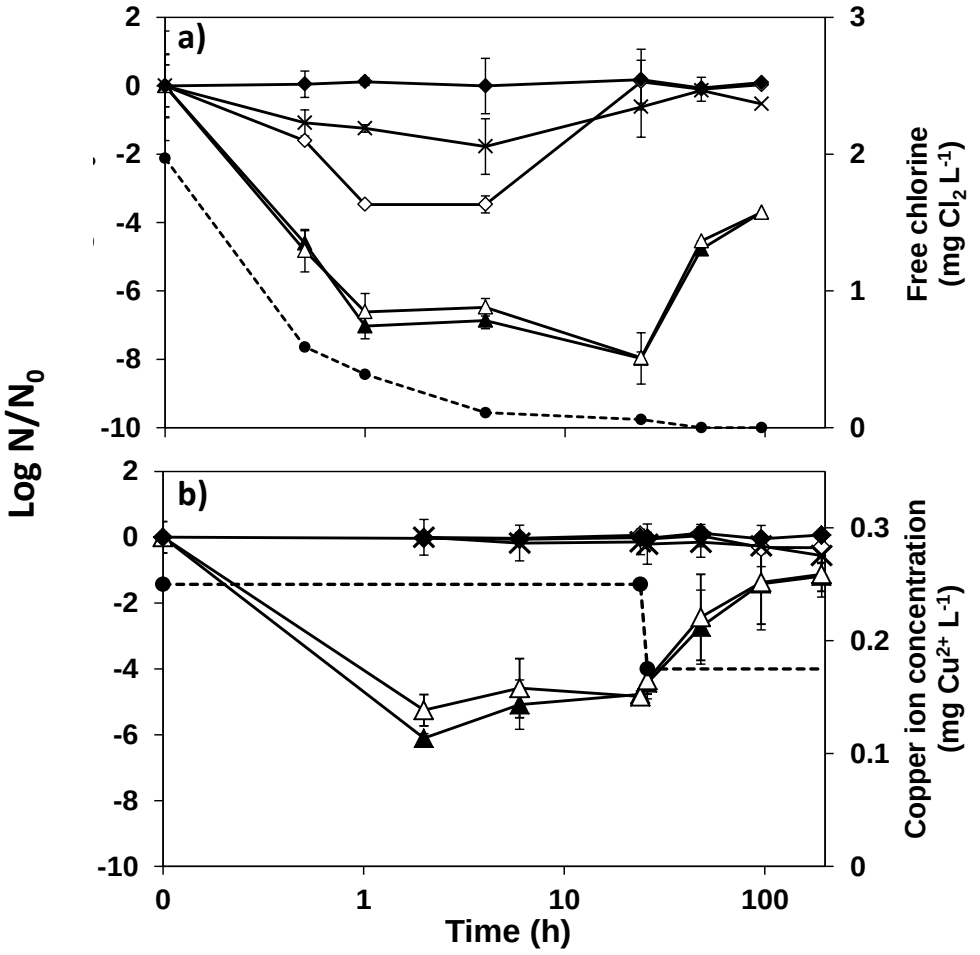

Figure 4-3 : Cell survival of *P. aeruginosa* PA14 in buffered sterile water (pH = 7.3) as a function of time elapsed after application of 2.0 mg Cl_2 L^{-1} of free chlorine (a) and 4 µM copper ions (b). Cell survival is expressed as the logarithm of N/N_0, where N_0 is the initial cell count and N is the cell count at sampling time. Enumeration was done by culture (▲, $CFU \cdot ml^{-1}$), enzyme-based assay (∆, $MPN \cdot ml^{-1}$), qPCR (X, genomic units$\cdot L^{-1}$) and LIVE/DEAD staining (◊, viable cells and ♦, total cells). Viable cells are defined as cells with membrane integrity and total cells as the sum of viable and dead cells. Error bars indicate standard deviation (*n* = 3). Free chlorine concentration decay (●) and copper ion concentration (○) are presented on the secondary y axis. Copper ions were chelated with diethyldithiocarbamate at t = 24 h (b).

4.3.2 Effect of copper ions stress on *P. aeruginosa* culturability

The current World Health Organization guideline value for copper concentrations in drinking water is set at 2 mg Cu^{2+} L^{-1} (World Health Organization (WHO), 2008). In the United States, the Environmental Protection Agency fixed the maximum contaminant level goal for copper at 1.3 mg Cu^{2+} L^{-1} (United States Environmental Protection Agency (USEPA), 2009b)(United States Environmental Protection Agency, 2009). California has the lowest recommended level with an established public health goal of 0.3 mg L^{-1} (California Environmental Protection Agency et al., 2008). In the present study, copper stress was evaluated by adding 0.25 mg Cu^{2+} L^{-1}, representative of concentrations found in internal plumbing of large buildings in the study area (data not shown) and meeting recommended levels in drinking water. *P. aeruginosa* counts dropped from $3x10^7$ to $2.3x10^1$ CFU mL^{-1} (6.1 log) in culture and from $2.9x10^7$ to $1.6x10^2$ MPN mL^{-1} (4.7 log) as measured by the enzyme-based method within 2 hours of exposure to copper ions (Figure 4-3b). These results agree with prior reports of steep decline in culturable *P. aeruginosa* and persistence of a resistant sub-population at lower copper ions levels (Dwidjosiswojo et al., 2011; Huang et al., 2008; Teitzel & Parsek, 2003). Unlike free chlorine, ionic copper did not affect viability and no transition period was observed with fluorescent staining (suggesting the absence of significant membrane permeabilization, data not shown). Results obtained by qPCR were constant throughout the experiment and comparable with total cell count. Copper ions were neutralized after 24 hours of contact time by adding a chelating agent (100 µM diethyldithiocarbamate) that was demonstrated to have no inhibitory impact (data not shown). The quenching resulted in recovery of culturability within 3 days, with cell densities approaching initial concentrations. No measurable changes in total or viable cell counts were noted, minimizing the importance of cell multiplication in the apparent recovery of culturability of *P. aeruginosa* although some contribution from surviving cells is possible. These results are in agreement with a previous study documenting the reversible inhibition of *P. aeruginosa* culturability by copper ions (Dwidjosiswojo et al., 2011). Slightly higher losses of culturable cells (from 10^6 CFU mL^{-1} to below detection limit) than those observed here were reported following exposure of an environmental strain of *P. aeruginosa* to 0.635 mg Cu^{2+} L^{-1} copper, with complete recovery in 14 days (Dwidjosiswojo et al., 2011).

These results have significant implications for monitoring *P. aeruginosa* in drinking water of health care establishments because of the presence of copper in internal plumbing. While newer

copper piping might inhibit *P. aeruginosa* attachment initially, passivation of the material results in biofilm colonization after 1 or 2 years of utilization (Critchley et al., 2001; van der Kooij, Veenendaal, & Scheffer, 2005). Although the main source of copper in water is piping, brass present in valves, faucets, meters, and fittings can also leach copper in water during stagnation. In spite of the impact of brass elements, environmental conditions in the volume of water contained in the tap and its connecting pipe (< 50 mL) are most favorable to culturable state. Indeed, copper concentrations will be lower than in the connecting copper piping and free chlorine will be depleted. Therefore, the selection of sampling volumes carries direct implications for culturability and the interpretation of monitoring results. Recommended and typical sampling volumes vary between 50 mL and 500 mL (Chaberny & Gastmeier, 2004; Department of Health (DH) et al., 2013; Ferroni et al., 1998; Trautmann, M. et al., 2001), large enough to include water from the connecting pipe containing potentially high copper concentration. Cells from the initial stagnant volume would then be exposed to enough copper to enter a VBNC state, potentially leading to cell count underestimation when using standard culture methods. Bacteria collected in copper-rich water continue to be exposed to this inhibitor until processing for detection (up to 24 h), unless sufficient quencher or chelating agent are added.

4.3.3 Impact of free chlorine and copper ions stresses on enzyme-based detection compared to culture method

Pseudalert is a new enzyme-based assay used to provide a quick response detection of *P. aeruginosa* currently applied to pools, spas and bottled water. It is based on the same platform as Colilert, a US Environmental Protection Agency-approved kit used for the detection of *Escherichia coli*. The enzyme-based assay for *P. aeruginosa* offers an attractive alternative to culture and is currently undergoing validation for monitoring of *P. aeruginosa* at water points of use in healthcare establishments. Technical data from the manufacturer and recent publications provide information on the positive correlation with culture enumerations in pool samples and bottled water (Idexx Laboratories, 2010a, 2010b; Mannisto, 2012; Semproni et al., 2014). However, the influence of direct exposure to free chlorine or copper ions stress on the performance of the test has not been documented in drinking water on a wide range of cell concentrations.

Figure 4-4 shows that paired measurements for a control culture in early exponential phase using direct culturing and the enzyme-based methods are highly correlated (R^2 = 0.99, n = 24) as confirmed by the 95% prediction interval (Appendix 2, Figure A-2.1a). This correlation is observed over a wide range of concentrations and remains in agreement with the previously published dataset on pool and spa water samples (R^2 = 0.95, n = 14) established with lower detected concentrations of *P. aeruginosa* (Idexx Laboratories, 2010b). Overall, results obtained by the enzyme-based assay led to slightly higher estimates than direct culturing with a positive bias of 7% (Figure 4-4).

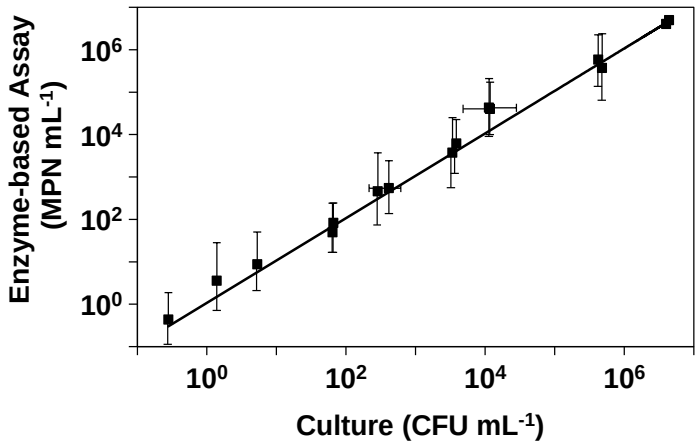

Figure 4-4 : *P. aeruginosa* PA14 cells concentrations suspended in buffered sterile water measured by enzyme-based assay in Most Probable Number (MPN) $\cdot mL^{-1}$ and standard culture methods in $CFU \cdot mL^{-1}$ (*n* = 24; y = 1.07 x; R^2 = 0.995). Error bars indicate 95% confidence range for MPN and standard deviation for culture.

Figure 4-5 presents paired measurements by enzyme-based assay and culture methods on stressed cells. Exposure to free chlorine did not modify the correlation observed in the absence of stress (R^2 = 0.99) (Figure 4-5). The 95% prediction intervals on the log transformed data without a forced intercept confirms that chlorine exposure does not modify the correlation between the enzyme-based and the culture results (Appendix 2, Figure A-2.1 a and b). However, for copper ion induced stress conditions, an apparent scatter from the regression line is noted (Figure 4-5) and the 95% prediction interval on the log transformed data without a forced intercept clearly shows the poor correlation in the presence of 0.25 mg Cu^{2+} mL^{-1} (Appenix 2, Figure A-2.1c). As no interference with the reagent was observed (data not shown), the increased response of the

enzymatic assay may be attributed to the interference of copper ions with the enzymatic hydrolysis of the substrate, possibly because of the enhanced production of the targeted enzyme (Teitzel et al., 2006).

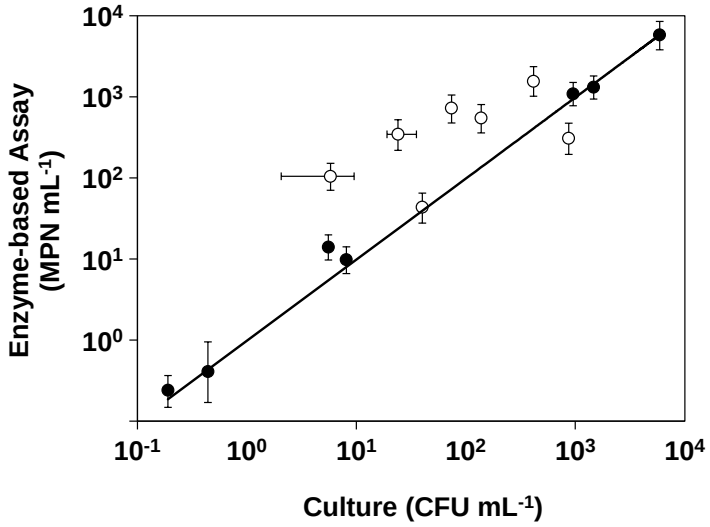

Figure 4-5 : *P. aeruginosa* PA14 cells concentrations measured by the enzyme-based assay in Most Probable Number (MPN) ·mL^{-1} and standard culture methods in CFU·mL^{-1} in presence of 2 mg L^{-1} initial chlorine concentration (•, n = 7; y = 0.99 x; R^2 = 0.99) and 4 µM copper (○, n = 7).

4.3.4 Implications for environmental monitoring

Culture-based methods are most commonly prescribed to monitor *P. aeruginosa*, and standards and guidelines in drinking water and clinical settings are still almost entirely expressed in CFU mL^{-1}. Results obtained in this study demonstrate the presence of viable *P. aeruginosa* cells in some water samples that would not be detected by standard plate count culturing or by an enzyme-based assay due to prior exposure to free chlorine or copper ion stress, a common situation within internal plumbing. Residual chlorine is often detected in municipal cold water and standard sampling protocols recommend the addition of sodium thiosulfate to neutralize residual chlorine upon sample collection (American Public Health Association (APHA) et al., 2012). Even with neutralization, bacteria previously exposed to free chlorine may not fully recover culturability before the start of culture, as samples are processed within 24h of sampling. Resulting counts may then be an underestimation, providing a false sense of security. The comparison of results from culture and the enzymatic assay suggest a greater sensitivity of the

enzymatic assay that, coupled with the quicker response time (< 26 hours), may provide additional surveillance value and contribute to prevention.

Results of this study reveal the presence of a large proportion of VBNC cells in the presence of common environmental stressors such as free chlorine and copper ions. It was shown that *P. aeruginosa* exposed to chlorine and copper ions are unlikely to be measured by standard culture methods, or even newer quicker response methods based on enzymatic reactions. This raises the question of the sanitary significance of the presence of *P. aeruginosa* in a VBNC state. Although some cells in the VBNC state are avirulent, the potential for VBNC cells to become infectious once resuscitated has been clearly documented (Dwidjosiswojo et al., 2011; Oliver, J. D., 2010). Conditions and time lag for such resuscitation vary greatly, and the time of recovery in nutrient-rich environments such as a sink drain or susceptible host is poorly documented. As disinfectant residuals are not stable or are often absent in internal plumbing, VBNC cells could regain culturability and infectivity between sampling events. Copper ion concentrations in large building drinking water systems can reach and surpass 0.25 mg L^{-1}. This is especially the case in large buildings such as hospitals, where copper is a commonly used pipe material and stagnation between uses can lead to elevated copper concentrations. These results also highlight the need to revisit the evaluation of Cu-Ag based-disinfection methods efficacy. Moreover, they highlight the importance of using an appropriate monitoring protocol, including sampling volume, sample treatment and analytical method to assess the risks to which patients are directly or indirectly exposed.

On the other hand, the use of qPCR as routine monitoring in hospitals is still infrequent. The main drawback cited against its wide use is its inability to distinguish between viable and dead cells, as all intact DNA can be amplified. Nevertheless, qPCR can still be used as a monitoring tool, especially to interpret changes to the baseline values in a drinking water system. Clearly, an increase in the qPCR signal indicates cell multiplication and proactive action could be taken to resolve the issue before it is detected by culture methods. In critical situation, this approach could complement culture and enzymatic methods and help reduce the risk associated with the presence of *P. aeruginosa* in water. Viability PCR is being developed, where an intercalating dye prevents the amplification of DNA in membrane compromised dead cells, but it still suffers from practical limitations. Given the demonstration of the potential of VBNC cells to regain virulence, viability PCR holds great promise for future monitoring improvements.

4.4 Acknowledgements

Authors would like to thank Jacinthe Mailly, Julie Philibert and Mélanie Rivard for their technical support. This work was funded by the NSERC Industrial Drinking Water Chair of Ecole Polytechnique and industrial partners. ED holds a Canada Research Chair.

CHAPTER 5. ARTICLE 4 – TEMPERATURE DIAGNOSTIC TO IDENTIFY HIGH RISK AREAS AND OPTIMIZE *LEGIONELLA PNEUMOPHILA* SURVEILLANCE IN HOT WATER DISTRIBUTION SYSTEMS

Legionella risk in a hot water distribution system is most often assessed via periodic water sampling in a few selected control points. Positive results will trigger investigation and response to the detected contamination. However, this approach is reactive and does not allow the anticipation of risk areas ahead of an actual contamination. This chapter proposes a proactive diagnostic approach to evaluate risk areas for *L. pneumophila* development within a large building hot water distribution systems based on temperature measurement and control. The proposed approach is based on results from a field sampling campaign in five different systems and a review of existing worldwide regulations and guidelines to control *Legionella*. This paper is published in *Water Reasearch*. Supplementary information is presented in Appendix 3.

TEMPERATURE DIAGNOSTIC TO IDENTIFY HIGH RISK AREAS AND OPTIMIZE LEGIONELLA PNEUMOPHILA SURVEILLANCE IN HOT WATER DISTRIBUTION SYSTEMS

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ABSTRACT

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

KEYWORDS

Legionella pneumophila, premise plumbing, viable but not culturable (VBNC), thermal control, temperature profile, culturability

5.1 Introduction

Legionella pneumophila (*Lp*) is an opportunistic pathogen that can proliferate in hot water distribution systems (HWDS) of large buildings, such as health care facilities (HCFs), where it can cause waterborne nosocomial pneumonias. Although its optimal growth temperature lies between 25 and 42°C (Yee & Wadowsky, 1982), *Lp* has been isolated from water systems at temperatures up to 60°C (Martinelli et al., 2000), and in cold water systems with temperatures below 20°C (Arvand et al., 2011). The presence of *Lp* in HCFs water systems is well

demonstrated, with reports of 10-50% positive hot water samples taken from taps and showers in Europe and the United States (Arvand et al., 2011; Bargellini et al., 2011; Martinelli et al., 2000; Serrano-Suarez et al., 2013; Stout et al., 2007). Risk characterization of water sources remains uncertain because of the lack of reliable dose response models (Buse et al., 2012) and therefore the difficulty to define an acceptable level of *Lp* contamination that would minimize risk. While the level of positivity for *Legionella* in health care facilities (HCF) HWDS has been proposed as a reliable predictive risk factor (Best et al., 1983; Lin et al., 2011), the specificity and sensitivity of the 30% positivity cut-off point has been recently questioned (Allen, J. G. et al., 2014; Allen, J. G. et al., 2012; Pierre, Stout, & Yu, 2014).

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

Although a multitude of possible system architectures are encountered, a simplified schematic of a hot water distribution system (HWDS) can be established (Figure 5-1) and should include: the number and characteristics of key systems components such as the calorifiers, reheating units and reservoirs, the distribution systems including principal, subordinate and tertiary flow and return loops and point-of-use devices (tertiary terminal end). A schematic and characterization of each HWDS within a premise must be established independently (BSR/ASHRAE, 2013; Health and Safety Executive (HSE), 2013; République Française, 2010a). This data is the foundation for interpreting monitoring results and identifying high risk areas.

A summary of the key elements from selected regulations and guidelines to implement temperatures control of *Lp* in large buildings, and when available, in HCFs is provided as supplementary material (Appendix 3, Table A-3.1). Approaches to control *Lp* in hot water

distribution systems (HWDSs) vary considerably, but all guides include objectives or obligations for optimal operating temperatures at critical points in the distribution systems. Also commonly specified are construction and operational standards, such as minimizing stagnation (recirculation loops, elimination of hydraulic and physical dead ends, etc.), recommendations on the use of devices and materials not promoting bacterial proliferation (construction material, flow, temperature, etc.) and requirements for microbiological monitoring in relation to pre-established criteria that define corrective actions.

In France, recently strengthened regulations determine mandatory minimum temperature and *Legionella* monitoring at defined critical control points: 1) hot water outlet and reservoir when present; 2) return loop; and 3) representative points-of-use considered at risk (farthest from the water heater or serving vulnerable patients) but the number of sampling points to be monitored is not specified (République Française, 2005, 2010a, 2010b) (Appendix 3, Table A-3.1). It is recommended that temperatures be monitored daily or continuously at hot water heater outlets and at each return loops, and weekly at service points in HCFs. Temperature measurements at points of use are conducted on flushed samples (2-3 min). In the United Kingdom, a risk management approach is proposed, with recommended preventive measures including system maintenance, elimination of stagnation or dead zones, reduction of aerosol formation, maintenance of adequate temperatures and use of materials unfavorable to biofilm development (Department of Health (DH) & Estates and Facilities Division, 2006b; Health and Safety Executive (HSE), 2013). Temperature control regimen is presented as the preferred initial approach for *Legionella* control (Appendix 3, Table A-3.1). Minimal monthly temperature monitoring is specified at control points including water heater outlet, return loops and sentinel taps. Sentinel taps include representative at-risk taps as well as the first and last taps of each return loops. The use of continuous temperature monitoring is recommended for the water heater outlets and the return loops. In addition, temperature at the tap should be monitored annually on a rotating basis covering 20% of taps yearly, to ensure the whole system is meeting required temperatures for *Legionella* control. It is not permissible to shut down pumped recirculation as it would lead to the loss of the required system temperatures. *Legionella* monitoring is not prescribed unless target temperatures cannot be achieved; however it is recommended in areas with highly vulnerable patients. Weekly flushing for several minutes is recommended for low usage taps.

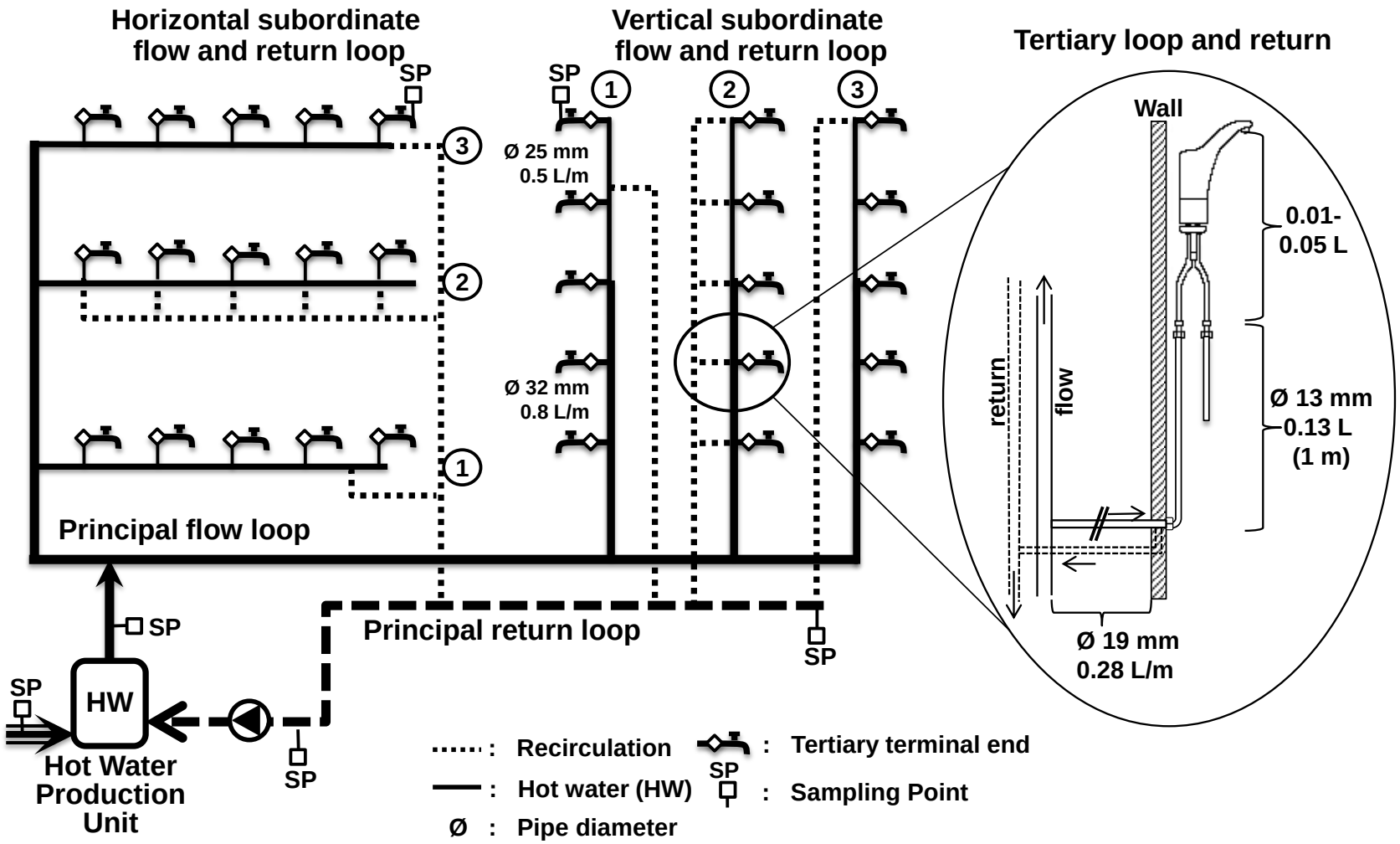

Figure 5-1 : Hot water distribution system general schematic including temperature control points. Three different types of vertical and horizontal distribution systems are represented: ① recirculation before the last tap; ② recirculation connected after each device; ③ recirculation connected after the last device.

