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An assessment of health hazards associated with the use of water mist systems as a cooling intervention in Australia

Edmore Masaka
Edith Cowan University

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An assessment of health hazards associated with the use of water mist systems as a cooling intervention in Australia

Edmore Masaka,

B. Tech (Env. Health), MPH, Grad Dip OHS

Supervisors:

Ass. Professor Sue Reed

Prof. Jacques Oosthuizen

Dr. Maggie Davidson

School of Medical and Health Sciences

Edith Cowan University

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy.

Date of submission: 26 July 2021

“Everything about microscopic life is terribly upsetting. How can things so small be so important?”

(Asimov & Shulman, 1988, p. 156)

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

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ABSTRACT

Water mist systems (WMS) installed and used for cooling ambient temperatures in public places fall within the category of premise plumbing. Premise plumbing refers to the water distribution networks that lie downstream of the water meter, and within buildings. The colonization of premise plumbing by opportunistic premise plumbing pathogens (OPPPs) such as *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium avium*, *Acanthamoeba* and *Naegleria fowleri* is emerging as a challenge for public health and water quality management. Contrary to other premise plumbing features like showers and domestic taps that have been implicated in various waterborne infections, the health risks associated with WMS are not well understood.

The primary aim of this thesis research was to advance understanding of the health risks associated with OPPPs in WMS used to cool ambient air temperatures for thermal comfort. A literature review was the foundation (1st study) of this research and aimed to characterise the state of knowledge about the health risks of OPPPs in WMS. The 2nd study, a questionnaire survey of 10 WMS owners and 22 Environmental Health Officers (EHOs) was conducted as formative research to understand and describe the characteristic features of WMS, as well as the knowledge, perspectives and practices of operators, and regulatory authorities that manage these systems within the context of public health legislation. Additionally, this formative research informed the research methodology for the 3rd study, a microbial investigation of 10 WMS, from which 30 bioaerosol (air), biofilm (surface) and water samples were collected, giving a total of ninety samples (N=90). Microbial samples were analysed by both culture-based (growth media) and culture-independent (polymerase chain reaction (PCR)) methods

to quantify and identify the presence of 5 representative OPPPs: *L. pneumophila*, *P. aeruginosa*, *M. avium* and free-living amoebae (FLA) including *Acanthamoeba* and *N. fowleri.* Data on water profile parameters of water temperature, water pH and concentration of free residual chlorine, total dissolved solids (TDS) and total organic carbon (TOC) were statistically analysed to determine their impact on the colonisation of OPPP in WMS.

This research identified a critical knowledge gap regarding the health risk of OPPPs in WMS (Study 1), as well as low levels of awareness amongst WMS managers and EHO's regarding the health risks associated with WMS including: non-existent or ad-hoc maintenance regimes, and lack of training and education about the systems (Study 2). Furthermore, the Study 3 research demonstrated colonisation of WMS by public health pathogens of concern including of *P. aeruginosa (49%)*, *L. pneumophila* serogroup (Sg) 2-14 (18%) and *L. pneumophila* Sg 1 (6 %), and *Acanthamoeba* (< 3 %). On the positive, neither *M. avium* nor *N. fowleri* were detected in the WMSs investigated. Free residual chlorine was negatively correlated with all OPPPs, except for *Acanthamoeba* indicating that this may be a critical variable in the safe operation of WMS. *L. pneumophila* Sg 2-14 and Sg 1 were strongly correlated with both TDS and TOC concentration, indicating that these indicators could be used as a warning for at-risk systems, as could elevated water temperature that was positively correlated with *P. aeruginosa*.

This research indicates that WMS present a potential health risk due to colonisation by *L. pneumophila* Sg 2-14, *L. pneumophila* Sg 1, *P. aeruginosa* and *Acanthamoeba,* and should be regulated under public health legislation for microbial contamination of air handling and water systems. Consideration should be given to reviewing existing

public health legislation to capture WMS used as a cooling intervention with consideration of other emerging OPPPs besides *Legionella* spp., as well as development of codes/guidelines for the auditing and operation of WMS, and the upskilling and training of operators and EHOs on their associated health risks. This research has practical applications in public health, as well as commercial WMS cleaning and maintenance businesses, and other industries that use WMS for air cooling and dust suppression.

ACKNOWLEDGEMENTS

I would like to thank my dear wife Batsi, and sons David and Victor. Although not directly involved with this work, your support, encouragement, and patience, often having to wait on me for hours throughout the night as I drove over 1200 km from Newman to Perth delivering samples to the laboratory was invaluable in my research journey.

I express my thanks and gratitude to my supervisory team of Ass. Prof. Sue Reed (Principal Supervisor), Prof. Jacques Oosthuizen (Supervisor) and Dr. Maggie Davidson (Supervisor). Thank you all for your thought-provoking guidance, amazing energy, dedication, and the ability to think outside the box. Your ability to provide me with both professional and technical guidance, whilst at the same time giving me freedom to be in control of this study has enabled me to learn and broaden my understanding of the research rigor. Sue, your eagle eye for detail, never ending ability to successfully navigate through challenges and deep knowledge and experience gave me a sure sense of confidence and direction. Jac, your relevant and practical advice, deep understanding of the discipline and extensive supervisory experience was a sure encouragement for me. Maggie, your amazing enthusiasm built on an amazing breadth of experience and knowledge in the field went a long way in getting me through. I would like to thank Edith Cowan University, the School of Medical and Health Sciences, Graduate Research School for the services rendered as I progressed in my research journey.

Many thanks to Trevor Chapman and the Western Australia Local Health Authority Analytical Committee for providing part of the funding required for my laboratory analysis. I would thank Lindsay Graham and the National Institute of Occupational Safety and Health (NIOSH) for providing the NIOSH BC 251 bioaerosol samplers used in this study. My thanks also go to Prof. Dennis Helsel for his insightful advice on how to deal with monitoring data that contains measurements below the detection limits. Thank you to all owners that allowed me to study their water mist systems and to all the members of the Northwest Environmental Health Group who agreed to participate

in the survey research. I also thank the Shire of East Pilbara for having allowed me to periodically take time off to carry out my research over a 4-year period.

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LIST OF PUBLICATIONS

Paper 1

Masaka, E., Reed, S., Oosthuizen, J., & Davidson, M. (2020). Health Risks Associated with the Use of Water Mist Systems as a Cooling Intervention in the Pilbara Region of Western Australia. *Journal of Environmental Health*, *83.9*, 16-22. Retrieved from https://www.neha.org/node/61884

(Impact Factor 0.521, Q3-Public Health, Environmental and Occupational Health – Schimago Journal Ranking (SJR)

Paper 2

Masaka, E., Reed, S., Oosthuizen, J., Davidson, M. (2021). Knowledge and awareness survey on health risks of using water mist systems as a cooling intervention (*Submitted to Journal of Environmental Health, Manuscript ID 2021-JEH-059*

(Impact Factor 0.521, Q3-Public Health, Environmental and Occupational Health – Schimago Journal Ranking (SJR)

Paper 3

Masaka, E.; Reed, S.; Davidson, M.; Oosthuizen, J. Opportunistic Premise Plumbing Pathogens. A Potential Health Risk in Water Mist Systems Used as a Cooling Intervention. *Pathogens* 2021, 10, 462. **https://doi.org/10.3390/** pathogens10040462

(Impact Factor 3.492, Q1 – Microbiology SJR)

STATEMENT OF CONTRIBUTION OF OTHERS

Paper 1

My contribution to this paper included the identification of the research question, development of the literature search criteria, conducting the preliminary literature search, article review and the synthesis of the literature. All these activities were undertaken under the standard PhD Supervision of Ass. Prof. Sue Reed, Prof. Jacques Oosthuizen and Dr. Maggie Davidson. I developed the first draft of this paper and incorporated comments raised by the above-mentioned supervisory panel in all subsequent drafts until the final one.

Paper 2

My contribution to this paper included the development of the research question and survey methodology, design, pilot testing and implementation of the survey questionnaires to collect respondents' data, and the analysis of the survey responses. All these activities were undertaken under the standard PhD Supervision of Ass. Prof. Sue Reed, Prof. Jacques Oosthuizen and Dr. Maggie Davidson. I developed the first draft of this paper and incorporated comments raised by the above-mentioned supervisory panel in all subsequent drafts until the final one.

Paper 3

My contribution to this paper included the development and implementation of the experimental design, materials and methods including the development and implementation of the sampling plan, putting in place arrangements for the laboratory analysis of samples, the interpretation of laboratory results and statistical analysis. All these activities were undertaken with normal PhD supervisory input from my panel of Ass. Prof. Sue Reed, Prof. Jacques Oosthuizen and Dr. Maggie Davidson. I developed the first draft of this paper and incorporated comments raised by the above-mentioned supervisory panel in all subsequent drafts until the final one.

Name	Signature	Date
Edmore Masaka		21 July 2021
Ass. Prof. Sue Reed		21 July 2021
Prof. Jacques Oosthuizen		21 July 2021
Dr. Maggie Davidson		21 July 2021

DEFINITIONS AND ABBREVIATIONS

EHO	Environmental Health Officers
FLA	Free Living Amoeba
Free Living Amoeba	One celled organism that can ingest bacteria, yeast, and other organisms as a food source can complete their life cycles in the environment without a human or animal host.
HBM	Health Belief Model
HSE	Health and Safety Executive
KAP	Knowledge Attitude and Practices
MAC	*Mycobacterium Avium* Complex
NTM	Non-Tuberculous *Mycobacteria*
Oligotrophic conditions	An environment that offers very low levels of nutrients and carbon to sustain normal microbial growth
Opportunistic premise plumbing pathogens	A group of emerging microorganisms capable of causing infections in people with weak immunity
OPPPs	Opportunistic premise plumbing pathogens
PAM	Primary amoebic meningoencephalitis
pH	Is the measure of how acidic or basic property of a liquid or a material
Premise plumbing	All sections of the water distribution network downstream of the main supply line and within buildings including the array of hot and cold-water features connected to it.

Scheme water	The potable water produced and supplied by a central treatment authority to domestic, industrial, and commercial premises
TDS	Total dissolved solids
TOC	Total organic carbon
Treated bore water	Water obtained from an underground aquifer that has undergone processing to render it suitable for an intended use such as drinking etc.
Water mist systems	Premise plumbing features that are installed in outdoor dining and entertainment areas to achieve evaporative cooling of ambient temperatures by the release of tiny water mists that flash evaporate in the surrounding areas.
WDS	Water distribution system
WMS	Water mist systems

THESIS OVERVIEW

This thesis has been constructed on five chapters that combine to make a coherent body of research.

Chapter 1

This chapter presents the general introduction. It describes the use and nature of water mist systems (WMS) as a cooling intervention and positions this thesis research in the perspective of earlier research in the field of opportunistic premise plumbing pathogens (OPPPs). The chapter also describes the issues related to the operation of WMS, including the operational and environmental factors that could be implicated in their colonisation by OPPPs. This chapter also discusses the theoretical framework holding and supporting the research, providing the foundation for the subsequent research described in chapters 2 – 4 by advancing the research questions investigated in the publications.

Chapter 2

Chapter 2 presents the literature review findings on the health risks associated with the use of WMS as a cooling intervention in public places (Study 1). These findings help to answer the following research question:

Research Question 1, *What is the state of knowledge about the health risks associated with the use of WMS as a cooling intervention in public places?*

This chapter evaluates/critiques the existing research on WMS health risks by interrogating existing peer-reviewed literature on WMSs, as well as similar plumbing systems, to identify the existing knowledge gaps addressed by this thesis research. This review also focuses on the usage and location of WMS, the OPPPs implicated in waterborne infections arising from the use of similar water mist systems, the effect of water profile parameters of temperature, free chlorine residual concentration, water pH, total dissolved solids (TDS), total suspended solids (TSS) and total organic carbon (TOC) concentration on OPPP colonisation, applicable public health regulatory regimes, and OPPP control strategies for WMS. The findings from this review helped

inform subsequent research activities described in Chapters 3 and 4. Appendix 1 is the copy of Chapter 2, the paper which has been published in the peer reviewed *Journal of Environmental Health (JEH)*.

Chapter 3

Chapter 3 discusses the findings from the two empirical questionnaire surveys administered to WMS owners and Environmental Health Officers (EHOs) to gather information on the operation and regulation of WMS systems respectively (Study 2) The findings helped to answer the following research question:

Research Question 2: *What are the characteristics of WMS used as a cooling intervention in public places, and the level of knowledge and awareness of the associated health risks among owners of these systems and EHOs involved in the regulation of environmental health?*

The questionnaire survey gathered information that helped to describe the types of WMS, and operation of ten systems in the study area. This garnered an understanding of the participants' knowledge, attitudes and practices regarding the health risks of OPPPs, determine if regulatory regimes for these systems had been developed and/or implemented, and inform the methodological design of the microbial investigation of WMS (Study 3). Appendix 2 is the manuscript format of paper 3 which has been submitted to the *Journal of Environmental Health* (Reference 2021-JEH-059).

Chapter 4

Chapter 4 discusses the experimental research which investigated the potential occurrence of microbial pathogens in 10 WMS. These pathogens included *Legionella pneumophila, Pseudomonas aeruginosa, Mycobacterium avium*, *Acanthamoeba* and *Naegleria fowleri* in 10 WMS (Study 3). The findings of this investigation helping to answer the following research question:

Research Question 3: *Can WMS be colonized by OPPPs, and are environmental conditions of water temperature, residual chlorine disinfectant concentration, water pH, TDS and TOC associated with the occurrence of OPPPs in WMS?*

Bioaerosol, biofilm and water samples were collected from WMS during three (3) sampling events in February, May, and August of 2019 giving a total of ninety (90) samples. These samples were analysed by both culture-based and culture-independent polymerase chain reaction (PCR) methods to detect the presence of the 5 representative OPPPs already discussed before. The water profile parameters of free residual chlorine, water temperature, water pH, total dissolved solids (TDS), and total organic carbon (TOC) were also measured, and statistically evaluated for correlations with the presence and concentration of OPPPs. Appendix 3 is the copy of Chapter 4 which has been published in the peer reviewed *Pathogens Journal*.

Chapter 5

Chapter 5 discusses the research findings and assesses the extent to which they support the main research questions and how they relate to existing research on OPPPs. The answers to each research question are determined based on the research findings and their significance. This chapter

critically reflects on the impact of the project. The new insights presented by the results of the study are advanced, including their impact on the existing operation and regulation of WMSs, as well as the training organisations and statutory organisations that oversee these systems. The limitations of the research are summarised to aid in the appropriate interpretation of the findings. Lastly the chapter

brings together all the project findings in a conclusion with recommendations and strategies for safely operating WMS. The conclusions based on the extent to which the findings have helped to answer each research question are presented. Recommendations based on three areas, policy, operation, and regulation of WMS are presented in this section.

The structure of the thesis, illustrating how the various studies come together as a coherent body of research is shown in Figure 1

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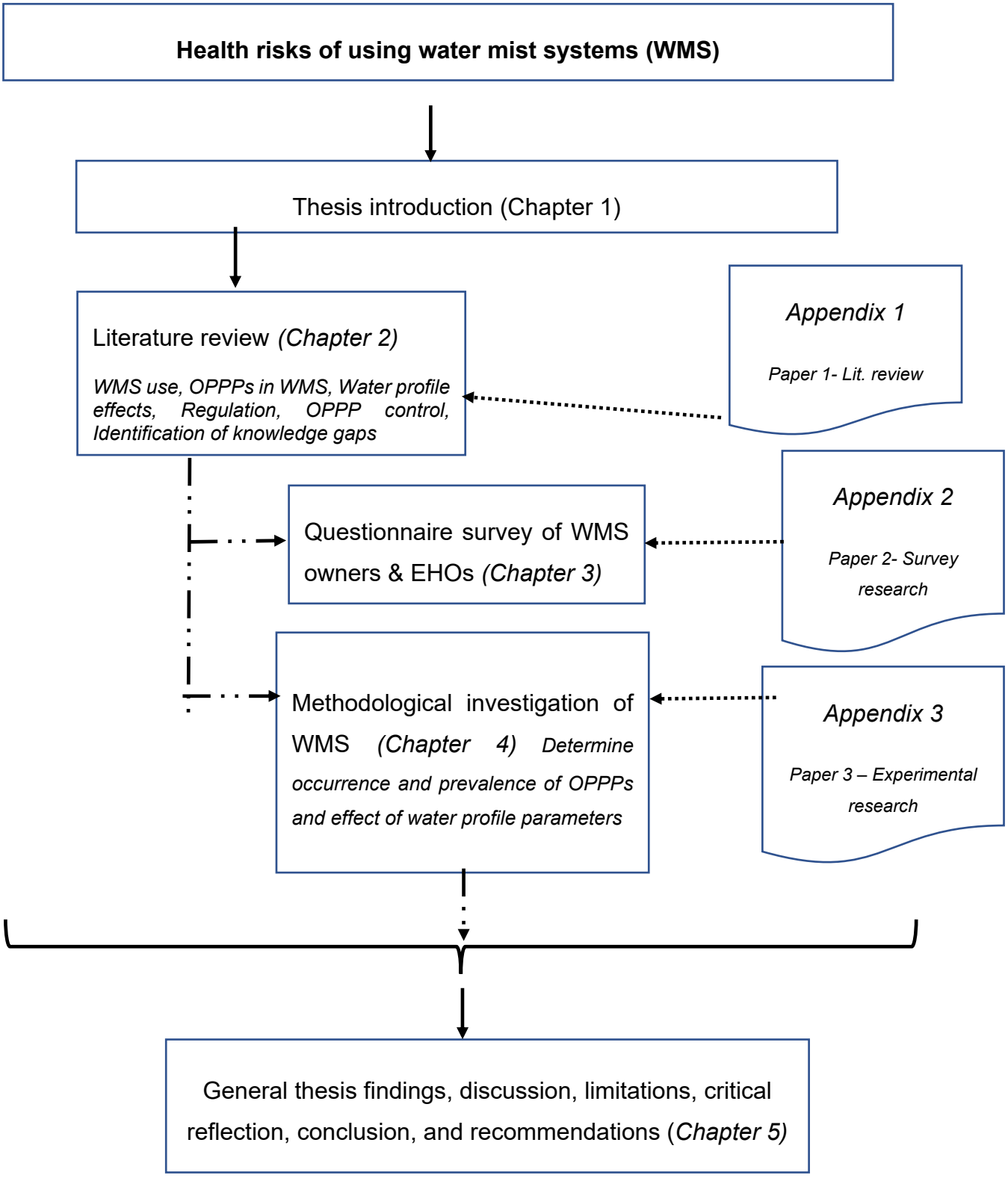

Figure 1. Thesis overview flowchart

CHAPTER 1 THESIS INTRODUCTION

This thesis explores the health risks associated with the use of water mist systems (WMS) used as a cooling intervention in public places due to colonisation by opportunistic premise plumbing pathogens (OPPPs). Water mist systems are premise plumbing features that are typically installed in outdoor dining and entertainment areas to achieve evaporative cooling of ambient temperatures by the release of tiny water mists that flash evaporate in the surrounding areas, (Ozmist, n.d.). Premise plumbing includes all those sections of the water distribution network downstream of the main supply line and within buildings (Wang et al., 2013). Opportunistic premise plumbing pathogens are a group of emerging pathogens that have become commensal in water distribution networks (Falkinham III et al., 2015) and premise plumbing systems (Liu et al., 2019), with their occurrence not necessarily being a result of external contamination, but of their extraordinary ability to adapt and regrow in these environments (Falkinham, 2015).

The best known OPPP species to colonise premise plumbing is *Legionella* spp., a ubiquitous bacterium naturally occurring in aquatic environments such as rivers and lakes, and now known to colonise artificial water features and building plumbing installations. Recent research in OPPPs has also determined that other microbial species including *Mycobacterium spp.*, *Pseudomonas spp.,* and free-living amoebic species of *Acanthamoeba*, *Naegleria spp.*, *Vermamoeba (Hartmanella)* are significant pathogens that colonise premise plumbing systems. Ever since the discovery of Legionnaires' disease among attendees to the 58th annual convention of the American Legion in July of 1976 (Winn, 1988), and its association with possible inhalation of water mists contaminated with the *Legionella* spp. bacteria, further research has established that OPPPs are an emerging public health concern in premise plumbing features (Ashbolt, 2015; Falkinham III et al., 2015).

Plumbing features such as showers (Bauer et al., 2008; Feazel et al., 2009), nebulizers (Allegra et al., 2016), faucets (Bollin et al., 1985; Charron et al., 2015), cooling towers (Carducci et al., 2010), fountains (De Boer et al., 2002), water taps (Donohue et al., 2014), air conditioning systems (Bennett et al., 2014), spas (Fallon &

Rowbotham, 1990; Glazer et al., 2007), and hot water systems (Borella et al., 2004; Borella et al., 2005) get colonised by *Legionella* spp. and other pathogens of public health concern including *P*. *aeruginosa*, *M. avium*, *Acanthamoeba* and *N. fowleri.*

Previous research has demonstrated that these pathogens can be dispersed as bioaerosols during the aerosolization of water from these premise plumbing features (Bollin et al., 1985; Fernstrom & Goldblatt, 2013). All these pathogens have been linked to outbreaks of diseases including legionellosis (Bennett et al., 2014; Hampton et al., 2016), amoebic keratitis in contact lens wearers (Taher et al., 2018), *P. aeruginosa* infections in hospital patients (Schneider et al., 2012), *M. avium* related pulmonary disease (Falkinham et al., 2008) and pulmonary amoebic meningoencephalitis (PAM) (Budge et al., 2013; Parsonson & Nicholls, 2016). The inhalation of contaminated water aerosols, as well as aspiration and skin contact have been identified as the main routes of exposure associated with these infections (Davis et al., 2016).

The incidence of OPPP related infections has been increasing over the years. *Legionella* spp. alone, is responsible for 2 to 15% of patients hospitalized globally with community-acquired pneumonia (Sakamoto, 2015). The USA has recorded a four and half times increase in legionellosis since 2000, and 32 cases of waterborne disease outbreaks were reported between 2011 and 2012, with 66% of the outbreaks being associated with *L. pneumophila* (Beer et al., 2015). In Australia, an average of 374 cases of legionellosis were notified annually between 2008 and 2018 (Australian Government Department of Health, 2019), with an incidence rate of 2 per 100,000 persons in 2015, dropping to 1 per 100,000 in 2019.

Unlike waterborne pathogens of faecal origin that often contaminate water distribution networks, OPPPs thrive in water environments that are hostile to most other pathogens (Falkinham et al., 2015), making it challenging to anticipate, control, monitor and prevent their regrowth. Environmental parameters of biofilm formation, elevated temperatures, low oligotrophic conditions, water pH, inadequate disinfectant residual, type of plumbing materials, and water stagnation act as drivers for the survival of these pathogens in premise plumbing features (Wang, 2013). Research has established the limitations of conventional water treatment methods in effectively

controlling OPPP regrowth in premise plumbing. Almost 95% of the microbiome in water systems reside in biofilms that are not usually targeted in routine water monitoring programs (Flemming et al., 2002). This means that most of these microorganisms are missed during the sampling of the water phase in which only 5% of the microbiome reside. The control of waterborne pathogens by maintaining an adequate concentration of free residual chorine is universally accepted as an effective measure to prevent the outbreak of waterborne diseases (Western Australia Environmental Health Directorate, 2016). However, research findings have indicated a unique tendency of OPPPs to resist destruction by water disinfectants such as chlorine (Canals et al., 2015). The importance of knowledge and skills in identifying hazards and health risks associated with any storage, reticulation and use of water has been well established and built into most standards, guidelines and risk management plans to prevent the outbreak of waterborne diseases, and the ability of health authorities to prevent the outbreaks (World Health Organisation, 2010). According to Julien et al. (2020), an understanding of premise plumbing factors that promote the multiplication of opportunistic pathogens is important to adequately evaluate the risks associated with these microorganisms. Their report adopted a risk management approach, and argues that a link exists between water throughput, plumbing design, materials, and water quality, and that such data is needed to inform comprehensive risk assessments.

Water mist systems belong to this class of water pipes called premise plumbing, which form a part of water distribution network downstream of the water meter or ring mains supply. The colonisation and proliferation of OPPPs in other premise plumbing features with similar operational conditions to those of WMS has already been confirmed by previous research (Barna et al., 2016; Brenda et al., 2018; Lu et al., 2017; Falkinham et al., 2015). However, no investigative research has been published on the potential of WMS used for ambient cooling to be colonized by OPPPs. Most of the research on the health risk associated with premises pluming focusses on the colonisation and regrowth of OPPPs in aerosol generating features such as cooling towers, showers, faucets, fountains, hot water systems and garden hoses. Furthermore, the research has been limited to indoor domestic plumbing systems

(Borella et al., 2004; Borella et al., 2005), hospital plumbing networks (Baird et al., 2011), and industrial water-cooling installations (Carducci et al., 2010).

Current literature reviewed predominantly concurred on the critical role played by environmental parameters such as water temperature, residual free chlorine, water pH, TDS, and TOC in enhancing the survival of OPPPs in premise plumbing systems. However, none of the researchers investigated the same phenomenon in WMS used as a cooling intervention in public places. The widely accepted role of biofilms in the establishment of ecological niches for OPPPs in premise plumbing features, and the enhanced resistance to disinfectants transferred to these pathogens by the biofilms is widely discussed. However, a gap in knowledge about the role of biofilms in WMS exists. The ecological role of free-living amoeba (FLA) in acting as a host and shield for OPPPs in WMS is also still unknown.

1.1 The research problem: Water mist systems

Water mist systems used as a cooling intervention in public places present a potential public health risk because of their shared characteristics with the above-mentioned premise plumbing features, which have been linked with outbreaks of infectious respiratory diseases such as Legionnaires' disease and other bacterial pneumonias, and for their potential to emit biologically contaminated aerosols that may be inhaled by exposed persons (Demirjian et al., 2015; Kanamori et al., 2016).

The assembly of a water mist system used as a cooling intervention in public places is shown in Figure 1.1. This consists of a high-pressure water pump connected to a network of high-pressure tubing. Hydraulic or pneumatic nozzles fitted to the tubing at graduated distances release tiny water aerosols that reduce ambient temperatures by flash evaporation as shown in Figure 1.2.

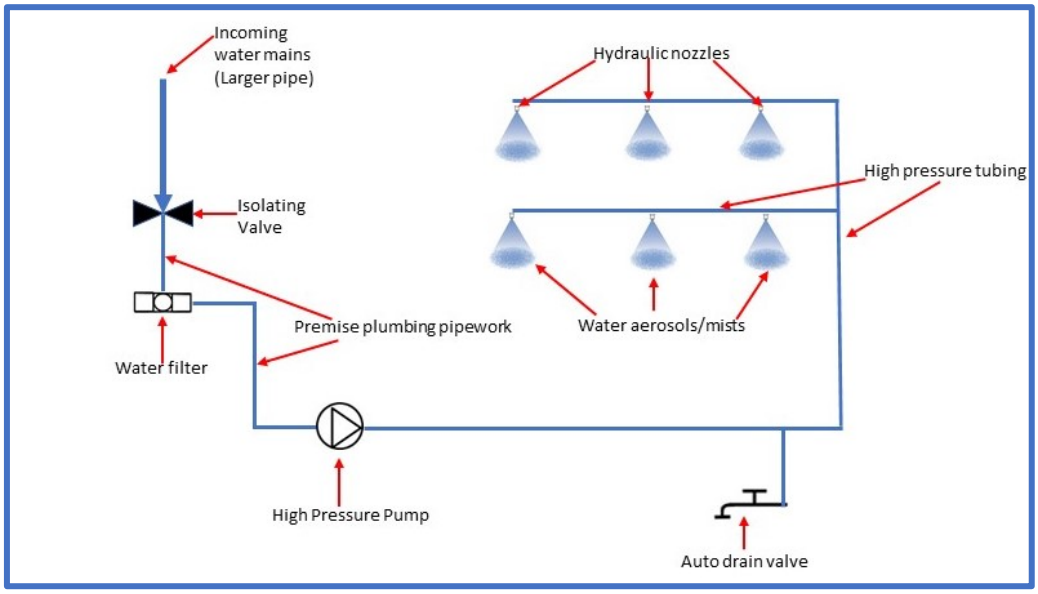

Figure 1.1 Schematic of a water mist system

Figure 1.2 Water mist system in operation

The climate of the north-western region of Australia is often dry and hot, Figure 1.3 (Bureau of Meteorology, 2016), making the use of conventional air conditioning systems expensive and often impractical in outdoor dining and entertainment areas. The use of WMS to achieve the desired temperature control in public places has been

increasing in recent times. Most of these WMS are easily available in local hardware shops where they are sold in kit form for easy installation by customers.

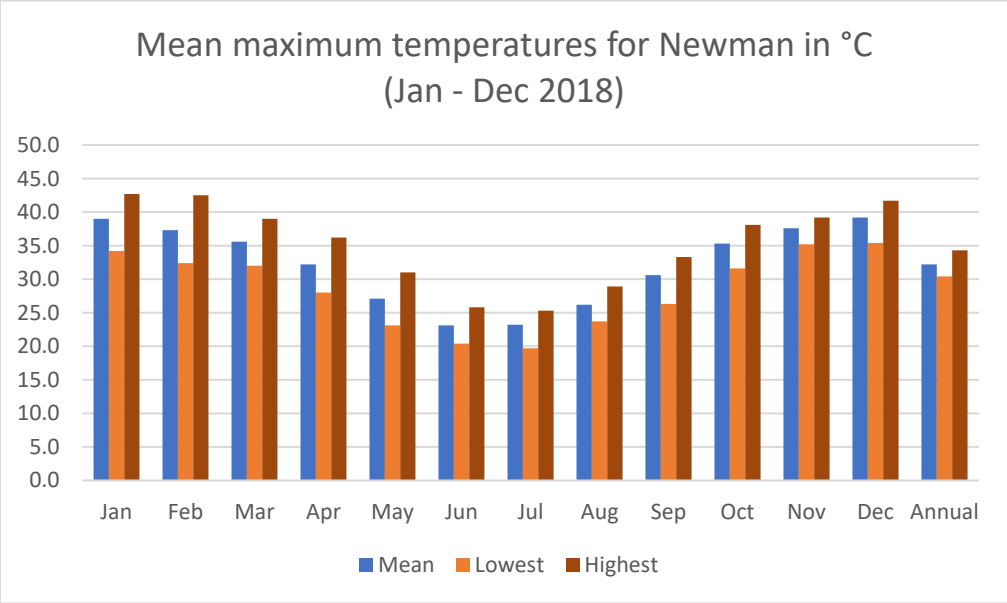

Figure 1.3 Mean maximum temperatures for Newman (Jan - Dec 2018). (Bureau of Meteorology, 2016)

Many of the remote locations where WMS are used do not have access to centrally produced and treated reticulated water supplies, thus owners use onsite bore water that is not subjected to a rigorous water quality management regime.

Furthermore, the WMS plumbing network is often installed outdoors where it is exposed to direct sunlight and elevated temperatures, often causing a rapid depletion of free residual chlorine levels and creating conditions conducive for the survival and growth of heterotrophic OPPPs (Agudelo-Vera et al., 2020; Bilinski et al., 2012).

The actual number of WMS installed in Australia and globally is unknown because such data is unavailable. Currently in Australia, building owners are not required to submit applications for approval to instal such systems, and existing environmental health inspection protocols do not include oversight over WMS. Therefore, there are no records of where these types of WMS are installed. WMS are also used for dust suppression in mining operations such as ore crushing and offloading decks, however,

these are different in terms of settings (occupational versus public), scale and mode of operation. In Australia, occupational settings fall under a different regulatory regime and are not the focus of this research. WMS are used in public places, and in private dwelling settings. Public places include entertainment, recreational and areas where members of the public gather for business, worship or similar purposes. These systems are also used in private settings, which can be divided into two categories namely: privately owned commercial operations such as mining camps, roadhouses, caravan parks and private dwellings. The installation and use of WMS in private dwellings is not the focus of this research. In all settings for which this research focuses on, data on the number of people exposed to WMS bioaerosols is not captured and therefore not available. Currently there is no guidance on the safe operation of these WMS, except the non-standard do-it-yourself installation instructions that come with off-the shelf kits.