Although all available regulations and guidelines provide information on various aspects of the implementation of a successful temperature control regimen, there is no consistent guidance on key elements such as the selection of sentinel points, the incorporation of *Lp* monitoring and the interpretation of the temperature monitoring results. Reports on the efficacy of the implementation of temperature control in health care facilities (HCFs) reveal limited success (Arvand et al., 2011; Bargellini et al., 2011; Blanc et al., 2005; Darelid, Lofgren, & Malmvall, 2002; Hruba, 2009; Lee, J. V. et al., 2011; Serrano-Suarez et al., 2013). Nevertheless, adjusting the temperature at the heater outlet to ensure water temperatures greater than 50-55^{o}C at distal outlets can be highly effective in reducing the proportion of positive swabs or water samples (Arvand et al., 2011; Blanc et al., 2005; Ezzeddine, Van Ossel, Delmee, & Wauters, 1989). Moreover, areas consistently positive for *Lp* were associated with poor hot water recirculation leading to temperature losses (Blanc et al., 2005). In most case studies, the actual conditions of application of the temperature control regimen are poorly documented with some information on temperatures only available for the water heater and return. The efficacy of temperature control regimens must be assessed by its ability to suppress *Lp* growth in the distal areas, as distal growth is highly significant (Cristina et al., 2014; Serrano-Suarez et al., 2013). On the other hand, there is increased risk of scalding for temperatures higher than 50°C at the tap (Moritz, A. R. & Henriques, 1947). Some countries specify maximum temperatures at the point-of-use to avoid scalding (Appendix 3, Table A-3.1), but newly updated regulation in United Kingdom require a risk assessment comparison between the risk of scalding and the risk of infection before limiting the hot water temperature below 50°C, a risk factor for *Legionella* proliferation.

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

5.2 Materials and methods

5.2.1 Hot water system characterization

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

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

5.2.2 Temperature profiling and water sampling at points-of-use

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

addition, three taps from system 5 were selected for a repeat temperature profile sampling. Residual chlorine was measured onsite (Pocket Colorimeter^{TM} II, Hach, USA) for all samples.

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

5.2.3 Impact of stagnation

The third part of the sampling campaign was conducted on 2 taps of system 5 to study the impact of stagnation on the detection of *Lp*. Stagnation is defined as the period during which the tap is not used and water remains idle within the piping. One liter of hot water was sampled in sterile propylene bottles with sodium thiosulfate (final concentration 1.1 mg/L) after 1 hour, 1, 2, 3, 5 & 10 days of stagnation. These stagnation times were chosen to represent various situations within a real system: 1h for the time between usage in a patient room; 1-day for patient daily care; 2 and 3 days for areas closed on the weekend (i.e. outpatients clinics); 5 and 10 days for an empty room in between patients or a temporary ward closure. *Lp* concentrations were measured by qPCR as described in section 5.2.4.

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

5.2.4 Microbiological analyses

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

Culture was conducted according to the standard AFNOR NF T90-431 procedure (Association Française de Normalisation (AFNOR), 2006). Briefly, 1 liter was filtered on sterile 0.4 µm polycarbonate membranes (47 mm diameter; Maine Manufacturing, LLC), which were then sonicated in 5 ml sterile water at 47 kHz for 1 min (Bransonic, Danbury, USA). Heat treatment (50°C, 30 min), acid treatment (pH=2; 5 min) and combination of both were performed on 3 separate 1 ml aliquots. Samples were plated on GVPC selective agar (Innovation Diagnostics Inc.) and incubated at 36°C for 10 days. Typical colonies that developed after 4-10 days were sub cultured on confirmation plates for 2-4 days at 36°C. Resulting colonies that developed on BCYE agar, but neither on blood agar nor on BCYE without cysteine were considered as *Legionella* spp. Confirmation for *Lp* was conducted using the *Legionella* latex test (DR0800, OXOID Limited). The calculated detection limit for the culture method was 50 CFU/L for both *Legionella* spp. and *L. pneumophila*.

Quantification by qPCR was performed on a Corbett Rotorgene 6000 using the iQ-Check Quanti *L. pneumophila* kit (Bio-Rad, Mississauga, Canada) with the following protocol: 15 min initial denaturation at 95°C followed by 50 cycles with denaturation at 95°C for 15 s, annealing at 57°C for 30 s, elongation at 72°C for 30s and final elongation for 15 min at 72°C (Bonetta et al. 2010). An internal control and four DNA standards ranging between 19 and $3.9x10^4$ genomic units (GU) were supplied with the kit. Sterilized water was used as negative control. DNA extraction was performed directly on filters using a bead beating method adapted from Yu and Mohn (1999). Briefly, 1L was filtered on 0.45 µm mixed cellulose ester and the filter was inserted into an extraction tube containing a garnet matrix and one 1/4-inch ceramic sphere (Lysing Matrix A, MP Biomedicals, Solon, USA). Lysing buffer was added to each tube prior to the bead beating step performed on a FastPrep MPBio-24, followed by ammonium acetate precipitation and successive ethanol washes.

5.2.5 Statistical analyses

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

5.3 Results and discussion

5.3.1 General system characterization

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

For systems 1-4, incoming water had chlorine residual of 0.30±0.03 mg Cl_2/L, pH of 7.77±0.05 and conductivity of 307±29 μS. For system 5, residual chlorine was higher, at 0.5±0.1 mg Cl_2/L, pH of 7.82±0.07 and conductivity of 288±13 μS. There was no additional disinfection treatment in any of the hot water systems studied and mean residual chlorine was 0.04±0.02 mg Cl_2/L for all systems.

5.3.2 Temperature monitoring

5.3.2.1 Water heater outlet

Most guidelines specify that target temperatures must be maintained at all times, but seldom do they specify the monitoring requirements of measurement frequency. Periodic temperature

readings, even daily measurements, do not provide insurance of temperature maintenance in the hot water distribution system (HWDS), unless the stability of the system's performance has been fully established. Systems seemingly providing water above 60°C based on daily measurements can actually produce lower temperature water for extended periods of time. In fact, the mean temperatures at the water heater outlet for four of the five systems studied were above 60°C, but online temperature monitoring revealed that production temperature was repeatedly below 60°C and reached down to 43°C in some cases (Table 5-1 and Figure 5-2).

System 1 consistently produced water above 60°C while systems 2, 3 and 5 regularly produced water below 60°C at certain periods of the day (Figure 5-2). For system 3, temperature was monitored weekly by the operators on Saturday mornings during low water demand providing an average of 62.5°C over a period of 24 months (Table 5-1). Nonetheless, when online monitoring was performed during a typical weekday, mean temperature was lower (57.8°C). It is also interesting to point out that even a very recently installed system (2011) equipped with a flash heating unit was also subject to periodic temperature drops (System 5, Figure 5-2). These observations demonstrate the need to use online monitoring to assess the temperature compliance of a HWDS compared to periodic manual readings of temperature. Daily variations in hot water demand in large HCFs with typical peak flow factors of > 6 (Bujak, 2010) can influence the temperature at the water heater outlet depending on the system's capacity. The extent and duration of the non-compliance of the hot water outlet temperature set point is important to consider and has been limited to the sporadic short duration (minutes) events in the German technical rules (Appendix 3, Table A-3.1).

Table 5-1 : HWDS Systems characterization through control points temperature and microbiological measurements

System No	Mean temperature ± SD, °C (*min;max*)				System mean heat loss (°C)	Point of use mean heat loss (°C)	Microbiological analysis, % positive		
							Culture		qPCR
	Water heater outlet	Principal return loop	Subordinate return loop	Point of use after 2 min			*Legionella* spp.	*L. pneumophila*	
1	62.2±0.9 (*58.8;64.0*)	57.2±0.1 (*57.0;57.4*)	-	54.8±6.8 (*50.0;59.6*)	5.0	7.4	6 % (1/17)	n.d. (0/17)	n.d. (0/17)
2	64.5±1.8 (*55.9;67.0*)	58.9±1.0 (*57.0;60.0*)	-	58.9±0.2 (*58.8;59.2*)	5.7	5.7	25 % (2/8)	n.d. (0/8)	13 % (1/8)
3	62.5±1.5 (*46.7;66.0*)	50.4±4.0 (*47.5;54.7*)	-	60.3±1.5 (*59.2;61.3*)	12.2	2.4	n.d. (0/5)	n.d. (0/5)	n.d. (0/5)
4	54.3±3.0 (*43.0;61.0*)	51.6±1.4 (*50.0;53.0*)	-	54.1±2.5 (*51;56.7*)	3.3	0	22 % (5/23)	22 % (5/23)	87 % (20/23)
5	61.6±1.9 (*46.1;70.4*)	53.9±0.6 (*49.1;56.1*)	**3&5**: 45.7±1.3 (*34.1-50.2*) **1&2**: 48.0±1.7 (*33.4-52.0*) **Kitchen**: 58.1±0.9 (*35.4-62*) **3**: 46.6±1.9 (*33.2-51.6*)	46.2±7.1 (*36;63.3*)	7.7	15.4	82 % (9/11)	27 % (3/11)	100 % (11/11)

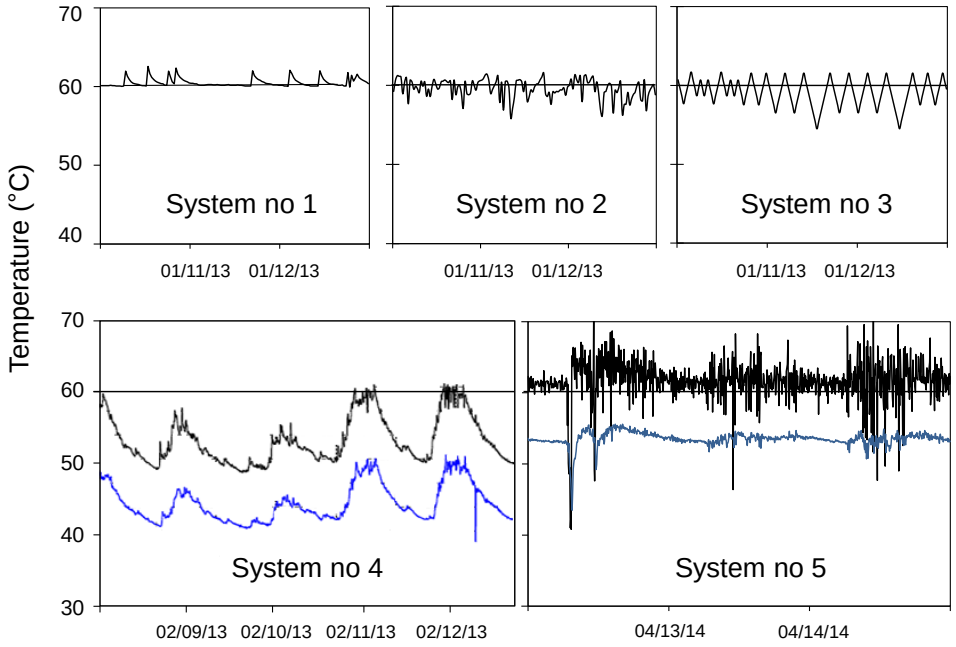

Figure 5-2 : Examples of continuous temperature monitoring at water heater outlet for each studied system (black line) and at the return loop for system 4 and 5 (blue line). No continuous monitoring data was available for systems 1- 3. Date format is MM/DD/YY. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

5.3.2.2 Return loops

The return loop at the point closest to the water heater is designated as the furthest point from the water heater and continuous temperature monitoring is often recommended (Figure 5-1). It is considered as an indicator of the system's capacity to maintain temperatures throughout the hot water distribution system (HWDS). In the five systems studied, the principal return loop temperatures ranged between 50.4 and 58.9°C with varying levels of blending from multiple return loops occurring upstream of the principal return control point (Table 5-1). Continuous monitoring for 2 months at the return loop manifold for combined returns of units 3&5 (45.7°C), units 1&2 (48.0°C), single return for the kitchen (58.1°C) and for unit 3 prior to merging with unit 5 (46.6°C) revealed wide differences compared to the overall combined return loop (53.9°C). Although a regulated control location (Appendix 3, Table A-3.1), temperature at the principal return loop is not indicative of the conditions in all subordinate loops within a complex HWDS if

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

Heat losses between the water heater outlet and a remote point will occur during stagnation (if recirculation is not effective or shut down for energy conservation purposes) or during circulation in the principal and subordinate flow and return loops. During low demand conditions, recirculation will dictate residence time and drive heat losses. Mean system heat losses were evaluated for each of the five studied systems (Table 5-1). For three of the five systems, temperature losses between the water heater and the principal return loop mean temperatures exceeded the target of ≤5°C set in several guidelines (Appendix 3, Table A-3.1). Heat losses during circulation can be minimized by reducing residence time. Water velocity can be set to meet desired maximum heat losses and general recommendations suggest maintaining a minimal velocity of 0.2 m/s (Blokker, Vreeburg, Schaap, & van Dijk, 2010; Centre scientifique et technique du bâtiment (CSTB), 2012), which would result in approximately 30 min residence time and 5°C heat losses in large health care facilities (HCF) insulated HWDS. Although insulation minimizes heat losses under flowing conditions, it is not sufficient to maintain high temperatures over prolonged periods of stagnation. Actually, slower heat losses during stagnation may lead to sustained optimal temperatures for *L. pneumophila* growth. Figure 5-3 shows that temperature decreased from 60°C to below 50°C within 30 min in fully insulated copper pipes and within 10 min for non-insulated pipe, both reaching room temperature after 3.5 hours. Periods of stagnation of 30 min or more are expected in the connecting piping upflow of points-of-use and in areas of inefficient recirculation.

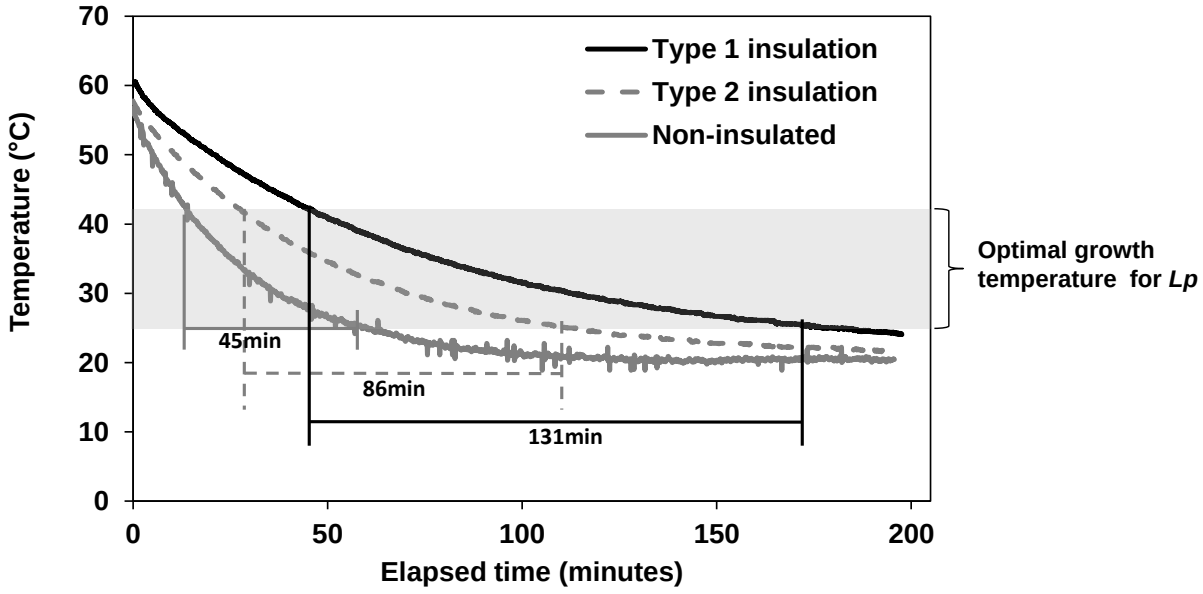

Figure 5-3 : Heat loss during stagnation of hot water in 1.25 cm diameter copper pipes with and without insulation at room temperature.

Existing standards and guidelines set design and operational obligations to control heat losses in hot water distribution systems (HWDS) to maintain at minimum target temperatures throughout the HWDS and to meet energy conservation goals, but these are generally only compulsory for new buildings. Recirculation flow rates should be calculated to maintain a <5^{o}C system heat loss or to ensure a minimum temperature of 50-55°C at the end of the return loop assuming adequate recirculation throughout the system (American Society of Plumbing Engineers (ASPE), 2008). The control points results required to evaluate heat loss goals compliance include the principal and subordinate return loops, the most distant point of the flow loop or all points of the system (Appendix 3, Table A-3.1). Monitoring results from the five HWDSs clearly show that the selection of the return loop reference point is critical. Heat loss evaluation from the principal return loop may mask major heat losses in subordinate flow and return loops, as we observed in system 5 with losses ranging from 3.5 to 16.3°C when evaluated for single or dual subordinate return loops (Table 5-1). Indeed, wide differences in temperature can occur between secondary return loops, and thus all return loops should be considered individually. The overwhelming importance given to temperature maintenance has also led to the specific banning of recirculation shutdown in Austria and United Kingdom (Appendix 3, Table A-3.1). The nightly shutdown of recirculation for energy conservation purposes is only allowed in two rules (Corporation des

maîtres mécaniciens en tuyauterie du Québec (CMMQ) & Régie du Bâtiment du Québec (RBQ), 2013a; DVGW German Technical and Scientific Association for Gas and Water, 2004) and only with the demonstration of unobjectionable hygienic conditions. Our results point out that the temperature losses of isolated subordinate loops during stagnation resulting from such shutdowns would quickly generate durable temperature conditions favorable to the growth of *Lp*. More importantly, such shutdowns during low or nil demand conditions expose the whole HWDS, instead of a relatively small volume (1,200L versus 90-300L in System 5) to these undesirable temperature conditions.

5.3.2.3 Temperature distribution at point-of-use

Sequential volume profiling results identify in which sections of the HWDS the heat losses take place, namely the tap and its connecting piping, the secondary piping, the distribution columns and/or the main feeder pipes. Profile variability for a given sampling point at different times and days was found to be small, with overall profile and maximum temperature reached being consistent over time despite variable temperature in the first liter (Appendix 3, Figure A-3.1). Temperature profiles obtained on the studied systems are summarized in three groups (Figure 5-4), with detailed profiles presented in Appendix 3, Figure A-3.2. Systems 1, 2 and 3 (Figure 5-4a) met recommendations for water heater outlet and return loop temperatures, with 86% of points reaching 55°C and all points being above 50°C after 2 minutes of flow, indicative of limited stagnant water volumes and effective recirculation. Ideal systems should have no or very little transition and reach equilibrium at recommended temperatures in order to maintain sufficient temperatures within the whole system. Despite reaching equilibrium temperature rapidly (<60s), system 4 could not achieve recommended temperature at the points-of-use with 57% of points never reaching 55°C although all above 50°C, mainly due to the insufficient water temperature at the water heater outlet (Figure 5-4b). System 5 shows a longer transition period before reaching temperature equilibrium and is unable to meet 55°C for 47% and 50°C for 19% of points, despite water heater and principal return loop temperatures meeting recommendations (Figure 5-4c).

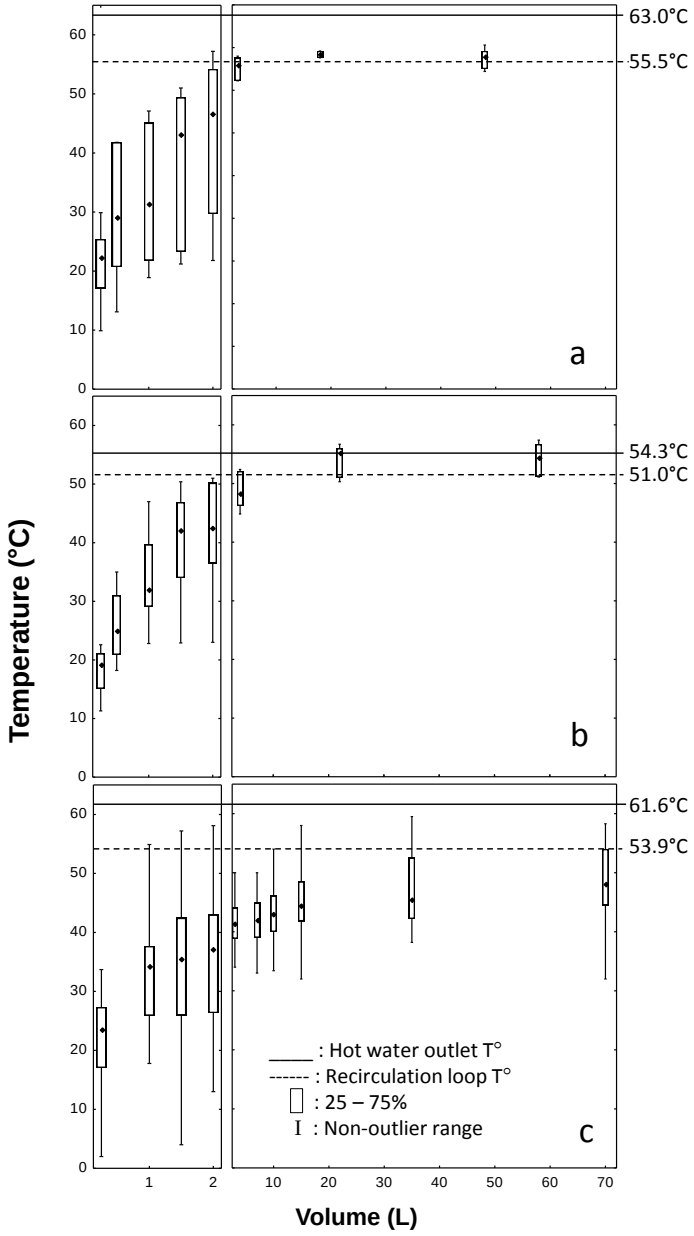

Figure 5-4 : Hot water temperature profiles at points-of-use as a function of volume for a) systems 1, 2 and 3 grouped, n = 7; b) system 4, n = 7; c) system 5, n = 36. Mean temperature at the hot water production unit outlet and at the return loop are shown for each system.

Additional temperature monitoring using surface thermocouples on subordinate flow and return pipes were conducted on system 5 (Appendix 3, Figure A-3.3). The ongoing temperature monitoring in subordinate flow and return loops in addition to the principal flow and return loop provided helpful information to identify local issues. For instance, broken valves in a shower faucet resulted in cold water entering the hot water feed pipe and riser. Fixing the device

increased the minimal temperature by an average 5°C in all 10 subordinate risers in this wing (Appendix 3, Figure A-3.3 a-c). A second example was insufficient recirculation causing a significant heat loss during night flow, which was corrected by the addition of a local pump on the subordinate return loop, after the furthest pair of risers (Figure A-3.3 d-g). These examples show the importance of characterizing local conditions and the potential of single faulty devices to influence temperature maintenance in large sections of hot water distribution systems (HWDS). Again, we conclude that relying on temperature maintenance in the principal return loop is not sufficient to identify such risk areas.