1.2 Theoretical framework

1.2.1 Opportunistic premise plumbing pathogens

Wang et al. (2013) have advanced the ecological niche model (Figure 1.4) to explain how OPPPs such as *L. pneumophila* survive within *Amoeba* and overcome microbial competition from other indigenous microbes in the system. Thomas et al. (2014) builds on this model, arguing that the presence of *Amoeba* in ordinary garden hoses also poses a health risk due to this FLA's ability to enhance the survival of *Legionella* spp.

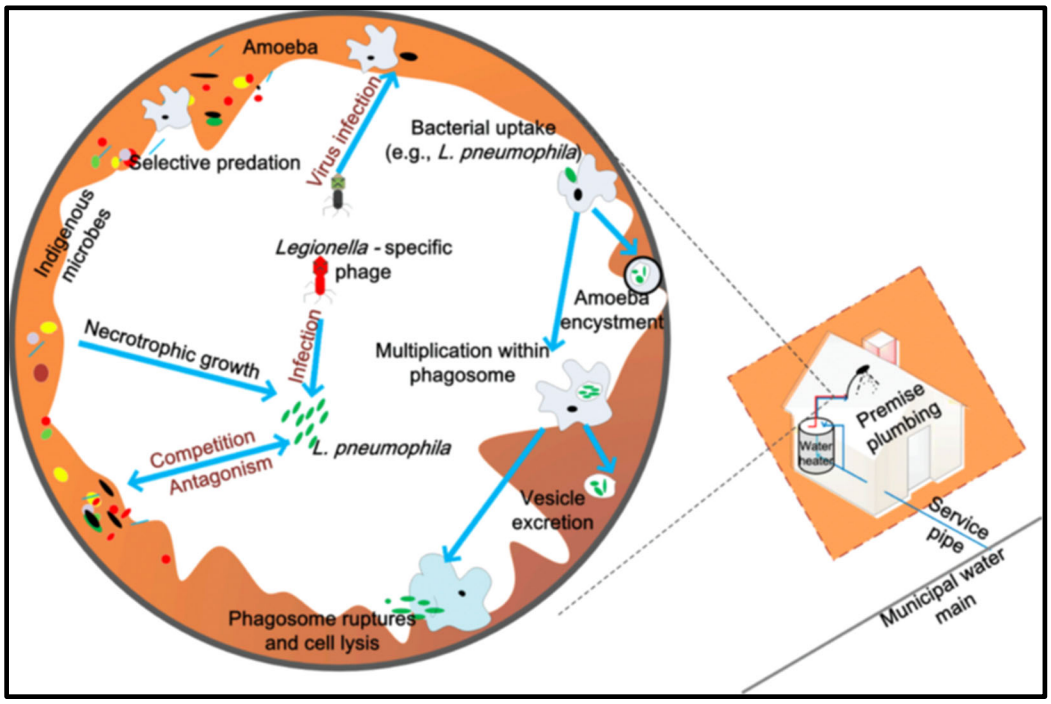

Figure 1.4 Ecological niche model for opportunistic premise plumbing pathogens (Wang et al, 2013)

1.2.2 Water quality

Ling et al. (2018), adopted the island biogeography model (Figure 1.5), explaining that premise plumbing water quality is influenced by factors such as reduced pipe diameters from the proximal mains supply pipework to the distal premise plumbing network, and the reduced residual free chlorine concentration past the mains supply. According to this model, these factors act as a geographic emigration limitation for the usual microbial flora that thrives in the water mains supply (proximal pipework) and will instead create an insular environment within the premise plumbing system (distal pipework) in which sturdy, naturally occurring OPPPs can thrive and regrow. This biogeography model is particularly relevant for the aims of this research, considering its focus on the impact of water quality on the resultant microbial flora that establishes itself in distal pipework such as WMS.

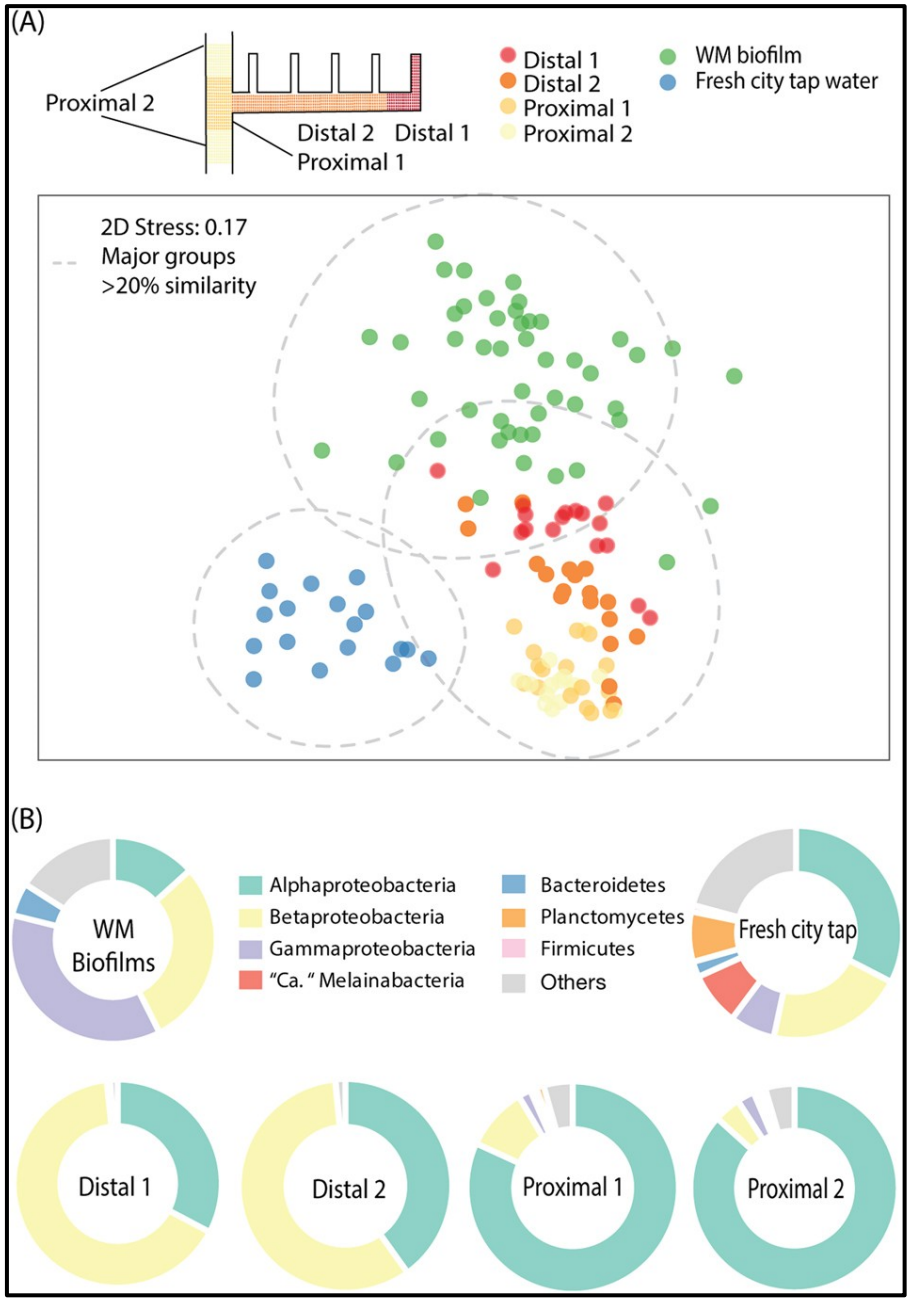

Figure 1.5 Biogeography model of drinking water microbiome assembly induced by water stagnation (Ling et al., 2018)

1.2.3 Bioaerosol science

Davis et al (2016) have explained that in situations where a water distribution system (WDS) is contaminated, the short-term inhalation exposures of people in domestic settings to airborne contaminants is possible. Baranovsky et al. (2018) built on this finding establishing that if water mists contaminated by OPPPs such as *Legionella*

spp. are inhaled, they may cause infections in exposed persons. The findings of Baranovsky et al. (2018) are the most relevant for the aims of this research given the emphasis it places on OPPP respiratory exposure.

1.2.4 Knowledge and skills

Glanz et al. (2008) have explained that the Health Belief Model (HBM) postulated in the 1950s was the foundation of the Knowledge Attitude and Practices (KAP) theory and acknowledged that people will engage in actions that improve their health based on what they perceive to be beneficial and valuable to themselves. Fan et al. (2018) expanded these early theories by arguing that knowledge is the foundation of behaviour changes, and that belief and attitudes drive these changes. Julien et al. (2020) then incorporate the importance of improving the knowledge and skills to be able to identify the impact of environmental parameters such as water stagnation and inadequate disinfection on the proliferation of OPPPs in premise plumbing. The findings of both Fan et al. (2018) and Julien et al. (2020) are the most relevant for the aim of this research, given the emphasis they put on the importance of knowledge and skills in driving behaviour change, and the need to understand factors that promote OPPP growth respectively. This justifies the assessment of human factors among WMS owners and EHOs to determine their ability to understand the health risks associated with the use of WMS and take actions to prevent their impact.

1.3 Research questions

To better understand the health risk of using WMS due to their colonisation by OPPP, the occurrence and prevalence of 5 representative OPPPs; *L. pneumophila*, *P. aeruginosa*, *M. avium*, *Acanthamoeba* and *N. fowleri* in WMS was investigated along with the influence of water temperature, free residual chlorine concentration, water pH, TDS, and TOC on the occurrence of these OPPPs. The WMS owners and EHO knowledge about the health risks of using WMS was also examined. To meet the research aim, the thesis addresses 3 research questions:

1.3.1 Question 1

What is the state of knowledge about the health risks associated with the use of WMS as a cooling intervention in public places?

1.3.2 Question 2

What are the characteristics of WMS used as a cooling intervention in public places and the level of knowledge and awareness of the associated health risks among owners of these systems and EHOs involved in the regulation of environmental health?

1.3.3 Question 3

Can WMS be colonized by OPPPs and are environmental conditions of water temperature, residual chlorine disinfectant concentration, water pH, TDS and TOC associated with the occurrence of OPPPs in WMS?

In summary, this thesis considered data from 3 studies to understand the health risks associated with the use of WMS used as a cooling intervention in public places. Participants for the survey research were drawn from a cohort of WMS owners and EHOs operating and working in the northwest (Pilbara) region of Western Australia, and 10 conveniently selected WMS were examined for the presence of OPPPs.

The findings will inform the review of existing public health legislation, with the aim of adopting a risk-management approach to ensure the effective and safe operation of WMS and furthermore to inform the development of guidelines to assist owners in the safe operation of WMS.

CHAPTER 2 HEALTH RISKS ASSOCIATED WITH THE USE OF WATER MIST SYSTEMS AS A COOLING INTERVENTION IN PUBLIC PLACES: A REVIEW OF THE LITERATURE

2.1 Introduction

Water mist systems (WMS) are defined as plumbing mechanisms installed in outdoor public places to reduce ambient temperatures. Small nozzles fitted to WMS atomize water into tiny aerosols that flash evaporate in the ambient atmosphere, reducing surrounding temperatures by as much as 10ºC (Ozmist, n.d.). These WMS are more energy efficient than conventional air conditioning systems (Wong & Chong, 2010). However, premise plumbing can promote the colonization and regrowth of opportunistic premise plumbing pathogens (OPPPs) including *L. pneumophila*, *M. avium*, *P. aeruginosa, Acanthamoeba and N. fowleri* (Falkinham, 2015; Whiley et al., 2014). These OPPPs cause opportunistic infections in people with compromised immunity, children and the aged (Falkinham et al., 2015).

Most of the WMS research has been experimental, focusing on design capabilities and the operational efficiency of the systems (Wong & Chong, 2010; Xuan et al., 2012). However, research on premise plumbing installations such as showers, water taps, and faucets has confirmed the presence of *L. pneumophila*, *M. avium*, *P. aeruginosa*, *Acanthamoeba*, and *N. fowleri* (Falkinham et al., 2015; Whiley et al., 2014). This literature review examines and describes the OPPP health risks associated with WMS systems in the Pilbara region of Western Australia. This region experiences extreme temperatures and has a higher use of WMS. The literature review focuses on the five major OPPPs implicated in aerosolised waterborne pathogens, namely, *L. pneumophila*, *M. avium, P. aeruginosa, Acanthamoeba* and *N. fowleri.*

2.2 Water misting systems

2.2.1 Water misting system location

Most WMS are located outdoors where they are exposed to elevated temperatures. The operation of WMS in these environmental conditions increases the water temperatures to levels that OPPPs such as *L. pneumophila* thrive (Lu et al., 2017). The densities of *Legionella* spp. and *Mycobacterium* spp. can increase with elevated water temperatures of 25 ºC – 40 ºC in showers and water taps (Lu et al., 2017). WMS located outdoors and exposed to elevated temperatures may be a risk for OPPPs.

2.2.2 Water misting system plumbing materials

The WMS used for cooling public places are made from varied materials such as polyvinyl chloride (PVC), polyethylene, nylon, or steel. The use of these plumbing materials can promote the regrowth of OPPPs (Wang, et al., 2012). These plumbing materials leach nutrients in the form of total organic carbon that promote biofilm formation on the internal surfaces of pipework and fittings (Rogers et al., 1994).

2.3 Water stagnation

Water stagnation has the effect of depleting residual free chlorine in water systems, resulting in the regrowth of OPPPs (Wang et al., 2013). When WMS are shut down during winter, there is potential for OPPP growth that can subsequently be aerosolized if the units are turned back on in summer without proper treatment. A whole lifecycle treatment plan must include the winter shutdown period and incorporate controls to prevent generation of contaminated aerosols.

2.4 Opportunistic premise plumping pathogens and opportunistic infections

The use of aerosol producing water systems is an emerging public health concern (Falkinham III et al., 2015; Wang et al., 2013) because they represent a potential exposure route to so called “opportunistic pathogens”, which can affect the health and wellbeing of exposed individuals, especially amongst vulnerable populations with predisposing risk factors, such as young children, the elderly, and HIV/AIDS patients (Falkinham et al., 2015). Key OPPPs associated with premise plumbing are *L.*

pneumophila, *M. avium*, *P. aeruginosa*, *Acanthamoeba* and *N. fowleri* (Bédard et al., 2016; Falkinham III et al., 2015).

2.4.1 *Legionella pneumophila*

Legionella pneumophila is associated with premise plumbing and several outbreaks of waterborne legionellosis (Bennett et al., 2014; Cohn et al., 2015). The bacteria colonize cooling towers, warm water baths, water fountains and showers, which when disturbed (aerosolized) can result in respiratory disease, and even death of exposed persons (Kim et al., 2015). This OPPP can grow inside amoebae (Liu et al., 2019) making it resistant to chlorine disinfection (Dupuy et al., 2011). *L. pneumophila* has been isolated from household plumbing (Barna et al., 2016; Brenda et al., 2018).

2.4.2 *Mycobacterium avium*

Mycobacterium avium belongs to a group of non-tuberculous *Mycobacteria* (NTM) called *Mycobacterium Avium* Complex (MAC). The MAC includes *M. avium* and *M. intracellulare* which are found in aquatic environments and soil, and transmitted via inhalation, ingestion, or inoculation (Rijhumal & Chai, 2015). The MAC causes various infections depending on the subspecies, its route of infection, as well as the immune health of the exposed person (Whiley et al., 2012). In people with compromised immunity, MAC causes pulmonary and soft tissue infections in healthy individuals (Falkinham, 2016). *M. avium* can cause cervical lymphadenitis in young women (Reuss et al., 2017) and pulmonary diseases in HIVAIDS patients (Falkinham , 2011).

Mycobacterium avium has been isolated from premise plumbing and potable water systems (Water Research Australia, 2014; Whiley et al., 2012), hospital plumbing (Baird et al., 2011) and household plumbing (Falkinham et al., 2008). This bacterium's ability to grow at temperatures >45 °C, paired with its chlorine resistance, enables it to thrive in water distribution systems (Falkinham et al., 2008). *M. avium* can colonize showerheads (Feazel et al., 2009), water taps and water-heaters (Wang et al., 2012), and subsequently be transmitted by the inhalation of contaminated aerosols (Falkinham III, 2013). Like *L. pneumophila, M. avium* can resist disinfection in premise plumbing by inhabiting amoebae (Steed & Falkinham, 2006). The misting systems in public places mimic showers in terms of elevated temperatures (Feazel et al., 2009;

Lu et al., 2017), as well as plumbing materials and potential for aerosol formation (Steed & Falkinham, 2006), making them a health risk for exposure to this OPPP.

2.4.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a versatile OPPP that can adapt and survive tough environmental conditions (Bédard et al., 2016). The bacterium favours moist environments and has low nutritional requirements because of its ability to metabolize different compounds (Yu et al., 2016). These properties enable *P. aeruginosa* to form biofilms with other microorganisms present in premise plumbing systems and confers resistance to disinfectants such as chlorine dioxide and monochloramines (Masak et al., 2014). Transmission of *P. aeruginosa* occurs through exposure to contaminated water by inhalation and immersion and can cause aggressive pneumonia in immunocompromised persons such as those with cystic fibrosis (Falkinham, 2015); as well as self-limiting ear and skin infections (Rossolini & Mantengoli, 2005). *P. aeruginosa* has been isolated from hospital water taps (Shareef & Mimi, 2008), showerheads and hydrotherapy pools (Caskey et al., 2018). The ubiquitous nature of this bacterium in the environment, combined with its hardiness and potential to form biofilms enhances its chlorine resistance (Zichichi et al., 2000). These factors make *P. aeruginosa* a particular concern in relation to misting systems that generate aerosols and are widely used in licensed clubs frequented by the elderly, and potentially immunocompromised individuals.

2.4.4 *Acanthamoeba*

Acanthamoeba is a protozoan that lives in varied environments, such as environmental and drinking water systems (Michel et al., 1997), tap and well water (Marciano-Cabral et al., 2010), hospital waters (Muchesa et al., 2015), aquatic facilities (Chang et al., 2010) and recycled water (Storey & Kaucner, 2009). *Acanthamoeba* is the causative agent of the central nervous system disease called granulomatous amoebic encephalitis (GAE), which affects people with weakened immunity. The species *Acanthamoeba*, can also cause infection of the corneal epithelium (Pruden et al., 2013). The microorganism can grow in domestic tap water (Codony et al., 2012), and has been isolated from hospital water supplies (Muchesa et al., 2015). A significant characteristic of this OPPP is its ability to engulf and shield other OPPPs

such as *L. pneumophila*, *P. aeruginosa* and *M. avium* from disinfection (Żbikowska et al., 2014), which makes it an essential target for WMS infection control strategies.

2.4.5 *Naegleria fowleri*

N. fowleri, the only pathogenic species of its genus, causes fatal primary amoebic meningoencephalitis (PAM). The infectious disease is transmitted by aspiration of contaminated water aerosols up the nasal passage (Yoder et al., 2012). This OPPP can live in premise plumbing, rainwater tanks as well as any system where warm water is present (Waso et al., 2018). The warm operational temperatures of WMS, coupled with their generation of inhalable water aerosols make them a source of this rare, but fatal infection.

2.5 Public health impact of opportunistic premise plumbing pathogens

The public health risk of OPPPs and their associated infectious diseases is significant. The ability of WMS to atomize water into aerosols is the key feature by which these systems achieve thermal comfort. The aerosols range in size from 0.3 to 10 µm and can be deposited into the lungs by inhalation where they can cause infections (Henningson & Ahlberg, 1994). The OPPPs range in size from 2 to 20 µm for *L. pneumophila* (Füchslin et al., 2010), 0.2 to 0.6 µm for *M. avium* (Vijay et al., 2017), 0.5 to 1.0 µm for *P. aeruginosa* (Deforet et al., 2015), 12 to 35 µm for *Acanthamoeba* (Khan & Siddiqui, 2012*)* and 15 to 20 µm for *N. fowleri* (Piñero et al., 2019). Most of these OPPPs fall within the size fraction that can be inhalable by people, as well as impact on skin and surfaces, creating a potential for exposure to pathogens. They can be ingested and can also cause skin irritation.

Legionella spp. alone, is responsible for 2 to 15% of patients hospitalized globally with community-acquired pneumonia (Sakamoto, 2015). In the USA, 32 cases of waterborne disease outbreaks were reported in the 2011/12 period, with 66% of the outbreaks being associated with *L. pneumophila,* and the incidence rate for waterborne *M. avium* disease over the same period was 647 cases per 100,000 persons (Beer et al., 2015*).*

In Australia, an average of 374 cases of legionellosis were notified annually between 2008 and 2018 (Australian Government Department of Health, 2019), with an incidence rate of 2 per 100,000 persons in 2015, dropping to 1 per 100,000 in 2019. The combined mandatory reporting of *L. pneumophila* and *L. longbeache* infections as legionellosis cases in Australia does not provide information about the incidence of infections caused by different *Legionella* spp. This obscures any trends associated with exposure routes, considering that one is soil-borne (*L. longbeache)* and the other is water-borne (*L. pneumophila).* A total of 143 cases of pulmonary amoebic meningoencephalitis (PAM) were reported across the USA. between 1962 and 2017 (Centres for Disease Control and Prevention, 2019). In Australia, 19 water-related PAM cases were recorded between 1960 and 1980 (Waso et al., 2018), and another 4 cases were reported in Queensland during the period 2006 to 2015 (Nicholls et al., 2016). The case rate for microbial keratosis, a disease caused by *Acanthamoeba* was 1 cases per 10,000 between 2005 and 2015 (Waso et al., 2018). Opportunistic infections caused by *M. avium* and *P. aeruginosa* may be underestimated because they are not notifiable in most countries (Falkinham et al., 2015). However, NTM diseases are notifiable conditions in the Australian states of Queensland and the Northern Territory. Pulmonary amoebic meningitis is notifiable in Western Australia (Australian Government Department of Health, 2019).

2.6 Water misting systems and factors promoting colonization by opportunistic premise plumbing pathogens

2.6.1 Biofilm formation

Biofilm potential is a significant risk factor for the incidence of respiratory/infectious disease outbreaks associated with WMS. Biofilms are complex heterogeneous colonies consisting of bacteria, fungi, protists, and other microbial organisms that grow as native communities in water systems (Wingender & Flemming, 2011). The formation of biofilms in premise plumbing systems increases their OPPP colonisation potential (Ashbolt, 2015; Falkinham, 2015; Pruden et al., 2013), and acts as a protection against disinfectants used to clean systems (Momba et al., 2000). The biofilms provide conducive and nutritive conditions for OPPP growth (Wang et al., 2013). Ninety-five percent of the microbiological population in drinking water systems

reside in biofilms as compared to approximately 5% in the water phase (Flemming et al., 2002). The OPPPs residing in biofilms of water systems can be released into the water phase where they can cause waterborne diseases (Flemming, 2011). Biofilm formation may occur due to extreme environmental conditions of temperature, pH, and pressure (Momba et al., 2000), and maintenance programs aimed at minimizing potential for their generation is essential. The sampling and analysis of biofilm samples from WMS used for cooling is recommended to provide an insight into their potential as sources of OPPPs.

2.6.2 Temperature

Elevated water temperatures in distribution systems promote the growth of OPPPs (Falkinham, 2015; Storey et al., 2004). The ability of microorganisms to survive at elevated water temperatures is a critical adaptation feature that enables *L. pneumophila*, *M. avium,* and *P. aeruginosa* to thrive in water systems (Falkinham III et al., 2015). The WMS used for cooling public places are exposed to high temperatures which can promote the regrowth of OPPPs (Storey & Kaucner, 2009). Determining the water temperature profile of WMS is needed to understand its influence on OPPP regrowth.

2.6.3 Presence of free-living *Amoeba*

The presence of amoeba in premise plumbing can aid the regrowth of OPPPs (Wang, 2013; Wang et al., 2013). The ability of free-living *Amoeba* (FLA) to amplify the number and virulence of OPPPs in engineered water systems is now widely recognised (Falkinham, 2015; Thomas & Ashbolt, 2010). Given the important part played by FLA, particularly *Acanthamoeba*, in the regrowth and amplification of OPPPs, as well as their virulence in water distribution systems, WMS used for cooling public places need to be investigated for this protozoan (Codony et al., 2012).

2.6.4. Opportunistic premise plumbing pathogens and resistance to chlorine disinfection

Chlorine is an effective water disinfectant (Western Australia Environmental Health Directorate, 2016), and remains as one of the most important public health

interventions of the century (Boorman, 1999). At the right pH (6.5 – 8.5), temperature (20 – 29 ºC) and turbidity, chlorine provides an adequate residual disinfectant effect (National Health and Medical Research Council, 2011). However, under certain environmental conditions, OPPPs can become resistant to chlorine and its derivatives (Canals et al., 2015; Codony et al., 2012), especially when part of a biofilm colony. Most WMS are connected to scheme water but several of those located in remote parts of the region use onsite borehole water supplies that are locally managed. Chlorination is the most common means of disinfection for Australian water supplies, with a minimum target of 0.5 mg/L residual chlorine recommended (National Health and Medical Research Council, 2011). Since chlorination is the main form of disinfection for water supplies connected to WMS, an investigation of its effectiveness in controlling the regrowth of OPPPs in these systems is warranted.

2.6.5. Opportunistic premise plumbing pathogens and low total organic carbon (TOC) concentration (Oligotrophic conditions)

Opportunistic premises plumbing pathogens can thrive in premise plumbing systems with low carbon concentrations (Falkinham III et al., 2015). Low-carbon or oligotrophic environments are characteristic of most premise plumbing (Wang et al., 2013). The nitrifying bacterial autotrophs present in low-carbon waters fix available carbon, making it available to heterotrophic organisms such as OPPPs to metabolize (Wang et al., 2013; Zhang & Edwards, 2009). Through this process, low-carbon water environments existing in WMS can select for *L. pneumophila* (van der Kooij et al., 2005), *P. aeruginosa* (Bédard et al., 2016) and *M. avium* (Falkinham et al., 2015). To better understand the impact of oligotrophic conditions on the ability of OPPPs to colonize and regrow in WMS, the sampling and analysis of water from these systems for TOC concentration is needed.

2.7. Other control methods for opportunistic premise plumbing pathogens in water misting systems

The resistance of OPPPs to disinfection presents significant challenges in using traditional approaches to control them, therefore alternative control approaches for OPPPs in premise plumbing may be needed. Control of OPPPs in water can be

achieved by reducing its turbidity (Falkinham III et al., 2015). Regular cleaning and disinfecting of nozzles may be an effective way of controlling OPPPs (American National Standards Institute, 2018; Health and Safety Executive, 2014). The installation of microbiological filters to WMS can control OPPPs (National Research Council, 2006). Like most bacteria, *L. pneumophila* and *P. aeruginosa* are susceptible to ultraviolet (UV) irradiation and can be controlled in premise plumbing by this method (Falkinham, 2015; Leoni et al., 2015), but it is noted that high turbidity will provide shelter for microorganisms. The introduction of non-pathogenic protozoa species that target OPPPs can achieve a probiotic control of these microorganisms in WMS (Wang et al., 2013). The addition of silver ions (Ag+), at a concentration of 40 µg/litre, has a bactericidal effect on *L. pneumophila* and many other microorganisms in plumbing systems without affecting humans, making them suitable for controlling OPPPs in WMS (Fewtrell, 2014).

2.8 Literature review summary

The use of WMS as a cooling intervention in public places should be considered a public health risk due to the potential for poorly managed systems to release microbial contaminated inhalable aerosols. These aerosols could contain pathogenic organisms, referred to as OPPPs, such as *L. pneumophila, M. avium, P. aeruginosa, Acanthamoeba* and *N. fowleri*. In addition to inhalation, the WMS aerosolization of contaminated water may result in deposition on skin, food, and other articles, causing a localized reaction (skin, eyes) or being ingested.

In this chapter, literature was reviewed to answer research question 1, *"What is the state of knowledge on the health risks of using WMS as a cooling intervention in public places?"*. Initially, it was expected that research articles focusing on WMS used specifically for evaporative cooling and their health risks would be identified, however, this review revealed a significant lack of research in this area. It was also established that there was limited research on the ecological role free-living amoeba (FLA) in acting as a host and shield for OPPPs in WMS, contrary to the extensive coverage of this aspect in all other premise plumbing features. Furthermore, this literature review revealed the concept of knowledge and skills as a risk factor for the safe WMS operation was scarcely mentioned in the existing literature. In view of the absence of

literature specifically investigating the health risks of using WMS as a cooling intervention in public places, research findings on OPPP colonisation in similar premise plumbing features (showers and hospital plumbing systems) was used as a foundation for investigating the potential of OPPPs to colonize and regrow in WMS. Based on this literature review summary, an investigation of the health risks associated with the use of WMS as a cooling intervention is warranted to better understand their public health impact and inform strategies to manage them.

CHAPTER 3 KNOWLEDGE AND AWARENESS SURVEY ON HEALTH RISKS OF USING WATER MIST SYSTEMS AS A COOLING INTERVENTION

3.1 Introduction

Building water systems such as air conditioning units, showers and fountains have been linked to outbreaks of waterborne diseases such as legionellosis caused by the plumbing pathogen *L. pneumophila*. The lack of maintenance of such systems, and the inadequate knowledge and awareness of the health risks associated with such aerosol producing systems have been implicated in several outbreaks (Bennett et al., 2014; Cohn et al., 2015).

The level of knowledge and the ability to comprehend issues that affect water quality is a key factor in addressing public health problems related to water distribution systems (Dean et al., 2016a). Furthermore, knowledge plays an integral part of conceptual models that aim to achieve higher levels of engagement with those involved in water use of any kind (Dean et al., 2016b). Research has also established a direct relationship between knowledge and the ability of people to support efforts and programs related to water quality (Salvaggio et al., 2013).

Of critical importance in safe system operation is knowledge of the effect of water stagnation and extended periods of water retention within plumbing systems on microbial colonization and proliferation by (Julien et al.,2020).

The level of knowledge and competency to safely operate WMS amongst the owners and operators is unknown. Similarly, the level of the same knowledge among the EHOs responsible for regulation of the WMS systems has not been determined. In addition to determining the knowledge of the health risks of using WMS among system owners and EHOs, this survey investigated the operational, administrative, and legislative factors that can influence the regrowth of opportunistic premise plumbing pathogens (OPPPs) in these features. The WMS used as a cooling intervention in public places are outside the scope of the *Health (Air handling and Water Systems) Amendment Regulations, 2013* (WA) that focus on heat exchange systems

incorporating heating plants and cooling towers as well as associated pipework and fittings. This means that their design, installation, and operation is currently unregulated, unless they were associated with a notifiable condition and would be required to be investigated as part of an outbreak.

Internationally, several other jurisdictions have already acknowledged the potential of water systems like WMS to promote *Legionella* spp. growth, as evidenced by their development of codes of practice, standards and technical guides emphasising the importance of knowledge, skills, and maintenance regimes in preventing the outbreak of waterborne diseases, particularly legionellosis (American National Standards Institute, 2018; enHealth, 2015; Health and Safety Executive, 2014).

This survey of WMS owners and EHOs builds on the findings of existing research, along with the provisions in standards and technical guides, to assess the level of knowledge, awareness, competence, and maintenance among WMS owners and EHOs. The results of this survey informed the design and implementation of an experimental investigation that determined the type and occurrence of OPPPs in WMS.

3.2 Ethics

This study received prior approval from the Edith Cowan University (ECU) Human Research Ethics committee (HREC), Approval Number 16337 MASAKA (Appendix 4). The written informed consent of all research participants was secured prior to their participation in the study.

3.3 Materials and methods

A cross-sectional descriptive survey of WMS owners and EHOs was carried out in the north-western region of Australia from 2018 -2019.

3.3.1 Study population

The study population for this survey research comprised of 27 EHOs and 15 WMS owners working and operating in the north-western part of Western Australia. Only EHOs employed in local governments and those working in non-governmental

organisations (NGOs) involved in environmental health were included in this study. Similarly, only owners of WMS installed and operated in public places were included in the study. After applying the inclusion and exclusion criteria discussed above, a total of 27 EHOs and 10 WMS owners were eligible for recruitment.