5.3.3 Legionella monitoring

Results of microbiological measurements for the five studied systems are presented in Table 5-1. Detection by qPCR was used in complement of culture detection as it has been shown to be efficient in monitoring changes in the bacterial numbers (Krojgaard, Krogfelt, Albrechtsen, & Uldum, 2011; Lee, J. V. et al., 2011). Culture positive samples for *Lp* were detected in systems 4 and 5 with 22 and 27% positivity respectively (detection limit = 50 CFU/L; quantification limit = 250 CFU/L). Culture positive samples results were low, with only one count above quantification limit at 600 CFU/L, located at a tap in system 5. Positivity increased above 80% for both systems when measured by qPCR and remained below detection limit for systems 1-3, except for one sample in system 2 (Table 5-1, Appendix 3, Table A-3.2). Systems in which water temperature was kept consistently above 60°C coming out of the water heater and maintained above 55°C across the network were below detection limit for *Legionella* by culture or qPCR. Such results strongly suggest that satisfactory management of temperature at control points in the studied systems resulted in lower prevalence. However, these results represent a water quality snapshot at a point in time and are not necessarily representative of microbial quality over time or at other locations in the HWDS. Several factors affecting *Lp* densities at a given point have been identified including intrinsic biological system heterogeneity, culturability, prior stagnation and sample volume. Napoli et al. showed variation of ≤ 20% concentrations of CFU/ml from one day to the next within a ward during repeated sampling over five consecutive days across eight units within a hospital (Napoli, Iatta, Fasano, Marsico, & Montagna, 2009). In the present study, confirmation sampling was conducted in two of the five HWDSs to investigate the temporal variability. Figure 5-5a shows results from repeated sampling conducted at three control points

(water heater outlet, principal return loop and a point-of-use) in systems 3 & 4. All samples were negative in qPCR and culture for system 1, whereas samples from system 4 were consistently positive in qPCR and to a lesser degree in culture (Figure 5-5a). Mean levels of *Lp* detected in system 4 were not significantly different between the 3 control points ($p > 0.05$). These findings are in agreement with recent reports of discrepancies between trends in *Lp* by qPCR and culture in suboptimal conditions for inactivation of viable but not culturable (VBNC) cells (Krojgaard et al., 2011; Lee, J. V. et al., 2011). Krojgaard et al. showed that qPCR levels can be used to verify the impact of corrective actions such as thermal shock and demonstrated non-detects qPCR results as a predictor of low risk.

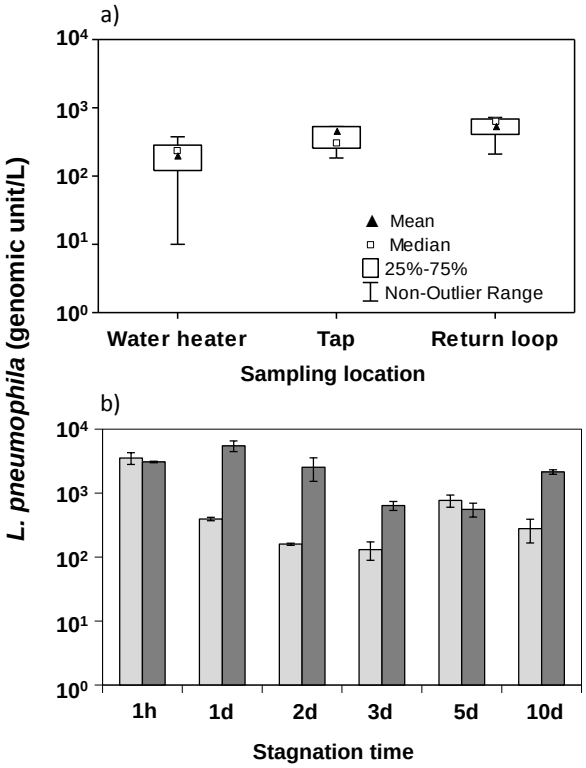

Figure 5-5 : Variability of *L. pneumophila* concentration measured by qPCR (a) in system 4, for repeat sampling events without prior stagnation (n = 5, Jan-Oct 2013) (b) in system 5, after different water stagnation times for tap A (light gray) and tap B (dark gray) (n = 2, Nov-Oct 2012).

Another factor that may influence levels of *Legionella* in water is the duration of stagnation prior to sampling. Recent evidence reported an increase in bacterial concentrations after various stagnation times (overnight to 14 days) in the cold water distribution system of a large building

(Lautenschlager et al., 2010; Lipphaus et al., 2014). A steady increase was observed in the first 12 hours of stagnation whereas longer stagnation time did not lead to further increase (Lautenschlager et al., 2010). In the present study, hot water was sampled from two taps at different stagnation times and *Lp* concentration was evalutated by qPCR (Figure 5-5b). The taps were not found to be statistically different when comparing mean results and no correlation was established between the mean *Lp* concentration and the stagnation time. However, the stagnation times were longer than 12 hours, except for the 1h stagnation and samples were taken from the hot water systems. To our knowledge, there is no reported data on the impact of stagnation on bacterial concentrations in hot water. These results suggest that *Lp* concentrations in the first liter of hot water at the tap may not be affected by stagnation time.

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

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

Volume sampled, typically 1L or more for *Lp*, plays an important role in data interpretation, either for temperature measurements or microbiological detection where the detection limit of the method improves with the use of higher volume of samples. As illustrated on Figure 5-1,

sampling the first liter will collect water from the tap and connecting pipes, and might reach water from the subordinate return and flow pipes depending on the configuration. For example, 8 meters of a 13 mm diameter pipe are required to reach 1L. If a larger sample volume is required to do multiple analyses (i.e. culture and qPCR or simultaneous detection of other waterborne opportunistic pathogens), it should be kept in mind that water will become less representative of the point-of-use.

5.3.4 Value of Temperature Control in *Lp* Risk Management

The implementation of a water safety plan is the recommended approach for preventive risk-management related to drinking water (World Health Organization (WHO), 2011) and temperature control is widely recognized as the first risk mitigation measure for *Legionella* control in hot water distribution systems (HWDS) (Appendix 3, Table A-3.1).

Maintaining sufficient temperatures at all critical points, including the subordinate return loops, and minimizing volumes of uncontrolled temperature in the terminal ends appear essential to a successful system wide thermal control of culturable and VBNC *Legionella*. Most studies report on the results of temperature control based on prevalence measured by culture-based detection methods. Although lower prevalence is generally observed after temperatures are increased, limited efficacies are often reported. An early study observed 50% reduction of tap positivity following an increase in temperature from 45 to 60°C at the water heater outlet, although an elevated number of taps located in patient rooms remained positive (Ezzeddine et al., 1989). Water temperature at the tap ranged between 30 and 56°C after a few minutes of flushing, demonstrating the system's inability to provide elevated temperatures in all areas. A similar reduction in % positive taps from 60-90% to 30-40% was reported in a hospital when water heater temperature was raised from 50 to 65°C, in that case providing temperatures >50°C at most outlets (Blanc et al., 2005). Importantly, the remaining positive outlets were situated in an area with inadequate recirculation. A third field study documented a successful reduction of *Legionella* positive taps from 100% to a mean value of 12% maintained over 10 years following the hot water temperature increase from 45 to 65°C (Darelid et al., 2002) This temperature regimen was implemented following an outbreak and resulted in water temperatures between 56 and 61°C at the tap after 5 minutes flushing. Recent field studies support the importance of maintaining elevated temperatures at distal locations (estimated by the temperature after 1 minute

of flushing), with 4–11% of positive at T≥55°C vs 14-82% for T<55°C (Arvand et al., 2011; Bargellini et al., 2011; Hruba, 2009). Those observations show that the efficiency of thermal inactivation in complex recirculated full scale HWDS is enhanced when temperature exposure is sufficient in all areas of the HWDS. However, significant distal amplification of *Legionella* can occur as evidenced by long term full scale sampling results (Cristina et al., 2014; Serrano-Suarez et al., 2013) and a number of taps may remain positive for *Legionella*.

The limitations of thermal control in HWDS raise questions on the validity of the existing threshold temperatures of 50-60°C. Pioneer work evidenced the consistent susceptibility of 40 *Lp* isolates to temperature, with 1 log reduction achieved in 2.3-5 min at 60°C and 8 log reduction after 25 min as estimated by culturability (Stout, Best, & Yu, 1986). Recent findings show that elevated temperatures between 55 and 70°C will produce VBNC cells that cannot be detected by culture methods. Laboratory studies conducted on HWDS samples confirm the suppression of culturability at T≥55°C as evidenced by the presence of *Lp* when measured by qPCR and viable qPCR (Lee, J. V. et al., 2011; Mansi et al., 2014). Despite a rapid loss of culturability at temperatures >55°C, some *Lp* strains can resist in the VBNC state for periods of 30-60 minutes at temperatures between 55 and 70°C (Allegra et al., 2008; Allegra et al., 2011; Epalle et al., 2015). Furthermore, the development of heat resistant *Lp* strains was observed over time for groups of strains isolated in hospital water systems submitted to periodic extreme temperature (24h @ 65°C a few times a year), while no such resistance was observed for strains isolated from the system where heat shock treatments (70°C 30 minutes) were sparingly applied. Finally, the efficacy of thermal disinfection on biofilm, the main reservoir of *Lp* in HWDS (Buse, Lu, Struewing, & Ashbolt, 2014), is at best scarce and reports limited and non-lasting efficacy of 70°C for 2 hours on culturable *Legionella* spp. (Saby, Vidal, & Suty, 2005). These findings stress that high temperature regimen provide *Lp* control not *Lp* eradication and the importance of maintaining a constant temperature regimen throughout the system to provide adequate contact time and avoid growth.

We propose a system wide risk classification to assess risk in an HWDS based on published reports and our findings (Table 5-2). In addition to monitoring temperature at critical control points, the evaluation criteria also include the percentage of time that temperature is maintained at the hot water production unit or return loops. Indeed, exposure to temperature should be considered instead of temperature alone, as regulated for chemical disinfection (Concentration X

Time concept). Subordinate return loop temperatures are used to evaluate the system's heat loss within each sector of the building. Temperature exposure in the subordinate flow and return loop is estimated based on temperatures measured after 1 minute of flushing and serves to determine risk in specific areas. When evaluating the five systems against the proposed risk classification (Table 5-2), results from the characterization of the HWDS combined with the temperature profiles at point-of-use were good predictors of areas at risk for *Lp* detection (Table 5-1). In light of these findings and considering the presence of VBNC *Legionella* at temperature ranging between 55 and 70°C (Epalle et al., 2015), the set points proposed in existing regulations and guidelines and selected for the proposed risk classification approach appear minimal and should be met at all times. The development of heat resistant strains following periodic heat shock also supports the maintenance of a steady thermal preventative inactivation regimen instead of relying on periodic curative thermal shock (Allegra et al., 2011). The apparent limited success of HWDS in large buildings may have been caused by inconsistent maintenance of sufficiently elevated temperatures in all areas of the building because of inadequate recirculation and/or low set-points.

Table 5-2 : Proposed risk classification based on temperature control points

Risk of amplification	Temperature criteria					Studied system classification
	Water heater outlet	Return loop	Taps or points of use	Heat loss		
				Mean	Max	
Very low	≥ 60°C At all times	> 55°C	> 55°C after 1 min	< 5°C	< 5°C	1
Low	≥ 60°C 90%	> 55°C	> 55°C after 1 min	< 5°C	< 10°C	
At risk	≥ 60°C < 90%	> 50°C	> 50°C after 2 min	<10°C	>10°C	2, 3
High risk	≥ 60°C < 50%	< 50°C	< 50°C after 5 min	> 10°C	> 10°C	4, 5

Regulations and guidelines all recommend the identification of representative sampling points for *Lp* sampling and temperature monitoring at designated control points. However, the rationale for frequency and number of sites for temperature monitoring is not evident and the limited number of proposed control points implies that the HWDS is well balanced. Furthermore, there is little

guidance for follow-up action to identify the cause of temperature losses. To remediate this shortfall, a diagnostic flowchart for the initial assessment of *Legionella* risk within an existing HWDS is proposed using temperature measurements and profiles at the water heating unit, return loops and critical points (Figure 5-6). We propose a step approach starting from the principal return and flow loop system that indicates the overall system risk level, then moving progressively to the subordinate flow and return loops to identify large building areas or sectors at risk, and finally to the tertiary terminal ends, to identify local issues with defective faucets or showers. The diagnostic flowchart also proposes a staged response in terms of corrective and preventative actions, including *Lp* monitoring. Critical control points, defined as the water heater outlet, the principal return loop and representative at risk points-of-use (not reaching control temperature, farthest from the water heater or serving vulnerable patients) are prioritized for sectors or systems identified at risk by the initial risk assessment (Figure 5-6). This step approach can help direct efforts towards high risk areas and optimize resource allocation, especially costly *Lp* monitoring. Nevertheless, an ongoing *Lp* monitoring strategy and schedule should be put into place through a water safety plan once initial assessment is completed and corrective measures have been completed.

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

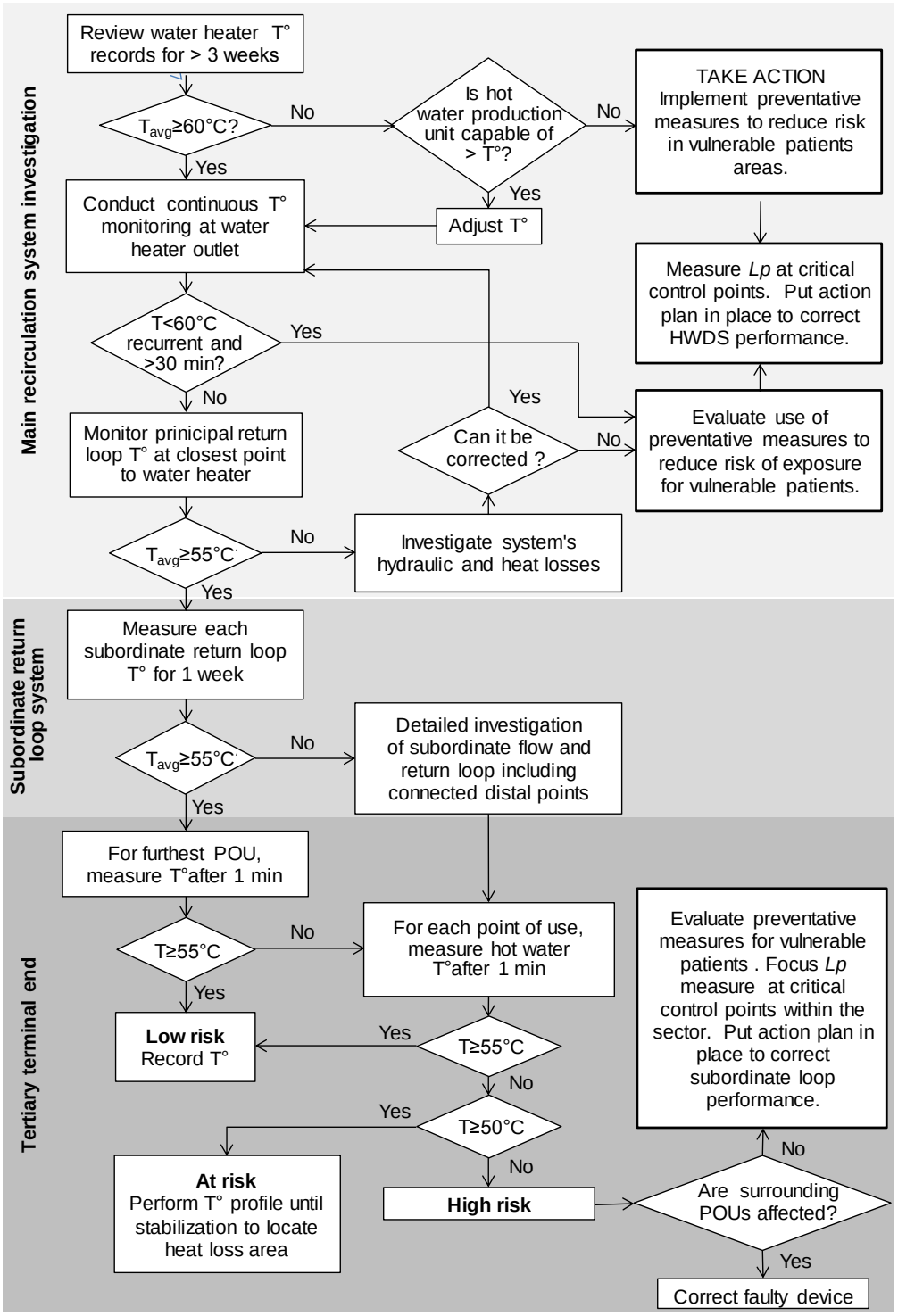

Figure 5-6 : Diagnostic flowchart for the initial assessment of Legionella risk in an existing HWDS

5.4 Conclusions

- A step approach combining temperature monitoring of the hot water distribution system (HWDS) main components and temperature profiling at points-of-use can be used to determine the susceptibility of overall hot water distribution system and specific areas of large buildings to *Legionella* proliferation. When multiple subordinate return loops are present, temperature should be monitored at each subordinate return loop prior to the principal return loop. Monitoring temperature representative points or even all points on a rotating basis (e.g. 20%/year) is time consuming and yet insufficient for rapidly detecting faulty equipment such as defective valves.
- The impact of faulty thermostatic devices extends far beyond the terminal connecting piping and can affect large areas of buildings, placing significant volumes of hot water at risk. Faulty return valves should be rapidly identified and repaired or replaced. A change observed in results from continuous temperature monitoring of the subordinate return loop can provide useful information to identify the occurrence of a faulty device.
- Temperature monitoring will help understand the hydraulics, quantify the thermal losses of the recirculating system and identify the distribution columns that need balancing. A systematic diagnostic is necessary to identify areas most at risk in hydraulically unbalanced HWDS or in older buildings where original plans and drawings may not be available or renovations and rearrangements have occurred.
- Systems assessment and monitoring should also take into account area specific hydraulic conditions within the building, including closed units, low usage and configuration of the overall system.
- Temperature profiling should be performed at a large number of points confirming the extent (volume) and nature (systemic or distal) of undesirable temperatures in HWDS, guiding *Lp* monitoring decisions. The staged approach based on inexpensive and easily implemented temperature profiling can optimize resources and funds allocation by directing efforts towards high risk areas.
- Although necessary, *Lp* monitoring is costly and time-consuming, and should be targeted to enable decision making for infection control. Our staged approach can guide corrective

system interventions and serve as a basis to justify preventive risk reduction actions and select sampling points for *Lp* monitoring.

5.5 Acknowledgements

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CHAPTER 6. ARTICLE 5 – POST-OUTBREAK INVESTIGATION OF *PSEUDOMONAS AERUGINOSA* FAUCET CONTAMINATION BY QUANTITATIVE POLYMERASE CHAIN REACTION AND ENVIRONMENTAL FACTORS AFFECTING POSITIVITY

Pseudomonas aeruginosa outbreaks and hospital water systems contamination have been reported. Most studies report the number of positive points-of-use and use culture detection methods to evaluate positivity. Although some studies have been conducted across the building premise plumbing, they seldom identify environmental factors leading to contamination and amplification of *P. aeruginosa* in the water system. This chapter presents a post-outbreak investigation in a hospital cold water system by quantitative polymerase chain reaction and culture detection methods. Multiple environmental parameters related to the sink were investigated, leading to the identification of factors significantly impacting water positivity. This paper was submitted in *Infection Control and Hospital Epidemiology*.

As part of this research, a better understanding of the impact of the type of faucet on *P. aeruginosa* positivity was necessary. This was addressed through the research project of Dominique Charron, who published an article on the impact of electronic faucets on the contamination by *P. aeruginosa*. This article was published in *Infection Control and Hospital Epidemiology* and is presented in Appendix 4. I co-authored this publication with Ms. Charron.

POST-OUTBREAK INVESTIGATION OF *PSEUDOMONAS AERUGINOSA* FAUCET CONTAMINATION BY QUANTITATIVE POLYMERASE CHAIN REACTION AND ENVIRONMENTAL FACTORS AFFECTING POSITIVITY

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ABSTRACT

OBJECTIVE. To perform a post-outbreak prospective study of the *Pseudomonas aeruginosa* contamination at the faucets (water, aerator and drain) by culture and qPCR and assess influencing environmental factors.

SETTING. A 450-bed pediatric university hospital in Montreal, Canada.

METHODS. Water, aerator and drain swab samples were collected from faucets and analyzed by culture during the outbreak period and by culture and quantitative polymerase chain reaction (qPCR) for the post-outbreak investigation. Water microbial and physico-chemical parameters were measured, together with a detailed characterization of the sink environmental and design parameters.

RESULTS. The outbreak genotyping investigation identified drains and aerators as the source of infections. The implementation of corrective measures was effective, but a post-outbreak sampling revealed 50% positivity in water by qPCR compared to 7% by culture. *P. aeruginosa* was recovered in the water, the aerator and the drain in 21% of sinks. Drain alignment and water microbial quality were significant factors for water positivity, whereas aerator positivity was a significant variable to predict the load of *P. aeruginosa*, with an average 2 log higher in faucets with a positive aerator.

CONCLUSIONS. *P. aeruginosa* contamination in various components of the sink environment was detected several years after the resolution of an outbreak. Although the contamination may not be detectable by culture in the water, *P. aeruginosa* is present and can recover its culturability under favorable conditions. The importance to have clear maintenance protocol of the water systems, including the drainage component of the system, is highlighted.

6.1 Introduction

Pseudomonas aeruginosa is a source of outbreaks, especially in intensive care units (ICU) (Jefferies et al., 2012). Several of these outbreaks have been directly or indirectly linked to water systems (Aumeran et al., 2007; Bert et al., 1998; Durojaiye et al., 2011; Ferroni et al., 1998; Halabi et al., 2001; Hota et al., 2009; Merrer et al., 2005; Reuter et al., 2002; Romano et al., 2013; Schneider et al., 2012; Trautmann, M. et al., 2001; Vianelli et al., 2006; Yapicioglu et al., 2011). In ICUs, 30-50% of *P. aeruginosa* infections are associated with water (Exner, 2012). A multicentric prospective study recently established tap contamination in patient's room as an important environmental risk factor for *P. aeruginosa* acquisition (Venier et al., 2014).

Several factors promote water contamination, including the type of faucets (Blanc et al., 2004; Charron et al., 2015; Halabi et al., 2001), the presence and type of aerators on the faucets (Walker, J. T. et al., 2014), the volume of mixed hot and cold water (Charron et al., 2015), the alignment of the sink drain (Ehrhardt et al., 2006), construction or renovation settings, ICU vs non-ICU wards (Ehrhardt et al., 2006; Reuter et al., 2002). Once contaminated, eradication of *P. aeruginosa* in the water system is reported to be challenging and often results in replacing the devices (Bert et al., 1998; Durojaiye et al., 2011; Halabi et al., 2001; Hota et al., 2009; Merrer et al., 2005; Romano et al., 2013; Schneider et al., 2012; Yapicioglu et al., 2011) or installing point-of-use 0.2 μm filters (Aumeran et al., 2007; Schneider et al., 2012; Vianelli et al., 2006).

Although cultivation is the reference method, it may not reveal background contamination ready to flare up when a change occurs, favoring culturability and growth. Environmental stressors present in water such as chlorine and copper decrease culturability without necessarily decreasing viability (Bédard et al., 2014; Dwidjosiswojo et al., 2011). Most studies describing environmental contamination have been conducted with culture detection method. Often, *P. aeruginosa* could not be isolated from water but was recovered from biofilm swabs. The use of quantitative polymerase chain reaction (qPCR) for the detection and measurement of bacteria in drinking water is not routinely used yet. However, this method could offer an interesting alternative for assessing the underlying contamination of systems and assess risk areas.

The present study describes a follow up investigation of the water system contamination by *P. aeruginosa* a decade after an outbreak in a neonatal ICU (NICU). The objectives were to 1) describe the investigation of an outbreak and corrective measures implemented; 2) conduct a

follow up investigation of the water system using qPCR and cultivation methods to evaluate the level of contamination and 3) identify the factors that contribute to the persistent contamination of the water system by *P. aeruginosa* despite corrective measures. To our knowledge, this is the first report of *P. aeruginosa* occurrence in hospital water systems by qPCR.

6.2 Methods

The investigation was performed at CHU Sainte-Justine, a 450-bed pediatric university hospital in Montreal, Canada. The outbreak took place between January 2004 and November 2005, in both intensive and intermediate care rooms. All taps were equipped with aerators. Distribution and draining systems including faucets, aerators and sinks were approximately 50 years old. Over the past 15 years, construction and renovation have required several prolonged interruptions in water supply.

Environmental sampling was conducted between March and April 2005, within and outside of the NICU. Water samples of 375 mL were collected from 69 taps in sterile containers after cleaning the aerator with 10% sodium hypochlorite and flushing for 30 seconds. A mix of hot and cold water was collected simultaneously. A total of 40 water samples were also collected from the hot and cold distribution system following the same procedure. The samples were centrifuged 30 minutes at 10,000 rpm, the pellets resuspended and inoculated on blood agar plates (BAP) and MacConkey agar plates (MacAP). Swabs were collected from sink drains (57 samples), the faucets inner surface (56 samples), the inside surface of the aerators (16 samples) and environmental surfaces and solutions (100 samples) with sterile cotton swabs dipped in Trypticase Soy Broth (TSB). Swabs were incubated in TSB at 35°C for 24h and plated onto BAP and MacAP. All plates were incubated for 5 days at 35 °C and resulting colonies were identified according to standard laboratory procedures. Screening of 250 healthcare workers from the NICU was also conducted looking for hand dermatitis.

Available *P. aeruginosa* isolates from the 2005 investigation were genotyped by electrophoresis using the Genepath reagent kit (Bio-Rad) to demonstrate a potential link between clinical and environmental isolates, One colony of each morphotype was selected per plate. Pulsed-field gel electrophoresis was carried out using 1% certified agarose (Bio-Rad)). Patterns with at least one

band difference were considered distinct. The correlation between the different patterns was performed using Tenover's criteria (Tenover et al., 1995).

The following outbreak corrective measures were introduced: sterile water used for patient care, alcohol-based gel treatment following hand washing, wearing of gloves for direct contact with body secretions, installation of point-of-use filters (0.2 μm), good practices in milk preparation, replacement of drain pipes and establishment of procedure prohibiting storage of medical material under, or within a perimeter of 30 cm around a sink (Figure 6-1).

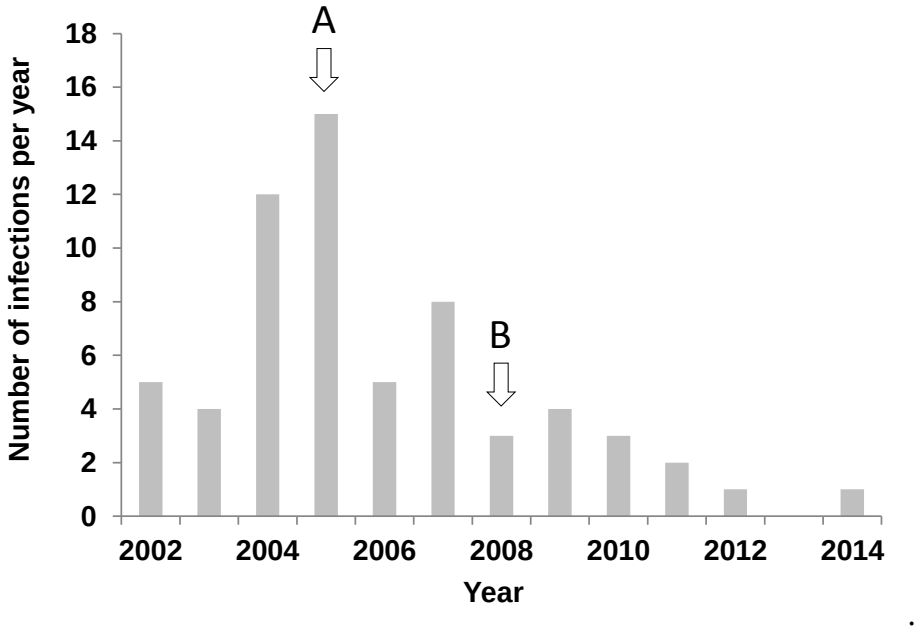

Figure 6-1: Epidemiological curve of *P. aeruginosa* infections in NICU between 2002 and 2014. Implementation of corrective measures is indicated by (A) and replacement of drains by (B).

A post-outbreak investigation was conducted in July 2013 in various areas of the hospital, including NICU. A total of 28 faucets were sampled as follows: 1) a swab from the drain, 2) 1L of first flush cold water in sterile polypropylene bottle with 1.1 mg/L sodium thiosulfate and 3) a swab of the aerator. Three additional faucets were sampled for water only. Cultivation, heterotrophic plate counts (HPC), qPCR and viable and total cell counts were determined on the water samples. Cultivation and qPCR were performed on swabs. HPC were determined on R2A agar at 22°C, after 7 days of incubation (American Public Health Association (APHA) et al., 2012). Viable and total cell counts were performed using LIVE/DEAD BacLight Kit (Molecular

Probes). Cultivation was performed according to ISO16266:2006 (International Organization for Standardization (ISO), 2006) as previously described (Charron et al., 2015). Briefly, the water samples were filtered (0.45 µm) and the filters incubated on cetrimide agar with 15 mg/L nalidixic acid at 37.5°C and colonies counted after 24h and 48h. Presence of *P. aeruginosa* by qPCR was assessed by targeting the *gyrB* gene (Corbett Rotor-Gene 6000) for 50 cycles: 10 min initial denaturation (95 °C), denaturation (95°C, 30s), annealing and elongation (60°C, 90 s) (Lee, C. S. et al., 2011). DNA was extracted after filtration of 450 mL on 0.45 µm mixed cellulose esther filter, using a bead beating method followed by ammonium acetate precipitation and ethanol washes, as before (Bédard et al., 2015).

Each faucet and its environment was characterized in detail, including the type of activating device, the connecting pipe material, the faucet internal diameter, the faucet alignment to drain and the drainage efficiency.

Statistical analysis (z-test and multivariate adaptative regression spline [MARSpline]) were performed with Statistica10 (StatSoft). MARSpline regression is a nonparametric analysis in which continuous, categorical, and nominal variables can be added to the model and from which a better fit from a few or all variables is proposed. Significance level was set at p=0.05.

6.3 Results

The epidemiological curve in the NICU before, during and after the outbreak is shown on Figure 6-1. Twenty-seven *P. aeruginosa* infections were reported during the time of the outbreak (2004-2005) and 6 patients died as a consequence of the infection.

Water characteristic as measured in 2005 and in 2013 are presented in Table 6-1. Corrosion deposits were visible on faucets, aerators, sink traps and mixing valves. Observations of the water drainage system demonstrated several accumulations of carbonate scale and biofilm along the drains causing important narrowing of the pipes (Figure 6-2). Clogging of the drains resulting in water stagnation into the sinks was frequently reported by the hospital staff.

Table 6-1 : Mean tap water microbiological and physico-chemical characterization

	During outbreak [2005] (n=4)	Post-outbreak [2013] (n=28)
HPC (CFU/mL)	$5.2\pm3.7x10^{1}$	$3.7\pm10x10^{2}$
Viable (Bact/mL)	$1.1\pm0.3x10^{5}$	$0.9\pm1.5x10^{5}$
Total (Bact/mL)	$1.8\pm0.3x10^{5}$	$1.9\pm2.6x10^{5}$
Residual chlorine (mg Cl_2/L)	n/a	0.05±0.05
Total copper (ug /L)	n/a	570±140
Hot water temperature (°C)	n/a	53±10

a b

Figure 6-2: Transversal view of a drain with accumulations of limestone scale and biofilm (a) and a clean drain (b).

P. aeruginosa was recovered by cultivation from 32/57 (56%) sink drains, 3/56 (5%) faucet swabs and 5/16 (31%) aerators during outbreak investigation. A total of 66 isolates of *P. aeruginosa* were available for PFGE testing, resulting in the identification of 51 different genotypes and variants: seven exclusively from clinical isolates and 32 exclusively from environmental isolates. The dominant genotype (no 4) and probably related variants (only one band difference) were shared by twelve clinical and five environmental isolates including four aerators and one drain. Two other genotypes were shared between the environment and clinical isolates: genotype no 5 was shared by a patient isolate and a swab from the faucet and genotype 16 was shared by one patient isolate and swabs from two drains. All water samples during the

outbreak and swabs from other environmental surfaces and solutions were negative. Survey done on healthcare worker hands was unrevealing.

The post outbreak investigation results are presented in Table 6-2. Results obtained by qPCR detection revealed dramatically higher positivity for all sampling sites. *P. aeruginosa* was detected in the water and the biofilm from the corresponding aerator and drain for 6 faucets and 12 faucets had 2 positive sites (water/aerator, water/drain or aerator/drain) (Table 6-2).

Figure 3 shows the mean concentrations of copper and residual chlorine and the hot water temperature at sampled taps for *P. aeruginosa* positive and negative water from faucets as evaluated by qPCR method (Table 6-2). The impact of various environmental parameters on *P. aeruginosa* detection is presented in Table 6-3. For each parameter, the positivity in the water and corresponding aerator and drain swabs are presented. Three additional faucets were sampled for water only and included for the water samples analysis. In order to evaluate the effect of all factors combined on the *P. aeruginosa* load detected in water by qPCR, a multivariate regression (MARSpline) was conducted on all variables from Table 6-3 together with hot water temperature, copper and residual chlorine concentration. Only the aerator positivity was found to be a significant variable in the model response to predict level of contamination of the water.

Table 6-2 : Proportion of *P. aeruginosa* culture and qPCR positive post-outbreak samples for each type of sampling site and for two or more corresponding sampling sites

				Proportion of sinks with corresponding positive samples for two or more sampling locations			
	Water	Aerator	Drain	Water & aerator	Water & drain	Aerator & drain	Water, aerator & drain
Culture	2/28 (7%)	1/28 (3.5%)	16/28 (57%)	1/2 (50%)	0/2 (0%)	0/1 (0%)	0/2 (0%)
qPCR	14/28 (50%)	18/28 (64%)	25/28 (89%)	9/14 (64%)	10/14 (71%)	14/18 (78%)	6/14 (43%)

Table 6-3 : Summary of *P. aeruginosa* occurrence and percentage measured by qPCR in water, aerator swab and drain swab samples grouped by sink environmental design parameters

	Water		Aerator swab		Drain swab	
	N	%	N	%	N	%
Faucet activating device						
Manual with two levers	5/13	38%	6/13	46%	10/13	74%
Manual with one lever	5/8	62%	5/6	83%	5/6	83%
Foot operated	6/10	60%	5/9	56%	9/9	100%
p-value	*0.1*		*0.06*		*0.2*	
Connecting pipes						
Flexible hoses	4/6	67%	4/6	67%	3/6	50%
Copper pipes	11/21	52%	10/18	56%	17/18	94%
Flexible hoses and copper pipes	1/4	25%	2/4	50%	4/4	100%
p-value	*0.2*		*0.3*		*<0.05*	
Faucet internal diameter						
≥ 1 cm	10/21	48%	10/20	50%	17/20	85%
< 1 cm	6/10	60%	6/8	75%	7/8	88%
p-value	*0.3*		*0.1*		*0.4*	
Faucet alignment to drain						
Behind	8/10	80%	6/9	67%	9/9	100%
Direct	2/5	40%	4/5	80%	4/5	80%
Forward	6/12	50%	6/10	60%	8/10	80%
Side	0/3	0%	0/3	0%	2/3	67%
p-value	*<0.05*		*0.2*		*0.1*	
Heterotrophic plate counts						
< 10 CFU/mL	3/11	27%	4/11	37%	10/11	91%
≥ 10 CFU/mL	10/17	59%	12/17	71%	14/17	82%
p-value	*0.05*		*<0.05*		*0.3*	
Speed of water drainage in sink						
Good	6/14	43%	6/13	46%	11/13	85%
Average	8/13	62%	8/11	73%	9/11	82%
Poor	2/4	50%	2/4	50%	4/4	100%
p-value	*0.2*		*0.1*		*0.4*	
Room usage						
Patient care	13/17	76%	9/14	64%	13/14	93%
Other	3/14	21%	7/14	50%	11/14	79%
p-value	*<0.05*		*0.2*		*0.2*	

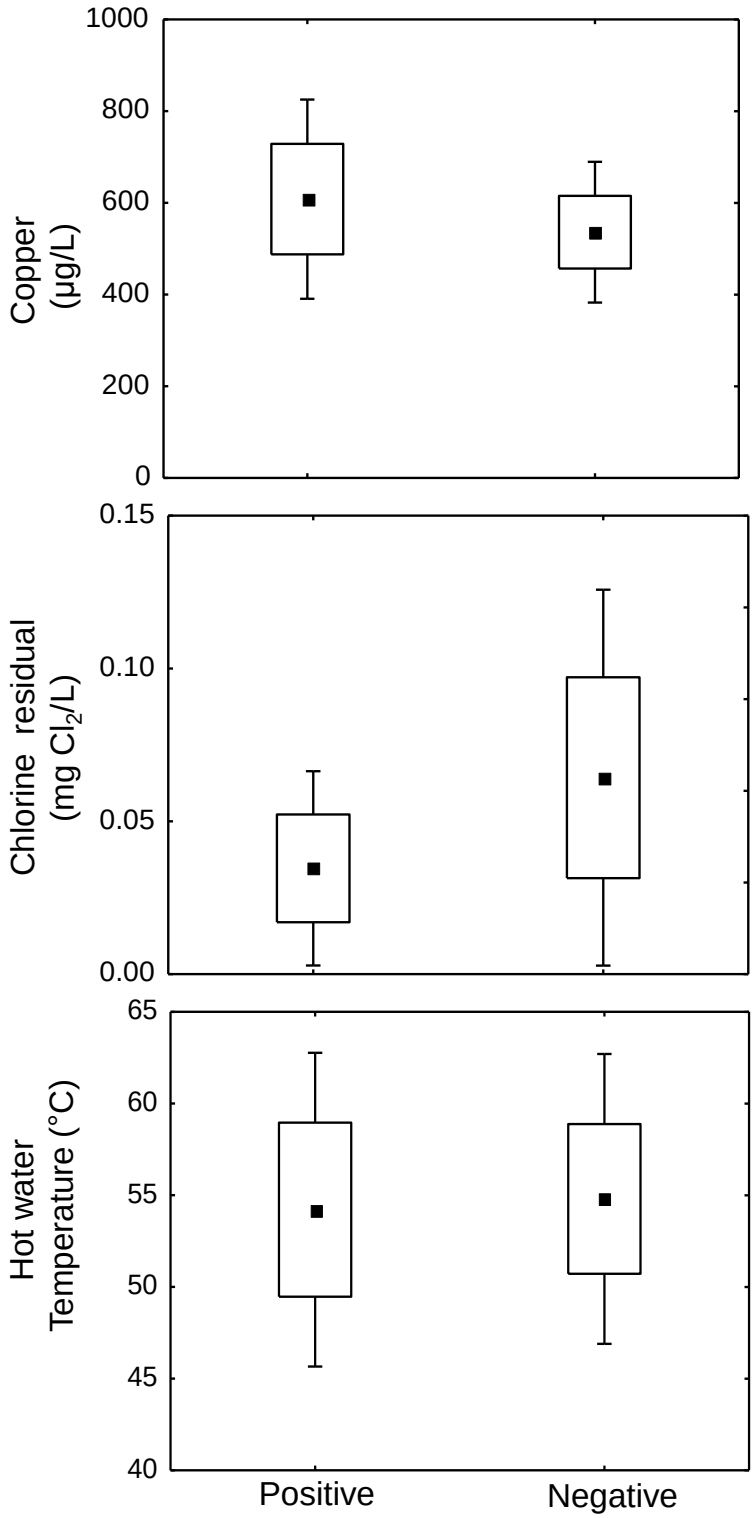

Figure 6-3 : Mean values for copper concentration, chlorine residual and hot water temperature for positive and negative *P. aeruginosa* water samples as measured by qPCR (n=28).

6.4 Discussion

The environmental survey and the genotyping results confirmed the water system as the source of the neonatal outbreak. Common strains were identified between the clinical and water system samples, notably between 12 patients and the sink aerator where the milk bottles were prepared. A drastic reduction in the number of cases was observed after the implementation of corrective measures preventing the transmission to patients (Figure 6-1) without addressing the root cause of the contamination. The high level of aerators positivity with 4 of the 5 positive aerators harboring strains shared with clinical isolates led to the hypothesis of retrograde contamination from the drain was proposed. The accumulation of biofilm and scale depicted on Figure 6-2 and the reported recurring problems associated to drain clogging could be the source of backsplashing of contaminated drains on the faucet aerators. Splashing was observed on material stored in the close vicinity of some sinks. Some investigators have proposed that, in highly contaminated sink drains, water splashes contaminate hands, environment, health care materials as well as faucets in a retrograde way (Hota et al., 2009). To address the hypothesized root cause of the system contamination (i.e. water drainage defect), drains in the NICU were replaced and storage of material underneath the sink or within a perimeter of 30 cm around the sink was prohibited.

Despite the established link between aerators and drain swabs and clinical cases, *P. aeruginosa* could not be isolated from water. This may be explained by the water sampling protocol used during the outbreak. Samples were collected after flushing the tap for 30 s and detection was performed by traditional culture methods. Recent evidence of higher levels of HPC and *P. aeruginosa* in the first flush volume within the faucet compared to water from the system has been shown (Cristina et al., 2014; Lipphaus et al., 2014). Also, bacteria present in the water distribution system are exposed to stressors such as chlorine, copper and high temperatures (Bédard et al., 2014; Dwidjosiswojo et al., 2011). Since samples were a mix of hot and cold water, the presence of those three stressors could have influenced culturability and therefore the ability to detect the presence of *P. aeruginosa* by culture in water samples. The post-outbreak sampling campaign was conducted on the cold water system only, without preflushing. The detection was performed by both culture and qPCR, to improve detection of cells stressed by drinking water conditions and hereby establish the water contamination. This is the first report of *P. aeruginosa* occurrence in hospital water measured by qPCR in water and biofilm samples. We

have demonstrated that drinking water stressed cells are not detectable by culture but are measured by qPCR and can recover culturability when provided with favorable conditions (Bédard et al., 2014).

Total and viable cell counts were comparable between the two sampling campaigns, but HPCs were 1 log higher during post-outbreak sampling measurements. This is likely attributed to the different sampling protocol, where first flush was sampled in the post-outbreak campaign. Lautenschlager et al. reported a 31-fold increase in culturability for water sampled in the first flush (0.62%) compared with water from the system (0.02%) (Lautenschlager et al., 2010).

The follow up water system investigation showed the persistence of a low level *P. aeruginosa* contamination in the system. The modified sampling protocol led to 2/28 positive water samples by culture, an increase compared to outbreak investigation results but still a very low positivity. Water positivity increased drastically when measured by qPCR. As revealed by the MARSpline analysis, the aerator positivity was a significant variable to predict detected levels of *P. aeruginosa* qPCR. When the aerator was positive, mean concentrations of *P. aeruginosa* in water were 2 logs higher than in faucets where the aerator was not positive, showing the link between the aerator and the water contamination.

Mean copper concentration in water was not significantly different between qPCR positive and negative samples (Figure 6-3a). In this study, copper concentration in water was higher (570 µg/L) than the concentration reported to inhibit *P. aeruginosa* growth (250 µg/L) (Bédard et al., 2014; Dwidjosiswojo et al., 2011), but lower than those reported as lethal to *P. aeruginosa* (Teitzel & Parsek, 2003). Furthermore, studies have shown development of resistance after prolonged exposure to copper ions, with *P. aeruginosa* cells maintaining their viability despite elevated copper concentrations. Growth is observed until the minimum inhibitory concentration of 127 mg/L is reached, more than 100-fold the concentrations present in the sampled water (Teitzel & Parsek, 2003). Copper concentrations observed in drinking water would therefore affect culturability without affecting the viability. These cells have the capacity to recover culturability once copper stress is reduced (Dwidjosiswojo et al., 2011).

Mean residual chlorine (Figure 6-3b) was slightly more elevated in negative samples, but was not found to be a significant factor for qPCR positivity as analysed by MARSpline. Hot water temperature mean values were also comparable between positive and negative samples for *P.*

aeruginosa (Figure 6-3c). Temperature in hot water system can affect water and aerator positivity if it is high enough to perform thermal disinfection. In the present study, hot water temperature were below 65°C, a temperature lower than required for thermal disinfection of water and biofilm (Park et al., 2011; Van der Mee-Marquet et al., 2005).

Further analyses performed on qPCR positive water, aerator and drain swabs samples revealed the impact of environmental parameters (Table 6-3). The type of faucet activating device, the faucet internal diameter and the speed of drainage did not lead to statistically different *P. aeruginosa* positivity of neither the water nor the aerator and drain biofilm. The water and the aerator positivity were not significantly impacted by the connection pipe material, although it was reported as significant in previous studies (Charron et al., 2015; Walker, J. T. et al., 2014). As stated previously, copper affects culturability of *P. aeruginosa* and previous studies evaluated positivity based on culture detection method. The present study suggests that viable and total *P. aeruginosa* water contamination is not significantly influenced by the connecting material.

However, faucet alignment to drain configuration led to significantly different positivity of the water. Results suggest that water from faucet aligned behind the drain have higher rate of contamination by *P. aeruginosa* than other configurations. All three faucets aligned aside from the drain were negative for *P. aeruginosa*, but the sampling size is too small to conclude. Previous studies have associated direct water flow into the drain as a source of retrograde contamination of the faucet and changed the sink design to avoid alignment between the faucet and the drain (Breathnach et al., 2012; Hota et al., 2009; Schneider et al., 2012). However, to our knowledge, no other study reported the impact of the actual positioning of the faucet to the drain on positivity. Drains have high rates of contamination by *P. aeruginosa* (Döring et al., 1991; Hota et al., 2009; Schneider et al., 2012) and the risk of retrograde contamination can be managed by ensuring appropriate design of the sink and maintenance of efficient drainage of the sink. Further investigation with a larger number of each configuration will be needed to determine the optimal drain positioning with relation to the faucet.

Samples from taps with HPC higher than 10 CFU/mL had twice more positivity for *P. aeruginosa* in the water and aerator biofilm samples. Heterotrophic plate counts are used as an indicator of the general microbial quality of the water in main distribution systems (Bartram et al., 2003). Water positivity was also significantly different if the faucet sampled was in a patient

room or in outside patient rooms, with higher positivity observed in patient rooms. Detailed utilization survey of both faucets and drains in sampled rooms would help provide an explanation for this finding. Ehrhardt et al. reported higher positivity for NICU faucets (71%) vs faucets samples outside of NICU (12.5%) (Ehrhardt et al., 2006), but there was no mention of the faucet usage in NICU compared to outside..

Our study was subjected to a number of limitations. First, because of the large number of parameters investigated, some categories had limited number of samples. Second, results apply to one hospital system and may vary in a different setting. Third, the genotyping of the strains in the post outbreak sampling campaign could not be performed. The sampling was conducted in absence of active clinical cases.

Results from this study revealed presence of a low *P. aeruginosa* contamination in various components of the sink environment several years after the resolution of an outbreak. This confirms the difficulty to eradicate *P. aeruginosa* from the plumbing components once contaminated. Importantly, although the bacteria may not be detectable in the water by traditional culture methods, *P. aeruginosa* is present and can recover its culturability under favorable conditions. In a hospital environment, this suggests that failure to maintain good practices or disrupting events such as renovation may act as a promoting factor leading to an increased concentrations and risk of patient exposure. These results demonstrate the importance of defining a clear and detailed protocol to determine the precise maintenance required for water systems, including the wastewater system.

6.5 Acknowledgements

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CHAPTER 7. GENERAL DISCUSSION

This chapter highlights the main findings issued from this research project. The overall objective was to improve our capacity to control contamination and amplification of opportunistic pathogens in hospital premise plumbing to reduce risk exposure, especially for vulnerable individuals. Figure 7-1 summarizes the different steps of the research work and the specific objectives pursued. The first step was to understand two key aspects of the premise plumbing ecology: 1) where to find bacteria within the premise plumbing; and 2) what are the main factors affecting sampling results. Once these aspects were established, the second step was to identify key environmental factors promoting bacteria in water through field investigations of hospital water systems. Based on these results and current literature, the last step was to develop a risk classification system together with a risk characterization tool for hot water systems. Recommendations for ongoing practical risk mitigation measures will be covered as part of the conclusions and recommendations chapter.