3.3.2 Sample size determination and recruitment of participants

The Qualtrics sample size calculator (Smith, 2010) was used to determine the sample sizes for the EHOs and WMS owners' surveys, respectively. The population size, a 5 % margin of error, 95 % confidence level and a standard deviation of .5 were applied in calculating the sample sizes. The population size of EHOs working in the study area was calculated from a publicly available register of EHOs working in the north-western region of Western Australia, and the one for WMS owners was determined from the public registers of public and private commercial places kept by the local governments in the study area. It is widely accepted that no sample is perfect (Smith, 2010), therefore determining the confidence interval is important to determine how much error to allow. Pursuant to this, a 5 % margin of error, or 95 % confidence level was set. Questionnaire responses will inevitably exhibit a level of variance and determining a standard deviation that will moderate this expected variation is important (Smith, 2010). A standard deviation of 0.5 was therefore set prior to the administration of the surveys. After applying these parameters, a priori sample size of 10 WMS (100% of the eligible population size) and 26 EHOs was determined.

Participant lists were prepared from the publicly available register of the EHOs, public and private commercial establishment owners. Email invitations that incorporated a link to the survey were sent out using Qualtrics. The provision of a link assured the participants of anonymity. The survey questionnaire included an information sheet that explained the purpose the research, what the results will be used for, their right to decline to participate and confirmation of their willingness to do so. The email also mentioned a deadline for the survey completion and submission. At least one reminder email was automatically programmed into the Qualtrics for those unable to submit by the due date. The survey response rate for the EHO survey was 84.6%, and 100% for the WMS owners.

3.3.3 Survey questionnaires

3.3.3.1 Survey questionnaire for WMS owners

A questionnaire for WMS owners was developed based on the design, operational, maintenance and risk management recommendations of the Health and Safety Executive (HSE)*'s Legionnaires disease Technical Guidance HSG 274 Part 2, The control of Legionella* spp. *in hot and cold-water systems* (Health and Safety Executive, 2014), the *American National Standard Institute's ANSI/ASHRE Standard 188 - 2008* (American National Standards Institute, 2018) and Australia's *enHealth Guidelines for Legionella Control in the operation and maintenance of water systems in health and aged care facilities* (enHealth, 2015). The questionnaire was structured and contained single and multiple response questions to gather information on the type, location, water source, aerosolization, operational and maintenance aspects of WMS, as well as the level of knowledge and perceptions on the associated health risks.

This questionnaire was pilot tested with 4 WMS owners operating outside the study area to assess its feasibility in terms of the time it takes to complete, the clarity of questions and the consistency of coding to ensure accurate result interpretation (Jesús García de Yébenes Prous et al., 2009).

A Kappa index score of 0.26 for the WMS owners' questionnaire was calculated from the pilot test, demonstrating a moderate reliability as a data collection instrument (Jesús García de Yébenes Prous et al., 2009). Data from the pilot test were not included in the final analysis.

3.3.3.2 Survey questionnaire for Environmental Health Officers

The survey questionnaire for EHOs was developed based on the same criteria used to design the one for WMS owners except for the addition of questions on regulatory and inspection regimes for WMS installation and operation.

This questionnaire was pilot tested on 5 EHOs who do not work in the study area for the same reasons advanced in 3.3.3.1. A Kappa index score of 0.25 for the questionnaire demonstrated moderate reliability for data collection (Jesús García de

Yébenes Prous et al., 2009). The pilot data was also excluded from the final analysis of the survey results.

3.3.4 Data analysis

Data was analysed using the Minitab version 18 statistical software package. Prior to analysis, the categorical variables were coded 1 or 0 to facilitate data analysis (Alkharusi, 2012). The Fisher's exact test was used to measure association between variables because of its inability to be affected by small sample sizes (McDonald, 2009). A small sample size is likely to lower the possibility of picking up a real effect (Button et al., 2013). A confidence level of 95% (.05) was used to determine the significance of any association between variables. Results were presented as percentages, frequency tables, pie charts, bar graphs, and doughnuts.

3.4 Results

In this section, the results of the WMS owners survey are presented first followed by the results of the EHOs survey.

3.4.1 Water mist system owners survey results

3.4.1.1 Owners' responses to the type, use, and water sources used in WMS

Table 3.1 shows the WMS owners' description of their systems, purpose of use, type of nozzles used to aerosolise water and the source of water used. More than half of the WMS are used in public places for temperature reduction, and 90% of the systems use hydraulic nozzles to aerosolise and disperse water obtained from scheme water.

Table 3.1 Owners' responses to the type, use, and water sources for WMS

Survey Question	Responses	Frequency of responses	Percentage n = 10
Type of WMS used	Public	5	50%
	Private	3	30%
	Private and Public	2	20%
	Total	10	100%
Reasons for using WMS * *(Multiple response)*	Temperature reduction	7	47%
	Air humidification	1	7%
	Improve comfort	6	40%
	Others	1	7%
	Total	15	100%
Type of nozzles used on WMS	Pneumatic	1	11%
	Hydraulic	9	90%
	Total responses	10	100%
Source of water used in WMS	Bore-treated	0	0%
	Bore-untreated	0	0%
	Scheme	10	100%
	Surface	0	0%
	Reverse osmosis	0	0%
	Rainwater	0	0%
	Total responses	10	100%

3.4.1.2 Owners' responses to knowledge, health risks and importance of using WMS

Sixty percent of the WMS owners did not know that aerosolization of water in the WMS could expose people to the risk of inhaling OPPPs. However, 30% of them indicated that biological risks could arise from using these systems. These results indicate that most of the WMS owners (70%) were aware of the public health importance of WMS, with elevated temperatures and poor maintenance being the factors they thought could promote the growth of pathogens.

3.4.1.3 Owners' responses to WMS maintenance arrangements

Figure 3.1 indicates the WMS owners' perception of the importance of maintaining their systems, the frequency of maintenance undertaken, observation of biofilm

formation and whether maintenance schedules were used or not. Ninety percent of them perceived that adequate disinfection and adherence to manufacturer's specifications were the 2 most important maintenance aspects (Panel B of Figure 3.1). However, 70% did not observe biofilm formation in their systems (Panel C of Figure 3.1). Half of the WMS were occasionally or never maintained (Panel A of Figure 3.1), and only 40% of those who did have a cleaning and maintenance schedule in place (panel D of Figure 3.1)

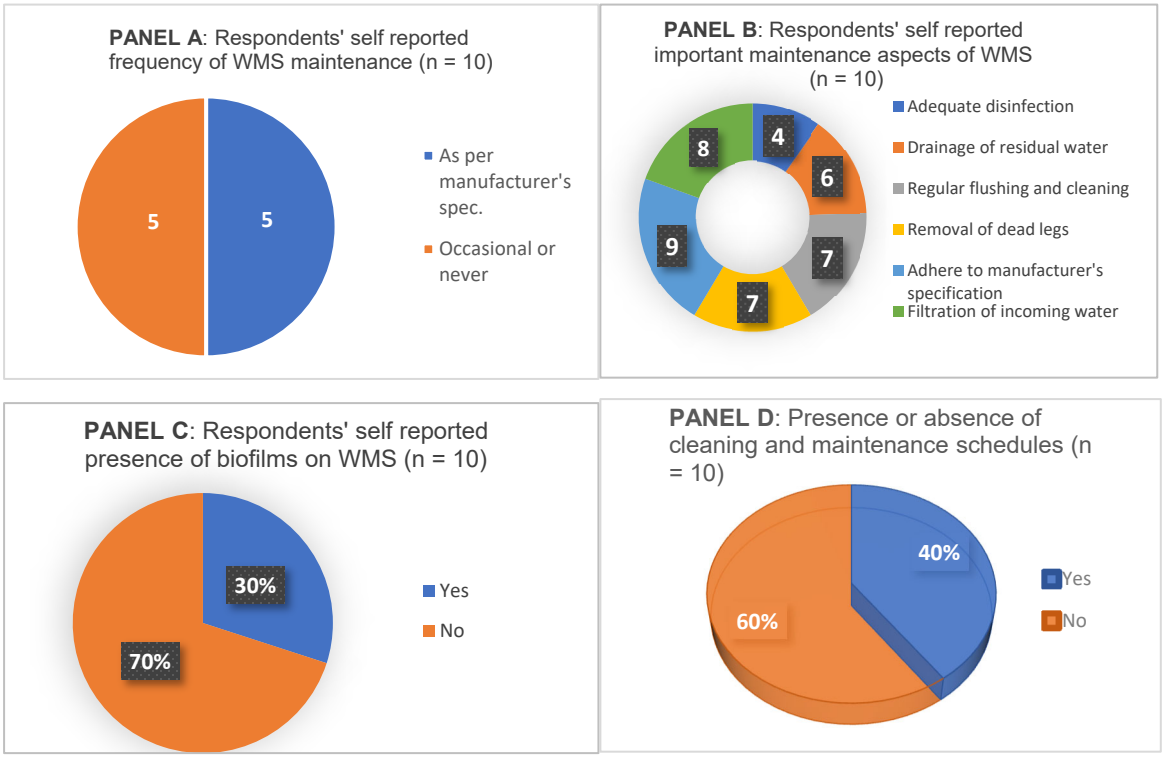

Figure 3.1 Owners' responses to WMS maintenance arrangements

3.4.1.4 Owners' responses to WMS training and operational competence

Regarding their status of training and competence in operating WMS, only 1 operator confirmed receiving in-house training, and 30% perceived themselves to be incompetent in operating these systems.

3.4.2 Environmental Health Officers survey results

3.4.2.1 EHO responses to the number, location, use, and water sources of WMS

The results in Table 3.2 indicate that the number of WMS used in each surveyed EHOs local government area was 1 to 5 (86%), with most of these being located outdoors (95%) and used for temperature reduction (76%). The EHOs reported the use of different sources of water in WMS with scheme water (59%) and treated bore water (36%) being the 2 common sources. However, 100% of the WMS owners indicated that only scheme water was used, a result that was statistically significant *(p = 0.03, Fisher's exact test)*. The results also confirm the seasonal use of WMS (86%) against frequent use (9%), a result that was statistically significant, *(p = 0.01, Fisher's exact test)*. All the EHOs indicated that hydraulic nozzles were used to aerosolise and disperse water aerosols, factors that are significant for public health when using water systems.

Table 3.2 EHO responses to the number, location, use, and water sources of WMS

Question	Responses	Frequency of responses	Percentage
No. of WMS in local government area	Nil	1	5%
	(1-5)	19	86%
	(6-10)	1	5%
	>10	1	5%
	Total	22	100%
Location of WMS	Outdoors	21	95%
	Indoors	1	5%
	Outdoors and Indoors	0	0%
	Total	22	100%
Use of WMS *	Temperature reduction	22	76%
	Air humidification	4	14%
	Decoration	1	3%
	Others	2	7%
	Total	29	100%
Source of water used in WMS	Bore-treated	8	36%
	Bore-untreated	0	0%
	Scheme	13	59%
	Surface	0	0%
	Reverse osmosis	1	5%
	Rainwater	0	0%
	Total	22	100%
WMS frequency of use	Often (> 4hrs per day)	2	9%
	Seasonal (Summer only)	19	86%
	Infrequent (No regular pattern)	1	5%
	Total	22	100%
Type of WMS nozzles	Pneumatic	2	9%
	Hydraulic	20	91%
	Total	22	100%
WMS aerosolisation	Yes	21	95%
	No	1	5%
	Total	22	100%

3.4.2.2 EHO responses to knowledge and awareness of WMS health risk questions

Panel A of Figure 3.2 indicates a low level of knowledge (77%) about the health risks of using WMS amongst the EHOs with those knowledgeable being able to identify only *Legionella* spp.*, Amoeba* spp. and *Pseudomonas* spp. as the specific health risks of using WMS (Panel B of Figure 3.2). None of the EHOs indicated knowledge of the

health risk posed by *N. fowleri* and *Mycobacterium* spp. The low level of knowledge about the health risk of using WMS amongst the EHOs (77%) was not significantly different from that of the WMS owners (*p = 0.37, Fisher's exact test).*

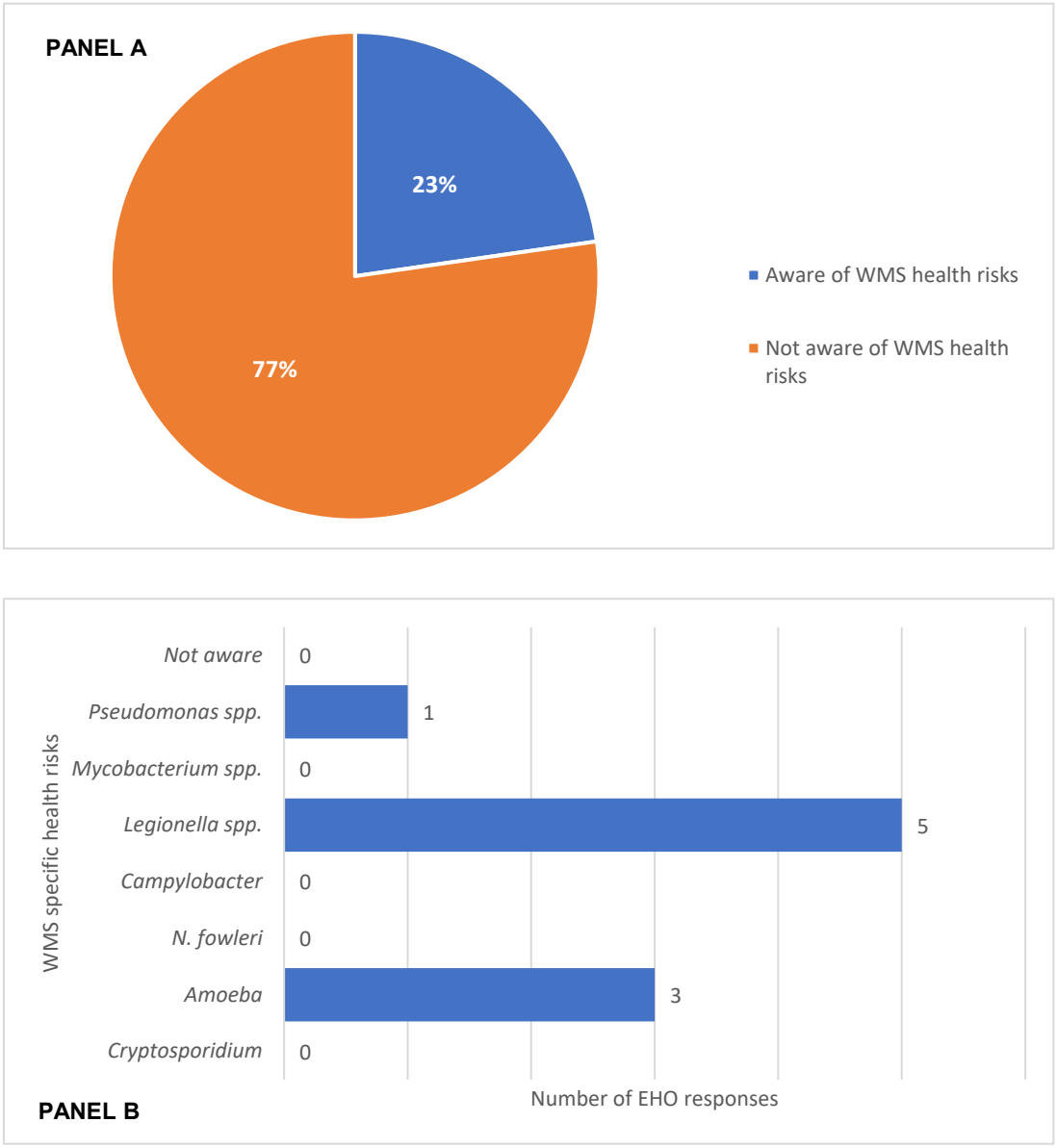

Figure 3.2 PANEL A (EHO awareness of WMS health risks) PANEL B (EHO awareness of WHS health risk type)

3.4.2.3 EHO responses to the presence of a regulatory and inspection regime

The lack of a regulatory and inspection regime for the WMS installed and used in public places was a notable finding as indicated by 100% of the EHOs (Table 3.3),

even though over a fifth of them reported that they had received public complaints about the use of these systems, and 23% of them indicating that the complaints were related to biological contamination.

Table 3.3 EHO responses to the presence of a regulatory and inspection regime

Question	Responses	Frequency of responses	Percentage
WMS approval/licensing process in place	Yes	0	0%
	No	22	100%
	Total	22	100%
WMS inspection regime in place	Yes	0	0%
	No	22	100%
	Total	22	100%
Frequency of regulatory inspection	Once per year	0	0%
	Twice per year	0	0%
	> Twice per year	0	0%
	Not applicable	0	0%
	Don't know	22	100%
	Total	22	100%
Public complaints received	Yes	5	23%
	No	17	77%
	Total	22	100%
Nature of public health complaints	Biological	5	23%
	Chemical	0	0%
	Other	1	5%
	None	16	73%
	Total	22	100%

3.5 Discussion

3.5.1 Water mist system location, purpose of use, water source and aerosolization potential

According to WMS owners' respondents, about 50% (n = 5) of their systems are located outdoors and used for cooling in public places (Table 3.1). This finding was corroborated by the majority of the EHOs 95% (n = 21) who confirmed outdoor location of these water systems as shown in Table 3.2.

Public places are captured under the *Health (Miscellaneous Provisions) Act, 1911* (WA) as places where people gather for various purposes including entertainment and recreation. These premises were the focus of this study. The installation and use of WMS in public settings presents a potential risk of exposure to OPPPs that may contaminate tiny water mists released by these systems. In health risk assessment terms (enHealth, 2015), WMS installed and operated in public places constitute a risk to people who interact with contaminants released into the ambient air.

Temperature reduction was the main reason for using WMS in public places as reported by the majority of the WMS owners, 70%, (n = 7) (Table 3.1) and corroborated by most of the EHOs, 76% (n = 22), as indicated in Table 3.2, a result that was not significant (*p = 0.37, Fisher's exact test*).

This is not surprising considering the extreme temperatures experienced in the north - western part of Australia (Bureau of Meteorology, 2016). Water mist systems can reduce ambient temperatures in outdoor settings (Ozmist, n.d.). These findings are consistent with those of a Singaporean study which established that water mist fans can reduce ambient temperatures and improve thermal comfort (Wong & Chong,2010). The uptake of these systems is expected to increase due to the projected increase in mean maximum temperatures caused by climate change (Loechel et al., 2011). Elevated temperatures can promote the growth of *Legionella* spp. bacteria in building plumbing systems (Lu et al., 2017), a phenomenon that may become common in WMS installed and used in this region. The examination of WMS to determine the possible presence of *Legionella* spp. and other OPPPs is needed.

Investigating, characterizing, and understanding the potential regrowth of OPPPs in WMS is important to assist in developing conceptual site models (CSM) that may be used in designing control measures (National Environment Protection Council, 2010).

The ability to release bioaerosols is one of the critical risk factors for any water system assessed for contaminated bioaerosol release (Health and Safety Executive, 2013). The formation and release of tiny water mists is achieved by small nozzles that atomise the water under either hydraulic or pneumatic pressure (Farnham et al., 2015). Ninety percent (n = 9) of the WMS owners (Table 3.1) reported that the WMS installed and used in their areas used hydraulic nozzles for the atomisation and dispersion of water

mists into the ambient atmospheres, a finding that was corroborated by 91% of the EHOs (n = 20) (Table 3.2). However, this result was not statistically significant (p = 1.0, Fisher's exact test). Hydraulic pressures applied to WMS at a force of 1000 psi, can produce and disperse tiny and respirable water mists as small as 3 – 10 µm (Henningson & Ahlberg, 1994). This finding is important considering that the inhalation of bioaerosols contaminated with OPPPs such as *Legionella* spp. has been associated with illnesses (Russo et al., 2018). The study of WMS used as a cooling intervention is important to determine their potential to produce and release contaminated and inhalable bioaerosols.

The type and source of water used in WMS can have significant downstream effects on water quality for consumers as evidenced by the adoption of a preventive strategy that addresses all pollution risks from catchments to consumers (National Health and Medical Research Council, 2011). All 10 WMS owners indicated that scheme water was used in their systems (Table 3.1). However, 59 % (n = 13) and 36% (n = 8) of the EHOs respectively, indicated that scheme and treated bore water was used in the WMS (Table 3.2). The difference in the WMS owners and EHOs responses was statistically significant *(p = 0.03, Fisher's exact test)*. This difference could be attributed to a knowledge gap between the two groups. Through their training and experience in water quality, EHOs possess adequate knowledge to correctly evaluate water sources and quality. However, the difference in terminology used by EHOs and WMS could also result in this observed difference in knowledge of the water sources. In the case of water obtained from underground aquifers, underlying bedrocks can influence water chemistry by leaching mineral elements (Adabanija et al., 2020) that can promote biofilm formation and the regrowth of OPPPs (Ji et al., 2015). A comparative study of OPPP occurrence in potable and recycled waters established that in addition to microbial ecology, water chemistry significantly increased occurrence of these microorganisms (Garner et al., 2018). These findings justify the need to identify and investigate the source of water used in WMS, to help understand its contribution to the regrowth of OPPPs.

3.5.2 Knowledge of WMS health risks, skills, and operational competence

The knowledge of health risks is a key driver of behaviour change to take corrective measures to mitigate the risk (Fan et al., 2018). To emphasize the importance of knowledge in managing the risk of *Legionella* spp. in water systems, competence, which is a combination of knowledge and skills, has been incorporated as a requirement in several guidelines for the effective management of OPPPs, especially *Legionella* spp. in water systems (enHealth, 2015; Health and Safety Executive, 2013).

Only 36% (n = 4) of the WMS owners knew about the associated biological risks even though 70% (n = 7) perceived that their systems are of public health significance . This finding was corroborated by the results of the EHO survey that indicated that 77 % (n = 17) of them did not know the health risks associated with using WMS (Panel A of Figure 3.2), with *Legionella* spp., *Amoeba* spp. and *Pseudomonas* spp. being the only OPPP species that the remaining 23% (n = 5) of the EHOs knew could regrow in WMS (Panel B of Figure 3.2).

The observed difference in the level of knowledge about the health risks of WMS between the WMS owners and EHOs was not significantly different *(p = 0.36, Fisher's exact test)*. The observed low level of knowledge amongst the WMS owners and EHOs about the type of health risks and OPPPs that can colonize and regrow in WMS is concerning, considering the potential of widespread exposure of people who patronize these public places. A study to understand the factors associated with this low level of knowledge about the health risks of using WMS in public places in required.

Understanding operational risks that can promote OPPP growth in premise plumbing such as WMS is important in effectively managing this emerging public health concern (Julien et al., 2020). Poor maintenance, 22% (n = 8) followed by operational temperatures above 25 °C ,19% (n =7) were factors mostly identified by WMS owners as being important for OPPP growth in WMS. However, although a fifth of the respondents knew that poor maintenance could lead to OPPP growth in their WMS, only 50% (n = 5) of them reported that they carry out maintenance of their systems regularly according to the manufacturer's specifications (Panel A of Figure 3.1). The formation of biofilms on the internal surfaces of premise plumbing is well known for promoting OPPP growth and shielding these pathogens from the bactericidal effects

of any residual disinfectants, particularly chlorine and its derivatives (Huang et al.,2020; Feazel et al., 2009; Tang & Bae, 2020). Only 14% (n = 5) of the WMS owners were aware of biofilms as a risk factor for OPPP growth, with a third of them reporting having observed this phenomenon in their WMS (Panel C of Figure 3.1), a difference that was not significant, *(p = 0.65, Fisher's exact test).*

It has been stated earlier that the knowledge of health risks is a key driver of behaviour change to take corrective measures to mitigate a risk (Fan et al., 2018), and the acquisition of these attributes is achieved through training and practice. Approximately 90%, (n =9) of the WMS owners reported that they had not received any training in the operation of their systems (Table 3.3), a situation that could have made 40% (n = 4) of them perceiving that they were only slightly competent in operating the same as indicated in Table 3.3.

The systematic use of cleaning and maintenance schedules is important in preventing opportunistic pathogen growth in building water systems and cooling towers (American Society for Testing Materials, 2008; Rangel et al., 2011). Sixty percent (n = 6) of the WMS owners reported that they do not have any cleaning and maintenance schedules for their WMS (Panel D of Figure 3.1). This result could be related to the lack of regular maintenance already identified in this study. However, an insignificant association between the respondents' failure to carry out regular maintenance and the lack of cleaning and maintenance schedules was determined *(p = 1.0, Fisher's exact test)*.

The reported inadequacy of training and regular maintenance of WMS is concerning considering its demonstrated importance in managing OPPP growth in similar premise plumbing systems. The lack of maintenance on an air conditioning installation was identified as the major cause of the United Kingdom's largest legionellosis outbreak that occurred in Cumbria in 2002 (Bennett et al., 2014) and was also implicated in a similar outbreak at a Melbourne aquarium in 2000 (Greig et al., 2004). It is important to carry out a study to fully understand and recommend effective training programs and maintenance regimes for WMS used as a cooling intervention in public places.

The infrequent use of any water mist system causes water stagnation (Feazel et al., 2009) and depletion of residual disinfectants (Bowman & Mealy, 2007). It was reported by 86% (n = 19) of the EHOs that the WMS in their jurisdictions were operated

seasonally, and only 2 and 1 respondent reported frequent use (>4hrs per day) and infrequent use respectively (Table 3.2). The difference in the reported frequency of use by the EHOs was significant, *(p = 0.01, Fisher's exact test).* The seasonal use of WMS in the north-western part of Australia is a function of large variations in the mean winter and summer air temperatures (Bureau of Meteorology, 2016). Water temperatures in premise plumbing systems increase during the hot summer seasons, to levels optimum for the growth of OPPPs. A summer increase in Legionnaires' disease in the Netherlands was associated with the proliferation of *L. pneumophila* in premise plumbing during this period (Brandsema et al., 2014). An Australian study investigating OPPP growth in water distribution systems across the country established a seasonal variation in the occurrence of *L. pneumophila* in recycled water schemes across different climatic zones (Storey & Kaucner, 2009). Therefore, it is important that a study of WMS in north-western Australia be undertaken to understand the impact of their infrequent use and climatic variations on the occurrence of OPPPs.

3.5.3 Regulation and inspection of water mist systems

Several governments and jurisdictions have developed legislation, standards, and guidelines to effectively manage the public health risk posed by water systems that can be colonised by OPPPs and are able to generate and release contaminated bioaerosols into the environment. The *HSE's Code of Practice on the control of Legionella in water systems* is legally enforceable under the country's health and safety legislation (Health and Safety Executive, 2013), just as the Australian Standard *AS 3666 on cooling water systems* (Standards Australia, 2011a) is enforceable under Western Australia's *Health (Miscellaneous Provisions) Act 1911* (WA).

All the EHOs surveyed, 100% (n = 22) reported that a licensing and approval system for WMS was not in place, and that they did not inspect the installed WMS as part of their public health regulatory activities (Table 3.3). This lack of a regulatory oversight over the water mist systems is surprising considering that 23%, (n = 5) of these EHOs reported that they received public complaints about biological contamination of the same (Table 3.3). This lack of a regulatory regime to ensure that WMS are safely installed and operated to protect public health can be attributed to the inadequacy of current legislation to capture these systems, and the absence of standards that specify

minimum design and installation criteria. The existing guidelines focus on *Legionella* spp. control in cooling towers, hospitals and aged care settings (enHealth, 2015), and industrial settings such as cooling towers (NSW Department of Health, 2004; Standards Australia, 2011a), disregarding the public health risks of some emerging OPPPs of concern including, *M. avium, P. aeruginosa, Acanthamoeba* and *N. fowleri*. A study of these WMS is needed to inform the development of a legislative framework and operational guidelines for these systems.

3.6 Conclusions

In this chapter, the results of a questionnaire survey of WMS and EHOs that use and are expected to regulate these features in public places respectively were presented and discussed. Firstly, this survey research intended to understand the level of knowledge about health risks and skills required to operate WMS, secondly it intended to gather information to describe the design, location, operation, and regulatory framework of the WMS used as a cooling intervention in public places. The study also intended to identify the factors that may be associated with colonisation of WMS by OPPPs and then using these findings to inform the design and implementation of an experimental study to examine and assess these risks.

Besides demonstrating that most of the WMS were located outdoors, operated infrequently, generated and released aerosols, showed evidence of biofilm formation, and were not regularly maintained; the research unequivocally showed that the level of awareness and knowledge of the health risks that could be associated with WMS was low among the system owners and EHOs.

Furthermore, the survey research results demonstrated the lack of a regulatory framework for WMS, even though respondents acknowledged receiving some public health complaints related to WMS. The findings in this chapter will inform the review of existing environmental health legislation to incorporate the monitoring of WMS and the development of guidelines to assist owners of such systems to safely operate them. Finally, the findings presented in this chapter provided some methodological considerations for the experimental design of the research project presented and discussed in Chapter 4.

CHAPTER 4 OPPORTUNISTIC PREMISE PLUMBING PATHOGENS, A POTENTIAL HEALTH RISK IN WATER MIST SYSTEMS USED AS A COOLING INTERVENTION

4.1 Introduction

Water mist systems (WMS) are premise plumbing installations used for cooling and are typically installed in outdoor areas to produce and release water aerosols that flash evaporate in the surrounding air, resulting in a sudden reduction of ambient temperatures. Premise plumbing refers to all the water distribution and storage infrastructure within buildings and downstream from the water meter. Water mist systems present a potential public health risk because of their shared characteristics with other aerosol generating premise plumbing systems such as cooling towers, spa pools and showers that have been associated with outbreaks of infectious respiratory diseases caused by OPPPs such as Legionnaires' disease and bacterial pneumonia (Demirjian et al., 2015; Kanamori et al., 2016). These systems produce microscopic inhalable aerosols (0.3–10 µm) (Henningson & Ahlberg, 1994), which if produced from contaminated water sources, can cause debilitating and fatal respiratory infections. Microorganisms that colonize and regrow in these premise plumbing systems are often referred to in the literature as opportunistic premise plumbing pathogens (OPPPs) and are part of the normal microbiome of premise plumbing (Falkinham III et al., 2015), which includes showers (Bauer et al., 2008), garden hoses (Thomas et al., 2014), water taps and faucets (Cassier et al., 2013), hot water systems (Borella et al., 2005), spa pools (Leoni et al., 2015) and air conditioning units (Bennett et al., 2014).

Several characteristics common to premise plumbing that can enhance the risk of microbial colonization and proliferation are oligotrophic conditions, water stagnation and long periods of water retention within plumbing systems (Julien et al., 2020). Plumbing materials and components, disinfection methods, system corrosion, water quality/source and elevated temperatures are known to influence the survival of these pathogens in premise plumbing (Falkinham, 2015; Julien et al., 2020). Other features that enhance the survival of OPPPs include their ability to form and colonize biofilms, survival inside free-living amoeba (FLA), and resistance to disinfectants (Ashbolt,

2015). *Acanthamoeba* has a significant ability to engulf other OPPPs, and through this process shields them from disinfectants such as chlorine, and at the same time confer increased virulence to these OPPPs, that are then able to multiply in premise plumbing (Ashbolt, 2015). Opportunistic pathogens commonly isolated from premise plumbing include *L. pneumophila, M. avium, P. aeruginosa, Acanthamoeba* and *N. fowleri* (Wang et al., 2013). These opportunistic pathogens represent an increased public health risk of *L. pneumophila* infection in persons with compromised immunity (Pruden et al., 2013), as well as the elderly and smokers (Heymann & American Public Health, 2015).

Exposure to contaminated waters is an important pathway for infection with OPPPs with inhalation, aspiration and nasal irrigation being the major routes of exposure (Falkinham et al., 2015). Various pneumonic and respiratory tract illnesses have resulted from the inhalation of water mists <10 µm contaminated with bacterial pathogens such as *L. pneumophila* (Haupt et al., 2012; Russo et al., 2018), *M. avium* (Falkinham, 2011; Falkinham et al., 2008), *P. aeruginosa* (Bédard et al., 2015; Schneider et al., 2012) and the aspiration of water contaminated with *N. fowleri* has resulted in a rare but fatal disease called primary amoebic meningoencephalitis (PAM) (Budge et al., 2013; Parsonson & Nicholls, 2016), and infection by *Acanthamoeba* has been associated with diseases of the eyes called acanthamoeba keratitis and granulomatous amoebic encephalitis (GAE) (Taher et al., 2018).