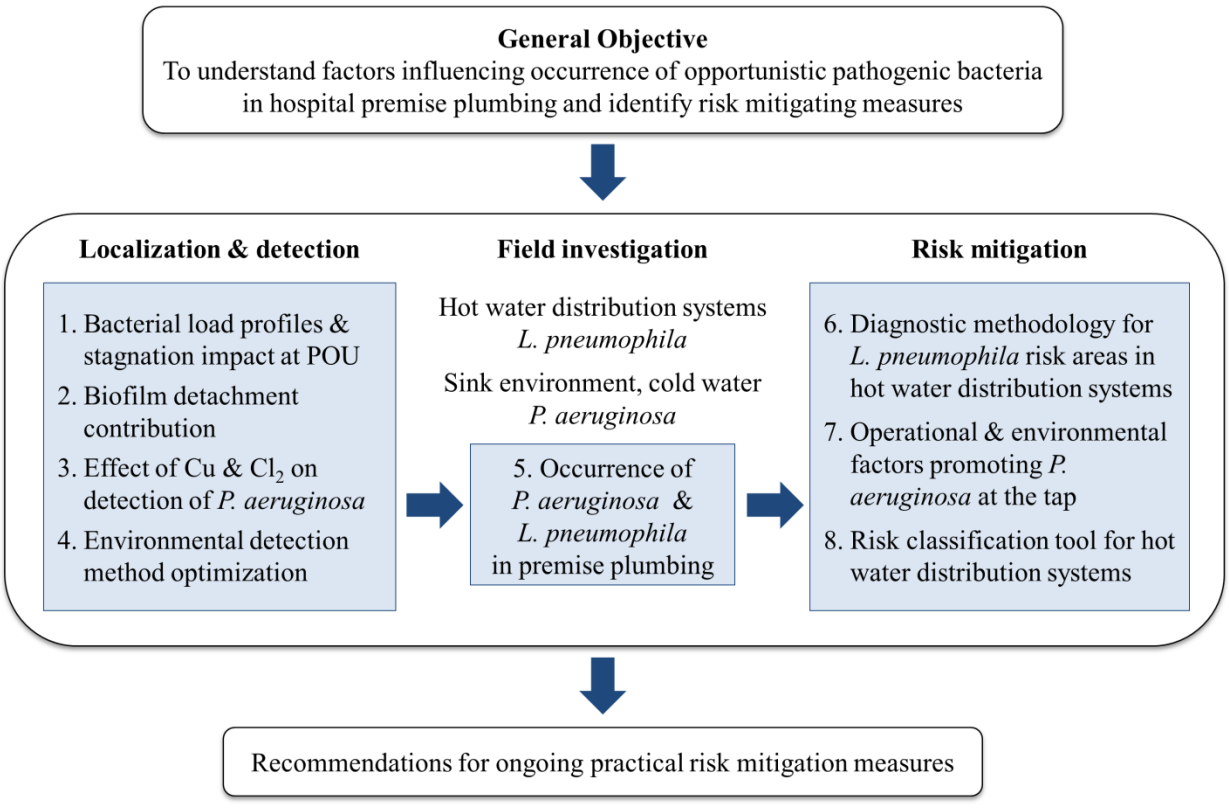

Figure 7-1 : Summary of the research conducted

7.1 Where to find bacteria within the premise plumbing

In premise plumbing, especially in large buildings, there are numerous locations to choose from for sampling. In order to optimize cost and time, only a few points are generally selected for microbiological analysis. The choice of sampling point locations becomes critical to be representative of the whole system, and requires a detailed knowledge of the water system layout together with a thorough understanding of the microorganism's ecology (Environment Agency, 2005).

7.1.1 Distal vs system contamination

There is no consensus if it is better to sample the first volume of water or to flush before collecting water when sampling for opportunistic pathogens. If the objective is to measure the device contribution, the first volume of water is more representative. On the other hand, if the objective is to evaluate the distribution system, prior flushing is required in order to reach the water flowing within the system. A short (1-2 min) pre-flushing is used for both *L. pneumophila* and *P. aeruginosa* (Department of Health (DH) et al., 2013; Health Facilities Scotland (HFS), 2011).

In Chapter 3, amplification between the municipal water inlet and the water at the faucet was observed for heterotrophic plate counts (>5 logs) but minimal for total viable cells (1.6 log). For *L. pneumophila* and *P. aeruginosa*, there was no detection in the municipal water and concentrations up to $6x10^2$ CFU/L and $3x10^3$ CFU/L respectively were recovered in water sampled at the faucet (Chapters 5 & 6). This distal amplification has also been observed in previous studies (Cristina et al., 2014; Serrano-Suarez et al., 2013). Heterotrophic plate count profiles at the faucet demonstrated that more than 50% of culturable cells in the first liter of cold water were recovered from the small volume of water contained within the faucet (15mL). Cell culturability is increased within the faucet as compared to cells from up flow piping (Figure 3-4). This is likely attributed to the proportion of culturable cells in the biofilm (Buse et al., 2014; Rogers et al., 1994b; van der Kooij et al., 2005). Based on findings on the contribution of the biofilm to the water contamination (Chapter 3), it is possible that the bacterial load increases as the water makes its journey through the pipes. Device utilization in the building creates an irregular water demand, inducing stagnation periods of variable duration depending on the

localization within the building. For each stagnation period, results suggest bacteria are released from the biofilm in proportion to the surface-to-ratio of the pipe. This is an important finding in the understanding of the distal contamination and for improving devices design to minimize bacterial contamination. As shown on Figure 3-3, small parts with large projected surfaces are present in faucets. A reduction of these projected surfaces would contribute to reduce the surface-to-volume ratio.

7.1.2 Bacterial load profiles

Still in Chapter 3, a clear declining in HPC profile was observed inthe first 10 liters at the tap, especially in cold water. The important surface-to-volume ratio within the tap compared to its connecting pipes was hypothesized as the explanation for the steep HPC decline observed in the first 50 mL collected. The HPC concentration profiles observed in the connecting pipes and the copper pipe section connecting the device to the subordinate loop were attributed to two factors with opposite effect on cells culturability. First, this terminal section of piping serves only one device and experiences true stagnation between uses of this device, despite water utilization patterns in the building. Such conditions should promote cells culturability present in the faucet. The second factor is the important presence of copper piping, reported to support lower HPC concentrations in the biofilm compared to plastic and elastomeric materials (Buse et al., 2014; Moritz, M.M. et al., 2010; Rogers et al., 1994b). In addition, the culturable fraction in the biofilm grown on copper was reported to be lower than on plastic and elastomeric materials, with 0.9% compared to 15-18% (Moritz, M.M. et al., 2010). The lower culturability of biofilm cells in the copper piping section could explain the lower culturable bacterial load recovered in the water corresponding to this section (Figure 3-4).

More stable profiles observed for viable cell counts from the first liter of water sampled (Figure 3-2), in line with previous studies reporting that the biofilm total cell count was not significantly different for copper or plastic surfaces (Lehtola, M.J. et al., 2004; Wingender & Flemming, 2004). Likewise, the impact of stagnation on viable cell profiles was not as pronounced as observed for HPC profiles. In Chapter 3, it was shown that HPC concentrations were predominantly influenced by the biofilm cells released during stagnation. The increased number of cells in HPCs ($4x10^2$ CFU in the first 15 mL) is however minimal compared to the mean viable cell counts measured in the first 10L ($2.7x10^4$ bact/mL), explaining the smaller impact of

stagnation on viable cell counts. This is an important finding, as short stagnation periods of stagnation are of regular occurrence in healthcare facilities and can impair the water microbial quality as evaluated by HPCs. However, such stagnation episodes have minimal effect on the viable bacteria present in the water.

7.1.3 Hot vs cold water systems

In large buildings, both hot and cold water systems are at risk of bacterial growth. In hot water, heat losses may create areas with ideal growth temperature for bacteria. Forced recirculation may reduce this effect, but hydraulic balancing is a challenge for existing large hospital systems delivering multiple floors and multiple wards. In cold water, there is no forced recirculation and water movement is created only through demand at single devices. Cold water can stagnate for long periods of time, especially in the device connecting pipes, and temperature will equilibrate with ambient room temperature. In Chapter 3, similar profiles were observed for viable and total cells in cold and hot water. When considering specific bacteria, both systems might however not present the same risk factors. *L. pneumophila* is most frequently recovered from hot water systems whereas *P. aeruginosa* is most often recovered in cold water. Nonetheless, *L. pneumophila* has been reported in cold water systems (Arvand et al., 2011; Donohue et al., 2014) and *P. aeruginosa* may proliferate in biofilm from suboptimized hot water plumbing, especially in the device connection piping where water may stagnate and reach temperatures as low as 35°C, ideal for *P. aeruginosa* growth. One limitation of our study was to focus on the occurrence of *L. pneumophila* in hot water distribution systems and of *P. aeruginosa* in cold water systems. Although most of the control points will be in the hot water system, the cold water system should be checked on an annual basis (Health and Safety Executive (HSE), 2013).

7.2 How to find the bacteria present in the system

Findings from this thesis also show the importance of understanding how the sampling protocol and the detection methods selected may influence bacteria recovery and results interpretation.

7.2.1 Optimal sampling protocol

The sampling volume was shown to be critical in interpreting results obtained from sampling (Figure 3-5). This observation stresses the importance to select the appropriate sampling volume

and maintain it throughout the monitoring period. When putting in place a sampling campaign for an outbreak investigation or a risk assessment study, the sampling volume should be defined to suit both the initial investigation and the ongoing monitoring to enable results comparison between sampling events. It should also consider the objective is to evaluate the distal contamination or the contamination present in the system. Based on results from this research project, the selection of a smaller sampling volume on first flush would be preferable for the evaluation of distal contamination to increase chances of bacteria recovery. More than 50% of HPC recovered in the first 2L from a tap were in the first 15mL (Chapter 3). When conducting similar experiments for *P. aeruginosa* detection in a positive tap, 47% of the bacteria were recovered in the first 25 mL (n=2, Appendix 4). Sampling a smaller volume on first flush would allow collection of a volume of water that was less exposed to stressors (chlorine and copper) and maximize chances of recovery. On the other hand, the first volume can be considered to be more indicative of local conditions (i.e. a specific device rather than representative of all points-of-use) The optimization of the sampling volume on first flush for *P. aeruginosa* and *L. pneumophila* is desirable and should include the advantages and disadvantages of smaller sampling volumes.

The selection of a short stagnation time prior to sampling would be more representative of the regular exposure of the patient or user. Results have shown that prior stagnation of the cold water can influence the concentration recovered from the water. No stagnation at all prior to sampling would reduce the recovery, with bacteria stressed from the premise plumbing conditions with copper and chlorine (Chapter 3 & 4). If prior stagnation is not possible, sampling should at a minimum be conducted around the same time of the day every time, with a preference for the early morning, prior to water being used for patient care.

7.2.2 Detection methods

Culture is the most commonly used method for the detection of opportunistic pathogens in water systems from healthcare facilities. However, stressors inherent to drinking water environment will affect culturability and therefore recovery of bacteria present in the water system. In this research project, the impact of chlorine and copper concentrations present in the water was demonstrated for *P. aeruginosa*. As observed in Chapter 4, a chlorine residual between 0.3 and 2 mg Cl_2/L was sufficient to suppress cell culturability. However, viability was only partially affected and immediate recovery was observed once residual chlorine disappeared. Two

important findings can be drawn from these results. First, chlorine disinfection is effective to reduce culturable population by several logs, however typical disinfection curve show a tail off, where a small portion of the population can survive chlorination, as observed for *P. aeruginosa* (Behnke et al., 2011; Xue et al., 2013) and for *L. pneumophila* (Mansi et al., 2014). Second, although cells are under detected by culture due to previous exposure to chlorine, there is no clear answer with regards to the risk associated with these cells. The ability of VBNC cells to regain culturability and infectivity has been reported (Li et al., 2014), but there is still much unknown on the time and conditions required for this recovery. Even if the VBNC cells cannot directly infect susceptible hosts, they are still present in the system and may recover if the disinfection is not optimal or if there are unplanned outages. This effect is compounded by the irregular and highly variable flow patterns encountered in hospitals that may impact the maintenance of the disinfectant residual, the temperature or the hydraulics at each point-of-use, especially in a hospital with wing or unit closures.

As presented in Chapter 4, copper was an important environmental stressor, decreasing cells culturability without affecting their viability. At concentration tested (250 µg/L), culturability decreased by 6.1 log in the span of 2 hours. When collating data from the study presented in Appendix 4 and the data from Chapter 6, the mean copper concentration measured in faucets from hospital premise plumbing was 500 µg/L (n=172), considerably higher than tested concentrations. Only 6% of the sampled taps had copper concentrations below 250 µg/L, with a 20% positivity for *P. aeruginosa* by culture vs a 3% positivity rate for the remaining taps with higher copper concentrations. Despite the small number of water samples with less than 250 µg/L copper, this data suggests an impact of the copper concentration in water on sample positivity as evaluated by culture. This raises a question with regards to the large number of taps that were negative for *P. aeruginosa* in water and had copper concentrations >250 µg/L. These taps may in fact have been positive for *P. aeruginosa*, but undetected by culture methods due to the elevated copper concentrations in water. This is further supported by results obtained on the subset of 31 water samples evaluated by culture and qPCR methods (Chapter 6). The mean copper concentration was 545 µg/L, and a positivity rate of 6% was observed by culture compared to 52% when measured by qPCR. Samples that were positive by culture corresponded to the highest levels detected by qPCR amongst tested samples. Furthermore, the mean copper concentration was not significantly different between negative and positive qPCR results (Figure 6-3). These

results suggest that copper concentration is one of the environmental factors that reduce *P. aeruginosa* culture detection in water from faucets, resulting in an underestimation of the actual load in the water.

Culturability detection has also been shown to be impacted by elevated temperatures. For example, *L. pneumophila* was not recovered by culture for samples where T>55°C (Lee, J. V. et al., 2011; Mansi et al., 2014), but it was still detected in the VBNC state (Allegra et al., 2008; Allegra et al., 2011; Epalle et al., 2015) and by qPCR (Lee, J. V. et al., 2011; Mansi et al., 2014). Overall, detection by qPCR in water samples was not susceptible to temperature, chlorine and copper concentrations values measured samples. This is likely because these stressors were present at levels sufficient to suppress culturability but not enough to impair viability. In addition, results from Chapter 3 and 5 have shown that qPCR detection is not impacted by stagnation. *L. pneumophila* concentrations measured by qPCR were not significantly different for the different stagnation times studied, suggesting no impact of stagnation on recovery by qPCR. This is in line with findings from Chapter 3 showing that viable cell profiles were not as much influenced by stagnation than HPC profiles. This is an important advantage when sampling in healthcare facilities, where prior controlled stagnation is difficult to achieve without involving a lot of time and resources.

Detection by qPCR presents an interesting complement to culture detection in samples where the above environmental stressors are present. However, several studies reported a lack of correlation between culture and qPCR results, with positivity generally higher by qPCR than by culture (Whiley & Taylor, 2014) in systems with suboptimal conditions for inactivation of VBNC cells (Krojgaard et al., 2011; Lee, J. V. et al., 2011). The discrepancies observed between qPCR and culture are attributed to the combination of an underestimation by culture and an overestimation by qPCR due to amplification of all intact DNA, including dead cells. Although qPCR does not differente culturable, viable and dead cells, it has been shown that qPCR levels can be used to verify the impact of corrective actions and demonstrated non-detect by qPCR as good predictors of low risk (Krojgaard et al., 2011). Results of an ongoing monitoring sampling can be used as a monitoring tool. Once the baseline of the system is established, increases in qPCR results will be an indicator of cell growth compared to normal operation and trigger investigation in the area where the change was detected. Results from Chapter 5 suggest that qPCR results were consistent

between repeated sampling conducted on control points for positive and negative systems (Figure 5-5).

7.3 Environmental factors promoting bacterial amplification

Environmental factors specific to premise plumbing may have a promoting effect on bacterial load in the water distribution system. As part of this research project, two key factors were investigated in more details to assess their impact on the water contamination at the tap: variable hydraulic conditions (i.e. periodical stagnation of variable duration, recirculation, flow rates) and high surface-to-volume ratio. In addition, factors specific to the sink environment were also investigated, especially the aerator, the drain, the volume of mixing and the type of device. In this section of the discussion, the findings linking these factors to the increased risk of contamination are covered.

7.3.1 Hydraulic

Recent guidelines stress the need to properly manage hydraulics to ensure homogenous temperature and biocidal control in all areas of the HWDS (Centre scientifique et technique du bâtiment (CSTB), 2012), and system balancing under varying demand should be ensured. A system that is not hydraulically balanced will result in a reduction of temperatures and increased biofilm formation in areas with reduced flow. Temperature and hydraulic management go hand in hand since temperature cannot be maintained at all points in the distribution system unless proper water circulation is ensured. In Chapter 5, a stagnation period of 30 min for insulated pipes was sufficient to drive a temperature drop from 60°C to below 50°C. Such stagnation periods are a regular occurrence for devices and connection piping in healthcare facilities, providing favorable conditions for bacteria to recover from temperature stress. To minimize the volume of water experiencing stagnation, a minimal water velocity of 0.2 m/s is recommended (Blokker et al., 2010; Centre scientifique et technique du bâtiment (CSTB), 2012). For a large hot water distribution system (>1000 L) serving multiple floors and building wings, maintaining this velocity would translate into an approximative 30 min residence time for water in the flow and return loops and 5°C heat loss for systems in circulation. Stagnation, variable flowrates, and suboptimized recirculation are key elements that can prevent consistent delivery of temperature and residual disinfectant at the point of use. Results from the hot water distribution systems

investigations highlight the difficulty to maintain balanced hydraulic in systems of older buildings, where several additions and modifications have been done over the years. Detailed plans of the premise plumbing were not always representative of the current configuration and the localization of faulty sectors difficult to achieve. Based on findings from this research project, the use of temperature monitoring through profiling at the tap and continuous monitoring of the subordinate flow and return loops was proposed as a tool to help understand the hydraulics, quantify the thermal losses of the recirculating system and identify the distribution columns that need balancing.

7.3.2 High surface-to-volume ratio

The important contribution of the biofilm to the bacterial load in the water was demonstrated through the observed correlation between the surface-to-volume ratio in the plumbing and the HPC concentration in water (Figure 3-6). There are two mechanisms involved in cell release from biofilm into water from the premise plumbing (Wingender, 2011):

1) Dispersion: During stagnation, water flow interruption creates a change in the environment dynamic and the equilibrium between the biofilm and the water might be perturbed. In a mature biofilm, live cells may be released in water to colonize other surfaces. Cells released during this process are mostly culturable cells, able to go colonize new surfaces.

2) Erosion and sloughing: The cessation of stagnation is marked by a flow of water, susceptible to erode the biofilm cells that are closest to the bulk water interface. These are the only susceptible cells to this form of detachment. Daughter cells produced at the interface and cells not embedded in the biofilm matrix are prone to this phenomenon. These types of cells would also likely be culturable.

In premise plumbing, *L. pneumophila* and *P. aeruginosa* are adapted organisms that can persist and multiply in the biofilm (Wingender, 2011) and would therefore be susceptible to be released in the water phase following stagnation.

The demonstration of the exponential correlation between the surface-to-volume ratio and the bacterial load in the water brings a different outlook on the installation of smaller diameter pipes to reduce the water flow or to minimize the volume of stagnant water that is not recirculated

(specific to the device). According to the National Plumbing Code, installation of piping with an internal diameter of 6.3 mm is permitted for faucet connections. The surface-to-volume ratio for these connections increases to 6.3. This ratio is comparable to the one observed by Cartier et al 2012 in newer faucets with reduced internal volume (Cartier, Nour, Richer, Deshommes, & Prévost, 2012). Although already suspected, the confirmed contribution of the biofilm to the bacterial load increase at distal points of a water distribution system is key information to orient future work. Because of the limited number of faucets sampled, the sampling should be repeated on a larger sample size. Nonetheless, current findings can already be used to evaluate bacterial control strategies in an established system, interpret the short term effect of disinfection treatments applied to systems and provide insight for future premise plumbing design. In Europe, some manufacturers are already working on improving the internal surfaces of faucets to minimize roughness and area (Walker, J. & Moore).

7.3.3 Sink environment characteristics

As part of the field investigation for *P. aeruginosa* in cold water, each faucet and its environment was characterized in detail, including the type of activating device, the connecting pipe material, the faucet internal diameter, the faucet alignment to drain and the drainage efficiency (Chapter 6). This section of the discussion focuses on reporting the findings with regards to aerators, drains, and type of devices.

Aerators. Aerators vary in design and in material. Literature showed higher contamination of complex structure aerators made of plastic material (Walker, J. T. et al., 2014). These structures provide an increased surface for biofilm colonization and remain wet for prolonged periods of time between uses, depending on the faucet design. As reported in Chapter 6, a high positivity rate (64%) was observed for aerator biofilm when measured by qPCR, compared to 3.5% by culture. Based on findings from Chapter 4, this result is not surprising as several of the aerators were made of metal and visible copper oxide deposits were present. The metal aerators probably inhibited the culturability of the bacteria and limited their growth. However, results by qPCR showed their important presence and the risk of exposure in a growth promoting environment such as complex structures and plastic materials. Furthermore, *P. aeruginosa* concentrations in water were 2 log higher for sinks with a positive aerator. These findings stress the importance of better understanding the role of the aerator in faucet and water contamination. The choice of an

aerator or laminator device should be based on the following criteria to reduce biofilm promoting conditions and risk of exposure: 1) simple structure to minimize surface available for colonization; 2) materials not promoting growth; 3) no flow restriction to allow proper flushing of the faucet and 3) no splashing or aerosolization. Furthermore, the source of the aerator contamination can be from the water or from retrograde contamination from the drainage system.

Drains: Drains have been suggested as a source of cross contamination by several authors (Breathnach et al., 2012; Hota et al., 2009; Levin et al., 1984; Maltezou et al., 2012; Schneider et al., 2012). In our investigation, more than 50% of drains were positive for *P. aeruginosa* by culture and close to 90% by qPCR. Drains can act as an important reservoir for waterborne pathogens as they provide ideal conditions for biofilm growth. Although self-disinfecting drains can be installed, they are costly and should be prioritized for highly vulnerable patient areas (Schneider et al., 2012). Risk mitigation from the water drainage system involves: 1) managing the design and maintenance protocols to avoid physical contact between the drain content and the water delivery system, the staff or the patients; 2) ensure proper utilization of wash hand station, avoiding discharge of contaminated fluids into the drain. With regards to design, previous studies have associated direct water flow into the drain as a source of retrograde contamination of the faucet (Breathnach et al., 2012; Hota et al., 2009; Schneider et al., 2012). These findings brought changes to the sink design to avoid alignment between the faucet and the drain. Guidelines in Scotland refer to the potential aerosolization from the drain if taps discharge directly into a drain hole that can cause splashing and recommend the tap outlet to not point directly into the drain (Health Facilities Scotland (HFS), 2007). In our study, we documented the faucet alignment to drain configuration for each sampled sink. Drain alignment was significant factor for *P. aeruginosa* water positivity. In the studied systems, two principal configurations were observed when facing the sink: the drain was positioned to the side of the faucet or the drain was aligned with the faucet. For drains aligned with the faucet spout, the positioning was either behind, directly under or in front of the faucet spout. Results suggest that water from faucet aligned and positioned behind the drain present a higher rate of contamination than other configurations. Depending on the flow rate and faucet discharging angle, the water stream may become in contact with the drain if they are positioned within the same axis. Only sinks with a drain positioned to the side of the faucet had negative water samples for *P. aeruginosa*. However, the number of observations in our study limits the strength of our conclusions and further

investigation should be conducted. Positioning the drain with an offset to the side from the water stream coming from the faucet avoids direct flow of water into the drain and therefore, the generation of contaminated aerosols susceptible to deposit on the aerator and incorporate into the biofilm. Proper utilization of hand wash stations is also a key mitigation measure, reducing the risk of pathogen incorporation and proliferation into the biofilm. A study following an outbreak with *Elizabethkingia meningoseptica*, an opportunistic pathogen that can form biofilms and survive in water, was associated to the misuse of hand hygiene sinks (Balm et al., 2013). Hand hygiene sinks that were used for disposal of patient fluids or rinsing patient care items were more often contaminated than other sinks. Recently, it was suggested to reconsider the location of waste clinical material disposal site as part of an infection prevention approach (Walker, J. & Moore). Results from our field investigation revealed significantly higher water positivity, for *P. aeruginosa* for faucets located in patient rooms (Chapter 6, Table 6-3). In light of our preliminary observations during field investigation, we hypothesize that the type of usage of the sink might play a role in the higher contamination by *P. aeruginosa*. To our knowledge, there is no other report comparing faucet positivity for *P. aeruginosa* in patient rooms' vs other sites. Another study showed higher positivity in ICU patient rooms vs non-ICU patient rooms during an ICU outbreak investigation (Ehrhardt et al., 2006). These findings provide further insights in corrective and preventive measures that can be implemented to minimize the risk of retrograde contamination from the drain to the faucet and its environment. **Type of device**: There are numerous types of faucets available for hand washing stations. They can be divided in three large categories based on their mode of activation: electronically activated faucets, manual or conventional faucets and pedal or foot-operated faucets. In the review presented in Chapter 1, a summary of reported percent positivity by *P. aeruginosa* for various manual and electronic faucet devices as well as sink drains is was presented (Table 2-1). Despite the numerous studies reporting electronic faucets has contaminated, little information on the specific characteristics from the electronic faucets. Based on the findings from this research project and literature reports, four factors associated with electronic faucets can increase the risk of infection: 1) the presence of a thermostatic mixing valve; 2) the presence of complex parts in the mixing valve of some models, providing large surface area at mitigated temperatures; 3) the use of materials promoting biofilm growth, again reported in some models with complex mixing valves; 4) the activation requiring the hands to be placed under the spout to trigger the flow. The last factor

exposes the hands of the users to the first volume contained in the tap, shown to be highly contaminated (Chapter 3 & Appendix 4). Findings from this project show that extensive flushing is not required to significantly decrease bacterial load exposure and that the simple fact of manually activating the levers to open the water flow is sufficient time to flush the first 15 to 50 ml of water contained in the tap. Nonetheless, some electronically activated faucets have simple designs and minimal presence of plastic or elastomeric materials compared to manually activated faucets. One of the key learning from this research project is the complexity and multiplicity of factors contributing to faucet contamination. It is not as simple as choosing the best mode of activation, but it is rather a question of understanding the internal design and materials of the selected taps as well as minimizing the volume of stagnant mixed hot and cold water.

7.4 Risk assessment and diagnostic tools for hot water systems

Risk associated with the presence of *L. pneumophila* in healthcare facilities hot water distribution system is often unknown until cases occur and an investigation is required. The ongoing monitoring frequency for *Legionella* varies over a wide range, from weekly to annually depending on the country (Appendix 3, Table A-3.1). Monitoring is done through culture detection, which may not reveal residual population stressed by chlorine, temperature or copper (Chapter 5). In addition, microbiological sampling is costly, and results are highly dependent on the sampling protocol and environmental conditions prevailing at the time of sampling.

In parallel, all guidance documents include objectives or obligations to maintain optimal operating temperatures at critical points in the hot water distribution systems. It is recommended that temperatures be monitored at hot water heater outlets and at each return loops from a continuous to a monthly basis, depending on the country, and from weekly to monthly at representative points-of-use in healthcare facilities. However, despite key control points meeting temperature requirements, the detailed investigations conducted as part of this project have revealed suboptimal temperatures in subordinate sectors (Table 5-1). These areas are at risk for the presence of *L. pneumophila* but would not be identified if monitoring is only conducted at key control points, as specified in several guidelines. Based on findings from this research and on a review of existing guidelines and regulations, we proposed a diagnostic flow chart for existing building based on temperature monitoring (Figure 5-6). The step approach combining temperature monitoring of the hot water distribution system (HWDS) main components and

temperature profiling at points-of-use can be used to determine the overall susceptibility of the hot water distribution system and to identify specific areas of large buildings at risk for *Legionella* proliferation. It is complementary to current monitoring guidelines suggesting continuous monitoring at water heater outlet and return loop and periodic temperature measurement for a few representative points-of-use at a frequency varying from monthly to annually (Appendix 3, Table A-3.1). Temperature monitoring will help understand the hydraulics, quantify the thermal losses of the recirculating system and identify the distribution columns that need balancing. A systematic diagnostic is necessary to identify areas most at risk in hydraulically unbalanced HWDS or in older buildings where original plans and drawings may not be available or renovations and rearrangements have occurred. Monitoring temperature representative points or even all points on a rotating basis (e.g. 20%/year) is time consuming and yet insufficient for rapidly detecting faulty equipment such as defective valves. A single faulty device has been shown to have important effect on temperature of immediate surrounding piping (Appendix 3, Figure A-3.2) but would remain undetected if current guidance temperature monitoring approach is followed given the low monitoring frequency at points-of-use. Continuous monitoring for each return loop will allow detection of risk areas; further investigation by monitoring subordinate flow and return loops in the identified sectors will help identify more precisely areas at risk. Continuous temperature monitoring on individual risers will help detect broken equipment and enables rapid investigation and resolution, preventing the establishment of optimal bacterial growth conditions for prolonged periods of time in between periodic monitoring. Once deficient sectors are identified, temperatures profiles can be used to locate culprit area in the premise plumbing.

To help the interpretation of the results generated through the diagnostic method, a system risk classification was also proposed as part of this research. There is little guidance available for the interpretation of the temperature monitoring results. The selection of classification criteria was based on results from this research project and reported in the literature. Most regulations and guidelines suggest 60°C as the minimal temperature for the water coming out of the water heating unit. Results from Chapter 5 show that for systems where temperatures were above 60°C at the water heating unit exit and 55°C across the network, there was no detection of *L. pneumophila* neither by culture nor qPCR methods. Although higher temperatures would increases control of *L. pneumophila*, we need to think in terms of contact time between the

bacteria and the elevated temperature. The log reduction of *L. pneumophila* is a function of contact time at a given temperature (Allegra et al., 2008; Stout et al., 1986). This is why in addition to a minimum temperature at the water heater outlet, the percentage of time the temperature is met together with the total heat loss in the system are important. These criteria provide conditions to maximise the contact time at temperatures above 55°C throughout the system, except for the distal volume. The shorter the connection between the point-of-use and the subordinate flow and return loop, the quicker the system temperature will be reached at the faucet. If the recirculation is effective, the distal volume should be minimal and flushed within one minute, corresponding to a volume of 6 L for faucets with an average flow rate of 6 L/min (Charron et al., 2015; Facility Guidelines Institute (FGI), 2013). A larger connecting volume will lead to longer time to achieve system's temperature and increase the level of risk. The proposed risk classification system needs to be applied on a larger scale to validate the different criteria. However, it is a step forward to help healthcare facilities interpret temperature monitoring data and prioritize areas where corrective measures should be implemented.

CONCLUSIONS AND RECOMMENDATIONS

This research project sought to improve our capacity to limit the proliferation of opportunistic pathogens in hospital premise plumbing through a better control of operational and environmental factors. Several questions were initially raised: Where are the bacteria located in the premise plumbing? Do short stagnation periods impact bacterial load in the water? Is the presence of common drinking water inhibitors masking the presence of bacteria when measured by traditional culture methods? Which factors lead to the amplification of *P. aeruginosa* at the point-of-use? How can *L. pneumophila* be controlled to reduce the risk of healthcare related infections? The results from this research bring multiple elements to answer these questions. The following conclusions were reached regarding the hospital premise plumbing general microbial contamination:

- The strong correlation observed between the surface-to-volume ratio of the pipes and the bacterial concentration in the bulk phase suggests the release of bacteria from the biofilm as the main contributor to the increase in culturable cells concentrations after one hour stagnation.
- Similar bacterial load profiles and concentrations were recovered from hot and cold water systems. Both water systems present operational and environmental factors susceptible to promote amplification of culturable cells at distal sites.
- More than 50% of the culturable bacteria in a one liter sample are recovered from the first 15 mL collected when opening the tap. This information highlights the importance of the sampling volume and its role in interpreting measured bacterial loads.
- A short 250 mL flush before using a tap will significantly reduce exposure to the elevated initial bacterial load observed in the bacterial profiles. However, this practice is difficult to implement with electronically activated faucets where hands will be exposed to the first volume of water while activating the faucet.

The literature review on *P. aeruginosa* in drinking water systems revealed its adaptability to the premise plumbing environment and its capacity to integrate the biofilm from the faucet and the drain. Despite the difficulty to eradicate it once established in the biofilm, its presence in water

could not always be detected by culture. The effect of drinking water stressors on the culturability of *P. aeruginosa* was evaluated and led to the following conclusions:

- *P. aeruginosa* exposed to chlorine and copper ions are unlikely to be measured by standard culture methods, or even newer quicker response methods based on enzymatic reactions. This finding highlight the importance of using an appropriate monitoring protocol, including sampling volume, sample treatment and analytical method to assess the direct and indirect risks of exposure to *P. aeruginosa* in premise plumbing. Copper ion and residual chlorine concentrations in the systems investigated surpassed the concentrations suppressing culturability.
- The optimization of a qPCR method including a DNA extraction protocol tailored to premise plumbing water samples was possible and allowed the detection of *P. aeruginosa* despite culture suppressing operational conditions: temperatures above 50°C, presence of a residual chlorine (between 0.3 and 2 ppm) and mean copper concentrations of 500 µg/L.
- Detection by qPCR represents an interesting monitoring tool to complement culture, especially to interpret changes to the baseline values in a drinking water system. An increase in the qPCR signal indicates cell multiplication and proactive action could be taken to resolve the issue before it is detected by culture methods and reduce the risk associated with the presence of *P. aeruginosa* in water.

As part of the research, field investigations were conducted in the cold water system (targeting *P. aeruginosa),* and in the hot water distribution system (targeting *L. pneumophila*). Results from the investigation of the cold water system at the faucet corroborated findings obtained from the lab-scale study, and led to the following conclusions:

- *P. aeruginosa* contamination in various components of the sink environment was detected several years after the resolution of an outbreak, illustrating the difficulty to eradicate *P. aeruginosa* from the plumbing components once established.
- A low *P. aeruginosa* contamination of water was detected by culture while qPCR detection revealed 50% positivity of water from faucets sampled. Although the bacteria may not be detectable in the water by traditional culture methods, *P. aeruginosa* is present and can recover its culturability under favorable conditions. In a hospital

environment, this suggests that failure to maintain good practices or disrupting events such as renovation may act as a promoting factor leading to an increased concentrations and risk exposure for individuals and surrounding patient care material.

- *P. aeruginosa* was recovered in the water, and in the biofilm from the aerator and the drain for 21% of sinks. Drain alignment, the type of device and water microbial quality were significant factors for water positivity, whereas aerator positivity was a significant variable to predict the load of *P. aeruginosa*. An appropriate choice of device as well as room layout will contribute to minimizing the colonization of taps by *P. aeruginosa* and the risk of exposure for patients.

In hot water distribution systems, results from the multiple systems investigated led to the development of a risk diagnostic flow chart and a risk classification table for the presence of *L. pneumophila*. The following conclusions can be drawn from this portion of the work:

- A step approach combining temperature monitoring of the hot water distribution system (HWDS) main components and temperature profiling at points-of-use can be used to determine the susceptibility of overall hot water distribution system and specific areas of large buildings to *Legionella* proliferation. This approach can guide corrective system interventions and serve as a basis to justify preventive risk reduction actions and select sampling points for *L. pneumophila* monitoring.

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

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

The conclusions of the present work led to the formulation of recommendations for risk mitigation of opportunistic pathogen proliferation in hospital premise plumbing:

- At the sink:
  - Faucet design should minimize: the surface area in contact with water, the stagnant mixed hot and cold water volume and the presence of plastic or elastomeric materials.
  - Thermostatic mixing valves should be installed on faucets only if a risk assessment has evaluated that its use by vulnerable patients causes them to be at risk of scalding. If a thermostatic valve is to be installed, then it should be integral to the body of the device to minimize the stagnant volume.
  - The length of the connection piping under the sink, from the device to the wall, should be minimized to reduce the volume of water in presence of a larger surface-to-volume ratio.
  - Flow straighteners and aerators should be avoided as much as possible, as recommended by the Department of Health in UK (Department of Health (DH) et al., 2013).
  - A drain cleaning program should be implemented to avoid plugging due to low usage or low flow.
  - Putting hands under the first flush of water should be avoided.
- Specific to the hot water distribution system:
  - Water recirculation should not be interrupted periodically for energy savings; it is critical to maintain constant temperature regimen throughout the systems to prevent prolonged periods at temperatures promoting bacterial growth. Maintaining high temperatures provides *L. pneumophila* control, not eradication.
  - Stagnant volume in the terminal end piping up flow from the device should be minimized by connecting the return loop as close as possible to the device.
  - Temperature should be monitored continuously at each subordinate return loop prior to the principal return loop.
  - Temperature profiling should be performed at a large number of points confirming the extent (volume) and nature (systemic or distal) of undesirable temperatures in HWDS to

guide *L. pneumophila* monitoring decisions. The staged approach based on inexpensive and easily implemented temperature profiling can optimize resources and funds allocation by directing efforts towards high risk areas.

- Risk assessment and sampling:
  - A water safety plan should be implemented for each healthcare facility. The water safety plan will drive an engineering risk assessment of the water system and should include a risk assessment step prior to modifications to the system (device or plumbing).
  - For systems with in-building disinfection, disinfectant application should be done diligently and monitored closely to avoid periods of disinfectant depletion. In presence of disinfectant within the water, the use of qPCR detection for ongoing water monitoring should be considered for early detection of opportunistic pathogen increases.
  - To maximize the recovery of planktonic bacteria, sampling should be performed during periods of no use or low use of water.
  - Ideally, first volume of water at the tap and after flushing samples should be collected to assess whether the contamination is distal (a device) or systemic. For distal sampling without flushing, the sampling volume should be kept to 1L or less to remain representative of the point of use and to increase chances of recovery.
- New buildings:
  - A commissioning procedure for water systems in new or renovated building should be developed to assess opportunistic pathogen risk prior to building occupation. The procedure could be based on the diagnostic flow chart proposed in this work and expanded to include both cold and hot water systems.
  - The room design should include the following considerations: 1) minimize the number of taps to avoid underused water outlets and low throughput; 2) choose sink design to avoid splashing from water flowing into the drain; 3) if splashing is unavoidable, position the bed and patient care material outside of the splashing radius.

This doctoral work also sparked new questions and ideas for future research venues. It would be interesting to:

- Evaluate the impact of copper-silver ionization disinfection on *P. aeruginosa* and *L. pneumophila* using detection methods able to detect viable cells since copper suppresses culturability.
- Investigate the hydraulic and temperature distribution within the pipes for systems when cold water is added to the hot water coming out of the heater. In some systems, the hot water heater delivers water at temperatures higher than 60°C and the water temperature is then levelled to 60°C by adding cold water prior to hot water distribution in the system.
- Evaluate further the impact of hot water distribution system temperature regimen on the establishment and proliferation of other opportunistic pathogen in the water systems, including *P. aeruginosa* at the faucet.
- Pursue hot water distribution system hydraulic investigations to better understand the impact of secondary recirculation pumps added in sectors where temperatures cannot be maintained. Early results have shown flow inversion in sectors with a secondary pump.
- Define an ongoing monitoring program to quickly detect faulty devices such as showers and electronic faucets with broken valves or recirculation pumps.
- Study the impact of ward closures on microbial water quality and the biofilm at the points-of-use.
- Understand the effect of high water flow rates on the cell release from the biofilm into the water during short periods of stagnation.
- Verify the effect of preventive and curative disinfection treatments applied in hospital premise plumbing on the biofilm bacterial density and diversity.

The role of the built environment as a source of healthcare acquired infections is increasingly recognized within hospitals. Sustained research efforts will help to further improve our understanding of these complex systems, where multiple variables influence the proliferation of opportunistic pathogens. A multidisciplinary outlook and a root cause analysis approach are necessary to develop and implement successful risk management plans.

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APPENDICES

APPENDIX 1 : SUPPLEMENTARY MATERIAL, ARTICLE 2: IMPACT OF STAGNATION AND BIOFILM ON BACTERIAL LOAD PROFILES IN WATER FROM PREMISE PLUMBING OF A LARGE BUILDING

Journal: Environmental Science and Technology

Title: Impact of stagnation and biofilm on bacterial load profiles in water from premise plumbing of a large building

Authors: Emilie Bédard, Céline Laferrière, Eric Déziel and Michèle Prévost

Number of pages: 3

Number of figures: 2

Figure A-1.1

Figure A-1.2

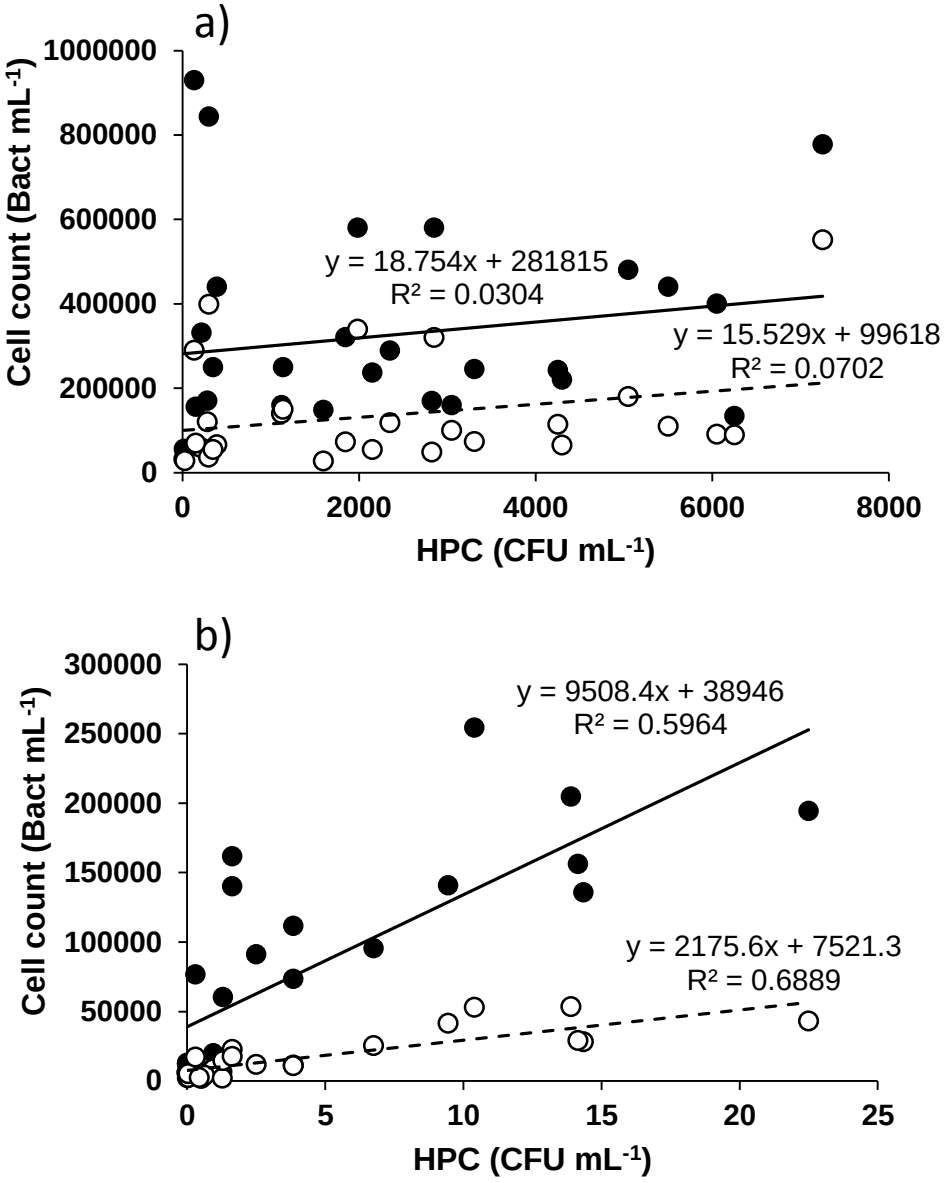

Figure A-1.1: Heterotrophic Plate Counts correlation with total cell counts (full circle) and viable cell counts (empty circle) in tap water at a) first flush volume (15 mL) and b) after 2 minutes flush.

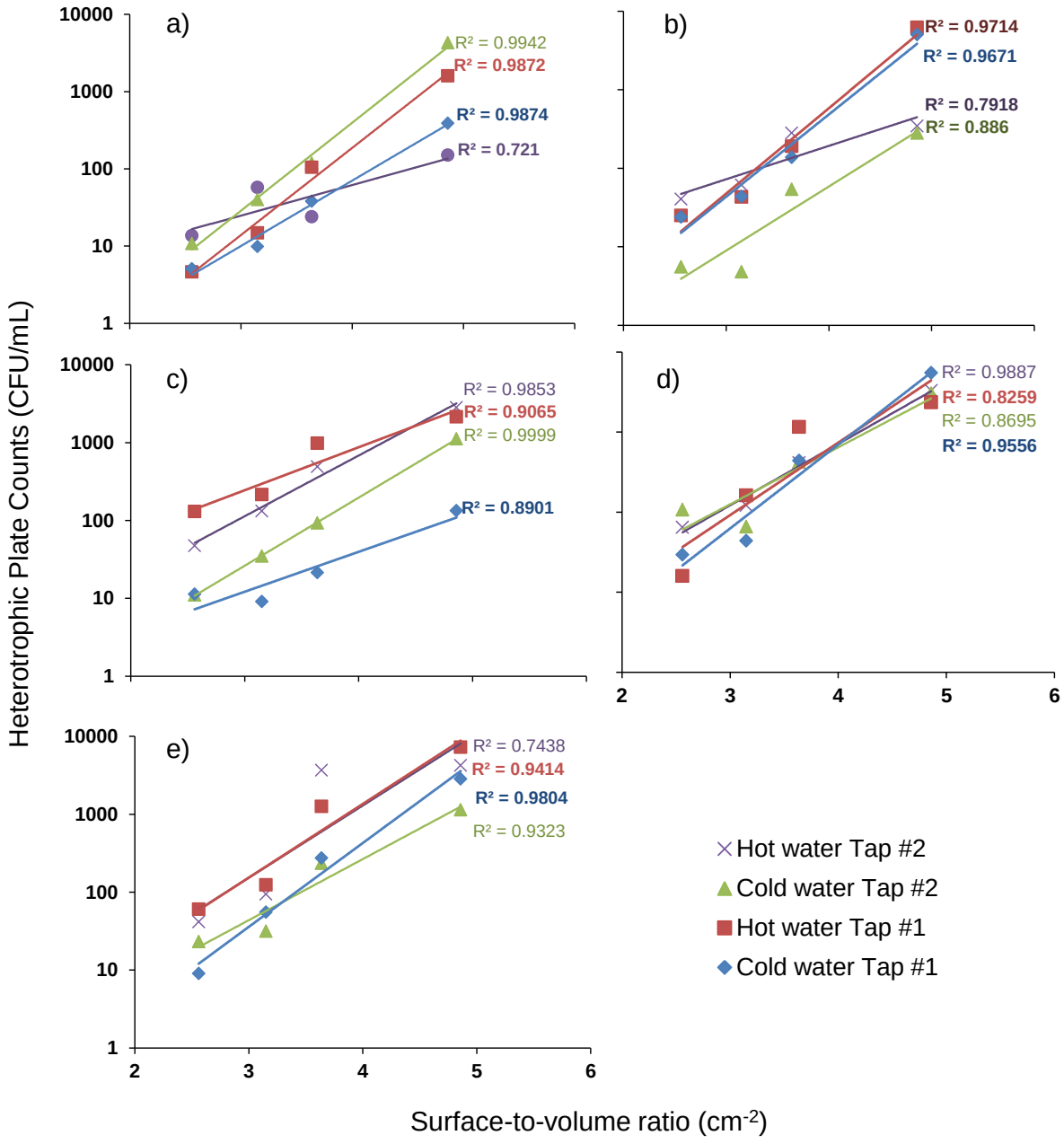

Figure A-1.2: HPC concentration as a function of surface-to-volume ratio in cold and hot water at two different taps (Tap #1, Tap #2) for controlled stagnation time of 24h (a), 48h(b), 72h (c), 120h (d) and 240h (e)

APPENDIX 2 : SUPPLEMENTAL INFORMATION, ARTICLE 3: RECOVERY OF *PSEUDOMONAS AERUGINOSA* CULTURABILITY FOLLOWING COPPER- AND CHLORINE-INDUCED STRESS

Journal: FEMS Microbiology Letters

Title: Recovery of *Pseudomonas aeruginosa* culturability following copper- and chlorine-induced stress

Authors: Emilie Bédard, Dominique Charron, Cindy Lalancette, Eric Déziel and Michèle Prévost

Number of pages: 2

Number of figures: 1

Figure A-2.1

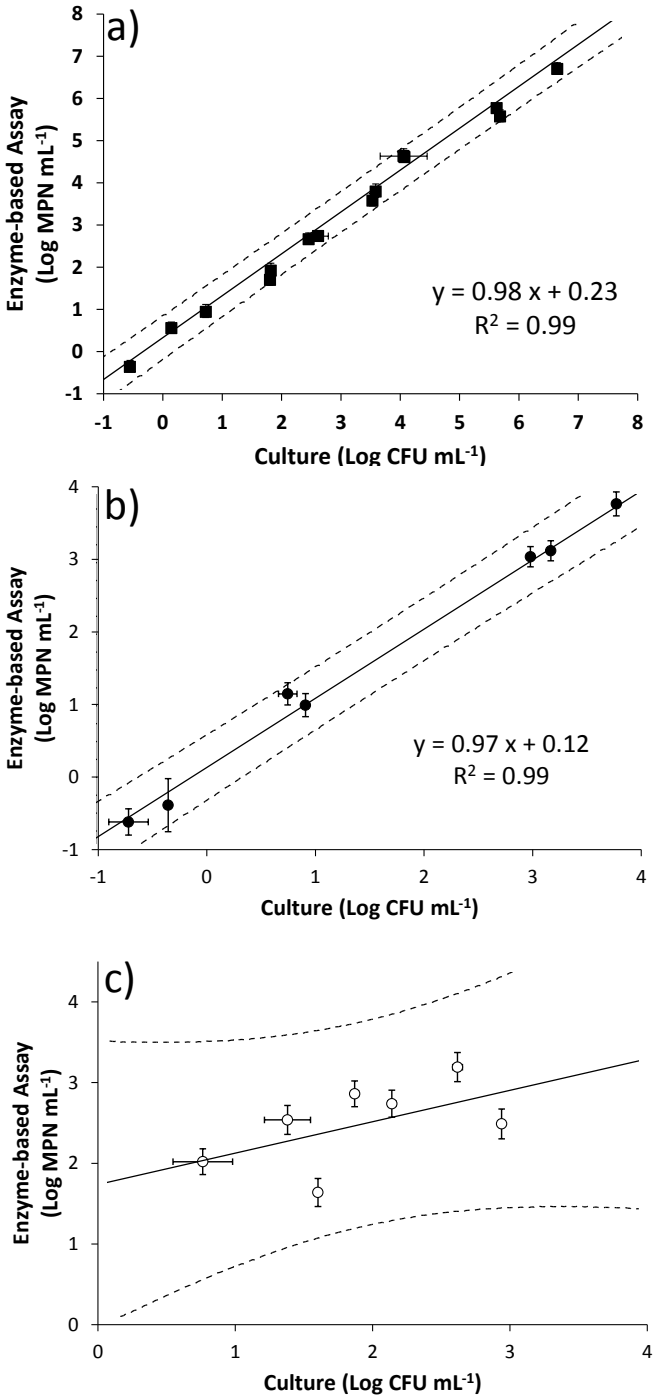

Figure A-2.1 Log transformed data for paired measurements using culture and the enzyme-based assay in absence of chlorine or copper, same data as presented in Fig. 2 (a), in presence of free chlorine (b) and copper ions (c), same data as presented in Fig. 5. Linear regression is represented by the black line with the equation and R^2 value. Prediction interval (95%) is represented by the dotted lines. Error bars indicate standard deviation.