Although a body of knowledge exists on the presence of OPPPs in premise plumbing features such as showers, water taps, hot water systems, etc., no such study has investigated the potential of WMS used for ambient cooling to be colonized by OPPPs. Currently, there is no literature explaining the environmental characteristics that promote the growth and persistence of OPPPs in these systems. In this study, we investigated the potential occurrence of five selected OPPPs in WMS, namely, *L. pneumophila, P. aeruginosa, M. avium, Acanthamoeba* and *N. fowleri,* to determine the health risks associated with the use of such systems, and to determine whether there is any correlation between the occurrence of the OPPPs in the WMS with residual disinfection, water temperature, water pH, TDS and total organic carbon (TOC).

4.2 Methods Section

In this section, the procedures used to collect bioaerosol, biofilm and water samples from WMS are described including the storage and transportation of the same. Analytical methods employed to detect and isolate *L. pneumophila* Sg 1 and 2-14, *P. aeruginosa, M. avium, Acanthamoeba* and *N. fowleri* are described.

4.2.1 Materials and Methods

To determine the health risks associated with the use of WMS as a cooling intervention in public places, samples of water, biofilm and bioaerosols were collected and analysed for the presence of the target OPPPs. The Qualtrics sample size calculator was used to determine the number of WMS from which samples were collected. Prior to determining the sample size, the population size, a margin of error, confidence level and a standard deviation that ensures less variability were determined. A population size of 10 WMS installed and used in public and private commercial settings determined from the register of public places compiled and maintained by the local governments in the area. Exploratory visits were made by the researcher to these public places to confirm the presence and operation of WMS. Bearing in mind that no sample is perfect, a 5 % margin of error was determined, and a 95 % confidence level applied. To ensure less variability in WMS sampled, a standard deviation of 0.5 was applied in calculating the sample size. A sample size of 10 per sample type per sampling event was determined after applying these defined parameters to the Qualtrics sample size calculator (Qualtrics, 2020; Smith, 2010), A total of 30 water samples, 30 biofilm samples and 30 bioaerosol samples were collected from 10 WMS located in the northwestern part of Australia over three sampling events (February, May, and August) in 2019.. The samples were analyzed at EcoDiagnostic, an Australian laboratory accredited by the National Association of Testing Authorities (NATA).

Ethics approval to conduct this study was obtained from the Edith Cowan University (ECU) Human Research Ethics committee (HREC), Approval Number 16337 MASAKA. Informed consent was obtained from all participants involved in the study.

4.2.1.1 Bioaerosol samples

Bioaerosol samples were collected using the NIOSH BC251 –2 stage bioaerosol samplers to which was connected conductive polypropylene filter cassettes loaded with 37 mm polytetrafluoroethylene (PTFE) filters of 3 µm pore size. The sampling was undertaken in accordance with the method described by Coleman et al., (2018). One and half meters of Teflon tubing was used to connect the bioaerosol samplers to SKC AirCheck XR 5000 air sampling pumps that were operated at 3.5 L/minute for a maximum of 30 min to collect positional samples. Before each sampling session, the airflow through the sampler was calibrated, and the flow rate checked after each sampling session, using the SKC Defender 510 Dry Cal standard primary calibrator. Air temperature and humidity was recorded during the sampling process using a Lascar EL-USB-2 humidity and temperature meter and wind speed was also recorded during the sampling process using a Meteos Anemo-Thermometer with a 54 Mm Propeller. The bioaerosol samples were stored and transported on ice at <4°C to EcoDiagnostic laboratory for analysis using both culture-based and molecular methods for *M. avium, P. aeruginosa, and N. fowleri*, *Legionella* sp*p.* (including *L. pneumophila* Sg 1 and Sg 2–14) and *Acanthamoeba*.

This research project was aimed at detecting the potential for people to be exposed to the OPPPs in public places where WMS are used. The personal monitoring of members of the public visiting these public places was not conducted because it is not viable, practicable or ethical. Furthermore, there are no standard methods to measure the personal exposure for these organisms, with available equipment being large, and weighty. The focus of this research on detecting potential presence of pathogens in WMS themselves prior to their release into ambient atmospheres informs the adoption of preventative approaches to control the hazard at source.

4.2.1.1.1 Bioaerosol sample preparation

The inside walls of the NIOSH BC 25 L, 15 mL and 1.5 mL tubes were rinsed with a solution of ATL and proteinase K. The PTFE filters were aseptically removed from the cassettes, placed inside the rinse solution and vortexed. This solution (with the filter paper) was incubated at 60°C for 30 min to achieve lysis. Two separate aliquots of this solution (440µL) were loaded onto the QIAsymphony instrument (QIAGEN) for

DNA extraction. Two extracts of 200 µL each eluded from the QIAsymphony instrument were combined and filtered using an AMICON Ultra DNA concentrator. They were checked for inhibition at the neat dilution using a PPC qPCR assay and then analyzed neat to detect *M. avium* (qPCR), *Legionella* spp. (PCR), *P. aeruginosa* (qPCR), *Acanthamoeba* (PCR) and *N. fowleri* (qPCR). The qPCR results were expressed qualitatively as detected or not detected. In the absence of a standard method for detecting OPPPs in bioaerosols, validated inhouse PCR and qPCR methods were used as described under analytical methods.

4.2.1.2 Biofilm samples

4.2.1.2.1 Biofilm sample collection

Biofilm samples were collected from the WMS using swabs stored in E-Swab vials containing 1 mL of liquid and sodium thiosulfate to inactivate any residual disinfectants. Swabbing was done following the requirements of the Centers for Disease Control and Prevention (CDC)'s "*Sampling procedure for biofilms in Legionella outbreak investigations"* (Centres for Disease Control and Prevention, 2015). The swabbing was done from the inside walls of WMS pipes and sprinkler nozzles. These swabs were put back into the E-Swab vials and transported on ice at 4 °C to EcoDiagnistic laboratory for analysis.

4.2.1.2.2 Biofilm swab sample preparation

One hundred micro liters (100 µL) of the sample were plated to culture for *Legionella* spp. and *P. aeruginosa* and 100 µL being plated for confirmation. One millilitre (1 mL) each of this preparation was used to culture for *Acanthamoeba* and *N. fowleri* with confirmation being done by PCR. Some samples required dilutions (1:10, 1:100, etc) to account for the high concentration of background flora. Deoxyribonucleic acid (DNA) was extracted from the swab solution (400 µL and eluted into 200 µL) to detect *M. avium*.

4.2.1.3 Water Samples

Water samples were collected from the WMS, stored, and transported to the analyzing laboratory following the requirements of "*AS 2013–2012, Water Quality—Sampling for*

microbiological analysis" (Standards Australia, 2012). Sterile plastic bottles (500 mL) treated with sodium thiosulfate to deactivate any available disinfectants were used to collect water samples for microbiological testing for the presence of *L. pneumophila, P. aeruginosa, M. avium, Acanthamoeba and N. fowleri*. The bottles were stored and transported on ice at 4°C to a NATA laboratory for analysis, except for the amoeba samples that were transported at ambient temperature (Codony et al., 2012). A calibrated industrial HM Digital TDS and water temperature thermometer with a measuring range of 0–80°C, and accuracy of ±2%, was used to measure water temperature and total dissolved solids. A Palintest Pooltest 9 Premier water testing unit was used to measure the free chlorine residual disinfectant level, pH and temperature profile of the water samples.

4.2.1.3.1 Water Sample Preparation and Analysis

All manipulations associated with sample preparation, culture media, materials and apparatus, enumeration techniques and their selection were conducted as described in "*AS/NZS.1: 2007-Water microbiology: Method 1. General information and procedures (ISO8199:2005, MOD)"* (Standards Australia and New Zealand, 2007). All samples were handled by trained laboratory staff. *N. fowleri* plates for confirmation were handled in a biosafety cabinet (BSC).

4.3 Results

4.3.1 Occurrence of Opportunistic Premise Plumbing Pathogens in Water Mist Systems

Figure 4.1 shows the frequency of OPPP occurrence in all WMS samples (bioaerosol, water and biofilm). A total of 64 (71%) of WMS samples analysed tested positive for OPPPs, with *P. aeruginosa* being found in 40 (44%) of the total samples. *L. pneumophila* Sg 2–14 was detected in 16 (18%) of the total samples and *L. pneumophila* Sg 1 was isolated from 5 (6%) of the total samples. Only three of the total samples analysed returned a positive reading for *Acanthamoeba*. None of the 90 samples analysed tested positive for both *M. avium* and *N. fowleri.*

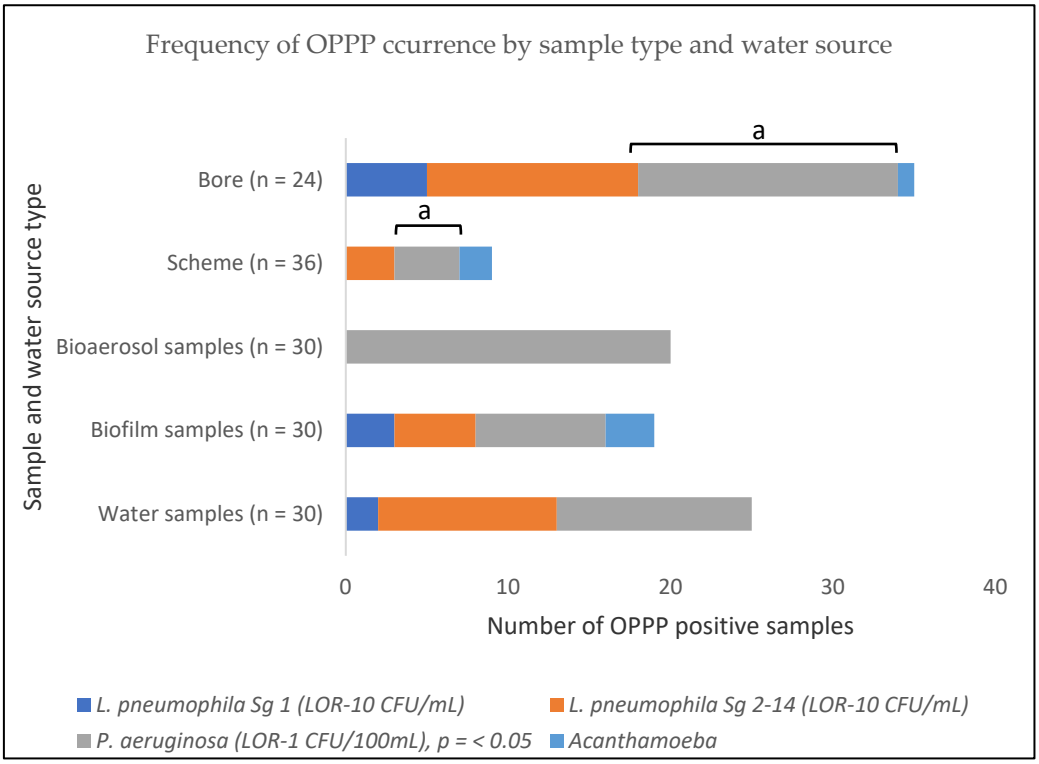

Figure 4.1 The frequency of OPPPs positively identified by sample type and water source

All samples, except bioaerosol samples, were initially identified via culture-based methods, which were then confirmed via molecular methods like the analysis of the bioaerosol samples: PCR/qPCR sensitivities were: *L. pneumophila* −1.6 Genomic Units/mL, *P. aeruginosa* 5–10 GU/10 mL, *Acanthamoeba* 5–8 gene copies/µL.

4.3.2 The Concentration of Detected OPPPs

The results of this study, as presented in Table 4.1, show that the concentration of all the OPPPs detected in WMS samples analysed by microbiological culture methods was higher in biofilm samples than in water samples, with *L. pneumophila* Sg 1 detection in biofilms being 30 × higher than in water. The biofilm concentration of *L. pneumophila* Sg 2–14 was three times higher than that of water and *P. aeruginosa* in biofilm samples was eight times higher than in water. The PCR results indicated the presence of *P. aeruginosa* in the bioaerosols only.

Table 4.1 Opportunistic premise plumbing pathogen concentration by sample type

Opportunistic Pathogen Detected	OPPP Concentration Level	OPPP Concentration Range by Sample Type		
		Biofilm (CFU/mL)	Water (CFU/mL	Bioaerosol qPCR
L. pneumophila (Sg 1)	Lowest	1000	100	Not detected
	Highest	3000	100	Not detected
L. pneumophila (Sg 2- 14)	Lowest	100	10	Not detected
	Highest	1000	300	Not detected
P. aeruginosa	Lowest	10	3	Detected
	Highest	2000	350	Detected

* PCR and/or qPCR analysis conducted for the detection of OPPPs in bioaerosol samples, results expressed as either detected/not detected.

4.3.3 The Frequency and Distribution of OPPPs

The frequency and distribution of OPPPs differed by the WMS sample type and water source as shown in Figure 4.1. Bioaerosol samples had a higher occurrence of *P. aeruginosa* (67%) than water samples (40%), and biofilm samples (70%). This occurrence of *P. aeruginosa* significantly differed by sample type χ^2 (2, N = 90) = 10.08, $p < 0.05$. Conversely, *L. pneumophila* Sg 2–14 occurred more frequently in water samples (37%), than in biofilm samples (17%), however, this difference was not statistically significant χ^2 (2, N = 90) = 3.07, $p > 0.05$. There was no association between the occurrence of *L. pneumophila* species and *P. aeruginosa* in biofilms and water samples χ^2 (1, N = 41) = 0.02, $p > 0.05$. V = 0.000.

No *L. pneumophila* Sg 2–14 was detected in the bioaerosol samples. Only 3 biofilm and 2 water samples tested positive for *L. pneumophila* Sg 1. *Acanthamoeba* was detected in three biofilm samples. *M. avium* and *N. fowleri* were not detected in any of the samples analyzed.

4.3.4 Opportunistic Premise Plumbing Pathogen Occurrence in water samples

The percentage occurrence of *L. pneumophila* Sg 2–14 in bore water samples as shown in Figure 4.1 was four times higher than in scheme water; however, the results of a Kruskal-Wallis mean ranks test of the individual occurrences showed that they did not differ significantly, $H\ (1) = 1.84$, $p > 0.05$. *L. pneumophila* Sg 1 was only detected in five bore water samples.

The results of a Kruskal-Wallis mean ranks test showed a significantly higher percentage occurrence of *P. aeruginosa* in bore water than in scheme water, $H\ (1) = 13.87$, $p < 0.05$. *Acanthamoeba* was detected in only 2 out of the 36 water samples obtained from systems fed with scheme water and in only one of the water samples obtained from systems fed with bore water.

4.3.5 Seasonal Occurrence of Opportunistic Premise Plumbing Pathogens

Seasonal differences in the occurrence of OPPPs in all samples (N = 90) were investigated, however, no statistical difference was observed in the occurrence of *L. pneumophila* Sg 1, *L. pneumophila* Sg 2–14 and *P. aeruginosa* in WMS across the three seasonal sampling periods (February, May, and August) as indicated by the following results of a Kruskal- Wallis mean rank test for the three OPPPs: *L. pneumophila* Sg 1, $H\ (2) = 0.77$, $p = 0.68$; *L. pneumophila* Sg 2–14, $H\ (2) = 0.89$, $p = 0.64$ and *P. aeruginosa*, $H\ (2) = 0.08$, $p = 0.96$.

4.3.6 Water Temperature

Temperature for all water samples ranged between 21.7 °C to 38.9 °C with the highest being recorded in February and the minimum in May. The results of a Kruskal-Wallis test showed that the mean ranks of water temperature in February were significantly higher than in May and August/September $H\ (2) = 23$, $p < 0.05$. Based on the results of this study, the occurrence of *P. aeruginosa* in WMS tends to increase with an

increase in the water temperature *rs* = 0.31, $p < 0.05$. No correlation was observed between water temperatures and the occurrence of all other OPPPs detected in the WMS namely, *L. pneumophila* Sg 1 *rs* = 0.08, $p > 0.05$, *L. pneumophila* Sg 2–14 *rs* = 0.09, $p > 0.05$ and *Acanthamoeba rs* = 0.04, $p > 0.05$.

4.3.7 Water pH

The pH for all the water samples showed a small range variation (7–7.9). There was no significant difference in the mean ranks of water pH across the three sampling sessions $H (2) = 0.87$, $p > 0.05$.

4.3.8 Total Dissolved Solids (TDS)

The highest TDS concentration was 399 mg/L and was recorded from a bore water sample during the May sampling event. The lowest concentration of 240 mg/L was measured from a scheme water sample during the first sampling event in February. The mean rank concentration of TDS in bore water samples was 6% (18.6 mg/L) higher than in scheme water (340.3 mg/L). This difference was statistically significant $H (1) = 16.78$, $p < 0.05$. No significant difference was noted for the mean ranks of TDS concentration across the three-sampling events $H (2) = 5.33$, $p = 0.07$.

4.3.9 Free Chlorine Residual

The concentration of free chlorine residual measured across the three sampling events ranged from 0.0 to 0.76 mg/L, a variance that reflects the complexity of these plumbing systems. The maximum concentration of free chlorine was measured in scheme water during August, with the minimum concentration in this water supply being 0.01 mg/L. Two-thirds of all bore water samples tested across the three sampling events had no free chlorine residual. All scheme water samples tested positive for free chlorine residual. This difference in free chlorine residual between bore and scheme water samples was significant, $H (1) = 19.95, p < 0.05$. No significant difference in residual chlorine concentration was observed in the water samples across the three-sampling events $H (2) = 0.26, p = 0.88$.

4.3.10 Total Organic Carbon (TOC)

Seventy percent (21 out of 30) of the water samples had TOC concentrations less than the detection limit of <1 mg/L and 17 of these were collected from the scheme water supply. The highest measured TOC concentration was 3 mg/L. The mean ranks of TOC concentration in the water samples collected across the seasons were not significantly different *H (2) = 3.5, p = 0.17*. However, the TOC concentration in the bore water samples was significantly higher than in the scheme water samples, *H (1)* = 7.11, *p* = 0.01.

4.4 The Relationship between water profile parameters

To determine the strength and direction of the association between the water profile parameters discussed above, the nonparametric Spearman's rho (r_s) test was used rather than the parametric Pearson test because of the absence of distribution normality in the data sets and the presence of outliers. Table 4.2 presents the Spearman rho correlation results among the water profile parameters. A significant negative monotonic correlation was determined between free chlorine residual and TDS,*rs* (30) = −0.566, *p* < 0.05 and TOC, *rs* (30) = –0.523, *p* < 0.05. Total organic carbon concentration had a significant and positive monotonic correlation with TDS, *rs* (30) = 0.549, *p* < 0.05. However, there was no significant correlation observed between water temperature and all other water profile parameters, and the same applied to water pH.

Table 4.2 The relationship between water profile parameters

Spearman Rho (ρ) Correlation between Water Profile Parameters						
Water Profile Parameter	**Statistical Test and Sample Size**	**Free Chlorine Residual**	**Water Temperature**	**Water pH**	**Total Dissolved Solids**	**Total Organic Carbon**
Free chlorine residual	Spearman rho ρ	1	−0.185	−0.065	−0.566	−0.523
	Significance (2 tailed)	.	0.328	0.735	**0.001**	**0.003**
	N	30	30	30	30	30
Water temperature	Spearman ρ Correlation	−0.185	1	0.111	−0.089	−0.198
	Significance (2 tailed)	0.328	.	0.558	0.639	0.293
	N	30	30	30	30	30
Water pH	Spearman ρ Correlation	−0.065	0.111	1	0.068	0.279
	Significance (2 tailed)	0.735	0.558	.	0.720	0.136
	N	30	30	30	30	30
Total dissolved solids	Spearman ρ Correlation	−0.566	−0.089	0.068	1	0.549
	Significance (2 tailed)	**0.001**	0.639	0.720	.	**0.002**
	N	30	30	30	30	30
Total organic carbon	Spearman ρ Correlation	−0.523	−0.198	0.279	0.549	1
	Significance (2 tailed)	**0.003**	0.293	0.136	**0.002**	.
	N	30	30	30	30	30

4.5 Relationship between Water Profile Parameters and the Occurrence of OPPPs in Water Mist Systems

The possible correlation between the water profile parameters and the occurrence of OPPPs in the WMS was determined using the Spearman *rho* correlation test which has been used in similar studies (Liu et al., 2019). The results of this analysis are shown in Table 4.3. Residual chlorine had a significantly weak and negative monotonic correlation with the occurrence of all OPPPs except with *Acanthamoeba*, rs (30) = 0.067, $p > 0.05$.

Table 4.3 The relationship between water profile parameters and the occurrence of OPPPs in WMS

Spearman Rho Correlation Analysis between OPPPs and Residual Chlorine, Water Temperature, pH, Total Dissolved Solids, and Total Organic Carbon					
Opportunistic Pathogen Detected	**Residual Chlorine (mg/L)**	**Water Temperature (°C)**	**Water pH (pH Units)**	**Total Dissolved Solids (mg/L)**	**Total Organic Carbon (mg/L)**
L. pneumophila (1)	−0.327 (p = 0.011)	0.080 (p = 0.543)	0.074 (p = 0.038)	0.268 (p = 0.038)	0.392 (p = 0.002)
L. pneumophila (2–14)	−0.401 (p = 0.002)	0.098 (p = 0.456)	0.002 (p = 0.987)	0.418 (p = 0.001)	0.393 (p = 0.002)
P. aeruginosa	−0.423 (p = 0.001)	0.313 (p = 0.015)	0.123 (p = 0.348)	0.480 (p = 0.000)	0.242 (p = 0.062)
Acanthamoeba	0.067 (p = 0.611)	0.035 (p = 0.789)	−0.062 (p = 0.637)	−0.057 (p = 0.663)	0.022 (p = 0.868)

The occurrence of all OPPPs did not correlate with water temperature except for *P. aeruginosa, rs (30) = 0.31, p < 0.05.* A weak and positive relationship was also observed between TDS concentration and *L. pneumophila* Sg 1, rs (30) = 0.27, $p < 0.05$, *L. pneumophila* Sg 2–14, rs (30) = 0.42, $p < 0.05$ and *P. aeruginosa*, rs (30) = 0.48, $p < 0.05$. The occurrence of both *L. pneumophila* Sg 1 and Sg 2–14 demonstrated a weak positive relationship with TOC, rs (30) = 0.39, $p < 0.05$ and rs (30) = 0.39, $p < 0.05$, respectively.

4.6 Discussion of Identifications of OPPP's

The occurrence of OPPPs in WMS used as a cooling intervention in public places has not been investigated, therefore, little is known about their ability to colonize and regrow in these systems, and whether water profile parameters of temperature, free chlorine residual concentration, pH, TDS and TOC can influence this occurrence. The culture and molecular analysis of 30 biofilm, 30 water and 30 bioaerosol samples collected from 10 WMS confirmed a percentage occurrence of 44% (n = 90) for *P. aeruginosa,* 18% (n = 90) for *L. pneumophila* Sg 2–14, 6% (n = 90) for *L. pneumophila* Sg1,3% (n = 90) for *Acanthamoeba* and zero for *M. avium* and *N. fowleri.* This is the first study to investigate the occurrence of these OPPPs in WMS used as a cooling intervention in public places.

Higher concentrations of all OPPPs were detected in WMS biofilm samples than in water and bioaerosol samples, supporting the argument that biofilms play a significant role in OPPP regrowth and survival in water systems. These results are consistent with other studies (Barna et al., 2016; Donohue et al., 2014; Liu et al., 2019; Shareef & Mimi, 2008; Wang et al., 2012). *Pseudomonas aeruginosa* was detected at higher concentrations in WMS biofilms when compared to all the other detected pathogens, a factor which can be attributed to the pathogen's known ability to colonize and thrive better in biofilms than in the water phase (Soto-Giron et al., 2016). In interpreting these results, it is important to note that the actual concentration of the OPPPs detected by culture methods could be even higher due to the possible presence of viable but non culturable organisms (VBNC) that may fail to grow under culture conditions (Li et al., 2014). This phenomenon is particularly relevant for *P. aeruginosa*, an opportunistic pathogen that can be affected into the VBNC state by low temperatures during sample transportation (Dwidjosiswojo et al., 2011).

Another reason for the higher numbers of the OPPPs in the WMS biofilms could be the latter's ability to shield the former from the effect of the chlorine disinfectant used in these systems. Higher disinfection resistance has been demonstrated for the following OPPPs resident in biofilms; *M. avium*, *P. aeruginosa* (Tang & Bae, 2020), *L. pneumophila* (Huang et al., 2020) and *Acanthamoeba* (Dupuy et al., 2011).

A presence of *P. aeruginosa* (67%, n = 30) was detected in WMS bioaerosol samples, indicating that these systems may present a risk of pneumonic infections caused by the inhalation of *P. aeruginosa* (Dean & Mitchell, 2020), which has been established in several other studies (Dean & Mitchell, 2020; Shareef & Mimi, 2008). This high detection of *P. aeruginosa* can be attributed to its ability to adapt and thrive better in various environments, such as the one induced by bioaerosol sampling processes. This finding is consistent with another study of *Pseudomonas* spp. occurrence in premise plumbing (Bédard et al., 2016). Furthermore, research in laboratory models has demonstrated that *P. aeruginosa* is able to remain airborne for periods greater than 45 min (Clifton et al., 2010), whereas *L. pneumophila* is reported to remain airborne for only 3 min (Séverine Allegra et al., 2016) after dispersal. By expressing a mucoid phenotype in air, *Pseudomonas* spp. can withstand desiccation common with bioaerosol sampling using filtration (Clifton et al., 2010). Therefore, *P. aeruginosa* can exist in higher concentrations in ambient atmospheres making it easier to capture during bioaerosol sampling compared to *L. pneumophila*. Further research to investigate this phenomenon in WMS is needed.

The OPPP's *L. pneumophila* Sg 2–14 and Sg 1 were detected in WMS, confirming that these systems could be a health risk for legionellosis should water aerosols they release when in operation be contaminated by these pathogens, a finding consistent with other studies (Greig et al., 2004; Haupt et al., 2012; White et al., 2013). When analyzing for *L. pneumophila* Sg 2–14, only 18% of samples were positive which is greater than another study (11%) (Katsiaflaka et al., 2016) and higher than the levels of *L. pneumophila* Sg 1 (6%), which were detected in several other studies (Donohue et al., 2014; Greig et al., 2004).

A 3% (n = 90) occurrence of *Acanthamoeba* in WMS water and biofilm samples was detected in this study, which positively correlated with free chlorine residual. The positive detection of *Acanthamoeba* in these WMS presents a health risk as described in several studies (Katsiaflaka et al., 2016; Kilvington et al., 2004; Ku et al., 2009; Liu et al., 2019; Taher et al., 2018), not only because of its pathogenicity, but for its ability to shield other pathogens such as *L. pneumophila* and *M. avium* from destruction by disinfectants such as chlorine (Thomas et al., 2010).

The OPPP's *M. avium* nor *N. fowleri* were not found in any samples; water (30), biofilms (30) or bioaerosols (30). Although not isolated in any samples, the potential presence of *M. avium* and *N. fowleri* in WMS cannot be completely ruled out, since studies of similar systems have demonstrated that this pathogen can regrow in premise plumbing (Liu et al., 2019; Thomas et al., 2010; Waso et al., 2018). The low sample volumes collected (250 mL) could have resulted in the extracted gene copies being less than the qPCR method's limit of detection. Sample volumes of 1 L have previously been used to successfully detect these pathogens from water samples (Gebert et al., 2018; Morgan et al., 2016), hence higher sample volumes may be needed for any future studies.

The occurrence of *L. pneumophila* species, *P. aeruginosa*, and thermophilic amoebic species including *Acanthamoeba* in premise plumbing systems tend to vary with seasons (Perrin et al., 2019). There was no statistical difference across seasons, a result which could be attributed to a loss in statistical power due to the smaller sample size (Baveja & Prabhav, 2017). The mean water temperature measured in the WMS across the three sampling events (29.9 °C) was optimum for the growth of all the targeted OPPPs and could have influenced this result, a finding that is consistent with another study which investigated the critical factors responsible for OPPP growth in premise plumbing (Wang, 2013).

A correlation was established between the occurrence of targeted OPPPs in WMS and the use of bore water, with this relationship being significant for *P. aeruginosa, $H(1) = 13.87$, $P < 0.05$*. One of the factors that could give rise to elevated levels of *P. aeruginosa* and *L. pneumophila* Sg 2–14 in the bore water samples could be the increased levels of iron in the shallow aquifers this water is drawn from (Western Australia Department of Water, 2009). Typically, bore water sources in Northern Australia tend to have a higher level of dissolved minerals such as iron, and can also alter the pH of underground water, resulting in the corrosion of pipework and increased colonization of plumbing systems by iron eating bacteria, a finding that is consistent with several other studies (Charles Darwin University, 2019; Rogers et al., 1994). There was no significant difference in the water pH measured across the three sampling events, a finding that could be attributed to the similarity in the chemistry of

the source water, which is a shallow aquifer system influenced by infiltration from surface waters (Western Australia Department of Water, 2009).

Although the primary source of all the water used in the WMS is drawn from the same aquifer, this study research observed a significant variation in the TDS concentration of bore water and scheme water, $H (1) = 16.78, p < 0.05$, a result which is not surprising considering that this parameter is usually higher in ground water sources (Adabanija et al., 2020).

The positive relationship between the formed biofilms and occurrence of *L. pneumophila* observed in this study is consistent with other studies (Buse et al., 2017; Wang, 2013), except for the weak correlation with *Acanthamoeba* which may be due to the possible parasitic colonization of free-living amoeba by *L. pneumophila* at water temperatures > 25°C.

A significant amount of research on OPPP occurrence has demonstrated that elevated water temperatures typical in premise plumbing systems is a critical factor in their survival (Falkinham, 2015; Julien et al., 2020; Ohno et al., 2008; Perrin et al., 2019). However, this study did not demonstrate any correlation between water temperature and the occurrence of all detected OPPPs except with *P. aeruginosa,* $rs (30) = 0.31, p < 0.05$, a finding different from several other studies (Agudelo-Vera et al., 2020; Falkinham, 2015; Perrin et al., 2019). The correlation with *P. aeruginosa* occurrence is consistent with existing literature (Lu et al., 2017). Furthermore, *P. aeruginosa* can adapt to various environmental conditions including surviving temperatures ranging from 10–42 °C and antagonism from other OPPPs (Bédard et al., 2016). Several reasons could be attributed to this phenomenon, particularly the higher-than-normal annual mean maximum temperatures in the study area that were 32.7°C in February 26°C in May and 29.2°C in August, time periods that aligned with the three sampling episodes conducted during our study, and with the higher winter temperatures typical of the tropics where this study area is located (Bureau of Meteorology, 2016)

Most of the water mist systems are situated outdoors and are reticulated by uninsulated pipework which absorbs elevated levels of radiant heat, resulting in elevated water temperatures that promote the growth of OPPPs as described in a study of temperature variation on OPPPs in domestic plumbing (Buse et al., 2017). In

interpreting the results of this study, it is important to acknowledge that most of the water temperatures recorded ranged between 21.7°C to 38.9°C, a zone known to be optimal for the growth of the detected OPPPs. This meant that assessing the effects of temperature on the detected OPPPs at levels below their optimum growth zone was not possible, considering the tendency of these pathogens to adhere to a threshold related response at temperature extremes (Wang, 2013).

A significant negative correlation was established between free residual chlorine concentration and the occurrence of most detected OPPPs. This highlights its effectiveness against most OPPPs, except *Acanthamoeba,* a finding consistent with several studies (Canals et al., 2015; Falkinham, 2015; Huang et al., 2020; Marchesi et al., 2011; Wang, Masters, et al., 2012). The monochloramine disinfectant used in the WMS is more effective over other forms of chlorine disinfectants because of its longer lasting residual effect, a finding that is consistent with other studies (Dupuy et al., 2011; Lautenschlager et al., 2010; Liu et al., 2019; Nguyen et al., 2012). The positive correlation of residual chlorine and *Acanthamoeba* is consistent with the findings of another study (Liu et al., 2019). This could be attributed to several reasons including the possible existence of the cystic form of *Acanthamoeba* detected during our study, which is known to confer resistance to the monochloramine disinfection as previously demonstrated in a previous study (Mogoa et al., 2011).

The TOC concentration in the WMS water samples was exceptionally low, with 70% (n=30) being lower than the detection limit of <1 mg/L, although it was positively correlated with the occurrence of *L. pneumophila* Sg 1, rs (30) = 0.39, $p < 0.05$ and *L. pneumophila* Sg 2–14, rs (30) = 0.39, $p < 0.05$. The low concentration of TOC in WMS is consistent with the findings of several studies of premise plumbing systems that promote the regrowth of these pathogens (Proctor et al., 2017; Wang, 2013).

Microbiological risk control strategies advocated in guidelines developed to control *Legionella* spp. in engineered water systems, including evaporative cooling systems, could be applied to WMS because of the similarities that exist between this pathogen and other OPPPs detected in this study. The Health and Safety Executive's Legionnaires' disease Technical Guidance HSG 274 Part 2 (Health and Safety Executive, 2014), American National Standard Institute's ANSI/ASHRE Standard

188–2008 (American National Standards Institute, 2018) and Australia's enHealth Guidelines for *Legionella* Control in the operation and maintenance of water systems in health and aged care facilities (enHealth, 2015) mandate the implementation of the following control strategies for *Legionella* spp.: risk assessment of water systems for effective design and construction; prevention of water stagnation, implementation of effective maintenance programs and adequate disinfection of water used. These steps avoid the growth of *Legionella* spp*.* bacteria in these systems, strategies that could be applied to prevent OPPPs growing in WMS investigated in this study

4.7 Analytical Methods

4.7.1 Detection and Measurement of *Legionella pneumophila*

The detection of *L. pneumophila* in water samples was undertaken according to the requirements of "*AS 3876:2017-Waters-Examination for Legionella* spp*., including Legionella pneumophila"* (Standards Australia, 2017). A volume of 0.1 mL of the untreated sample was aseptically inoculated onto 90 mm diameter plates of BCYE and MWY agar and incubated in humid conditions at 32°C ± 2°C for 7–10 days. The plates were examined visually on the fourth and last day for *Legionella* spp. colonies that showed iridescence and a change in morphology to granular and similar edges. The presumptive *Legionella* spp. colonies were picked and sub-cultured onto BCYE and BCYE-Cy agar plates and incubated in humid conditions at 32°C ± 2°C for 3 days. The colonies that grew on the BCYE but failed to do so on the BCYE-Cs were interpreted to be *Legionella* spp*.*

The confirmation of *L. pneumophila* was performed using a validated inhouse multiplex PCR method (EDP-312). The growing colonies from the BCYE agar plates were lysed in 100 µL of HP water at 95°C for 5 min to achieve lysis. The purification of the DNA from the prepared isolates was done using the QIAsymphony DNA Mini Kit (192) (QIAGEN) and following the manufacturer's instructions. The detection of *L. pneumophila* was done by amplifying the following primers and probe sets specific for *ssrA, mip* and *wzm,* and based on existing literature (Collins et al., 2015), Legsp-F (5′-NGG CGA CCT GGC TTC-3′) and Legsp-R (5′-GGT CAT CGT TTG CAT TTA TAT TTA-3′), and Lp-mip-F2 (5′-TTG TCT TAT AGC ATT GGT GCC G-3′) and Lp-mip-R

(5′-CCA ATT GAG CGC CAC TCA TAG-3′), and Lp-wzm-F(5′-TGC CTC TGG CTT AGC AGT TA-3′) and Lp-wzm-R(5′-CAC ACA GGC ACA GCA GAA ACA-3′). These primers and probes were used as previously described and were tested for specificity by spiking a sample of pure water with *Legionella* spp. and running a standard PCR and algarose-gel electrophoresis was applied to test for end product specificity. The PCR was then run in a Rotor-Gene Q (QIAGEN) machine following the manufacturer's instructions under the following cycling conditions: initial denaturation cycle of 1 min at 95°C followed by 30 cycles for denaturation at 95°C for 5 s, 30 cycles of annealing at 60°C for 10 s, extension at 72°C for 15 s and then an end holding cycle for 7 min at 72°C. The presence of matching patterns for *L. pneumophila* were observed as follows: PCR fragments 79 bp (10 % tolerance), 110 pb (10% tolerance) and 124 (5% tolerance). The lack of any matching pattern indicated the absence of *Legionella* spp. and the presence of a single matching pattern of 110 pb (10% tolerance) indicated presence of *Legionella* spp. The presence of *L. pneumophila* Sg 2–14 was indicated by 2 matching patterns of 110 pb (10% tolerance) and 124 (5% tolerance) and *L. pneumophila* Sg 1 by all 3 matching patterns.

4.7.2 Detection and Measurement of *Pseudomonas Aeruginosa*

The detection and enumeration of *P. aeruginosa* in water samples was done according to the requirements of "*AS/NZS 4276.13.2008 Method 13: Pseudomonas aeruginosa—Membrane filtration method"* (Standards Australia and New Zealand, 2008). One hundred milliliters (100 mL) of the sample was filtered through a 0.45 µm gridded cellulose acetate membrane filter. The prepared filters containing the filtrate were rolled onto prepared mPA-C agar plates that were then incubated in an inverted position in humid conditions at 41.5 °C ± 0.5 °C for 44 ± 4 hrs with any flat appearing colonies growing on the plates and depicting a light brownish outer rim to the green-black centre recorded as presumptive *P. aeruginosa*.

Confirmation of *P. aeruginosa* was determined by a modified and validated qPCR laboratory inhouse method (AS 4276.13 EDP-306). DNA was extracted from the bacterial isolates obtained from the incubated plates using QIAsymphony DNA Mini Kit (192) (QIAGEN) and following the manufacturer's instructions. The purity of the DNA was achieved by using the commercially available QIAsymphony DNA Kit

(QIAGEN) and following the manufacturer's instructions. *P. aeruginosa* detection was done by amplification in a Roto-Gene Q (QIAGEN) machine and following the manufacturer's instructions.

The following amplicon sequences described in literature (Khan & Cerniglia, 1994) were used: forward ETA1: 5′-GAC AAC GCC CTC AGC ATC ACC AGC-3′ and reverse ETA2: 5′-CGC TGG CCC ATT CGC TCC AGC GCT-3′ with a product result of 396 bp. A total volume of 25 µL was used for the PCR. The LightCycler instrument (QIAGEN) was used to achieve the following cycling conditions: 1 denaturation cycle at 95 °C for 3 min, 35 cycles with each one made up of 1 m at 94°C, 68°C for 90 s, 72°C for 1 min and an extension cycle of 10 min at 72°C.

4.7.3 Detection and Measurement of *Acanthamoeba* and *Naegleria fowleri*

A validated in-house EcoDiagnostics laboratory method (EDP-315) was used to detect and enumerate *Acanthamoeba* and *N. fowleri*. Two hundred and fifty milliliters (250 mL) of the sample, spiked with *E. coli,* were concentrated by centrifugation for both *Acanthamoeba* and *Naegleria* spp. The supernatant was poured off, and the pellet was resuspended in the remaining volume. One hundred microliters of the remaining volume were then spread plated onto non nutrient agar (NNA) plate and incubated at 42°C for 48 h for *Naegleria*, and at 25°C for 3 days for *Acanthamoeba*, and the presence of amoeba was confirmed using microscopy. Any plaques were picked for confirmation of *Naegleria* spp. by PCR, and then for *N. fowleri* and *Acanthamoeba* by qPCR and PCR, respectively.

For *N. fowleri* confirmation, the cells picked from the incubated NNA agar plates were aseptically transferred into 20 µL of lysis buffer for DNA extraction using the QIAsymphony DNA extraction kit and following the manufacturer's instructions. The PCR and qPCR were run using the *Naegleria* spp. specific primers and *N. fowleri* specific primers previously described in literature (Pélandakis et al., 2000) and (Puzon et al., 2009), respectively. The *Naegleria* spp. PCR amplicon used was sequenced as follows: *Naegleria* spp. forward primer 5′-GAA CCT GCG TAG GGA TCA TTT and reverse primer 5′-TTT CTT TTC CTC CCC TTA TTA-3′ and *N. fowleri* forward primer 5′-GTG AAA ACC TTT TTT CCA TTT-3′ and reverse primer 5′-TTT CTT TTC CTC CCC TTA TTA-3′. The qPCR cycling conditions were: 1 cycle for initial activation at

95°C for 5 min, followed by 60 cycles for denaturation at 95°C for 10 s and then 60 cycles for combined annealing and extension at 95°C for 45 s. Successful PCR amplification was confirmed by the following cycle threshold results in controls; Positive (Ct ≤ 36), Negative (Ct ≥ 37) and NTC control (Ct ≥ 37).

For *Acanthamoeba* confirmation, the twin amplicons JDP1 and JDP2 sequenced respectively as follows: forward primer 5′-GGCCCAGATCGTTTACCGTGAA and reverse primer 5′-TCTCACAAGCTGCTAGGGAGTCA were used for DNA amplification as described in literature (Schroeder et al., 2001). The cycling conditions for *Acanthamoeba* included 1 cycle for initial denaturation at 95°C for 5 min, followed by 40 cycles for denaturation at 95°C for 30 s, 40 cycles for annealing at 56°C for 30 s, 40 cycles for extension at 72°C for 1 min and then 1 cycle for holding at 72°C for 7 min. An *Acanthamoeba* PCR amplification was considered successful if the negative control showed no evidence of contamination indicated by the absence of an amplicon band and when the positive control showed a band in line with the expected amplicon of 500 bp ± 25% which was then considered positive for *Acanthamoeba* and indicated as detected per volume of 250 mL or 1 mL.

4.7.4 Detection and measurement of *Mycobacterium avium*

The detection of *M. avium* was done using qPCR and *M. avium* specific primers, previously designed and used in literature (Uppal et al., 2002), that target the amplification of the 16S rRNA gene and the IS1311 genetic construct as follows: *Mycobacterium* spp. forward 5′-ATAAGCCTGGGAAACTGGGT-3′ and reverse 5′-CACGCTCACAGTTAAGCCGT3′ with a product target of 484 bp and *M. avium* complex forward 5′-GCGTGAGGCTCTGTGGTGAA-3′ and reverse 5′ATGACGACCGCTTGGGAGAC-3′ with a product target of 608 bp. One hundred milliliters (100 mL) of the sample were filtered. The resultant filtrate was placed into 2 mL of ATL and ProtK and incubated at 60°C for 30 min, and then 400 µL was extracted using the QIAsymphony instrument. A 2 µL aliquot of the DNA sample was added to 48 µL of PCR mixture prepared as previously described in literature and ran into a LightCycler 2.0 Machine (QIAGEN) operated according to the manufacturer's instructions. The following cycling conditions were applied: 1 denaturation cycle at 95°C for 8 min to achieve activation followed by 29 amplification cycles made up of

denaturation for 60 s at 95°C, annealing for 60 s at 40°C, extension for 35 s at 72°C and the last extension cycle for 10 min at 72°C, (Appendix 5) A standard PCR and algarose-gel electrophoresis was applied to test for end product specificity.

4.8 Data and Statistical Analysis

The continuous water profile data (free chlorine residual concentration, water temperature, water pH, total dissolved solids (TDS) and total organic carbon) was log-transformed and box and whisker plots were used to determine normality before the application of statistical tests. All microbiological culture results for *L. pneumophila* Sg 1, L. *pneumophila* Sg 2–14 and *P. aeruginosa* were reported as colony forming units per milliliter (CFU/mL). The polymerase chain reaction *(PCR)* test results for *M. avium*, *Acanthamoeba*, and *N. fowleri* were reported as detected or not detected and the quantitative polymerase chain *(qPCR)* test results for the bioaerosol samples were reported as detected or not detected.

All sampling results containing censored data reported by the laboratory as being below the detection limits were handled by a non-parametric method advanced by Helsel (2011). Using this method, each of the non-detect values were assigned a value of −1 before the application of the Kruskal-Wallis hypothesis test of significance (Helsel, 2011). This test orders and ranks the data points to indicate the existence of any differences or patterns. This non-parametric test for data sets with non-detects has greater power than parametric tests when the data do not conform to a normal distribution and is preferred over substitution methods that tend to introduce invasive data, often influencing statistical scores (Conover & Conover, 1980).

Most of the water profile data were not normally distributed, so the Kruskal-Wallis test of statistical differences between variables (H statistic) was used as an alternative to the one-way analysis of variance (ANOVA). All the OPPP occurrence data was also not normally distributed; therefore, the Spearman rho test and the Chi-square test of association were applied where appropriate to measure the extent of association between water profile variables, and the occurrence of OPPP. Before the application of the Spearman's rho test, OPPP occurrence data was coded to 'detected' where a pathogen had been isolated and 'not detected' where the converse was true. The

detected and not detected variables were coded to ‘1’ and ‘0’, respectively, to facilitate statistical testing. A significance value of $p < 0.05$ was used to accept or reject the null hypothesis. The Minitab version 18 statistical package was used for all statistical analysis.

4.9 Conclusion

The findings of this study demonstrated that WMS used to cool ambient temperatures are a potential health risk due to colonization by OPPPs such as *L. pneumophila* Sg 1 and Sg 2–14, *P. aeruginosa,* and *Acanthamoeba*, and that factors such as free chlorine residual concentration, TDS concentration and TOC concentration can influence the regrowth of these pathogens in these systems. The current guidelines in Australia, developed partly due to public outrage following isolated outbreaks of *legionellosis*, focus more on the control of this pathogen in large facilities such as hospitals, aged care homes and shopping centers, ignoring the health risk posed by other emerging pathogens. Therefore, there is a need to develop guidelines covering a broader range of facilities that may expose people to airborne mists which may contain a range of opportunistic premise plumbing pathogens and review existing public health legislation with the aim of adopting a risk-management approach to ensure the effective control of health risks associated with WMS. Further research is needed to understand the relationship between the water profile in WMS and the survival of OPPPs, and conditions that may result in the release of these pathogens from biofilms and their potential to be released as bioaerosols during aerosolization.

CHAPTER 5 RESEARCH FINDINGS, RESEARCH IMPLICATIONS, CRITICAL REFLECTION, CONLUSIONS AND RECOMMENDATIONS

In this chapter, an overall summary of the thesis research is presented, followed by a summary of the research findings and their implications. This is followed by a critical reflection of the research, conclusions and lastly some recommendations on strategies to better manage and control the health risks associated with the use of WMS as a cooling intervention in public places.

5.1 Thesis summary

Opportunistic premise plumbing pathogens are a significant emerging public health issue in instances where plumbing features promote their regrowth and release tiny aerosols that can be contaminated with these pathogens. This research presents some findings that help understand how WMS used as a cooling intervention in public places constitute a public health hazard due to their colonisation by OPPPs. The objectives of this research were to interrogate existing literature to assess what is known and not known about the health risk associated with WMS, assess the level of knowledge about these risks amongst the WMS owners and EHOs and to investigate the occurrence of 5 selected OPPPs in these systems namely. *L. pneumophila, P. aeruginosa, M. avium*, *Acanthamoeba* and *N. fowleri.* This was achieved by advancing the following research questions:

5.1.1 Research Question 1

Research Question 1: "What is the state of knowledge on the health risks of using WMS as a cooling intervention in public places?"

This question identified gaps in knowledge regarding the use of WMS as a cooling intervention in public places, and these became the basis for the research activities conducted to fill these gaps.

5.1.1.1 Research Question 1 findings

Most of the literature reviewed has been limited to indoor domestic plumbing systems, hospital plumbing networks and industrial water-cooling installations, and only focused on the health risks of premise plumbing related to the colonisation and regrowth of OPPPs in other aerosol generating features such as cooling towers (Carducci et al., 2010) , showers (Bauer et al., 2008; Feazel et al., 2009) , faucets (Bollin et al., 1985; Charron et al., 2015) , fountains (De Boer et al., 2002), hot water systems (Borella et al., 2004; Borella et al., 2005) and nebulisers (S. Allegra et al., 2016).

The influence of environmental parameters such as water temperature, residual free chlorine, water pH, TDS, and TOC in enhancing the survival of OPPPs in WMS is not addressed in existing literature, although this has already been determined in similar premise plumbing features (Falkinham, 2015; Julien et al., 2020; Ohno et al., 2008; Perrin et al., 2019).

The role played by biofilms and free-living amoebic species including *Acanthamoeba* in WMS used as a cooling intervention is scarcely addressed in existing literature. Through the process of phagocytosis, amoebic species engulf other OPPPs, thrive in biofilms formed on the internal surfaces of premise plumbing and in so doing shield them from the biocidal effects of disinfectants such as chlorine, and enhance their virulence (Canals et al., 2015; Falkinham, 2015; Huang et al., 2020; Marchesi et al., 2011; Wang et al., 2012).

No studies have been conducted to assess the knowledge and understanding of premise pathogen risk factors amongst WMS owners and regulatory officers such as EHOs. The only studies available relate to drinking water systems (Dinka, 2018; World Health Organisation, 2010). The importance of knowledge as a risk factor for premise plumbing pathogens in drinking water distribution has been highlighted by Julien et al. (2020).

5.1.2 Research question 2

Research Question 2: "What is the level of knowledge and awareness of the health risks of WMS among owners and EHOs?"

This provided the information that helped to understand the WMS owners and EHOs' level of knowledge about the health hazards associated with using WMS as a cooling intervention in public places. The findings also helped to understand the characteristics of the WMS used in the study area, and to demonstrate that there is no regulatory framework covering the installation and operation of these systems. This information helped to inform the methodological design of the subsequent investigation of the installed WMS.

5.1.2.1 Research Question 2 findings

Most of the WMS installed in the northwest of Australia are used as a cooling intervention, this conclusion was confirmed by the survey participants' response that temperature control was the main reason for installing and using them. This finding corroborates with the one made by Wong and Chong (2010) that WMS are more energy efficient than conventional air conditioning systems, and that they are effective and convenient for outdoor cooling as established by (Farnham et al., 2015; Wong & Chong, 2010).

Hydraulic nozzles are the most used method for aerosolization of water in WMS used as a cooling intervention in public places. By applying a force of 1000 psi, these nozzles can produce and disperse tiny and respirable water mists as small as 3 – 10 µm (Henningson & Ahlberg, 1994), making it possible for those aerosols contaminated with OPPP to reach the lungs of exposed persons where they can cause infections (Bollin et al., 1985; Fernstrom & Goldblatt, 2013).

The WMS are predominantly used as a cooling intervention, are operated seasonally, and in summer only making water stagnation and chlorine depletion significant risk factors for OPPP growth (Bowman & Mealy, 2007; Feazel et al., 2009).

Both scheme (conventional water treatment plant supply) and bore water are used in WMS. The bore water supplies are mostly privately owned and remotely located and

are not subject to any formal water quality monitoring requirements. Water quality management is important to ensure the reduction of microorganisms and maintenance of a water chemistry balance that will prevent the occurrence of OPPPs (Garner et al., 2018).

The majority of EHOs, who are the environmental regulatory officers responsible for these systems, were not aware of the health risks that may be associated with the use of WMS as a cooling intervention in public places, with a similar finding being established for the owners of the systems themselves. *Legionella* spp., *Amoeba* spp. and *Pseudomonas* spp. were the only OPPPs identified by only a fifth of the EHOs as significant in WMS. This finding was corroborated by another one which determined that almost all the WMS operators were not trained in the safe operation of the WMS. This is an interesting but concerning finding considering the well documented cases of OPPP infections resulting from the exposure of people to bioaerosols released by similar systems (Brandsema et al., 2014; Green et al., 2019; Hilborn et al., 2013; Zichichi et al., 2000), particularly its relevance to premise plumbing in drinking water systems as determined by Julien et al. (2020).

Most of the WMS operators did not identify adequate disinfection of the water used in WMS as the most important maintenance factor to prevent the regrowth of OPPPs, although they knew that water temperature could contribute to the multiplication of these pathogens in their systems.

The findings of inadequate knowledge about the health hazards of WMS among both the EHO and WMS owners respondents reinforces the Health Belief Model and KAP theories construct as argued by Fan et al., 2018 and discussed in Section 1.2.4 of this thesis, that knowledge is the foundation of behaviour change, and that belief and attitudes drive these changes. Additionally, this KAP theory clearly supports these findings since they highlight the importance of improving the knowledge and skills of the respondents to be able to identify not only the inherent environmental hazards of using WMS, but the relevance of effective maintenance regimes (Julien et al., 2020).

The finding that many of the WMS operators did not have any maintenance and cleaning procedure in place for their WMS could have contributed to their inability to know that adequate disinfection was the first line of defence against OPPP growth as

highlighted by several standards and guidelines on the control of *Legionella* spp. in water systems (American National Standards Institute, 2018; enHealth, 2015; Health and Safety Executive, 2014).

All the survey participants reported that they did not approve nor licence the installation of WMS used as a cooling intervention in public places, and that they did not conduct any periodic inspection of the same. As such, there exists a significant gap in existing public health legislation to capture these water systems, and the absence of standards or guidelines to assist the owners to operate their systems in a manner that prevents the regrowth and possible exposure of the public to OPPPs. Currently there are no guidance limits for airborne exposure to the pathogens investigated in this research. This is due to the challenges of characterising exposure risk profiles because of the diversity of the biological agents and their toxicity and compounded by the absence of standardised methods for their sampling and laboratory analysis (Eduard et al., 2012). There is a need to explore the use of molecular methods that employ genus or species-specific qPCR primers to detect these bacterial OPPPs bioaerosols as a way evaluating personal and occupational exposure where possible to inform the development of future exposure limits or guidelines applicable to specific settings (Eduard et al., 2012; Mbareche et al., 2019).

5.1.3 Research question 3

Research Question 3: "Can WMS be colonized by OPPPs?", and "Are environmental conditions of water temperature, residual chlorine disinfectant, water pH, TDS and TOC associated with the occurrence of OPPPs in WMS?"

This question examined the actual occurrence of OPPPs in the WMS used to cool public places, including environmental factors that influence such occurrence. This research question provided empirical evidence to demonstrate that WMS used as a cooling intervention in public places are a potential health hazard due to their colonization by OPPPs.

5.1.3.1 Research Question 3 findings

The findings demonstrated that some OPPPs can colonise WMS used as a cooling intervention in public places. A total of 64 (71%) of WMS samples analysed tested positive for OPPPs, with P. aeruginosa being found in 40 (44%) of the total samples. *L*. *pneumophila* Sg 2–14 was detected in 16 (18%) of the total samples and *L. pneumophila* Sg 1 was isolated from 5 (6%) of the total samples. Only 3 samples tested positive for *Acanthamoeba*. The biogeography model discussed in section 1.2.2, fully supports these findings. The model states that sturdy, naturally occurring OPPPs can thrive in the distal pipework of water distribution systems due to the effect of pressures such as water stagnation, small pipe diameters and reduced free chlorine residual concentration (Ling et al., 2018). WMS investigated in this study are part of the distal pipework, and the OPPPs detected in them are naturally occurring and able to resist the stresses identified in this model.

The occurrence of the detected OPPPs was concentrated in biofilms rather than in water samples, with *L*. *pneumophila* Sg 1 being 30 × higher than in water, and *L*. *pneumophila* Sg 2–14 being three times higher in this sample matrix than in water. This finding has significant implications for strategies employed to control FLA and all OPPPs in WMS and is comparable with similar studies of other premise plumbing features (Feazel et al., 2009; Liu et al., 2019; Mathys et al., 2008).

No other OPPP was detected in bioaerosol samples except *P*. *aeruginosa* which was present in 67 % of this sample matrix. Davis et al., (2016) theorises that contaminated water distribution systems can potentially expose people in domestic settings to inhalation exposures of pathogenic microorganisms, and Baranovsky et al., (2018) established that water mists contaminated with *Legionella* spp. can be inhaled and cause infections in exposed people. Both these theories discussed in section 1.2.3 were inadequate in explaining the non-detection of some of the OPPPs measured as present in water samples.

The detection of *P*. *aeruginosa* in WMS bioaerosols is a health risk to exposed persons because it is known to cause aggressive pneumonia in immunocompromised persons such as those with cystic fibrosis (Falkinham, 2015); as well as self-limiting ear and skin infections (Rossolini & Mantengoli, 2005).

Although *L. pneumophila* Sg 2–14, *L. pneumophila* Sg 1 and *Acanthamoeba* were also detected in water, they were not measured as present in bioaerosols. This variance could be explained in terms of the differential abundance of these OPPPs in the water sample matrix (Lin & Peddada, 2020). At 350 CFU/mL in water, *P. aeruginosa* was the most abundant when compared with *L. pneumophila* Sg 2–14 (300 CFU/mL), and *L. pneumophila* Sg 1(100CFU/mL). Lin & Peddada, (2020), argue that the sampling fraction of a microorganism obtained remains constant between the ecosystem and the sample. This could explain the detection and abundance of only *P. aeruginosa* in the bioaerosol matrix. Another reason for this phenomenon could be attributed to the varying survivability of microorganisms in the air. Research in laboratory models have demonstrated that *P. aeruginosa* can remain airborne for periods greater than 45 minutes (Clifton et al., 2010), making it easily captured by a sampling train, whereas *L. pneumophila* spp. remain airborne for less than 3 minutes (Séverine Allegra et al., 2016) after dispersal. *P. aeruginosa* achieves this feat by expressing a mucoid phenotype in air that resists desiccation usually associated with microbes dispersed in air (Clifton et al., 2010).

Currently there is neither a standard method for the sampling of bioaerosols, a standard analytical method for the detection in bioaerosols of the OPPPs investigated in this study. The ability of these OPPPs to cause infections in exposed people has already been discussed, therefore further research is required to quantify the risk of exposure to these pathogens. However, the ethical challenges, practicability and viability associated with personal exposure monitoring of people in public places where these systems are installed negate these efforts. The personal exposure monitoring of people exposed to these pathogens in similar WMS installed and used in occupational settings for dust suppression and similar purposes may be practical under existing occupational health and safety legislation. Bore water tended to promote the regrowth of L. *pneumophila* Sg 2–14 better than scheme water, with its occurrence being four times higher in the former, a finding that is corroborated by the other finding which determined that most of the bore water tested had no free chlorine residual.

The concentration of free chlorine residual concentration in the WMS water tended to reduce with an increase in the concentration of TDS and TOC, a finding which could

reduce the efficacy of chlorine disinfectant, and in so doing promote the regrowth of OPPPs in WMS used as a cooling intervention in public places.

The efficacy of chlorine as a disinfectant against all detected OPPPs was demonstrated with a negative relationship being established, except for *Acanthamoeba*. This finding corroborates the results of several studies that have demonstrated the ability of this FLA to shield other pathogens from the biocidal effects of disinfectants (Canals et al., 2015; Huang et al., 2020; Marchesi et al., 2011; Wang, Masters, et al., 2012). The ecological niche model for OPPPs advanced by Wang et al., 2013, and discussed in section 1.2.1 fully supported analysis of research question number 3. This model explains how OPPPs such as *L*. *pneumophila* survive within *Amoeba* and overcome microbial competition from other indigenous microbes in the system. The detected presence of *Acanthamoeba* in the WMS, its observed positive correlation with free chlorine residual concentration could be an indication of its ability to shield other pathogens such as *L. pneumophila* from destruction by disinfectants such as chlorine (Thomas et al., 2010). This finding is important considering that chlorine is the commonly used water disinfectant in Australia (National Health and Medical Research Council,2011).

This study did not demonstrate any correlation between water temperature and the occurrence of all detected OPPPs except with *P. aeruginosa*. This finding is different from other other similar studies (Agudelo-Vera et al., 2020; Falkinham, 2015; Perrin et al., 2019). One reason that could have contributed to this is the constantly higher-than-normal annual mean maximum temperatures in the study area that were 32.7°C in February 26°C in May and 29.2°C in August (Bureau of Meteorology, 2016). The temperature profile of Newman, a location in which these WMS are located is given in Figure 1. These temperatures fall in the ideal zone for the growth of most OPPPs across all seasons, making their occurrence across all sampling events possible, a phenomenon that could have resulted in the lack of any significant difference across the 3 sampling events. Previous research has already demonstrated that temperature is a selective environmental parameter for OPPP occurrence in premise plumbing (Agudelo-Vera et al., 2020; Falkinham, 2015; Perrin et al., 2019), making is valuable to expect the same effect on WMS installed and operated in similar tropical regions.

5.1.4 Implications of the study

The findings of this study have contributed to a better understanding that WMS used as a cooling intervention can be colonised by OPPP, and hence they constitute a significant health risk to those people who get exposed to aerosols released during their operation. This study has also produced findings that show that the occurrence of OPPPs in these systems is subject to environmental, operational, and legislative drivers that can be effectively managed to mitigate the health risks posed by these WMS. The findings also concur with some conceptual as well as applied implications for all those in the domain of water quality management generally, and especially for WMS operators, EHOs, environmental health regulatory agencies and environmental health training institutions. These implications are:

5.1.4.1 For WMS owners/operators

The study has shown that, although the installation and use of WMS as a cooling intervention in public places achieves noticeable benefits at lower energy costs, it also presents significant health risks due to their colonisation by OPPPs. Underpinned by the ecological niche model for OPPPs, these WMS promote the growth of these pathogens. Having adequate knowledge of the associated health risks, adequate training, and skills to operate WWS in a way which prevents the growth of OPPPs is critical to avoid exposing patrons, staff and members of the public that may be in close proximity to these systems.

5.1.4.2 For EHOs and environmental regulatory authorities

The findings of this study have indicated that WMS used as a cooling intervention in various local government areas are located outside with pipework exposed to extremes of temperature, the water distributed in these systems is often inadequately disinfected and operated by untrained people in the absence of robust water quality and maintenance systems. The study subsequently demonstrated that pathogenic *L. pneumophila Sg 1, L. pneumophila Sg 2-14, P. aeruginosa* and *Acanthamoeba* colonise and regrow in these WMS, and that they are not subjected to any environmental health monitoring/inspection due to the absence of a regulatory framework to support preventative measures.

These findings confirm that these WMS are premise plumbing features that must be included in any environmental health monitoring programme to protect public health. To achieve this goal, existing legislation needs to be reviewed to provide a legal framework that facilitates this outcome.

5.1.4.3 For Environmental health training institutions and universities research centres

The study findings have demonstrated a lack of knowledge among EHOs and WMS owners about the health risk associated with the use of WMS as a cooling intervention, including the environmental conditions that can promote the colonisation and regrowth of OPPPs. As such universities offering EHOs education should include the design and operation of WMS used as a cooling intervention in their existing training courses. Technical and Further Education (TAFE) and other Registered Training Organisations (RTO) that offer training to tradespeople and run other short technical courses should incorporate WMS design, operation and maintenance in their existing plumbing and water quality courses.

The study showed that the relationship between environmental parameters and OPPPs in WMS can be complex, with pathogens such as *L*. *pneumophila* Sg 1 and Sg 2-14 favourably growing in biofilms than in other sample matrices, *P. aeruginosa* being the only OPPP present in bioaerosols, *Acanthamoeba* being positively correlated with chlorine residual, and TDS and TOC affecting chlorine disinfectant concentration. As such, universities and water research institutes should conduct further research to better understand these phenomena in WMS used in several commercial and industrial applications, particularly those that expose members of the public to aerosolised water from WMS.

5.1.5 Study limitations and opportunities for future research

As this study progressed, it became apparent that some limitations to the study were worth noting, however some of these limitations present opportunities for further research:

5.1.5.1 Limitations

5.1.5.1.1 Small sample size –

The sample sizes for the WMS owners (10) and EHOs (22) surveys were small, making it likely to lower the possibility of picking up a real effect (Button et al., 2013). To mitigate against this phenomenon, the F-statistic, suitable for small sample sizes was used to evaluate any association between variables. This means that generalisation of the study results should be done with caution. However, the novel nature of this research makes the findings valuable and important for informing future investigations.

5.1.5.1.2. Self-reported data biases

The self-reported data obtained using the survey questionnaires could not be validated for selective memory, telescoping, attribution and exaggeration biases. Further research with larger sample sizes to enable statistical validation and generalisation is recommended. However, the survey questionnaires were pilot tested to identify this phenomenon and adjustments were made to the questionnaires prior to the participants being asked to complete them, as described in section. This ensured that questions that would introduce recall these biases were either replaced or amended (Althubaiti, 2016).