APPENDIX 3 : SUPPLEMENTARY INFORMATION, ARTICLE 4: TEMPERATURE DIAGNOSTIC TO IDENTIFY HIGH RISK AREAS AND OPTIMIZE LEGIONELLA PNEUMOPHILA SURVEILLANCE IN HOT WATER DISTRIBUTION SYSTEMS

Journal: Water Research

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

Authors: Emilie Bédard, Stéphanie Fey, Dominique Charron, Cindy Lalancette, Philippe Cantin, Patrick Dolcé, Céline Laferrière, Eric Déziel, Michèle Prévost

Number of pages: 8

Number of tables: 2

Table A-3.1

Table A-3.2

Number of figures: 3

Figure A-3.1

Figure A-3.2

Figure A-3.3

Table A-3.1: Regulations and recommendations for *Legionella* prevention and control within healthcare buildings

	Temperature control					Initial system characterization (risk assessment)	Sampling sites (T° or *Legionella*)	Monitoring frequency	References
	Water heater	Return loop	System	At point of use	Design specifications				
Australia	≥60°C	Not specified		≤ 45°C childhood & healthcare centers, schools and nursing homes; ≤ 50°C all other buildings	Water velocity ≤ 3 m/s; Max flow rate at point of use device ≤ 9 L/min; Minimize deadlegs, must be flushable during maintenance	Not specified for water systems, only for air handling systems	Not specified	*Legionella*: varies by state, from none to every month	Australian/New Zealand Standard (AS/NZS) 2011
Austria	≥60°C		≥55°C		Thermal disinfection must be possible (70°C); Water heater volume ≤ 1 day of consumption; Avoid stagnant areas in HWDS; **Shutting down recirculation is not permitted**	Yes (risk analysis)	CW; HW outlet; HW at representative POU; Record: time to reach stable T°, final T°, usage frequency, system heat loss	T° and *Legionella*: from weekly to annually, depending on system's risk classification	Austrian Standards Institute 2007
Canada	60°C*	≥ 55°C* (Quebec only)		Showers and bath : ≤49°C; ≤43°C for healthcare and nursing homes (Quebec only)	For systems with 30 m length or 4 stories, temperature must be maintained with recirculation or reheating units; Shutting down recirculation at night is permitted. * Only applies to new building and expansions	Not specified	Not specified	Not specified	NRC 2005, CMMQ/RBQ 2013a, b, RBQ 2014
China	≥60°C	Not specified	≥50°C	≤43°C : pediatrics, geriatric, psychiatric wards, elderly homes, residential care for persons with disabilities; ≥50°C for all others	60°C for ≥ 5min prior to discharge into HWDS; Minimize dead legs and stagnant water	Yes (water safety plan)	According to water safety plan	*Legionella*: According to water safety plan	Hong Kong and The Government of the Hong Kong Special Administrative Region 2012

Table A-3.1: Regulations and recommendations for *Legionella* prevention and control within healthcare buildings (continued)

	Temperature control					Initial system characterization (risk assessment)	Sampling sites (T° or *Legionella*)	Monitoring frequency	References
	Water heater	Return loop	System	At point of use	Design specifications				
Europe	≥60°C (1h/d);	Ideally ≥55°C, no less than 50°C	Not specified	50 – 55°C within 1 min.	Return loop T° always ≥50°C; Water heater T° not <50°C for more than 20 min/day	Yes (risk assessment)	CW, HW outlet, return loop. Use temperature measurements to determine sampling sites	T° and *Legionella*, frequency not specified	The European Working Group for Legionella Infections (EWGLI) et al. 2011
France	>55°C Reg; ≥60°C Reco	>50°C	>50°C	≤50°C in rooms for personal care	For HWDS volume ≥ 3L, needs recirculation; Eliminate dead legs; Maintain water velocity ≥ 0.2 m/s; Connecting pipe volume ≤ 3 L	Yes (risk analysis)	8 to 11 sites: CW, HW outlet, return loop, POUs (representative, greatest pressure loss, high risk patients areas)	Representative and greatest pressure loss POU (1/yr *Legionella, L. pneumophila* & 1/wk T°), return loop (1/yr *Legionella* & 1/d T°); after flushing HWDS not used for several weeks (*Legionella*).	Castex and Houssin 2005, République Française 2010a, b
Germany	≥60°C		>50°C		ΔT ≤ 5°C; For HWDS volume ≥ 3L, needs recirculating; T ≤ 60°C permitted at water heater for a few minutes only	Data not available	HW outlet, return loop, 1 sample / rising pipe	*Legionella*: 1/year depending on size of installation; increased repeated sampling required if contamination present	Deutsche Regierung 2001, DVGW German Technical and Scientific Association for Gas and Water 2004
Italy	>60°C (Reco)			≥50°C	Not specified	Not specified	HW, CW if T>20°C	*Legionella*, frequency not specified	Regiona Assessorato alla Sanità et al. 2002
Netherlands	>60°C		≥60°C (warm water)		Mixing taps must be capable of immediately shut down if cold water pressure is lost	Yes (risk analysis every 3 yrs & ≤ 3 months after a change)	Recirculation (T°); HW furthest POU from the source; before and after flushing.	*Legionella*: Every 3 months; if requirements are met, monitor T° only; T°:return loop continuous monitoring.	Dutch Working Party Infection Prevention 2007

Table A-3.1: Regulations and recommendations for *Legionella* prevention and control within healthcare buildings (continued)

	Temperature control					Initial system characterization (risk assessment)	Sampling sites (T° or *Legionella*)	Monitoring frequency	References
	Water heater	Return loop	System	At point of use	Design specifications				
Switzerland	>60°C**	>50°C	>55°C	≥50°C	**Water heated to at least 60°C for a minimum of 1 h, must be used within < 24 h; Reduce nb of points of use to a minimum; heat loss less than 5°C	Yes (risk evaluation)	Hot water heater and points of use (cold and hot water)	Temperature: every 2 months, after 2 minutes flush *Legionella*: 1 or 2X/yr depending on types of wards;	Office fédéral de la santé publique 2008
United Kingdom	≥60°C	≥50°C (for each loop)	≥55°C	In healthcare premises: ≥55°C within 1 min at single hot water outlets and inlets to mixing valves; ≥50°C in all other buildings	Cut offs ≤ 2 Ø; Deadleg volume ≤ 1.5 L (principal system) or 0.5 L (secondary circulation); For sporadically used outlets, flush and purge weekly; low used outlets should be installed upstream of frequently used outlets; **Shutting down recirculation is not permitted**	Yes (water safety plan)	CW, HW outlet, return loop, HW purge, sentinel outlets (first and last POU on recirculating HWDS)	Temperature: monthly for HW outlet, HW return loop, sentinel POU; Annually: 20% taps. *Legionella*: when T° or disinfectanct can't be met	British Standards Institution (BSI) 2011, Department of Health (DH) and Estates and Facilities Division 2006a, b, HSE 2009, 2013, HFS 2012a, b
USA	≥60°C	≥51°C	≥51°C	≥ 49°C hospital patient-care areas ≥ 43.3°C nursing-care facilities	Hot water T° at coldest point in hot water heater, storage tank or distribution system at or above 51°C	Yes (HACCP)	Not specified	Not specified	BSR/ASHRAE 2013, CDC 2003
World Health Organization	>60°C	>55°C	≥50°C	≥50°C after 1 min (except for where thermostatic mixers are installed)	Circulation system not more than 5°C below HW outlet T°; dead end length ≤ 10X pipe diameter or volume ≤ 3 L	Yes (water safety plan)	According to WSP	*Legionella*: according to WSP – frequency varies depending on system status	Cunliffe et al. 2011

CW : Cold Water; HW: Hot Water; POU: Point-of-use; T°: Temperature

Table A3-2: *Legionella pneumophila* sampling plan and qPCR results in systems 1 to 5

System	Sampling point	Sampling objective			Number of sampling events	qPCR results (GU/L)	
		Initial assessment	Stagnation times	Repeatability		Mean	Standard Deviation
1	Water heater outlet	X		X	5	ND	-
	Principal return loop	X		X	5	ND	-
	Tap 1	X		X	5	ND	-
	Tap 2	X			1	ND	-
	Tap 3	X			1	ND	-
2	Water heater outlet	X			1	ND	-
	Principal return loop	X			1	ND	-
	Tap 1	X			1	ND	-
	Tap 2	X			1	ND	-
	Tap 3	X			1	ND	-
	Tap 4				1	73	-
	Tap 5				1	ND	-
	Tap 6				1	ND	-
3	Water heater outlet	X			1	ND	-
	Principal return loop	X			1	ND	-
	Tap 1	X			1	ND	-
	Tap 2	X			1	ND	-
	Tap 3				1	ND	-
4	Water heater outlet	X		X	5	269	106
	Principal return loop	X		X	5	532	217
	Hot water reservoir	X			1	ND	-
	Subordinate loop return	X			1	2080	-
	Tap 1	X			1	352	-
	Tap 2	X			1	593	-
	Tap 3	X			1	382	-
	Tap 4	X			1	ND	-
	Tap 5	X			1	76	-
	Tap 6	X		X	5	453	325
	Tap 7	X			1	124	-
4	Water heater outlet	X			2	8386	970
	Principal return loop	X			1	4300	-
	Tap 1	X			1	34378	-
	Tap 2	X			1	4366	-
	Tap 3	X			1	3497	-
	Tap 4	X			1	2118	-
	Tap 5	X			1	4827	-
	Tap 6	X			1	7098	-
	Tap 7	X			1	16705	-
	Tap 8	X			1	2454	-
	Tap 9		X		6	878	1320
	Tap 10		X		6	2404	1819

ND : Non Detect

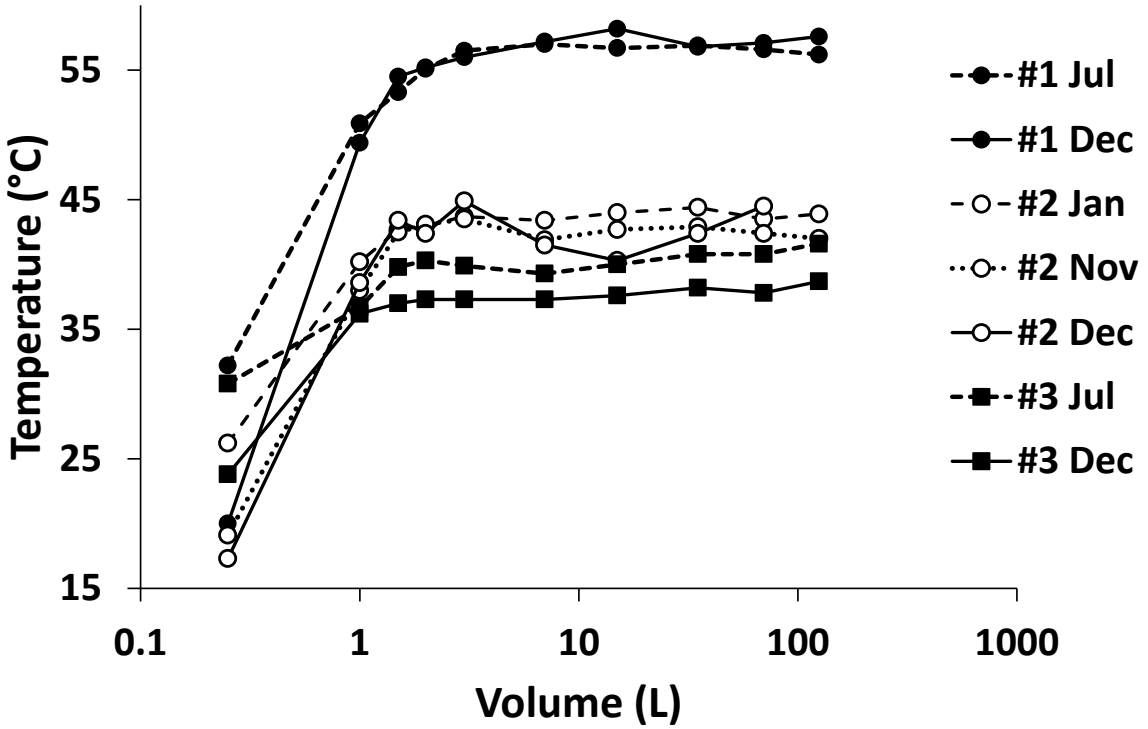

Figure A-3.1: Repeated temperature profiles in system no 5 at 3 different taps: Tap A (●), Tap B (○) and Tap C (■). Sampling was conducted at various months throughout the year 2012.

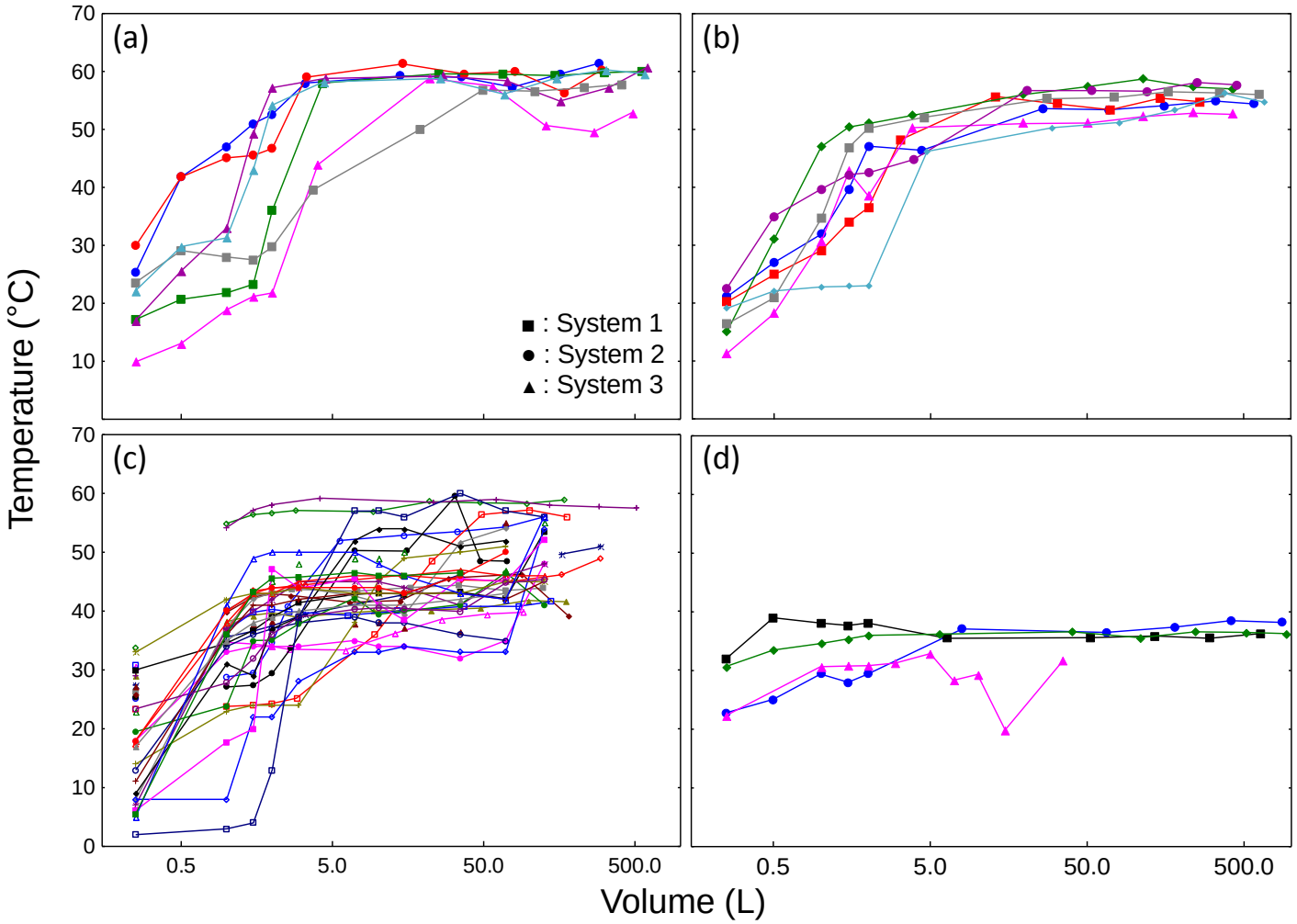

Figure A-3.2: Single sampling points temperature profile as a function of volume sampled in different hot water systems (a, b, c) and for mitigated taps (d). Systems represented are: (a) nos 1, 2, 3 combined; (b) no 4; (c) no 5. Figure (d) represents profiles of mitigated taps in system no 5.

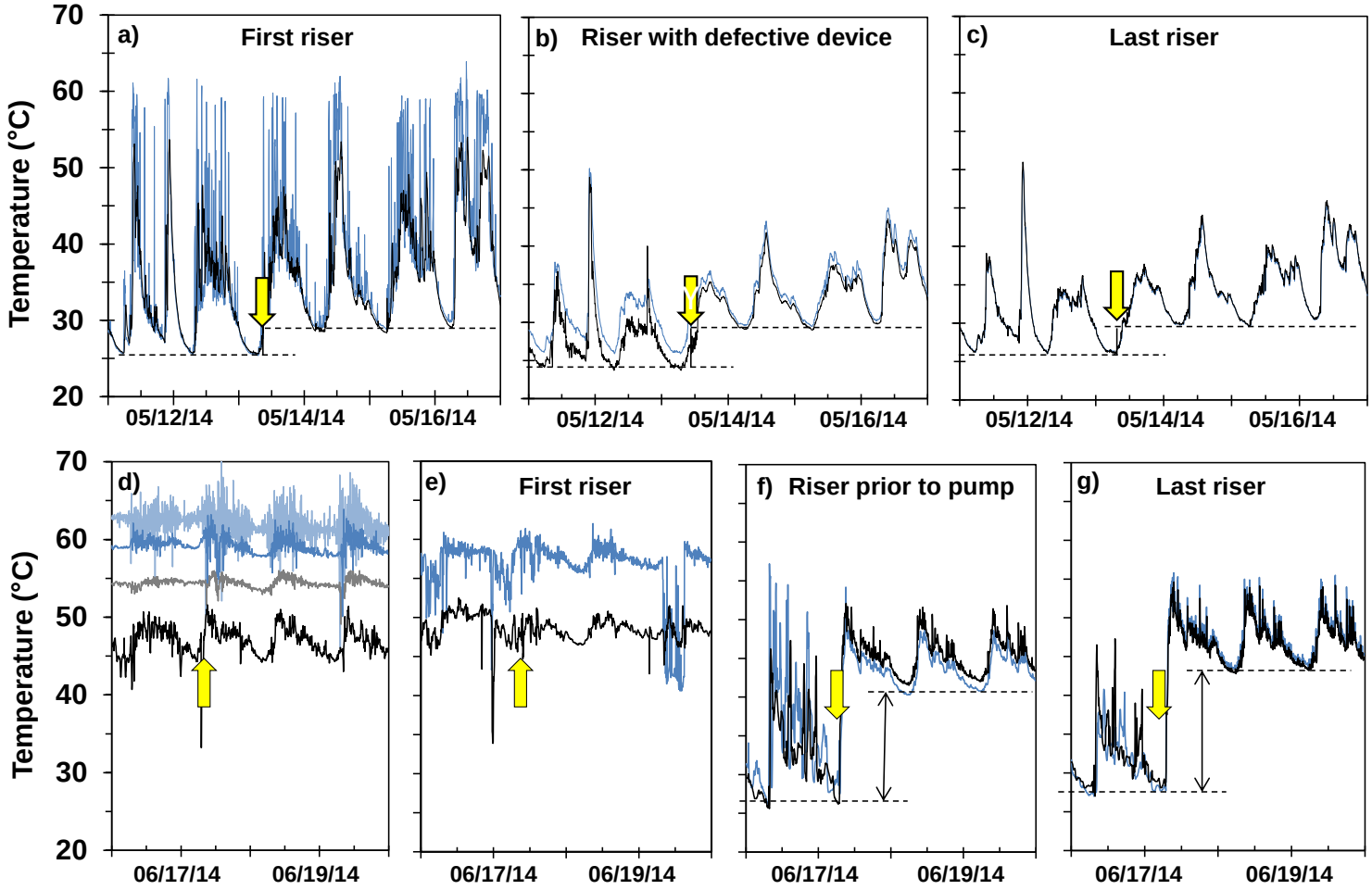

Figure A-3.3: Continuous temperature monitoring for hot water flow (dark blue) and return (black) in system 5 during 2 interventions on the system at different sampling locations: first riser into unit 3 (a, e), riser with intervention in unit 3 (b, f), last riser at the end of unit 3 (c, g), principal horizontal pipe coming into unit 3 (d). Light blue represents principal hot water flow at the heater outlet, dark grey represents principal return loop prior to water heater (3d).

APPENDIX 4 : IMPACT OF ELECTRONIC FAUCETS AND WATER QUALITY ON THE OCCURRENCE OF *PSEUDOMONAS AERUGINOSA* IN WATER: A MULTI-HOSPITAL STUDY

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INFECTION CONTROL & HOSPITAL EPIDEMIOLOGY

ORIGINAL ARTICLE

Impact of Electronic Faucets and Water Quality on the Occurrence of *Pseudomonas aeruginosa* in Water: A Multi-Hospital Study

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OBJECTIVE. To compare *Pseudomonas aeruginosa* prevalence in electronic and manual faucets and assess the influence of connecting pipes and water quality.

SETTING. Faucets in 4 healthcare centers in Quebec, Canada.

METHODS. Water samples from 105 electronic, 90 manual, and 14 foot-operated faucets were analyzed for *P. aeruginosa* by culture and enzymatic detection, and swab samples from drains and aerators were analyzed by culture. Copper and residual chlorine concentrations, temperature, and flow rate were measured. *P. aeruginosa* concentrations were analyzed in 4 consecutive volumes of cold water and a laboratory study was conducted on copper pipes and flexible hoses.

RESULTS. *P. aeruginosa* contamination was found in drains more frequently (51%) than in aerators (1%) or water (culture: 4%, enzyme detection: 16%). Prevalence in water samples was comparable between manual (14%) and 2 types of electronic faucets (16%) while higher for foot-operated faucets (29%). However, type 2 electronic faucets were more often contaminated (31%) than type 1 (14%), suggesting that faucet architecture and mitigated volume (30 mL vs 10 mL) influence *P. aeruginosa* growth. Concentrations were 100 times higher in the first 250 mL than after flushing. Flexible hoses were more favorable to *P. aeruginosa* growth than copper and a temperature of 40°C led to higher counts.

CONCLUSIONS. The types of faucets and connecting pipes, flow rate, and water quality are important parameters influencing the prevalence and the concentrations of *P. aeruginosa* in faucets. High concentrations of *P. aeruginosa* in the first 250 mL suggest increased risk of exposure when using the first flush.

Infect Control Hosp Epidemiol 2014;00(0):1–9

INTRODUCTION

Pseudomonas aeruginosa is a common cause of nosocomial infections, and 30% to 50% of hospital-acquired infections by *P. aeruginosa* are associated with water in intensive care units.[1,2] Electronically activated faucets (efaucets) have been increasingly installed to meet water conservation goals, with varying degrees of complexity in their architecture and numerous internal parts materials.[3,4]

A direct link between contamination of faucets and clinical cases has been established for electronic[4–7] and manual[2] faucets. Eleven peer-reviewed studies report the impact of efaucets on the positivity and level of contamination by *P. aeruginosa* in water[5,7–14] and swab samples.[4,6] Certain types of efaucet are more prone to contamination and more resistant to decontamination, leaving their replacement by manual faucets as the only option to resolve contamination.[5,9,11,13,14] The greater susceptibility of efaucets to contamination has led to recommendations limiting their installation.[15,16]

Comparative measurements of *P. aeruginosa* in faucets show that the type of aerator, the use of flexible connectors, and the presence of a separate mixing valve favor the growth of opportunistic pathogens in biofilm.[4,9,10] Other likely but not confirmed causes often cited include low flow rate, flow regime, stagnation volumes in the device, the type of material within the faucet, temperature of stagnation volume, configuration of upflow connecting pipes, and water usage pattern.[10,12,17] Only one study did not observe differences in contamination between efaucets and conventional faucets; the absence of difference was attributed to the short length of the connecting pipes (<25 cm).[8]

The last meters before the faucet are conducive to a significant increase of *Pseudomonas spp.*[18] and *P. aeruginosa*[19–21] detection. On the other hand, common environmental stressors present

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in water at the tap or within the distribution system, such as copper and chlorine, can influence the culturability of *P. aeruginosa* and induce a viable but nonculturable state.[22–24]

The objectives of this study were to (1) determine whether efaucets are more likely to be contaminated by *P. aeruginosa* than manual faucets, (2) assess the influence of connecting water pipes and environmental stressors on faucet positivity, and (3) locate the pipe sections most susceptible to the proliferation of *P. aeruginosa* within the cold water system.