5.1.5.1.3. Financial constraints

Financial constraints associated with the analytical costs for bioaerosol, biofilm and water samples collected from the WMS resulted in the sampling events being limited to 3 instead of 4 as initially intended. This resulted in less samples being analysed and could have influenced the statistical significance of some findings. Further research with adequate funding is recommended.

5.1.5.1.4. The effect of climatic variation on the occurrence of OPPPs in WMS

This study was based on 10 WMS located and operated in the climatic regions of the Pilbara: a tropical climate characterised by relatively high mean maximum temperatures across the seasons. The findings of this research did not show a climatic

variation in the occurrence of the OPPPs detected as well as with temperature, contrary to the findings of research in similar premise plumbing features (Lu et al., 2017). It is recommended that larger studies be conducted in climates with more distinct seasonal variations in temperature to determine the effect of climate variation on OPPP growth.

5.1.5.1.5 Occurrence of *M. avium* nor *N. fowleri* in WMS

This study did not detect the presence of *M. avium* nor *N. fowleri* in WMS used as a cooling intervention in public places, although studies of similar features have detected their presence. It is recommended that a similar study involving a bigger sample size be employed to examine if these OPPPs are also able to colonise and regrow in these systems.

5.1.5.1.6 Influence of environmental parameters on the microbiome ecology of WMS

This study showed some varied influence of environmental parameters on the occurrence of OPPPs in WMS, demonstrating the complexity of the inherent features that interact to create a niche for these pathogens to thrive. This phenomenon has been highlighted by previous research on OPPP occurrence in similar features, which has established that the microbiome of these pathogens can be varied, with some species dominating over others, and symbiotic relationships developing amongst other species (Ling et al., 2018). An opportunity for further research exists to determine the extent to which each of these environmental parameters influences the ability of some OPPP species detected in WMS to dominate over the others, since stronger correlations were demonstrated between the occurrence of some, but not all pathogens and environmental parameters.

5.1.5.1.7 Factors influencing the aerosolization of *P aeruginosa* and other OPPPs in WMS

Of all the OPPPs investigated in this study, only *P. aeruginosa* was detected in water aerosols released from the WMS, leaving an outstanding question as to why the other pathogens were absent in this sample matrix which is the main route of exposure for

OPPPs. It is recommended that further research be conducted to examine the mechanisms by which *P. aeruginosa* is released from the WMS biofilms and water phase into the aerosols, and whether these mechanisms could also apply to other OPPPs.

5.1.5.1.8 The efficacy of chlorine disinfection on *Acanthamoeba* in WMS

Acanthamoeba, now known for its phagocytic tendencies on OPPPs in similar systems, was detected in WMS. This FLA also exhibited a positive correlation with residual free chlorine, a concerning observation, since chlorine is the widely used disinfectant intended to prevent the regrowth of pathogens in the systems. Further research to understand the effectiveness of chlorine disinfection on *Acanthamoeba* in WMS is recommended.

5.1.5.1.9 The occurrence of OPPPs in industrial and commercial systems that aerosolise water into ambient atmospheres

Although this study investigated OPPP occurrence in WMS used as a cooling intervention in public places, similar systems are widely used in other industrial and mining operations such as dust suppression. It is recommended that similar studies of these systems in these industrial and mining settings be carried out to determine their health risks due to OPPP colonisation.

5.1.6 Critical reflection

This research has enabled me to synthesise a few principles and concepts that underlie OPPP regrowth in premise plumbing. Although OPPP growth in WMS can be based on the prevalence of similar growth conditions in other premise plumbing features, the OPPP microbiome that will eventually do so in these systems can be dependent on the dynamic interaction of various environmental factors. This insight indicates that if studied further in different settings and climates, unexpected and valuable results could be discovered. In as far as the thesis confirmed the growth of some OPPPs in these WMS, we need to be mindful of the potential influences of other factors such as the type of plumbing materials and water pressures that were excluded from this investigation, and that could further influence the occurrence and diversity of

these OPPPs. It is therefore possible to further investigate this phenomenon by studying the effect of several other environmental factors on OPPP regrowth in a model WMS, where all dependent variables can be manipulated, and effects observed over time.

This study has contributed to the understanding of health risks associated with the use of WMS as a cooling intervention in public places. However, as the study progressed and findings established, several recommendations can be made to help manage these risks and better protect the public particularly those with compromised immunity from possible OPPP exposure from WMS.

5.1.7 Conclusion

The conclusions of this research based on the 3 research questions investigated are stated below:

The health risks of using WMS as a cooling intervention has not been studied before, with OPPP research having been restricted to other similar premise plumbing features such as cooling towers, showers, hospital faucets, fountains etc. Furthermore, the important part played by adequate knowledge and skills in mitigating the risks of operating WMS is scarcely mentioned in existing research.

Most of the WMS used as a cooling intervention in north-western Australia tend to be located outdoors where elevated water temperatures result from direct exposure to the sun, are not regularly operated, generate, and release respirable aerosols, promote biofilm formation, are not regularly maintained, and are fed with water that is not always adequately disinfected, or contains impurities such as iron that may promote growth. These factors make such WMS ecological niches for OPPP colonisation and regrowth. The effect of water temperature, TDS and TOC concentrations on the occurrence of *P. aeruginosa* is a phenomenon of immense importance to operators of WMS in the north-western region of Australia where higher winter and summer temperatures are experienced during the greater part of the year (Bureau of Meteorology, 2016)

The findings showed that the WMS owners responsible for operating WMS, as well as the EHOs responsible for environmental health regulation do not have adequate knowledge about the health risks associated with the use of WMS as a cooling intervention in public places. Although some of them acknowledge the public health importance of WMS plumbing installations, their knowledge of conditions that can lead to the colonisation and regrowth of these pathogens in WMS was low.

The findings clearly showed the absence of a regulatory framework to ensure that the installation and operation of WMS used as a cooling intervention in public places is done in a manner that adequately manages the health risk posed by OPPP. The *Health (Air-handling and Water Systems) Regulations 1994 (WA)* does not include WMS used as a cooling intervention in public places within its scope, leaving a regulatory gap for these systems. The Australian Standard AS/NZS 3666.1:2011 (Standards Australia, 2011b), called up by several statutes, provides the minimum standards covering design and installation of air handling and water systems of buildings, however, this scope also fails to cover WMS used as a cooling intervention in public places.

The findings demonstrated that *L. pneumophila Sg 1, L. pneumophila Sg 2-14, P. aeruginosa* and *Acanthamoeba* can colonise and regrow in WMS used as a cooling intervention in public places. Opportunistic premise plumbing pathogen growth is enhanced in WMS biofilms, with *L. pneumophila* Sg 1 and Sg 2-14 being the most dominant in this environment. Aerosols released by WMS used as a cooling intervention can be contaminated by *P. aeruginosa.* The use of bore water with inadequate disinfectant concentrations promotes OPPP colonisation. The disinfectant levels can be reduced by elevated levels of TDS and TOC. The use of chlorine disinfectant in sanitising WMS may not adequately control the occurrence of *Acanthamoeba*, due to its ability to resist its biocidal effects. Based on these findings, WMS used as a cooling intervention are ecological niches for OPPP growth, hence effective strategies are needed to manage this health risk.

5.1.8 Recommendations

5.1.8.1 Policy

Currently, the design, installation, and operation of WMS in public places is not adequately regulated. It is recommended that the Environmental Health (EH) regulatory authorities in each Australian State could review existing EH legislation to capture WMS and put in place a registration and or licencing system for the same

There is no code of practice/guideline that provides information about the design, installation and operation of WMS used as a cooling intervention. Current guidelines deal with *Legionella* spp. management in aged care settings and do not address all potential OPPPs that can regrow in WMS. It is recommended that the West Australian Department of Health constitute a committee of experts drawn from EH, Water Quality management, Academia to develop a Code of Practice/Guideline that provides technically sound information on the design, installation and safe operation of WMS in public places focusing on Conceptual Site Models (CSM) that capture local characteristics.

Existing WMS are not subject to any periodic inspection/ assessment regime by environmental health staff. It is recommended that the Environmental Health unit in the West Australian Department of Health, in conjunction with Local Governments, develop and implement periodic inspection/monitoring of WMS installed in public places for compliance with legislation and/or a code of practice.

5.1.8.2 Knowledge, Awareness and Competence

The level of knowledge and awareness of the health risks associated with WMS in public places is low amongst both EHOs and system owners. It is recommended that Environmental Health Officer training institutions incorporate the safe design and operation of WMS into their current courses, and that the West Australian Department of Health develop and run short courses on the safe operation of WMS for system owners.

5.1.8.3 Operational and water quality management aspects

The results of this research indicated that disinfectant concentration in the WMS was below levels required to achieve effective sanitation, and that some pathogens showed resistance to chlorine. It is recommended that the Department of Health develops water quality management guidelines and factsheets that can be used by WMS owners to put in place effective water quality management plans at the local level. This needs to be complimented by periodic compliance sampling of water by the EHOs to ensure that effective sanitation is achieved and maintained. This monitoring program could be integrated with the DOHs current potable water quality management system.

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INTERNATIONAL PERSPECTIVES/SPECIAL REPORT

Health Risks Associated With the Use of Water Mist Systems as a Cooling Intervention in Public Places in Australia

Edmore Masaka, MPH
Sue Reed, MSc, MEngSc, PhD, COH, CIH
Jacques Oosthuizen, MMedSci, PhD, COH
Margaret Davidson, PhD
School of Medical and Health Sciences, Edith Cowan University

Abstract **The exposure of people to opportunistic premise plumbing pathogens (OPPPs) such as *Legionella, Mycobacterium*, and *Pseudomonas* in aerosolized water has been linked to opportunistic infections. Water mist systems (WMS) that are used to cool public places by flash evaporation of tiny water aerosols are gaining prominence in regions with hot climates in Australia. The potential of WMS to be colonized by OPPPs has not been adequately studied. The public health impact of OPPPs is significant, as *Legionella* accounted for 66% of waterborne disease outbreaks associated with drinking water systems in the U.S. in 2013–2014. *Legionella* infections caused by the inhalation of contaminated water aerosols in Europe increased from 1,161/year in 1994 to 4,546/year in 2004. As WMS are part of premise plumbing, they have structural characteristics that promote biofilm formation, growth of free-living amebae, inadequate disinfection levels, elevated water temperatures, and oligotrophic conditions—all of which promote OPPP inhabitancy. This special report highlights the potential public health risks of using WMS as a cooling intervention in public places and advocates for their regulation in places of public assembly and entertainment.**

Introduction

Water mist systems (WMS) are defined as plumbing mechanisms installed in outdoor public places to reduce ambient temperatures. Small nozzles fitted to WMS atomize water into tiny aerosols that flash evaporate in the ambient atmosphere, reducing surrounding temperatures by as much as 10 °C. These WMS are more energy efficient than conventional air conditioning systems (Wong & Chong, 2010). Premise plumbing promotes the colonization and regrowth of opportunistic premise plumbing pathogens (OPPPs), including *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa*, *Acanthamoeba*, and *Naegleria fowleri* (Falkinham, 2015; Whiley et al., 2014). These OPPPs cause opportunistic infections in children, older adults, and people with compromised immunity (Falkinham, Hilborn, et al., 2015).

Most of the research on WMS has been experimental, focusing on design capabilities and the operational efficiency of the systems (Wong & Chong, 2010; Xuan et al., 2012). Research on premise plumbing installations such as showers, water taps, and faucets, however, has confirmed the presence of *L. pneumophila*, *M. avium*, *P. aeruginosa*, *Acanthamoeba*, and *N. fowleri* (Falkinham, Hilborn, et al., 2015; Whiley et al., 2014). This special report examines and describes the OPPP risks associated with WMS systems in the Pilbara region of Western Australia. This region experiences extreme temperatures and has a higher use of WMS. We highlight the five major OPPPs implicated in waterborne diseases: *L. pneumophila*, *M. avium*, *P. aeruginosa*, *Acanthamoeba*, and *N. fowleri*.

Most WMS are located outdoors where they are exposed to elevated temperatures. Operation of WMS in these environmental conditions increases the water temperatures to levels at which OPPPs such as *L. pneumophila* thrive (Lu et al., 2017). The densities of *Legionella* and *Mycobacterium* species can increase with elevated water temperatures of 25–40 °C in showers and water taps (Lu et al., 2017). WMS located outdoors that are exposed to elevated temperatures can be a risk for OPPPs.

The WMS used for cooling public places are made from materials such as polyvinyl chloride (PVC), polyethylene, nylon, or steel. The use of these plumbing materials can promote the regrowth of OPPPs (Wang, Masters, et al., 2012). These plumbing materials leach nutrients that promote biofilm formation on the internal surfaces of pipework and fittings (Rogers et al., 1994).

Water stagnation causes disinfectant decay in water systems, resulting in the regrowth of

OPPPs (Wang et al., 2013). When WMS are shut down during winter, there is potential for growth of OPPPs that can subsequently be aerosolized if the units are turned back on in summer without proper treatment. A whole life cycle treatment plan must include the winter shutdown period and incorporate controls to prevent generation of contaminated aerosols.

Opportunistic Premise Plumbing Pathogens

The use of WMS is an emerging public health concern (Falkinham, Pruden, & Edwards, 2015; Wang et al., 2013) because they represent a potential source of exposure to so-called "opportunistic pathogens" that can affect the health and well-being of exposed individuals, especially among those with predisposing risk factors (e.g., children, older adults, and people with compromised immunity) (Falkinham, Pruden, & Edwards, 2015). Key OPPPs associated with premise plumbing are *L. pneumophila*, *M. avium*, *P. aeruginosa*, *Acanthamoeba*, and *N. fowleri* (Bédard et al., 2016; Falkinham, Pruden, & Edwards, 2015).

Legionella pneumophila

L. pneumophila has been associated with several outbreaks of waterborne legionellosis in premise plumbing (Bennett et al., 2014; Cohn et al., 2015). The bacteria colonize cooling towers, warm water baths, water fountains, and showers. If disturbed, the bacterial can aerosolize and result in respiratory disease and even death of exposed persons (Kim et al., 2015). *L. pneumophila* can grow inside amebae (Liu et al., 2019), making it resistant to chlorine disinfection (Dupuy et al., 2011); it has also been isolated from household plumbing (Barna et al., 2016; Byrne et al., 2018).

Mycobacterium avium

M. avium belongs to a group of nontuberculous *Mycobacteria* (NTM) called *Mycobacterium avium* complex (MAC). This complex includes *M. avium* and *M. intracellulare*, which are found in aquatic environments and soil, and transmitted via inhalation, ingestion, or inoculation (Rijhumal & Chai, 2015). MAC causes various infections depending on the subspecies, route of infection, and the immune health of the exposed person (Whiley et al., 2012). In people who previously had no symptoms, MAC causes pulmonary and soft tissue infections in healthy individuals (Falkinham, 2016). *M. avium* can cause cervical lymphadenitis in young women (Reuss et al., 2017) and pulmonary diseases in people with HIV/AIDS (Falkinham, Hilborn, et al., 2015).

M. avium has been isolated from premise plumbing and potable water systems (Water Research Australia, 2014; Whiley et al., 2012), hospital plumbing (Baird et al., 2011), and household plumbing (Falkinham et al., 2008). This ability of the bacterium to grow at temperatures >45 °C, paired with its chlorine resistance, enables it to thrive in water distribution systems (Falkinham et al., 2008). *M. avium* can colonize showerheads (Feazel et al., 2009), water taps, and water heaters (Wang, Edwards, et al., 2012), and subsequently be transmitted by the inhalation of contaminated aerosols (Falkinham, 2013). Like *L. pneumomphila*, *M. avium* can resist disinfection in premise plumbing by inhabiting amebae (Steed & Falkinham, 2006). WMS in public places mimic showers in terms of elevated temperatures (Feazel et al., 2009; Lu et al., 2017), plumbing materials, and potential for aerosol formation (Steed & Falkinham, 2006), making them a health risk for exposure to *M. avium*.

Pseudomonas aeruginosa

P. aeruginosa is a versatile OPPP that can adapt and survive tough environmental conditions (Bédard et al., 2016). This bacterium favors moist environments and has low nutritional requirements because of its ability to metabolize different compounds (Yu et al., 2016). These properties enable *P. aeruginosa* to form biofilms with other microorganisms present in premise plumbing systems, which confer resistance to disinfectants such as chlorine dioxide and monochloramines (Masák et al., 2014).

Transmission of *P. aeruginosa* occurs through exposure to contaminated water by inhalation and immersion and can cause self-limiting ear and skin infections (Rossolini & Mantengoli, 2005); it can also cause aggressive pneumonia in immunocompromised persons such as those with cystic fibrosis (Falkinham, 2015). *P. aeruginosa* has been isolated from hospital water taps (Shareef & Mimi, 2008), as well as from showerheads and hydrotherapy pools (Caskey et al., 2018). The ubiquitous nature of this bacterium in the environment, its hardiness, and potential for biofilm-produced chlorine resistance (Zichichi et al., 2000) make *P. aeruginosa* a particular concern in relation to WMS with their generation of aerosols and popularity in licensed clubs frequented by older adults and potentially immunocompromised individuals.

Acanthamoeba

Acanthamoeba is a protozoan that lives in varied environments, such as environmental and drinking water systems (Michel et al., 1998), tap and well water (Marciano-Cabral et al., 2010), hospital waters (Muchesa et al., 2015), aquatic facilities (Chang et al., 2010), and recycled water (Storey & Kaucner, 2009). *Acanthamoeba* is the causative agent of granulomatous amebic encephalitis (GAE), a central nervous system disease that affects people with weakened immunity. The species *Acanthamoeba* keratitis can also cause infection of the corneal epithelium (Pruden et al., 2013). The microorganism can grow in domestic tap water (Codony et al., 2012) and has been isolated from hospital water supplies (Muchesa et al., 2015). A significant characteristic of this OPPP is its ability to engulf and shield other OPPPs such as *L. pneumophila*, *P. aeruginosa*, and *M. avium* from disinfection (Zbikowska et al., 2014), which makes it an essential target for WMS infection control strategies.

Naegleria fowleri

N. fowleri, the only pathogenic species of its genus, causes fatal primary amebic meningoencephalitis (PAM). This infectious disease is transmitted by aspiration of contaminated water aerosols up the nasal passage (Yoder et al., 2012). This ameba can live in premise plumbing, rainwater tanks, and any system where warm water is present (Waso et al., 2018). The warm operational temperatures of WMS, coupled with their generation of inhalable water aerosols, make them a possible source of this rare but fatal infection.

Public Health Impact

The public health risk of OPPPs and their associated infectious diseases are significant. WMS create thermal comfort by atomizing water into aerosols. The aerosols range from 0.3–10 µm and can be deposited into the lungs by inhalation, where they can cause

infections (Henningson & Ahlberg, 1994). The OPPPs range from 2–20 µm for *L. pneumophila* (Füchslin et al., 2010), 0.2–0.6 µm for *M. avium* (Vijay et al., 2017), 0.5–1.0 µm for *P. aeruginosa* (Deforet et al., 2015), 12–35 µm for *Acanthamoeba* (Siddiqui & Khan, 2012), and 15–20 µm for *N. fowleri* (Piñero et al., 2019). Most of these OPPPs fall within the size fraction that are inhalable by people; or, they can land on skin and surfaces, creating another potential exposure route. They can also be ingested and cause skin irritation.

Legionella alone is responsible for 2–15% of patients hospitalized globally with community-acquired pneumonia (Sakamoto, 2015). In the U.S., 32 cases of waterborne disease outbreaks were reported between 2011 and 2012, with 66% of the outbreaks being associated with *L. pneumophila* (Beer et al., 2015). The incidence rate for waterborne *M. avium* disease over the same period was 647 cases/100,000 persons (Beer et al., 2015).

In Australia, an average of 374 cases of legionellosis were reported annually between 2008 and 2018 (Australian Government Department of Health, 2021), with an incidence rate of 1.50/100,000 persons in 2015, dropping to 1.2/100,000 in 2019. The combined mandatory reporting of *L. pneumophila* and *L. longbeachae* infections as legionellosis cases in Australia does not provide specific information about the incidence of infections caused by different *Legionella* species. This lack of specificity obscures any trends associated with exposure routes, considering that one is soilborne (*L. longbeachae*) and the other is waterborne (*L. pneumophila*).

A total of 143 cases of PAM were reported across the U.S. between 1962 and 2017 (Centers for Disease Control and Prevention, 2020). In Australia, 19 water-related PAM cases were recorded between 1960 and 1980. The case rate for microbial keratosis, a disease caused by *Acanthamoeba*, was 0.66 cases/10,000 between 2005 and 2015 (Waso et al., 2018). Opportunistic infections caused by *M. avium* and *P. aeruginosa* could be underestimated because they are not notifiable diseases in most countries (Falkinham, Hilborn, et al., 2015). NTM, however, are notifiable diseases in the Australian states of Queensland and the Northern Territory; PAM is a notifiable disease in Western Australia (Australian Government Department of Health, 2021). A total of 19 PAM cases were reported in Australia during 1965–1980 (Waso et al., 2018) and another 4 cases were reported in Queensland during 2006–2015 (Nicholls et al., 2016).

Factors That Promote Opportunistic Premise Plumbing Pathogen Colonization

Biofilm Formation

The potential of biofilm formation is a significant risk factor for the incidence of respiratory/infectious disease outbreaks associated with WMS. Biofilms are complex heterogeneous colonies consisting of bacteria, fungi, protists, and other microbial organisms that grow as native communities in water systems (Wingender & Flemming, 2011). Biofilms in premise plumbing systems provide conducive and nutritive conditions for OPPP growth, increase the potential for OPPP colonization, and inhibit disinfectants used to clean systems (Ashbolt, 2015; Falkinham, 2015; Momba et al., 2000; Pruden et al., 2013; Wang et al., 2013).

In drinking water systems, 95% of the microbiological population resides in biofilms compared with approximately 5% in the water phase (Flemming et al., 2002). The OPPPs residing in biofilms of water systems, however, can be released into the water phase where they can cause waterborne diseases (Flemming, 2011). Biofilm formation can occur as a result of extreme environmental conditions of temperature, pH, and pressure (Momba et al., 2000). Maintenance programs aimed at minimizing potential for their generation are essential. Sampling and analysis of biofilm samples from WMS used for cooling are recommended to provide an insight into their potential as sources of OPPPs.

Temperature

Elevated water temperatures in distribution systems promote the growth of OPPPs (Falkinham, 2015; Storey et al., 2004). The ability of microorganisms to survive at elevated water temperatures is an essential adaptation feature that enables *L. pneumophila*, *M. avium*, and *P. aeruginosa* to thrive in water systems (Falkinham, Pruden, & Edwards, 2015). The WMS used for cooling public places are exposed to high temperatures that can promote the regrowth of OPPPs (Storey & Kaucner, 2009). It is necessary to determine the water temperature profile of WMS to understand its influence on OPPP regrowth.

Presence of Free-Living Ameba

The presence of ameba in premise plumbing can aid the regrowth of OPPPs (Wang, 2013; Wang et al., 2013). The ability of free-living ameba to amplify the number and virulence of OPPPs in engineered water systems is widely acknowledged (Falkinham, 2015; Thomas & Ashbolt, 2011). WMS used in public places need to be investigated for free-living amebae, particularly *Acanthamoeba*, due to their virulence in water distribution systems and their role in the regrowth and amplification of OPPPs (Codony et al., 2012).

Resistance to Chlorine Disinfection

Chlorine is an effective water disinfectant and remains one of the most important public health interventions (Boorman, 1999; Government of Western Australia Department of Health, 2016). At the right pH (6.5–8.5), temperature (20–29 °C), and turbidity, chlorine provides an adequate residual disinfectant effect (Australian Government National Health and Medical Research Council, 2011). Under specific environmental conditions, however, OPPPs can become resistant to chlorine and its derivatives, especially when part of a biofilm colony (Canals et al., 2015; Codony et al., 2012).

Most WMS are connected to water treated at conventional water treatment plants; however, WMS located in remote parts of the region can use on-site borehole water supplies that are locally managed. Chlorination is the most common means of disinfection for Australian water supplies, with a minimum target of 0.5 mg/L residual chlorine recommended (Australian Governnment National Health and Medical Research Council, 2011). As chlorination is the main form of disinfection for water supplies connected to WMS, an investigation of its effectiveness in controlling the regrowth of OPPPs in these systems is warranted.

Low Total Organic Carbon Concentration Levels

OPPPs can thrive in premise plumbing systems with low carbon concentrations (Falkinham, Pruden, & Edwards, 2015). Low-carbon or oligotrophic environments are characteristic of most premise plumbing

(Wang et al., 2013). The nitrifying bacterial autotrophs present in low-carbon waters fix available carbon, making it available to heterotrophic organisms such as OPPPs to metabolize (Wang et al., 2013; Zhang & Edwards, 2009). Through this process, low-carbon water environments existing in WMS can select for *L. pneumophila* (van der Kooij et al., 2005), *P. aeruginosa* (Bédard et al., 2016), and *M. avium* (Falkinham, Pruden, & Edwards, 2015). To better understand the impact of oligotrophic conditions on the ability of OPPPs to colonize and regrow in WMS, analysis of water samples from these systems for total organic carbon concentration levels is needed.

Other Control Methods for Opportunistic Premise Plumbing Pathogens

The resistance to disinfection presents significant challenges in using traditional approaches to control OPPPs (Falkinham, Pruden, & Edwards, 2015); therefore, alternative control approaches for OPPPs in premise plumbing are needed. Control of OPPPs in water can be achieved by reducing turbidity (Falkinham, 2015). Additionally, regular cleaning and disinfecting of nozzles can be an effective way of controlling OPPPs (American National Standards Institute, 2018; Health and Safety Executive, 2014). The installation of microbiological filters to WMS can control OPPPs (National Research Council, 2006). Like most bacteria, *L. pneumophila* and *P. aeruginosa* are susceptible to UV irradiation and thus can be controlled in premise plumbing by this method (Falkinham, 2015; Leoni et al., 2015), but it has been noted that high turbidity inhibits disinfection. The introduction of nonpathogenic protozoa species that target OPPPs can achieve a probiotic control of OPPPs in WMS (Wang et al., 2013). The addition of silver ions at a concentration of 40 µg/L has a bactericidal effect on *L. pneumophila* and many other microorganisms in plumbing systems without affecting humans, making silver ions suitable for controlling OPPPs in WMS (Fewtrell, 2014).

Conclusion

The use WMS as a cooling intervention in public places should be considered a potential public health risk due to the potential for poorly managed systems to release inhalable aerosols contaminated with microbes. These aerosols could contain pathogenic organisms, referred to as OPPPs, such as *L. pneumophila*, *M. avium*, *P. aeruginosa*, *Acanthamoeba*, and *N. fowleri*. In addition to inhalation, the aerosolization of contaminated water in WMS can result in microbial deposition on skin, food, and other surfaces, resulting in a localized reaction (e.g., to skin or eyes) or ingestion. An investigation of the health risks associated with the use of WMS as a cooling intervention is warranted to better understand the public health impact and inform strategies to manage WMS.

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Corresponding Author: Edmore Masaka, School of Medical and Health Sciences, Edith Cowan University, 270 Joondalup Drive, Joondalup, Western Australia, 6027, Australia. Email: emasaka@our.ecu.edu.au.

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APPENDIX 2

Awareness of business owners regarding the health risks associated with the use of water mist systems in Australia

Abstract

Water mist systems (WMS) are commonly used to cool public places. They can be colonized by opportunistic premise plumbing pathogens (OPPPs) that cause infections in people with predisposing conditions. Adequate knowledge of health risks associated with WMS is important to avoid exposing people to OPPPs. A questionnaire survey of 10 WMS owners and 22 Environmental Health Officers was conducted in 2019 to assess their knowledge about the health risks of WMS. Most of the owners (60%, n = 10) and 77% of the EHOs, (n = 22) were not aware of the health risks. Only 50% (n = 5) of the WMS owners regularly maintain their systems, and 60% use maintenance and cleaning schedules. Most of the WMS owners (90%, n =10) had no training in the operation of the systems. The installation and operation of WMS as reported by all the EHOs surveyed is unregulated, (100%, n = 22).

Key words

Water mist system, health risk, opportunistic premise plumbing pathogens, *Legionella pneumophila, Pseudomonas aeruginosa, Mycobacterium avium, Naegleria. Fowleri, Acanthamoeba*

Introduction

Water mist systems (WMS) are a cooling intervention used in public places. They achieve environmental cooling by releasing tiny water mists that absorb the latent heat of the ambient air. These systems form part of premise plumbing, which is that part of a water distribution network installed downstream of the water meter and falling under the responsibility of property owners (Falkinham et al., 2015).

The water aerosols produced by WMS and similar misting systems may be < 2.5 µm, making them respirable and able to reach the alveoli regions of the lungs where they can cause infections in people with a weak immunity (Allegra et al., 2016). These systems can reduce the dry bulb air temperature by 8 – 12 $^{\circ}$C (Farnham et al., 2015).

The potential of WMS used as a cooling intervention to be colonized by opportunistic premise plumbing pathogens (OPPP) has been demonstrated in previous research undertaken by these authors (Masaka et al., 2021). Opportunistic premise plumbing pathogens are a group of microorganisms that have become adapted to surviving in premise plumbing networks and have been associated with some waterborne infections. Some common OPPPs include *Legionella pneumophila, Pseudomonas aeruginosa, Mycobacterium avium, Acanthamoeba* and *Naegleria. Fowleri* (Ashbolt, 2015; Aumeran et al., 2007).

The WMS used as a cooling intervention in public places are outside the scope of the *Health (Air handling and Water Systems) Amendment Regulations, 2013* (WA), a Western Australian statute regulating similar systems. Most of these systems are not connected to central water treatment facilities (scheme water), therefore owners tend to use poor quality water sourced from underground aquifers. These WMS are installed outdoors and above ground, resulting in water temperatures in the pipework being > 20° C (Agudelo-Vera et al., 2020), and ideal for the growth of OPPPs including.

The importance of knowledge, skills, and competence of operators has not been assessed. Falkinham III et al. (2015) and Liu et al. (2019) have acknowledged the importance of knowledge and competence in managing the risk of OPPPs in premise plumbing. Guidelines dealing with the prevention of *Legionella* growth in similar features acknowledge the importance of knowledge and competence to manage OPPP risks (enHealth, 2015; Health and Safety Executive, 2014). A higher knowledge of OPPP risks in WMS can increase the competence of operators to manage them. (Julien et al., 2020).

In this study, we investigated the knowledge and perceptions of both Environmental Health Officers (EHOs) and WMS owners working and operating in the northwestern part of Australia regarding the risk factors associated with OPPP growth. There is an increasing use of WMS in this region, a fact that can be attributed to their effectiveness in cooling ambient temperatures at a fraction of the costs associated with conventional air conditioning systems. Additionally, the climate in this region is characterized by extremes of temperatures during the summer season that falls between the months of August and March (Bureau of Meteorology, 2016), and is projected to become hotter due to the effects of climate change (Sudmeyer, 2016). Understanding the level of knowledge about the health risks of these WMS amongst the owners and also the Environmental Health Officers whose responsibility is to ensure public health safety from these environmental hazards is important.

Ethics

This study received prior approval from the Edith Cowan University (ECU) Human Research Ethics committee (HREC), Approval Number 16337 MASAKA. The written informed consent of all research participants was secured before they participated in the study.

Materials and methods

A cross sectional descriptive survey of WMS owners and Environmental Health Officers (EHOs) was carried out in the northwestern region of Australia from 2018 -2019.

Study population

The study population consisted of 27 Environmental Health Officers (EHOs) and 10 water mist system (WMS) owners working and operating in the north-western part of Western Australia. The population size of EHOs was drawn from a register of the North Western Environmental Health Group (NWEHG). Only those employed in local governments and working in non-governmental organisations (NGOs) were included in this study. The sample of WMS was drawn from a total of 15 who operate in the study area and were willing to participate. The Qualtrics sample size calculator (Smith, 2010) was used to determine the sample sizes for the EHOs and WMS owners' surveys respectively. The population size, a 5 % margin of error, 95 % confidence level and a standard deviation of .5 were applied in calculating the sample sizes. A priori sample size of 10 WMS (100% of the eligible population size) and 26 EHOs was determined.

Survey questionnaires

Two questionnaires, one for WMS owners (Appendix 1) and the other one for EHOs (Appendix 2) were developed as tools to collect data.

Survey of Environmental Health Officers

The survey questionnaire for EHOs was developed based on the requirements of the Health and Safety Executive (HSE)'s Legionnaire's disease Technical Guidance HSG 274 Part 2, and Australia's enHealth Guidelines for *Legionella* Control in the operation and maintenance of water systems in health and aged care facilities (enHealth, 2015). This questionnaire was

structured and contained questions to gather information on the level of knowledge and perceptions on the associated health risks of WMS, regulatory and monitoring regime, design and the operational. aspects of WMS.

Survey of WMS managers

The questionnaire for WMS managers was developed based on the same criteria used for the EHO one, except that it excluded the section on regulatory and monitoring regime since this is not their responsibility.

Questionnaire validation

Both questionnaires were pilot tested with 4 WMS owners and 5 EHOs based in the northern territory, a different geographical location with similar climatic conditions, to assess their feasibility in terms of the time it takes to complete, the clarity of questions, and the consistency of coding to ensure accurate result interpretation (Jesús García de Yébenes Prous et al., 2009).

A Kappa index score of 0.25 for the EHO questionnaire and 0.26 for the WMS owners' questionnaire was calculated from the pilot test, demonstrating moderate reliability for both instruments (Jesús García de Yébenes Prous et al., 2009). Data from these pilot tests were not included in the final analysis.

Data analysis

Data were analyzed using the Minitab version 18 statistical software package. Before analysis, the categorical variables were coded 1 or 0 to facilitate data analysis (Alkharusi, 2012). The Fisher's exact test was used to measure association between variables because of

its inability to be affected by small sample sizes (McDonald, 2009). A confidence level of 95% (.05) was used to determine the significance of any association between variables. Results were presented as percentages, frequency tables, pie charts, bar graphs and funnel graphs.

Results and discussion

WMS owners' knowledge and perception of the type of health risks associated with the use of WMS

Most of the WMS owners (70%), n = 10, perceive that their systems are of public health significance (Table 1), and yet only 4 (40%), n =10, of them knew about the associated biological risks. The rest of the respondents did not respond to this follow-up question because they did not know that WMS could be associated with health risks. The ability to comprehend particular health risks associated with these WMS requires one to have a basic level of knowledge and competence to do so.

WMS owners' knowledge of conditions that promote OPPP growth in WMS and relevant maintenance aspects

Most of the WMS owners who responded to the question about conditions that can promote the regrowth of OPPPs in WMS were knowledgeable about these, with poor maintenance 22 %, n =10, increased water temperature 19 %, n = 10, and frequency of use 19 %, n = 10 being the most significant aspects selected (Table 2). Although most of the respondents knew that poor maintenance could lead to OPPP growth in their WMS and were reasonably aware of the important activities necessary to achieve this, only 50% (5) of them reported carrying out maintenance of their systems regularly according to the manufacturer's specifications.

WMS owners' training and level of operational competence

Ninety percent (90%), n =10 of the owners reported that they had not received any training in the operation of the WMS (Table 3), and only one reported having undergone in-house training. The reported inadequacy of training and regular maintenance of WMS is concerning considering its demonstrated importance in managing OPPP growth in similar premise plumbing systems (Julien et al., 2020). Furthermore, the self-reported lack of competence in the safe operation of WMS could be a function of inadequate training or lack thereof (Figure 1). The absence of effective maintenance regimes for plumbing features capable of aerosolizing water was implicated in the United Kingdom's largest Legionellosis outbreak that occurred in Cumbria in 2002 (Bennett et al., 2014) and a similar outbreak at a Melbourne aquarium in 2000 (Greig et al., 2004).

EHOs' knowledge and awareness of WMS health risks

Almost 77%, n = 22, of the EHOs self-reported that they were unaware of the health risks associated with the use of WMS as a cooling intervention in public places (Figure 2). *Legionella, Amoeba,* and *Pseudomonas* species were the OPPPs the EHOs knew could regrow in WMS (Figure 3).

There was no observed difference in the level of knowledge about the health risks of WMS between the WMS owners and EHOs *($p = 0.36$, Fisher's exact test).* However, this low level of knowledge amongst the WMS owners and EHOs about the type of health risks and OPPPs that can colonize and regrow in WMS is concerning, considering the potential of widespread exposure of people who patronize these public places. The knowledge of health risks is a key driver of behavior change to take corrective measures to mitigate a health risk (Fan et al., 2018). To emphasize the importance of knowledge in managing the risk of *Legionella* in water systems, competence has been incorporated as a requirement in guidelines for the

effective management of *Legionella* in water systems (enHealth, 2015; Health and Safety Executive, 2013).

The type, use, operational and regulation of water mist systems

WMS owners' reported type of WMS

According to WMS owners' respondents, about 50% (n = 10) of the systems are used as a cooling intervention in public places. Public places are captured under the *Health (Miscellaneous Provisions) Act, 1911* (WA) as places where people gather for various purposes including entertainment and recreation. WMS installed and operated in public places constitute a risk to people who interact with contaminants released into the ambient air (enHealth, 2015), therefore an investigation to understand the potential regrowth of OPPPs in WMS is necessary to assist in developing conceptual site models (CSM) and controls (National Environment Protection Council, 2010).

WMS owners and Environmental Health Officers' reported use of WMS

Temperature reduction was reported by 70%, (n = 10) of WMS owners as the most common reason for using WMS, and by 76% (n = 22) of the EHOs, a difference that was not statistically significant (p = 0.37, Fisher's exact test). This is not surprising considering the extreme temperatures experienced in the northwestern part of Australia (Bureau of Meteorology, 2016). The uptake of these systems is expected to increase due to the projected increase in mean maximum temperatures caused by climate change (Loechel et al., 2011; Sudmeyer, 2016).

181

182 *EHOs' reported frequency of water mist system use*

183 The infrequent use of any water mist system results in water stagnation (Feazel et al., 2009).
184 Eighty-six percent, n = 22 of the EHOs self-reported that WMS in their jurisdictions were
185 operated seasonally, and only 2 and 1 respondents reported frequent use (> 4hrs per day) and
186 infrequent use respectively (Table 4). The infrequent use of WMS to cool ambient air in
187 public places is consistent with the seasonal variation in this study area where mean summer
188 temperatures often exceed 32 °C and the mean winter temperatures do not necessitate the use
189 of the same intervention (Bureau of Meteorology, 2016). The difference in the reported
190 frequency of use by the EHOs was significantly different, *(p = 0.01, Fisher's exact test).* A
191 summer increase in temperature has been associated with the proliferation of *L. pneumophila*
192 in premise plumbing (Brandsema et al., 2014). However, a study by these authors did not
193 establish a seasonal variation in the occurrence of OPPPs in WMS (Masaka et al., 2021).

194

195 *WMS owners and Environmental Health Officers' reported source of water used in WMS*

196 Most of the WMS owners (90%, n = 10) indicated that the water used in their WMS was
197 obtained from centrally managed water treatment plants. However, 59 % of the EHOs, n = 22
198 indicated that both scheme and treated borehole water obtained from underground aquifers
199 were used in the WMS. This difference in responses was significant *(p = 0.03, Fisher's exact*
200 *test)*. This difference could be attributed to a knowledge gap between the two groups. Water
201 from underground aquifers can influence water chemistry by leaching mineral elements
202 (Adabanija et al., 2020) that can promote biofilm formation and the regrowth of OPPPs (Ji et
203 al., 2015).

EHOs' reported type of nozzles fitted to WMS

The ability to release bioaerosols is one of the critical risk factors for any water system (Health and Safety Executive, 2013). The formation and release of tiny water mists are achieved by small nozzles that atomize the water under hydraulic or pneumatic pressure (Farnham et al., 2015). Almost 90% (n = 10) of the WMS owners and 91% (n = 22) of the EHOs self-reported that the WMS installed and used in their areas used hydraulic nozzles, a difference that was not statistically significant (*p = 1.0, Fisher's exact test*). This finding is important considering that the inhalation of bioaerosols contaminated with OPPPs has been associated with illnesses (Russo et al., 2018), and was the implicated mode of transmission in several outbreaks of Legionellosis where contaminated water mists were (Haupt et al., 2012; Quinn et al., 2015).

WMS owners' reported observation of biofilms in WMS

Biofilm formation in premise plumbing systems can influence the regrowth of OPPPs in water systems (De Sotto et al., 2020; Tang & Bae, 2020). Thirty percent (30%), n = 10 of WMS respondents observed the growth of biofilms in their systems (Table 5). However, there was no association between the respondents' knowledge of biofilm formation, 50%, n = 10, and their ability to identify this phenomenon in WMS, 30%, n = 10, *(p = 0.65, Fisher's exact test).* The ability to identify biofilms in WMS or similar features requires knowledge and skills of this phenomenon, however the low level of knowledge and competence already observed among the WMS owners (Table 2) could have impacted on this scenario.

227 *WMS owners' reported use of cleaning and maintenance schedules*

228 The systematic use of cleaning and maintenance schedules is important in preventing OPPP
229 growth in building water systems and cooling towers (American Society for Testing
230 Materials, 2008; New South Wales Government, 2004; Rangel et al., 2011). Table 5
231 indicates that 60% (n = 10) of the WMS owners do not use any cleaning and maintenance
232 schedules, a result which could be related to the earlier finding shown in Table 2 that only
233 50%, n = 10 carry out regular maintenance on these systems. However, an insignificant
234 association between the respondents' failure to carry out regular maintenance and the lack of
235 cleaning and maintenance schedules, *(p = 1.0, Fisher's exact test)* was determined. Lack of
236 maintenance of premise plumbing features has been implicated in some Legionellosis
237 outbreaks before (Bennett et al., 2014; Greig et al., 2004).

238

239 *EHOs' reported regulation and inspection of water mist systems*

240 Several governments have developed legislation, standards, and guidelines to effectively
241 manage the public health risk posed by premise plumbing that gets colonized by OPPPs and
242 can release contaminated bioaerosols into the environment. One hundred percent (100%), n =
243 22 of the EHOs self-reported that a licensing and approval system for WMS was not in place,
244 and that they did not inspect the installed WMS as part of their public health regulatory
245 activities (Table 6).

246 The absence of WMS regulation is surprising considering that 23%, n = 22 of the respondents
247 indicated that they received public complaints about the same. The *HSE's Code of Practice*
248 *on the control of Legionella in water systems* is legally enforceable under the United
249 Kingdom's health and safety legislation (Health and Safety Executive, 2013), just as the

Australian Standard *AS 3666 on cooling water systems* (Standards Australia, 2011) is enforceable under Western Australia's *Health (Miscellaneous Provisions) Act 1911* (WA). The absence of a regulatory regime for WMS is due to the inadequacy of this existing legislation, and standards. Most of the existing legislation focuses on the prevention of Legionellosis in aged care facilities and hospitals, ignoring other settings where WMS can be colonized by OPPPs. The lack of focus on other emerging OPPPs including M. avium, Acanthamoeba, N. fowleri is evident in the current guidelines.

Conclusions

This research indicated that the knowledge of health risks associated with the use of WMS is low among both owners and EHOs. The absence of maintenance and cleaning schedules for WMS operated seasonally, presents a significant risk for the colonization of these systems by OPPPs. There is an absence of a regulatory regime to ensure the safe installation and operation of WMS. The lack of formal training programs for WMS owners in the health risks associated with the use of WMS and also the safe operation of the same needs to be addressed to improve their competence and ability to manage these risks. These findings will inform the review of existing legislation to include WMS, the development of guidelines, and the development of training programs for both owners and EHOs. Furthermore, they will have implications for designers of WMS, not only in the recreational sectors, but also in industrial set ups where dust suppression in mining employs this technology.

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Opportunistic Premise Plumbing Pathogens. A Potential Health Risk in Water Mist Systems Used as a Cooling Intervention

Edmore Masaka *, Sue Reed, Maggie Davidson and Jacques Oosthuizen

Public Health and Occupational Health and Safety, School of Medical and Health Sciences, Edith Cowan University, Joondalup, WA 6027, Australia; s.reed@ecu.edu.au (S.R.); Ma.Davidson@westernsydney.edu.au (M.D.); j.oosthuizen@ecu.edu.au (J.O.)
* Correspondence: emasaka@our.ecu.edu.au; Tel.: +61-863-045-517

Abstract: Water mist systems (WMS) are used for evaporative cooling in public areas. The health risks associated with their colonization by opportunistic premise plumbing pathogens (OPPPs) is not well understood. To advance the understanding of the potential health risk of OPPPs in WMS, biofilm, water and bioaerosol samples (n = 90) from ten (10) WMS in Australia were collected and analyzed by culture and polymerase chain reaction (*PCR*) methods to detect the occurrence of five representative OPPPs: *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium avium*, *Naegleria fowleri* and *Acanthamoeba*. *P. aeruginosa* (44%, n = 90) occurred more frequently in samples, followed by *L. pneumophila* serogroup (Sg) 2–14 (18%, n = 90) and *L. pneumophila* Sg 1 (6%, n = 90). A negative correlation between OPPP occurrence and residual free chlorine was observed except with *Acanthamoeba*, *rs* (30) = 0.067, $p > 0.05$. All detected OPPPs were positively correlated with total dissolved solids (TDS) except with *Acanthamoeba*. Biofilms contained higher concentrations of *L. pneumophila* Sg 2–14 (1000–3000 CFU/mL) than water samples (0–100 CFU/mL). This study suggests that WMS can be colonized by OPPPs and are a potential health risk if OPPP contaminated aerosols get released into ambient atmospheres.

Keywords: water mist systems; opportunistic premise plumbing pathogens; legionella pneumophila; mycobacterium avium; pseudomonas aeruginosa; acanthamoeba; naegleria fowleri

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1. Introduction

Water mist systems (WMS) are premise plumbing installations used for cooling and are typically installed in outdoor areas to produce and release water aerosols that flash evaporate in the surrounding air, resulting in a sudden reduction of ambient temperatures. Premise plumbing refers to all the water distribution and storage infrastructure within buildings and downstream from the water meter. Water mist systems present a potential public health risk because of their shared characteristics with other aerosol generating premise plumbing systems such as cooling towers, spa pools and showers that have been associated with outbreaks of infectious respiratory diseases caused by OPPPs such as Legionnaires' disease and bacterial pneumonia [1,2]. These systems produce microscopic inhalable aerosols (0.3–10 µm) [3], which if produced from contaminated water sources, can cause debilitating and fatal respiratory infections. Microorganisms that colonize and regrow in these premise plumbing systems are often referred to in the literature as opportunistic premise plumbing pathogens (OPPPs) and are part of the normal microbiome of premise plumbing [4], which includes showers [5], garden hoses [6], water taps and faucets [7], hot water systems [8], spa pools [9] and air conditioning units [10].

Several characteristics common to premise plumbing that can enhance the risk of microbial colonization and proliferation are oligotrophic conditions, water stagnation and long periods of water retention within plumbing systems [11]. Plumbing materials and components, disinfection methods, system corrosion, water quality/source and elevated temperatures are known to influence the survival of these pathogens in premise

plumbing [11,12]. Other features that enhance the survival of OPPPs include their ability to form and colonize biofilms, survival inside free-living amoeba (FLA), and resistance to disinfectants [13]. *Acanthamoeba* has a significant ability to engulf other OPPPs, and through this process shields them from disinfectants such as chlorine, and at the same time confer increased virulence to these OPPPs, that are then able to multiply in premise plumbing [13]. Opportunistic pathogens commonly isolated from premise plumbing include *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa*, *Acanthamoeba* and *Naegleria fowleri* [14]. These opportunistic pathogens represent an increased public health risk of *L. pneumophila* infection in persons with compromised immunity [15], as well as the elderly and smokers [16].

Exposure to contaminated waters is an important pathway for infection with OPPPs with inhalation, aspiration and nasal irrigation being the major routes of exposure [17]. Various pneumonic and respiratory tract illnesses have resulted from the inhalation of water mists <10 µm contaminated with bacterial pathogens such as *L. pneumophila* [18,19], *M. avium* [20,21], *P. aeruginosa* [22,23] and the aspiration of water contaminated with *N. fowleri* has resulted in a rare but fatal disease called primary amoebic meningoencephalitis (PAM) [24,25], and infection by *Acanthamoeba* has been associated with diseases of the eyes called acanthamoeba keratitis and granulomatous amoebic encephalitis (GAE) [26].

Although a body of knowledge exists on the presence of OPPPs in premise plumbing features such as showers, water taps, hot water systems, etc., no such study has investigated the potential of WMS used for ambient cooling to be colonized by OPPPs. Currently, there is no literature explaining the environmental characteristics that promote the growth and persistence of OPPPs in these systems. In this study, we investigated the potential occurrence of five selected OPPPs in WMS, namely, *L. pneumophila*, *P. aeruginosa*, *M. avium*, *Acanthamoeba* and *N. fowleri*, to determine the health risks associated with the use of such systems, and to determine whether there is any correlation between the occurrence of the OPPPs in the WMS with residual disinfection, water temperature, water pH, TDS and total organic carbon (TOC).

2. Results

2.1. Occurrence of Opportunistic Premise Plumbing Pathogens in Water Mist Systems

To determine the occurrence of OPPPs in WMS, we collected 30 bioaerosol samples, 30 biofilm samples and 30 water samples from 10 WMS located in north western Australia. The samples were collected over three sampling events (February, May, and August) during 2019, representing the three climatic seasons of this region. These three seasons are summer, autumn and winter. During summer and the beginning of autumn, daily average temperatures go above 30 °C, often exceeding 35 °C for 6 months of the year, from October to March [27,28]. During the winter months, May–August, average temperatures are often above 20 °C. The annual rainfall rarely exceeds 350 mm [27,28]. These conditions are characterized by a higher rate of evaporation and are ideal for the growth of OPPPs. Both culture and molecular (PCR) methods were used to detect the presence of five representative OPPPs in the samples, namely *L. pneumophila*, *P. aeruginosa*, *M. avium*, *Acanthamoeba* and *N. fowleri*. The water profile parameters of free chlorine residual, temperature, pH, TDS and TOC were also measured and analyzed to determine their relationship with OPPP occurrence in the WMS. Figure 1 shows the frequency of OPPP occurrence in all WMS samples (bioaerosol, water and biofilm). A total of 64 (71%) of WMS samples analyzed tested positive for OPPPs, with *P. aeruginosa* being found in 40 (44%) of the total samples. *L. pneumophila* Sg 2–14 was detected in 16 (18%) of the total samples and *L. pneumophila* Sg 1 was isolated from 5 (6%) of the total samples. Only three of the total samples analyzed returned a positive reading for *Acanthamoeba*. None of the 90 samples analyzed tested positive for both *M. avium* and *N. fowleri.*

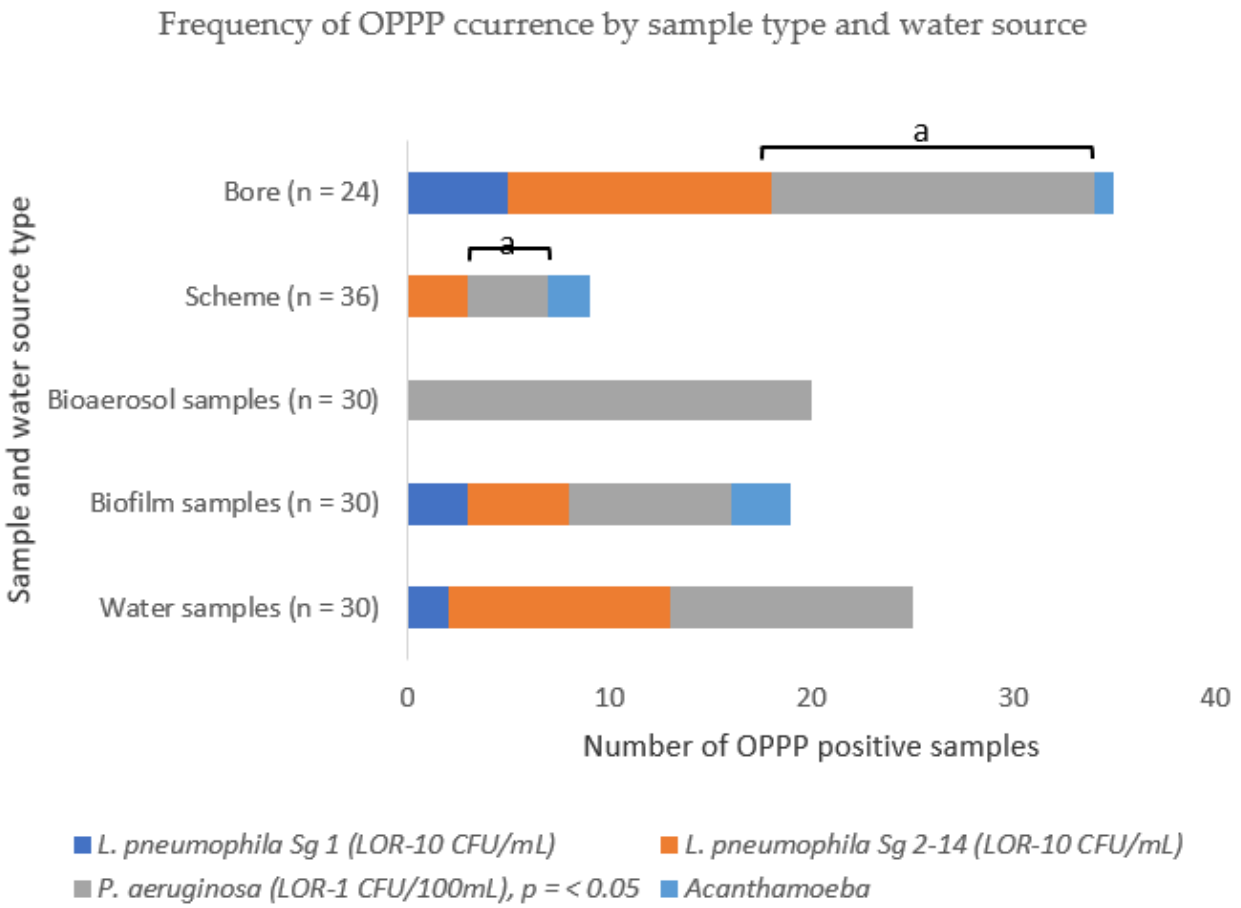

Figure 1. The frequency of OPPP positively identified by sample type and water source. All samples, except bioaerosol samples, were initially identified via culture methods, which were then confirmed via molecular methods similar to the analysis of the bioaerosol samples: PCR/qPCR sensitivities were: *L. pneumophila* −1.6 Genomic Units/mL, *P. aeruginosa* 5–10 GU/10 mL, *Acanthamoeba* 5–8 gene copies/µL. "a" = significant relationship.

2.2. The Concentration of Detected OPPPs

The results of this study, as presented in Table 1, show that the concentration of all the OPPPs detected in WMS samples analyzed by microbiological culture methods was higher in biofilm samples than in water samples, with *L. pneumophila* Sg 1 detection in biofilms being 30× higher than in water. The biofilm concentration of *L. pneumophila* Sg 2–14 was three times higher than that of water and *P. aeruginosa* in biofilm samples was eight times higher than in water. The PCR results indicated the presence of *P. aeruginosa* in the bioaerosols only.

Table 1. Opportunistic premise plumbing pathogen concentration by sample type.

Opportunistic Pathogen Detected	OPPP Concentration Level	OPPP Concentration Range by Sample Type		
		BIOFILM (CFU/mL)	Water (CFU/mL)	Bioaerosol qPCR *
L. pneumophila (Sg 1)	Lowest	1000	100	Not detected
	Highest	3000	100	Not detected
L. pneumophila (Sg 2–14)	Lowest	100	10	Not detected
	Highest	1000	300	Not detected
P. aeruginosa	Lowest	10	3	Detected
	Highest	2000	350	Detected

* PCR and/or qPCR analysis conducted for the detection of OPPPs in bioaerosol samples, results expressed as either detected/not detected.

2.3. The Frequency and Distribution of OPPPs Differed by Sample Type and Water Source

The frequency and distribution of OPPPs differed by the WMS sample type and water source as shown in Figure 1. Bioaerosol samples had a higher occurrence of *P. aeruginosa* (67%) than water samples (40%), and biofilm samples (70%). This occurrence of *P. aeruginosa* significantly differed by sample type χ^2 (2, N = 90) = 10.08, $p < 0.05$. Conversely, *L. pneumophila* Sg 2–14 occurred more frequently in water samples (37%), than in biofilm samples (17%), however, this difference was not statistically significant χ^2 (2, N = 90) = 3.07, $p < 0.05$. There was no association between the occurrence of *L. pneumophila* species and *P. aeruginosa* in biofilms and water samples χ^2 (1, N = 41) = 0.02, $p > 0.05$. V = 0.000. No *L. pneumophila* Sg 2–14 was detected in the bioaerosol samples. Only three biofilm and two water samples tested positive for *L. pneumophila* Sg 1. *Acanthamoeba* was detected in three biofilm samples. *M. avium* and *N. fowleri* were not detected in any of the samples analyzed.

2.4. Opportunistic Premise Plumbing Pathogen Occurrence by Water Source

The percentage occurrence of *L. pneumophila* Sg 2–14 in bore water samples as shown in Figure 1 was four times higher than in scheme water; however, the results of a Kruskal-Wallis mean ranks test of the individual occurrences showed that they did not differ significantly, *H (1)* = 1.84, $p > 0.05$. *L. pneumophila* Sg 1 was only detected in five bore water samples.

The results of a Kruskal-Wallis mean ranks test showed a significantly higher percentage occurrence of *P. aeruginosa* in bore water than in scheme water, *H (1)* = 13.87, $p < 0.05$. *Acanthamoeba* was detected in only 2 out of the 36 water samples obtained from systems fed with scheme water and in only one of the water samples obtained from systems fed with bore water.

2.5. Seasonal Occurrence of Opportunistic Premise Plumbing Pathogens

In this study, seasonal differences in the occurrence of OPPPs in all samples (N = 90) was investigated, however, no statistical difference was observed in the occurrence of *L. pneumophila* Sg 1, *L. pneumophila* Sg 2–14 and *P. aeruginosa* in WMS across the three seasonal sampling periods (February, May, and August) as indicated by the following results of a Kruskal- Wallis mean rank test for the three OPPPs: *L. pneumophila* Sg 1, *H (2)* = 0.77, $p = 0.68$; *L. pneumophila* Sg 2–14, *H (2)* = 0.89, $p = 0.64$ and *P. aeruginosa*, *H (2)* = 0.08, $p = 0.96$.

2.6. Water Temperature

Temperature for all water samples ranged between 21.7 °C to 38.9 °C with the highest being recorded in February and the minimum in May. The results of a Kruskal-Wallis test showed that the mean ranks of water temperature in February were significantly higher than in May and August/September *H (2)* = 23, $p < 0.05$. Based on the results of this study, the occurrence of *P. aeruginosa* in WMS tends to increase with an increase in the water temperature *rs* = 0.31, $p < 0.05$. No correlation was observed between water temperatures and the occurrence of all other OPPPs detected in the WMS namely, *L. pneumophila* Sg 1 *rs* = 0.08, $p > 0.05$, *L. pneumophila* Sg 2–14 *rs* = 0.09, $p > 0.05$ and *Acanthamoeba rs* = 0.04, $p > 0.05$.

2.7. Water pH

The pH for all the water samples showed a small range variation (7–7.9). There was no significant difference in the mean ranks of water pH across the three sampling sessions *H (2)* = 0.87, $p > 0.05$.

2.8. Total Dissolved Solids (TDS)

The highest TDS concentration was 399 mg/L and was recorded from a bore water sample during the May sampling event. The lowest concentration of 240 mg/L was measured from a scheme water sample during the first sampling event in February. The mean rank concentration of TDS in bore water samples was 6% (18.6 mg/L) higher than

in scheme water (340.3 mg/L). This difference was statistically significant $H\ (1) = 16.78$, $p < 0.05$. No significant difference was noted for the mean ranks of TDS concentration across the three sampling events $H\ (2) = 5.33$, $p = 0.07$.

2.9. Free Chlorine Residual

The concentration of free chlorine residual measured across the three sampling events ranged from 0.0 to 0.76 mg/L, a variance that reflects the complexity of these plumbing systems. The maximum concentration of free chlorine was measured in scheme water during August, with the minimum concentration in this water supply being 0.01 mg/L. Two-thirds of all bore water samples tested across the three sampling events had no free chlorine residual. All scheme water samples tested positive for free chlorine residual. This difference in free chlorine residual between bore and scheme water samples was significant, $H\ (1) = 19.95$, $p < 0.05$. No significant difference in residual chlorine concentration was observed in the water samples across the three sampling events $H\ (2) = 0.26$, $p = 0.88$.

2.10. Total Organic Carbon (TOC)

Seventy percent (21 out of 30) of the water samples had TOC concentrations less than the detection limit of <1 mg/L and 17 of these were collected from the scheme water supply. The highest measured TOC concentration was 3 mg/L. The mean ranks of TOC concentration in the water samples collected across the seasons were not significantly different $H\ (2) = 3.5$, $p = 0.17$. However, the TOC concentration in the bore water samples was significantly higher than in the scheme water samples, $H\ (1) = 7.11$, $p = 0.01$.

2.11. The Relationship between Water Profile Parameters

To determine the strength and direction of the association between the water profile parameters discussed above, the nonparametric Spearman's rho (*rs*) test was used rather than the parametric Pearson test because of the absence of distribution normality in the data sets and the presence of outliers. Table 2 presents the Spearman rho correlation results among the water profile parameters. A significant negative monotonic correlation was determined between free chlorine residual and TDS, $rs\ (30) = -0.566$, $p < 0.05$ and TOC, $rs\ (30) = -0.523$, $p < 0.05$. Total organic carbon concentration had a significant and positive monotonic correlation with TDS, $rs\ (30) = 0.549$, $p < 0.05$. However, there was no significant correlation observed between water temperature and all other water profile parameters, and the same applied to water pH.

Table 2. The relationship between water profile parameters.

Spearman Rho (ρ) Correlation between Water Profile Parameters						
Water Profile Parameter	**Statistical Test and Sample Size**	**Free Chlorine Residual**	**Water Temperature**	**Water pH**	**Total Dissolved Solids**	**Total Organic Carbon**
Free chlorine residual	Spearman rho ρ	1	−0.185	−0.065	−0.566	−0.523
	Significance (2 tailed)	.	0.328	0.735	0.001	0.003
	N	30	30	30	30	30
Water temperature	Spearman ρ Correlation	−0.185	1	0.111	−0.089	−0.198
	Significance (2 tailed)	0.328	.	0.558	0.639	0.293
	N	30	30	30	30	30
Water pH	Spearman ρ Correlation	−0.065	0.111	1	0.068	0.279
	Significance (2 tailed)	0.735	0.558	.	0.720	0.136
	N	30	30	30	30	30
Total dissolved solids	Spearman ρ Correlation	−0.566	−0.089	0.068	1	0.549
	Significance (2 tailed)	0.001	0.639	0.720	.	0.002
	N	30	30	30	30	30
Total organic carbon	Spearman ρ Correlation	−0.523	−0.198	0.279	0.549	1
	Significance (2 tailed)	0.003	0.293	0.136	0.002	.
	N	30	30	30	30	30

2.12. Relationship between Water Profile Parameters and the Occurrence of OPPPs in Water Mist Systems

The possible correlation between the water profile parameters and the occurrence of OPPPs in the WMS was determined using the Spearman *rho* correlation test which has been used in similar studies [27]. The results of this analysis are shown in Table 3. Residual chlorine had a significantly weak and negative monotonic correlation with the occurrence of all OPPPs except with *Acanthamoeba*, rs (30) = 0.067, $p > 0.05$.

Table 3. The relationship between water profile parameters and the occurrence of OPPPs in WMS.