MATERIAL AND METHODS

Samples were collected from 210 faucets and drains from April to August 2013 in 4 healthcare centers in the province of Quebec (Canada): 3 adult hospitals (405, 420, and 80 beds) and 1 pediatric hospital (450 beds). All hospitals were fed chlorinated surface water and no water quality events or outbreaks were reported during the study period. No onsite treatment was used in any of the hospitals. Most efaucets sampled were of simple architecture and equipped with a manual temperature control lever (no thermostatic mixing valves) (Table 1). For each faucet, environmental samples were collected: (1) a swab from the drain, (2) 1 L of first-flush cold water in sterile propylene bottle with 1% sodium thiosulfate, and (3) a swab of the aerator. Hot water temperature was measured after 1-minute flush, residual chlorine from the second liter, and water flow rate and copper ion concentration from first flush (50 mL) (resampled sites).

P. aeruginosa in water was detected by standard culture method (ISO 16266:2006[25]) and by enzyme detection (ED) using the Pseudalert kit (Idexx), an alternative detection method relying on cell multiplication and expressed as most probable number (MPN). For culture, 10 mL and 100 mL were filtered on 0.45-µm cellulose membrane in duplicate. Filters and environmental swab samples were placed on cetrimide agar with nalidixic acid (45.3 g/L Cetrimide Selective Agar [Remel], 10 mL/L glycerol [Fisher], 0.015 g/L nalidixic acid [Sigma-Aldrich]), incubated at 37.5°C for 48 h and counted after 24 h and 48 h. Residual chlorine concentrations were measured with a Pocket Colorimeter II (Hach). Total copper concentrations were measured by inductively coupled plasma mass spectrometry[26] after acid digestion of 0.5% HNO_3 for 24 h.

Laboratory-based studies were conducted on 2 brands of 12.5 mm–diameter certified (NSF61C) new braided flexible hoses and on 66 cm of new copper pipes. A length of 50.8 cm of Bélanger XCFC-420 PP (ELAST#1) and 76.2 cm of Uberhauss JLS38C12-030 (ELAST#2) were used. Preconditioned capped flexible (30 mL ELAST#1 and 45 mL ELAST#2) and copper pipes (100 mL) were then filled with sterile ultrapure phosphate-buffered water (pH = 7.3) spiked with a suspension of *P. aeruginosa* strain PA14[27] to obtain 1×10^5 cells/mL and maintained at temperatures of 20°C, 40°C, and 55°C for 24 h, 48 h, and 6 days.

Statistical analyses (χ^2 test and multivariate adaptive regression [MARSpline]) were performed with Statistica, version10 (StatSoft). MARSpline regression is a nonparametric analysis in which continuous, categorical, and nominal variables can be added to the model and from which a better fit from a few or all variables is proposed.

RESULTS

Table 2 shows the occurrence of *P. aeruginosa* in biofilm swab samples and water samples sorted by healthcare centers. In water, the occurrence of *P. aeruginosa* was also generally low but significantly higher with the ED method than the culture-based method.

Specific positivity rates of water samples for each type of faucets, connecting water pipes, and physicochemical parameters are presented in Table 3. Prevalence in water samples was comparable between electronic and manual faucets while slightly higher for foot-operated faucets for both methods.

Because multiple factors can affect the response both by culture and by enzymatic detection, a regression (MARSpline) was conducted on all available variables for all faucets combined (4 types) and for efaucets (2 types). Significant variables contributing to model response are shown in Table 4.

Results from the laboratory-based study on the impact of the connecting pipe's material on the persistence of

TABLE 1. Description and Characteristics of Analyzed Faucets

	Efaucets Type 1	Efaucets Type 2	Efaucets Type 3[a]	Foot-operated	Manual
Description	Equipped with manual local T° lever	Equipped with manual local T° lever	Without manual T° lever	Equipped wtih pedal	Equipped wtih single or dual lever
Location of lever	Faucet body	On the side of the sink	No lever	Pedal	Faucet body
Location of mixing valve	In faucet body	Under the sink	Under the sink	Under the sink	In faucet body
Electronic activation	x	x	x		
Thermostatic mixing valves			x		
Average mixed volume	10 mL	30 mL	Variable (>30 mL)	140 mL	10 mL

[a]Not in this study.

TABLE 2. Percentage of Positive Samples for *Pseudomonas aeruginosa* in Biofilm Swabs of Drains and Aerators by Culture and in 100 mL of Water Samples by Culture and Enzyme Detection

		Biofilm (swab)			
		Drain	Aerator	Water	
Healthcare centers	No. of faucets (n = 210)	Culture	Culture	Culture	Enzyme detection
1	68	65%	1.5%	3%	9%
2	60	40%	1.7%	5%	15%
3	52	60%	1.9%	4%	31%
4	30	27%	0%	7%	10%
Total all healthcare centers		51%[a]	1%[a]	4%[a]	16%[a]

[a] $P < .01$.

P. aeruginosa are presented in Figure 1a for water samples (MPN/mL) and Figure 1b for biofilm samples (expressed as level of abundance). Depending on the type of material, suspended densities decreased, remained stable, or increased at 40°C, while all materials but copper were colonized on swab samples.

Figure 2 shows the mean copper and residual chlorine concentration for all sampling and for positive and negative samples for *P. aeruginosa* measured by culture and ED methods. Overall mean concentration of copper was 461 µg/L ± 212 µg/L (range, 17–1,081 µg/L).

Figure 3a shows concentration profiles of *P. aeruginosa* in volumes sampled sequentially in 3 faucets connected with flexible hoses. Mean concentrations in the first-flush sample of 25 mL were 204 colony-forming units (CFU)/100 mL compared with 0.4 CFU/100 mL in the distribution systems. Copper concentration profiles showed substantial variations whether considering concentrations at a faucet (77–463 µg/L)

TABLE 3. Summary Statistics per Types of Faucets, Connecting Water Pipes, and Physicochemical Parameters with Occurrence and Percentage Positive for *Pseudomonas aeruginosa*

		Culture		Enzyme detection	
Data collections	Samples (total no.)	N	%	N	%
Type of faucets (n = 210)					
Electronic	105	5	5%	17	16%
Type 1 (manual local T° lever in faucet)	92	5	5%	13	14%
Type 2 (manual local T° lever on the side of the sink)	13	0	0%	4	31%
Manual	90	2	2%	13	14%
Foot-operated	14	2	14%	4	29%
Wall push button–activated	1	0	0%	0	0%
Connecting pipes (n = 210)					
Flexible hoses	118	5	4%	17	14%
Copper pipes	50	1	2%	9	18%
Flexible hoses and copper pipes	42	3	7%	8	19%
Temperature hot water (after 1 minute) (n = 210)					
≥42°C	129	6	5%	15	12%
<42°C	81	3	4%	19	23%
Copper (n = 134)					
≥250 µg/L	113	5	4%	21	19%
<250 µg/L	21	2	10%	4	19%
Chlorine (n = 160)					
≥0.2 mg/L	66	4	6%	8	12%
<0.2 mg/L	95	5	5%	22	23%
Flow rates (efaucets, n = 86)					
≥5 L/minute	67	2	3%	13	19%
<5 L/minute	19	3	16%	4	21%
Drain (n = 210)					
Faucet with positive drain	108	4	4%	14	13%
Faucet with negative drain	102	5	5%	20	20%

TABLE 4. Multivariate Adaptative Regression (MARSpline) Results Presenting Significant Variables for All Faucets (4 Types) and Specifically for Efaucets for Either Culture and/or Enzyme Detection as Response Variables

	Output		
Input - Variables	Response variable	Significant variables	R^2
All faucets - 4 types: manual, pedal, efaucet with local T° control in faucet, efaucet with To control on the side of the sink	Enzymatic detection (MPN/100 mL)	Temperature, aerator, connecting material, type of faucet, stagnation volume, Cu	0.42
Aerator (±), drains (±)			
Temperature, residual Cl_2, Cu concentration, stagnation volume	Culture (CFU/100 mL)	Aerator, type of faucet	0.51
Connecting material type (3)			
Efaucets - 2 types: with To control in faucet or on the side of the sink			
Aerator (±), drains (±)	Enzymatic detection (MPN/100 mL)	Cu, temperature, efaucet type	0.39
Temperature, residual Cl_2, Cu concentration, stagnation volume			
Connecting material type (3)			
Flow rates			

NOTE. CFU, colony-forming units; MPN, most probable number.

or peak concentrations from first and second flush, with varying chlorine concentrations (Figure 3b).

DISCUSSION

Reported prevalence for *P. aeruginosa* in manual and electronic faucets varies widely with positivity rates ranging from 0 to 100%.[4–14,19,28–30] These wide differences reflect the complexity and lack of consistency in the monitoring approaches, including sample type, sampling protocol, number of sampling sites, frequency, system configuration and characteristics, and sampling context. The present study investigated the following factors: mixed stagnant water volume, flow rate, aerator positivity, connecting pipe material, physicochemical parameters (Cl_2, Cu, temperature), and distal versus systemic contamination.

In this study, 2 types of efaucets were compared with manual and foot-operated faucets. Manual faucet positivity results obtained were within the range reported by previous large studies (>25 faucets) for water (1%–18%)[12,28–30] and swab (4%–48%)[2,4,31,32] samples. Water from all types of efaucets combined (Table 1) was not more frequently positive for *P. aeruginosa* than water from manual faucets (16% vs 14%). Interestingly, for positive water samples, maximum concentrations were higher in manual faucets (>300 CFU/100 mL by culture; 2,419 MPN/100 mL by ED) compared with efaucets (97.5 CFU/100 mL by culture; 308 MPN/100 mL by ED). Both types of efaucets studied were equipped with manual local temperature control levers, but the mixing valve was located either in the faucet body (type 1) or at a distance under the sink (type 2). The percentage of positive taps was higher (31%) in type 2 faucets with a larger volume of stagnant water (30 mL vs 10 mL) than in type 1 faucets (14%). Foot-operated faucets with a mixed volume of 140 mL were also more frequently positive (29%). The mixed volume was a significant contributor to contamination of water ($R^2 = 0.42$ for culture, $R^2 = 0.51$ for ED). These findings agree with prior reports of lower bacterial loads in water for efaucets with small mixed volume with local temperature control (7%) compared with type 3 faucets with larger mixed volume (74 %).[10]

Flow rates in efaucets can be as low as 2 L/min for water conservation goals. The lower flow rates can result in less efficient flushing and were suggested as a cause of higher contamination of efaucets compared with manual faucets with higher flow rates.[10] In this study, the mean flow rate for efaucets was 5.8 ± 1.7 L/min, comparable with manual faucet flow rates. This factor may explain in part the lower prevalence observed compared with other studies.

Despite its low positivity, the contamination of the aerator was a statistically significant variable predicting the concentration of *P. aeruginosa* in water by both methods (Table 4). The low level of colonization observed for aerator external swab samples was attributed to the simple structure of the aerators. Walker et al[4] observed 51% contamination of complex aerators whereas simple plastic and metal aerators were not contaminated.

Connecting pipe material was a significant contributor to the positivity and concentration of *P. aeruginosa* in water. Within positive samples, the percentage of faucets connected with flexible hoses was 1.9 times (ED) to 5.1 times (culture) higher than those connected with copper only (Table 3). This is in agreement with findings by Walker et al[4] showing that flexible hoses were more frequently contaminated than copper pipes (29% vs 5%). Plastic and elastomeric materials can support and favor *P. aeruginosa* biofilm development compared with copper.[33–35]

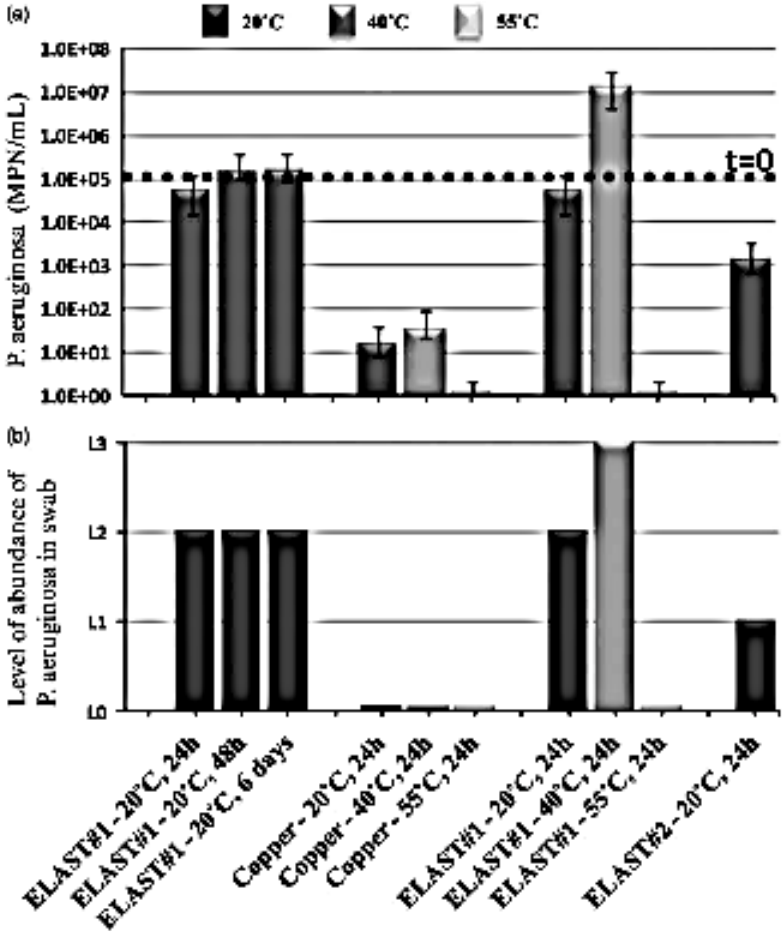

FIGURE 1. Mean *Pseudomonas aeruginosa* (most probable number [MPN]/mL) by enzymatic detection in water (a) and by culture for swab samples (b), in flexible hoses (ELAST #1 and ELAST #2) and in copper pipe sections incubated at different temperatures for 24 h to 6 days. Initial inoculum value of approximately 1×10^5 MPN/mL indicated by dotted line. Swab sample results are expressed as relative abundance (L0 = 0 colony-forming units [CFU]/swab, L1 = 1-10 CFU/swab; L2 = 10-100 CFU/swab, and L3 = >100 CFU/swab). All values at 55°C were below detection level.

Mean hot water temperatures were significantly ($P < .0001$) higher in manual faucets (55.0°C ± 7.6°C) than in efaucets (45.3°C ± 11.0°C). The 10°C difference observed can be explained (1) by a permanent cold water flow through the mixing valve for efaucets or (2) by mixing of hot and cold water supplies owing to defective or obstructed backflow valves (commonly reported by operators). Measured temperatures exceeded the ideal *P. aeruginosa* growth temperature upper limit of 42°C but clearly fell short of effective thermal inactivation conditions.[36] However, results from the laboratory-based study on connecting pipe materials show the importance of maintaining 55°C to control suspended and fixed *P. aeruginosa*. Thermal eradication of *P. aeruginosa* was reported as successful following flushing faucets at temperature of 70°C or higher for 30 minutes or longer.[13,37]

Copper was a significant contributor to concentrations detected by the ED method but not by culture (Table 4). It is noteworthy that significantly more positive water samples were detected with the ED method than by culture. The presence of elevated copper ion concentrations may affect the enzymatic response whereas it inhibits growth on culture media. The correlation between culture and ED results is altered in the presence of 63.5 to 250 µg of copper per liter.[23,34]

Temperature and copper concentrations in water were significant contributors to predict the positivity of *P. aeruginosa* by ED, especially when comparing type 1 and type 2 efaucets (Table 4). This highlights the importance of selecting efaucets that are designed to sustain high temperature flushing and that minimize the mixing chamber stagnant volume. However, higher hot-water-system temperature cannot ensure thermal

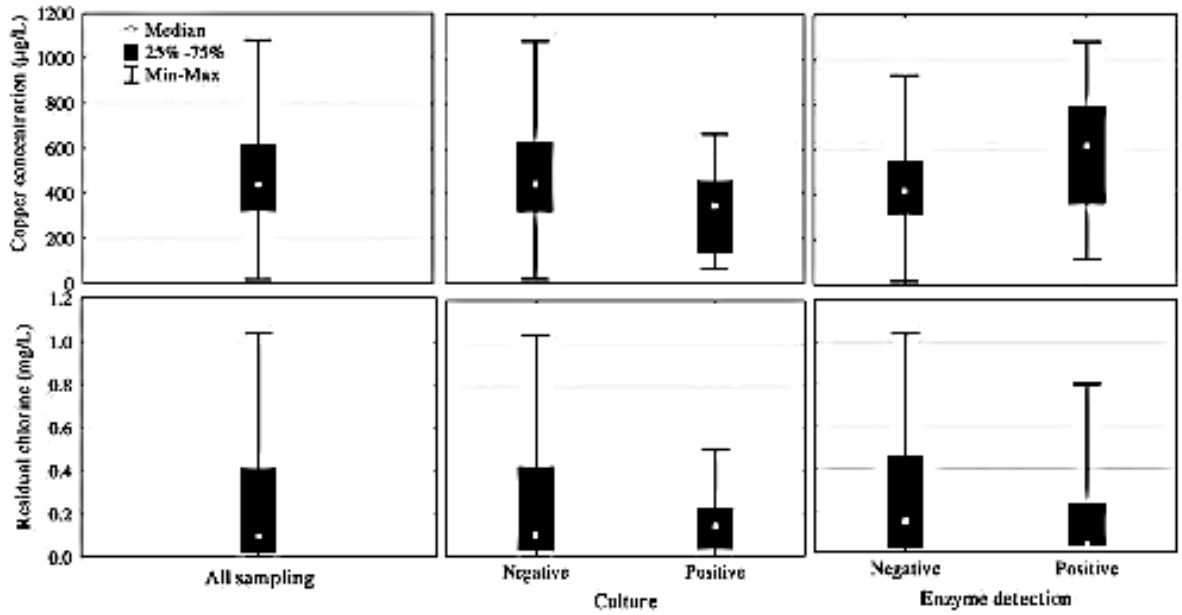

FIGURE 2. Mean copper (n = 160) and residual chlorine (n = 134) concentration for all sampling and for negative or positive *Pseudomonas aeruginosa* samples with culture and enzymatic detection method.

inactivation conditions sufficient to control distal growth of *P. aeruginosa* if efaucets have important stagnant volumes in mixing chambers.[38] The presence and significance of viable but nonculturable *P. aeruginosa* in premise plumbing induced by environmental stressors should be addressed in future research.

The absence of any significant impact of chlorine residual in cold water (0.23 ± 0.27 mg/L) was not surprising considering the demonstrated survival of *P. aeruginosa* to 0.5 mg Cl_2/L after 30 minutes[39] and reported inefficiency of low-dosage chlorine disinfection.[9,13,28]

Proliferation of opportunistic pathogens can be systemic within a building's water distribution system or localized at the point of use (distal). In the present study, *P. aeruginosa* contamination was more important at the faucet and in the connecting plumbing compared with water from the system. Similar results obtained in previous studies suggest the first liter as ideal to detect contamination,[18,19] with *P. aeruginosa* mean concentrations of 4.7 CFU/100 mL in the first liter reduced by 7-fold after flushing. In this study, the first 25 mL was more concentrated in *P. aeruginosa* (535 times by culture and 72 times by ED) compared with concentrations in flushed samples. The steep concentration gradient observed in Figure 3 reveals that level of contamination will vary depending on the volume sampled at a given faucet. Indeed, our weighted average concentrations in the first liters (11 CFU/100 mL and 51 MPN/100 mL) were comparable with concentrations reported by Cristina et al.[19] However, a sample volume of 1 L can mask the higher contamination of the first flush volume (25 mL) by factors of 9 (culture) and 6 (ED). The lower concentrations observed in the third volume sampled could be attributed to copper pipes in which water stagnates between uses. Low nutrient, low oxygen, and higher copper concentrations in this section will not be as favorable to biofilm. Finally, a significant concentration of *P. aeruginosa* was detected by ED after flushing, suggesting lower but sustained systemic contamination.

Retro-contamination from the drain has been proposed as a vector of faucet contamination.[31] In this study, the rate of contamination was significantly higher for drains (108/210) than for water (9/210) or aerators (3/210). This marked difference may be caused by suboptimal culture conditions to recover stressed environmental strains from water as opposed to drains.[40]

Our study is subject to a number of limitations. First, stagnation prior to sampling was not controlled. Second, a limited number of pedal-activated faucets were available for sampling, resulting in a smaller number of samples for this faucet type. Third, only 1 type of water quality was tested. Fourth, concentrations in drain biofilm are greater than in water, limiting result comparison.

Despite these limitations, results from this study reveal that simple-design efaucets with a smaller mixing chamber were not more susceptible to *P. aeruginosa* contamination than manual faucets. Multivariate regression models showed that aerator positivity, hot water temperature, copper concentrations, mixed

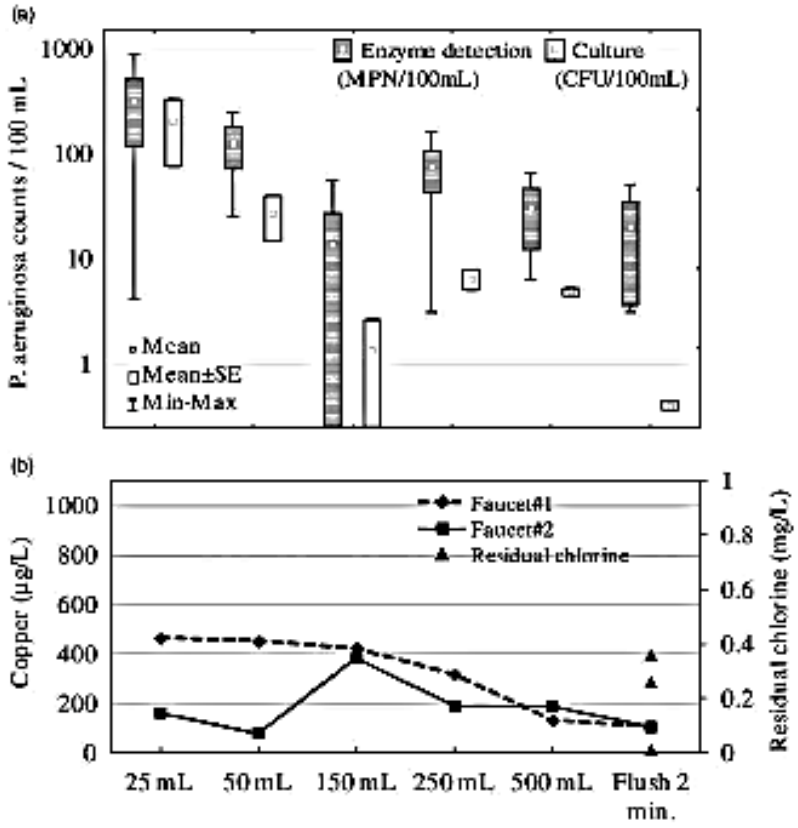

FIGURE 3. (a) Presence of *Pseudomonas aeruginosa* in water volumes taken in sequence (at 3 faucets) analyzed by culture (n = 2) and by enzymatic detection (n = 4); (b) copper (µg Cu/L) in water samples from sequential volumes and free chlorine concentrations (mg Cl_2/L) measured for each sampling event.

stagnation volume, and type of faucets and connecting material are significant contributors to the prediction of *P. aeruginosa* in water, highlighting the importance of documenting all parameters. Furthermore, the demonstration of a higher contamination in the first water volume raises an issue inherent to efaucets design. Healthcare workers will systematically expose their hands to the first 25 to 50 mL when activating an efaucet, whereas the same volume will have been flushed while the workers are turning the knobs of a manual faucet. This raises the importance of evaluating the impact of water-saving measures on the water quality and bacterial exposure for vulnerable patients.

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