Spearman Rho Correlation Analysis between OPPPs and Residual Chlorine, Water Temperature, pH, Total Dissolved Solids, and Total Organic Carbon					
Opportunistic Pathogen Detected	**Residual Chlorine (mg/L)**	**Water Temperature (°C)**	**Water pH (pH Units)**	**Total Dissolved Solids (mg/L)**	**Total Organic Carbon (mg/L)**
L. pneumophila (1)	−0.327 (p = 0.011)	0.080 (p = 0.543)	0.074 (p = 0.038)	0.268 (p = 0.038)	0.392 (p = 0.002)
L. pneumophila (2–14)	−0.401 (p = 0.002)	0.098 (p = 0.456)	0.002 (p = 0.987)	0.418 (p = 0.001)	0.393 (p = 0.002)
P. aeruginosa	−0.423 (p = 0.001)	0.313 (p = 0.015)	0.123 (p = 0.348)	0.480 (p = 0.000)	0.242 (p = 0.062)
Acanthamoeba	0.067 (p = 0.611)	0.035 (p = 0.789)	−0.062 (p = 0.637)	−0.057 (p = 0.663)	0.022 (p = 0.868)

The occurrence of all OPPPs did not correlate with water temperature except for *P. aeruginosa*, rs (30) = 0.31, $p < 0.05$. A weak and positive relationship was also observed between TDS concentration and *L. pneumophila* Sg 1, rs (30) = 0.27, $p < 0.05$, *L. pneumophila* Sg 2–14, rs (30) = 0.42, $p < 0.05$ and *P. aeruginosa*, rs (30) = 0.48, $p < 0.05$. The occurrence of both *L. pneumophila* Sg 1 and Sg 2–14 demonstrated a weak positive relationship with TOC, rs (30) = 0.39, $p < 0.05$ and rs (30) = 0.39, $p < 0.05$, respectively.

3. Discussion

The occurrence of OPPPs in WMS used as a cooling intervention in public places has not been investigated, therefore, little is known about their ability to regrow in these systems and whether water profile parameters of temperature, free chlorine residual concentration, pH, TDS and TOC can influence this occurrence. In this study, culture and molecular analysis of 30 biofilm, 30 water and 30 bioaerosol samples collected from 10 WMS confirmed a percentage occurrence of 44% (n = 90) for *P. aeruginosa,* 18% (n = 90) for *L. pneumophila* Sg 2–14, 6% (n = 90) for *L. pneumophila* Sg 1, 3% (n = 90) for *Acanthamoeba* and zero for *M. avium* and *N. fowleri.* As far as we know, this is the first study to investigate the occurrence of these OPPPs in WMS used as a cooling intervention in public places.

In this study, higher concentrations of all OPPPs were detected in WMS biofilm samples than in water and bioaerosol samples, supporting the argument that biofilms play a significant role in OPPP regrowth and survival in water systems. These results are consistent with other studies [29–33]. In our study, *P. aeruginosa* was detected at higher concentrations in WMS biofilms when compared to all the other detected pathogens, a factor which can be attributed to the pathogen's known ability to colonize and thrive better in biofilms than in the water phase [34]. In interpreting these results, it is important to note that the actual concentration of the OPPPs detected by culture methods could be even higher due to the possible presence of viable but non culturable organisms (VBNC) that may fail to grow under culture conditions [35]. This phenomenon is particularly relevant for *P. aeruginosa*, an opportunistic pathogen that can be affected into the VBNC state by low temperatures during sample transportation [36].

Another reason for the higher numbers of the OPPPs in the WMS biofilms could be the latter's ability to shield the former from the effect of the chlorine disinfectant used in these

systems. Higher disinfection resistance has been demonstrated for the following OPPPs resident in biofilms; *M. avium*, *P. aeruginosa* [37], *L. pneumophila* [38] and *Acanthamoeba* [39].

In this study, a presence of *P. aeruginosa* (67%, n = 30) was detected in WMS bioaerosol samples, indicating that these systems may present a risk of pneumonic infections caused by the inhalation of *P. aeruginosa* [40], which has been established in a number of other studies [10,18,21]. This high detection of *P. aeruginosa* can be attributed to its ability to adapt and thrive better in various environments, such as the one induced by bioaerosol sampling processes. This finding is consistent with another study of *Pseudomonas* occurrence in premise plumbing [41]. Furthermore, research in laboratory models has demonstrated that *P. aeruginosa* is able to remain airborne for periods greater than 45 min [42], whereas *L. pneumophila* is reported to remain airborne for only 3 min [43] after dispersal. By expressing a mucoid phenotype in air, *Pseudomonas* can withstand desiccation common with bioaerosol sampling using filtration [42]. Therefore, *P. aeruginosa* can exist in higher concentrations in ambient atmospheres making it easier to capture during bioaerosol sampling compared to *L. pneumophila*. Further research to investigate this phenomenon in WMS is needed.

L. pneumophila Sg 2–14 and Sg 1 were detected in WMS, confirming that these systems could be a health risk for Legionellosis should water aerosols they release when in operation be contaminated by these pathogens, a finding consistent with other studies [18,44,45]. When analyzing for *L. pneumophila* Sg 2–14, only 18% of samples were positive which is greater than another study (11%) [46] and also higher than the levels of *L. pneumophila* Sg 1 (6%), which were lower than in several other studies [33,44].

In this study, a 3% (n = 90) occurrence of *Acanthamoeba* in WMS water and biofilm samples was detected, with this occurrence being positively correlated with free chlorine residual. The positive detection of *Acanthamoeba* in these WMS presents a health risk as described in several studies [26,29,46–48], not only because of its pathogenicity, but for its ability to shield other pathogens such as *L. pneumophila* and *M. avium* from destruction by disinfectants such as chlorine [49].

This study did not detect any *M. avium* nor *N. fowleri* in any samples, water (30), biofilms (30) or bioaerosols (30). Although not isolated in any samples, the potential presence of *M. avium* and *N. fowleri* in WMS cannot be completely ruled out, since studies of similar systems have demonstrated that this pathogen can regrow in premise plumbing [29,49,50]. The low sample volumes collected (250 mL) could have resulted in the extracted gene copies being less than the qPCR method's limit of detection. Sample volumes of 1 L have previously been used to successfully detect these pathogens from water samples [51,52], hence higher sample volumes may be needed for any future studies.

The occurrence of *L. pneumophila* species, *P. aeruginosa*, and thermophilic amoebic species including *Acanthamoeba* in premise plumbing systems tend to vary with seasons [53]. This study did not show a statistical difference across seasons, a result which could be attributed to a loss in statistical power due to the smaller sample size [54]. The mean water temperature measured in the WMS across the three sampling events (29.9 °C) was optimum for the growth of all the targeted OPPPs and could have influenced this result, a finding that is consistent with another study which investigated the critical factors responsible for OPPP growth in premise plumbing [55].

Our study established a correlation between the occurrence of targeted OPPPs in WMS and the use of bore water, with this relationship being significant for *P. aeruginosa*, $H(1) = 13.87$, $p < 0.05$. One of the factors that could give rise to elevated levels of *P. aeruginosa* and *L. pneumophila* Sg 2–14 in the bore water samples could be the increased levels of iron in the shallow aquifers this water is drawn from [56]. Typically, bore water sources in Northern Australia tend to have a higher level of dissolved minerals such as iron, and can also alter the pH of underground water, resulting in the corrosion of pipework and increased colonization of plumbing systems by iron eating bacteria, a finding that is consistent with several other studies [57,58]. In this study, there was no significant difference in the water pH measured across the three sampling events, a finding that could

be attributed to the similarity in the chemistry of the source water, which is a shallow aquifer system influenced by infiltration from surface waters [56].

Although the primary source of all the water used in the WMS is drawn from the same aquifer, our research observed a significant variation in the TDS concentration of bore water and scheme water, $H\ (1) = 16.78$, $p < 0.05$, a result which is not surprising considering that this parameter is usually higher in ground water sources [59].

The positive relationship between the formed biofilms and occurrence of *L. pneumophila* observed in this study is consistent with other studies [55,60], except for the weak correlation with *Acanthamoeba* which may be due to the possible parasitic colonization of free-living amoeba by *L. pneumophila* at water temperatures > 25 °C [61].

A significant amount of research on OPPP occurrence has demonstrated that elevated water temperatures typical in premise plumbing systems is a critical factor in their survival [11,12,53,62]. However, this study did not demonstrate any correlation between water temperature and the occurrence of all detected OPPPs except with *P. aeruginosa*, $rs\ (30) = 0.31$, $p < 0.05$, a finding different from several other studies [12,53,62]. The correlation with *P. aeruginosa* occurrence is consistent with existing literature [63], Furthermore, *P. aeruginosa* can adapt to various environmental conditions including surviving temperatures ranging from 10–42 °C and antagonism from other OPPPs [41]. Several reasons could be attributed to this phenomenon, particularly the higher-than-normal annual mean maximum temperatures in the study area that were 32.7 °C in February, 26 °C in May and 29.2 °C in August, time periods that aligned with the three sampling episodes conducted during our study, and with the higher winter temperatures typical of the tropics where this study area is located [28].

Most of the water mist systems are situated outdoors and are reticulated by uninsulated pipework which absorbs elevated levels of radiant heat, resulting in elevated water temperatures that promote the growth of OPPPs as described in a study of temperature variation on OPPPs in domestic plumbing [60]. In interpreting the results of this study, it is important to acknowledge that most of the water temperatures recorded ranged between 21.7 °C to 38.9 °C, a zone known to be optimal for the growth of the detected OPPPs. This meant that assessing the effects of temperature on the detected OPPPs at levels below their optimum growth zone was not possible, considering the tendency of these pathogens to adhere to a threshold related response at temperature extremes [55].

This study established a significant negative correlation between free residual chlorine concentration and the occurrence of most detected OPPPs. This highlights its effectiveness against most OPPPs, except *Acanthamoeba,* a finding consistent with several studies [12,38,64–66]. The monochloramine disinfectant used in the WMS is more effective over other forms of chlorine disinfectants because of its longer lasting residual effect, a finding that is consistent with other studies [29,39,67,68]. The positive correlation of residual chlorine and *Acanthamoeba* is consistent with the findings of another study [29]. This could be attributed to several reasons including the possible existence of the cystic form of *Acanthamoeba* detected during our study, which is known to confer resistance to the monochloramine disinfection as previously demonstrated in a previous study [69].

This study determined that the TOC concentration in the WMS water samples was exceptionally low, with 70% (n = 30) being lower than the detection limit of <1 mg/L, although it was positively correlated with the occurrence of *L. pneumophila* Sg 1, $rs\ (30) = 0.39$, $p < 0.05$ and *L. pneumophila* Sg 2–14, $rs\ (30) = 0.39$, $p < 0.05$. The low concentration of TOC in WMS is consistent with the findings of several studies of premise plumbing systems that promote the regrowth of these pathogens [55,70].

Several microbiological risk control strategies advocated in guidelines developed to control *Legionella* species in engineered water systems, including evaporative cooling systems, could be applied to WMS because of the similarities that exist between this pathogen and other OPPPs detected in this study. The Health and Safety Executive's Legionnaire's disease Technical Guidance HSG 274 Part 2 [71], American National Standard Institute's ANSI/ASHRE Standard 188–2008 [72] and Australia's enHealth Guidelines

for *Legionella* Control in the operation and maintenance of water systems in health and aged care facilities [73] mandate the implementation of the following control strategies for *Legionella*: risk assessment of water systems for effective design and construction; prevention of water stagnation, implementation of effective maintenance programs and adequate disinfection of water used. These steps avoid the growth of *Legionella* bacteria in these systems, strategies that could be applied to prevent OPPPs growing in WMS investigated in this study.

4. Materials and Methods

To determine the health risks associated with the use of WMS as a cooling intervention in public places, a total of 30 water samples, 30 biofilm samples and 30 bioaerosol samples were collected from 10 WMS located in the northwestern part of Australia over three sampling events (February, May, and August) in 2019. For this investigative pilot study, the sample size for each sample type per sampling event was calculated using a confidence level of 95%, population size of 10 WMS and a margin of error of 5%, giving a sample size of 10 per sample type per sampling event. The samples were analyzed at EcoDiagnostic, an Australian laboratory accredited by the National Association of Testing Authorities (NATA).

Ethics approval to conduct this study was obtained from the Edith Cowan University (ECU) Human Research Ethics committee (HREC), Approval Number 16337 MASAKA. Informed consent was obtained from all participants involved in the study.

4.1. Bioaerosol Sampling

Bioaerosol samples were collected using the NIOSH BC251–2 stage bioaerosol samplers to which was connected conductive polypropylene filter cassettes loaded with 37 mm polytetrafluoroethylene (PTFE) filters of 3 µm pore size. The sampling was undertaken in accordance with the method described by Coleman, Nguyen [74]. One and half meters of Teflon tubing was used to connect the bioaerosol samplers to SKC AirCheck XR 5000 air sampling pumps that were operated at 3.5 L/minute for a maximum of 30 min to collect positional samples. Before each sampling session, the airflow through the sampler was calibrated, and the flow rate checked after each sampling session, using the SKC Defender 510 Dry Cal standard primary calibrator. Air temperature and humidity was recorded during the sampling process using a Lascar EL-USB-2 humidity and temperature meter and wind speed was also recorded during the sampling process using a Meteos Anemo-Thermometer with a 54 Mm Propeller. The bioaerosol samples were stored and transported on ice at <4 °C to EcoDiagnostic laboratory for analysis using molecular methods for *M. avium*, *P. aeruginosa*, and *N. fowleri*, *Legionella* species (including *L. pneumophila* Sg 1 and Sg 2–14) and *Acanthamoeba*.

Bioaerosol Sample Processing

The inside of the NIOSH BC 25 L, 15 mL and 1.5 mL tubes were rinsed (walls of the tube) with a solution of ATL and proteinase K. The PTFE filters were removed from the cassettes using a filter handling kit and placed inside this solution and vortexed, with a 70% ethanol solution being used to sterilize the forceps after each filter transfer. This solution (with the filter paper) was incubated at 60 °C for 30 min to achieve lysis. Two separate aliquots of this solution (440 µL) were loaded onto the QIAsymphony instrument (QIAGEN) for DNA extraction. The QIAsymphony instrument takes 400 µL of sample and extracts it, eluting into 200 µL. The two extracts were combined and filtered using an AMICON Ultra DNA concentrator, was checked for inhibition at the neat dilution using a PPC qPCR assay and then analyzed neat to detect *M. avium* (qPCR), *Legionella* spp. (PCR), *P. aeruginosa* (qPCR), *Acanthamoeba* (PCR) and *N. fowleri* (qPCR). The qPCR results were expressed qualitatively as detected or not detected. In the absence of a standard method for detecting OPPPs in bioaerosols, validated inhouse PCR and qPCR methods were used as described under analytical methods.

4.2. Biofilm Samples

Biofilm samples were collected from the WMS using swabs stored in E-Swab vials containing 1 mL of liquid and sodium thiosulfate to inactivate any residual disinfectants. Swabbing was done following the requirements of the Centers for Disease Control and Prevention (CDC)'s "*Sampling procedure for biofilms in Legionella outbreak investigations*" [75]. The swabbing was done from the inside walls of WMS pipes and sprinkler nozzles. These swabs were put back into the E-Swab vials and transported on ice at 4 °C to EcoDiagnistic laboratory for analysis.

Biofilm Swab Sample Preparation

One hundred micro liters (100 µL) of the sample were plated to culture for *Legionella* spp. and *P. aeruginosa* and 100 µL being plated for confirmation. One millilitre (1 mL) each of this preparation was used to culture for *Acanthamoeba* and *N. fowleri* with confirmation being done by PCR. Some samples required dilutions (1:10, 1:100, etc.) to account for the high concentration of background flora. Deoxyribonucleic acid (DNA) was extracted from the swab solution (400 µL and eluted into 200 µL) to detect *M. avium*.

4.3. Water Samples

Water samples were collected from the WMS, stored, and transported to the analyzing laboratory following the requirements of "*AS 2013–2012, Water Quality—Sampling for microbiological analysis*" [76]. Sterile plastic bottles (500 mL) treated with sodium thiosulfate to deactivate any available disinfectants were used to collect water samples for microbiological testing for the presence of *L. pneumophila*, *P. aeruginosa*, *M. avium*, *Acanthamoeba* and *N. fowleri*. The bottles were stored and transported on ice at 4 °C to a NATA laboratory for analysis, except for the amoeba samples that were transported at ambient temperature [77]. A calibrated industrial HM Digital TDS and water temperature thermometer with a measuring range of 0–80 °C, and accuracy of ±2%, was used to measure water temperature and total dissolved solids. A Palintest Pooltest 9 Premier water testing unit was used to measure the free chlorine residual disinfectant level, pH and temperature profile of the water samples.

Water Sample Preparation and Analysis

All manipulations associated with sample preparation, culture media, materials and apparatus, enumeration techniques and their selection were conducted as described in "*AS/NZS.1: 2007-Water microbiology: Method 1. General information and procedures (ISO8199:2005, MOD)*" [78]. All samples were handled by trained laboratory staff. *N. fowleri* plates for confirmation were handled in a biosafety cabinet (BSC).

4.4. Analytical Methods

4.4.1. Detection and Measurement of *Legionella pneumophila* Species

The detection of *L. pneumophila* in water samples was undertaken according to the requirements of "*AS 3876:2017-Waters-Examination for Legionella spp., including Legionella pneumophila*" [79]. A volume of 0.1 mL of the untreated sample was aseptically inoculated onto 90 mm diameter plates of BCYE and MWY agar and incubated in humid conditions at 32 °C ± 2 °C for 7–10 days. The plates were examined visually on the fourth and last day for *Legionella* colonies that showed iridescence and a change in morphology to granular and similar edges. The presumptive *Legionella* colonies were picked and subcultured onto BCYE and BCYE-Cy agar plates, and incubated in humid conditions at 32 °C ± 2 °C for 3 days. The colonies that grew on the BCYE but failed to do so on the BCYE-Cs were interpreted to be *Legionella* spp.

The confirmation of *L. pneumophila* was performed using a validated inhouse multiplex PCR method (EDP-312). The growing colonies from the BCYE agar plates were lysed in 100 µL of HP water at 95 °C for 5 min to achieve lysis. The purification of the DNA from the prepared isolates was done using the QIAsymphony DNA Mini Kit (192) (QIAGEN)

and following the manufacturer's instructions. The detection of *L. pneumophila* was done by amplifying the following primers and probe sets specific for *ssrA*, *mip* and *wzm*, and based on existing literature [80], Legsp-F (5′-NGG CGA CCT GGC TTC-3′) and Legsp-R (5′-GGT CAT CGT TTG CAT TTA TAT TTA-3′), and Lp-mip-F2 (5′-TTG TCT TAT AGC ATT GGT GCC G-3′) and Lp-mip-R (5′-CCA ATT GAG CGC CAC TCA TAG-3′), and Lp-wzm-F (5′-TGC CTC TGG CTT AGC AGT TA-3′) and Lp-wzm-R (5′-CAC ACA GGC ACA GCA GAA ACA-3′). These primers and probes were used as previously described [80] and were tested for specificity by spiking a sample of pure water with *Legionella* and running a standard PCR and algarose-gel electrophoresis was applied to test for end product specificity. The PCR was then run in a Rotor-Gene Q (QIAGEN) machine following the manufacturer's instructions under the following cycling conditions: initial denaturation cycle of 1 min at 95 °C followed by 30 cycles for denaturation at 95 °C for 5 s, 30 cycles of annealing at 60 °C for 10 s, extension at 72 °C for 15 s and then an end holding cycle for 7 min at 72 °C. The presence of matching patterns for *L. pneumophila* were observed as follows: PCR fragments 79 bp (10 % tolerance), 110 pb (10% tolerance) and 124 (5% tolerance). The lack of any matching pattern indicated the absence of *Legionella* spp. and the presence of a single matching pattern of 110 pb (10% tolerance) indicated presence of *Legionella* spp. The presence of *L. pneumophila* Sg 2–14 was indicated by 2 matching patterns of 110 pb (10% tolerance) and 124 (5% tolerance) and *L. pneumophila* Sg 1 by all 3 matching patterns.

4.4.2. Detection and Measurement of *Pseudomonas aeruginosa*

The detection and enumeration of *P. aeruginosa* in water samples was done according to the requirements of "*AS/NZS 4276.13.2008 Method 13: Pseudomonas aeruginosa—Membrane filtration method"* [81]. One hundred milliliters (100 mL) of the sample was filtered through a 0.45 µm gridded cellulose acetate membrane filter. The prepared filters containing the filtrate were rolled onto prepared mPA-C agar plates that were then incubated in an inverted position in humid conditions at 41.5 °C ± 0.5 °C for 44 ± 4 h with any flat appearing colonies growing on the plates and depicting a light brownish outer rim to the green-black centre recorded as presumptive *P. aeruginosa.*

Confirmation of *P. aeruginosa* was determined by a modified and validated qPCR laboratory inhouse method (AS 4276.13 EDP-306). DNA was extracted from the bacterial isolates obtained from the incubated plates using QIAsymphony DNA Mini Kit (192) (QIAGEN) and following the manufacturer's instructions. The purity of the DNA was achieved by using the commercially available QIAsymphony DNA Kit (QIAGEN) and following the manufacturer's instructions. *P. aeruginosa* detection was done by amplification in a Roto-Gene Q (QIAGEN) machine and following the manufacturer's instructions. The following amplicon sequences described in literature [82] were used: forward ETA1: 5′-GAC AAC GCC CTC AGC ATC ACC AGC-3′ and reverse ETA2: 5′-CGC TGG CCC ATT CGC TCC AGC GCT-3′ with a product result of 396 bp. A total volume of 25 µL was used for the PCR. The LightCycler instrument (QIAGEN) was used to achieve the following cycling conditions: 1 denaturation cycle at 95 °C for 3 min, 35 cycles with each one made up of 1 m at 94 °C, 68 °C for 90 s, 72 °C for 1 min and an extension cycle of 10 min at 72 °C.

4.4.3. Detection and Measurement of *Acanthamoeba* and *Naegleria fowleri*

A validated in-house EcoDiagnostics laboratory method (EDP-315), was used to detect and enumerate *Acanthamoeba* and *N. fowleri*. Two hundred and fifty milliliters (250 mL) of the sample, spiked with *E. coli*, were concentrated by centrifugation for both *Acanthamoeba* and *Naegleria* species. The supernatant was poured off, and the pellet was resuspended in the remaining volume. One hundred microliters of the remaining volume were then spread plated onto non nutrient agar (NNA) plate and incubated at 42 °C for 48 h for *Naegleria*, and at 25 °C for 3 days for *Acanthamoeba*, and the presence of amoeba was confirmed using microscopy. Any plaques were picked for confirmation of *Naegleria* sp. by PCR, and then for *N. fowleri* and *Acanthamoeba* by qPCR and PCR, respectively.

For *N. fowleri* confirmation, the cells picked from the incubated NNA agar plates were aseptically transferred into 20 µL of lysis buffer for DNA extraction using the QIAsymphony DNA extraction kit and following the manufacturer's instructions. The PCR and qPCR were run using the *Naegleria* specific primers and *N. fowleri* specific primers previously described in literature [83,84], respectively. The *Naegleria* spp. PCR amplicon used was sequenced as follows: *Naegleria* spp. forward primer 5′-GAA CCT GCG TAG GGA TCA TTT and reverse primer 5′-TTT CTT TTC CTC CCC TTA TTA-3′ and *N. fowleri* forward primer 5′-GTG AAA ACC TTT TTT CCA TTT-3′ and reverse primer 5′-TTT CTT TTC CTC CCC TTA TTA-3′. The qPCR cycling conditions were: 1 cycle for initial activation at 95 °C for 5 min, followed by 60 cycles for denaturation at 95 °C for 10 s and then 60 cycles for combined annealing and extension at 95 °C for 45 s. Successful PCR amplification was confirmed by the following cycle threshold results in controls; Positive ($Ct \leq 36$), Negative ($Ct \geq 37$) and NTC control ($Ct \geq 37$).

For *Acanthamoeba* confirmation, the twin amplicons JDP1 and JDP2 sequenced respectively as follows: forward primer 5′-GGCCCAGATCGTTTACCGTGAA and reverse primer 5′-TCTCACAAGCTGCTAGGGAGTCA were used for DNA amplification as described in literature [85]. The cycling conditions for *Acanthamoeba* included 1 cycle for initial denaturation at 95 °C for 5 min, followed by 40 cycles for denaturation at 95 °C for 30 s, 40 cycles for annealing at 56 °C for 30 s, 40 cycles for extension at 72 °C for 1 min and then 1 cycle for holding at 72 °C for 7 min. An *Acanthamoeba* PCR amplification was considered successful if the negative control showed no evidence of contamination indicated by the absence of an amplicon band and when the positive control showed a band in line with the expected amplicon of 500 bp ± 25% which was then considered positive for Acanthamoeba and indicated as detected per volume of 250 mL or 1 mL.

4.4.4. Detection and Measurement of *Mycobacteria avium*

The detection of *M. avium* was done using qPCR and *M. avium* specific primers, previously designed and used in literature [86], that target the amplification of the 16S rRNA gene and the IS1311 genetic construct as follows: *Mycobacterium* spp. forward 5′-ATAAGCCTGGGAAACTGGGT-3′ and reverse 5′-CACGCTCACAGTTAAGCCGT3′ with a product target of 484 bp and *M. avium* complex forward 5′-GCGTGAGGCTCTGTGGTGAA-3′ and reverse 5′-ATGACGACCGCTTGGGAGAC-3′ with a product target of 608 bp. One hundred milliliters (100 mL) of the sample were filtered. The resultant filtrate was placed into 2 mL of ATL and ProtK and incubated at 60 °C for 30 min, and then 400 µL was extracted using the QIAsymphony instrument. A 2 µL aliquot of the DNA sample was added to 48 µL of PCR mixture prepared as previously described in literature [86] and ran into a LightCycler 2.0 Machine (QIAGEN) operated according to the manufacturer's instructions. The following cycling conditions were applied: 1 denaturation cycle at 95 °C for 8 min to achieve activation followed by 29 amplification cycles made up of denaturation for 60 s at 95 °C, annealing for 60 s at 40 °C, extension for 35 s at 72 °C and the last extension cycle for 10 min at 72 °C. A standard PCR and algarose-gel electrophoresis was applied to test for end product specificity.

4.5. Data and Statistical Analysis

The continuous water profile data (free chlorine residual concentration, water temperature, water pH, total dissolved solids (TDS) and total organic carbon) was log-transformed and box and whisker plots were used to determine normality before the application of statistical tests. All microbiological culture results for *L. pneumophila* Sg 1, L. *pneumophila* Sg 2–14 and *P. aeruginosa* were reported as colony forming units per milliliter (CFU/mL). The polymerase chain reaction *(PCR)* test results for *M. avium*, *Acanthamoeba*, and *N. fowleri* were reported as detected or not detected and the quantitative polymerase chain *(qPCR)* test results for the bioaerosol samples were reported as detected or not detected.

All sampling results containing censored data reported by the laboratory as being below the detection limits were handled by a non-parametric method advanced by Helsel [85].

Using this method, each of the non-detect values were assigned a value of −1 before the application of the Kruskal-Wallis hypothesis test of significance [87]. This test orders and ranks the data points to indicate the existence of any differences or patterns. This non-parametric test for data sets with non-detects has greater power than parametric tests when the data do not conform to a normal distribution and is preferred over substitution methods that tend to introduce invasive data, often influencing statistical scores [88].

Most of the water profile data were not normally distributed, so the Kruskal-Wallis test of statistical differences between variables (H statistic) was used as an alternative to the one-way analysis of variance (ANOVA). All the OPPP occurrence data was also not normally distributed; therefore, the Spearman rho test and the Chi-square test of association were applied where appropriate to measure the extent of association between water profile variables, and the occurrence of OPPP. Before the application of the Spearman's rho test, OPPP occurrence data was coded to 'detected' where a pathogen had been isolated and 'not detected' where the converse was true. The detected and not detected variables were coded to '1' and '0', respectively, to facilitate statistical testing. A significance value of $p < 0.05$ was used to accept or reject the null hypothesis. The Minitab version 18 statistical package was used for all statistical analysis.

5. Conclusions

The findings of this study demonstrated that WMS used to cool ambient temperatures are a potential health risk due to colonization by OPPPs such as *L. pneumophila* Sg 1 and Sg 2–14, *P. aeruginosa,* and *Acanthamoeba*, and that factors such as free chlorine residual concentration, TDS concentration and TOC concentration can influence the regrowth of these pathogens in these systems. The current guidelines in Australia, developed partly due to public outrage following isolated outbreaks of *Legionella*, focus more on the control of this pathogen in large facilities such as hospitals, aged care homes and shopping centers, ignoring the health risk posed by other emerging pathogens. Therefore, there is a need to develop guidelines covering a broader range of facilities that may expose people to airborne mists which may contain a range of opportunistic premise plumbing pathogens and review existing public health legislation with the aim of adopting a risk-management approach to ensure the effective control of health risks associated with WMS. Further research is needed to understand the relationship between the water profile in WMS and the survival of OPPPs, and conditions that may result in the release of these pathogens from biofilms and their potential to be released as bioaerosols during aerosolization.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10040462/s1, File 1: PCR and qPCR primer sequencies and cycling conditions used during the study. File 2: Summary of analytical methods used during the study.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Edith Cowan University (ECU) Human Research Ethics committee (HREC), Approval Number 16337 MASAKA.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Primer sequences and reaction conditions used in nested PCR amplifications is contained in supplementary materials. The primary data used to generate results reported in this study are available on request from the corresponding author subject to applicable restrictions.

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APPENDIX 4

Project Number 16337 MASAKA ethics approval

From: Research Ethics <research.ethics@ecu.edu.au>
Sent: Tuesday, November 8, 2016 11:38 AM
To: 'emasaka@our.ecu.edu.au' <emasaka@our.ecu.edu.au>
Cc: 'edmore1028@yahoo.co.uk' <edmore1028@yahoo.co.uk>; Sue REED <s.reed@ecu.edu.au>; Jacques OOSTHUIZEN <j.oosthuizen@ecu.edu.au>; Research Assessments <researchassessments@ecu.edu.au>
Subject: 16337 MASAKA ethics approval

Dear Edmore

Project Number: 16337 MASAKA
Project Name: An assessment of risks associated with the use of water misting systems as a cooling intervention in public places in the Pilbara region of Western Australia.

Student number: [redacted]

The ECU Human Research Ethics Committee (HREC) has reviewed your application and has granted ethics approval for your research project. In granting approval, the HREC has determined that the research project meets the requirements of the *National Statement on Ethical Conduct in Human Research*.

The approval period is from 8 November 2016 to 15 December 2018.

The Research Assessments Team has been informed and they will issue formal confirmation of candidature (providing research proposal has been approved). Please note that the submission and approval of your research proposal is a separate process to obtaining ethics approval and that no recruitment of participants and/or data collection can commence until ethics approval has been granted, your research proposal has been approved and formal confirmation of candidature has been received.

All research projects are approved subject to general conditions of approval. Please see the attached document for details of these conditions, which include monitoring requirements, changes to the project and extension of ethics approval.

Please feel free to contact me if you require any further information.

Regards
Kim

Kim Gifkins, Senior Research Ethics Advisor, Office of Research & Innovation, Edith Cowan University, 270 Joondalup Drive, Joondalup, WA 6027
Email: **research.ethics@ecu.edu.au** Tel: +61 08 6304 2170 | Fax: +61 08 6304 5044 | CRICOS IPC 00279B

APPENDIX 5

PCR/qPCR Primer sequencies and cycling conditions used during the study

Pathogen	**Cycling conditions**	**PCR/qPCR Primer sequencies**
Legionella spp.	1. Initial denaturation cycle of 1 minute at 95 °C 2. 30 cycles for denaturation at 95 °C for 5s 3. 30 cycles of annealing at 60 °C for 10 s 4. Extension at 72 °C for 15s 5. End holding cycle for 7 minutes at 72 °C PCR fragments 79 bp (10 % tolerance), 110 pb (10% tolerance) and 124 (5% tolerance)	***PCR*** ***Collins et al., 2015*** *Legsp-F (5' – NGG CGA CCT GGC TTC -3');* *Legsp-R (5'- GGT CAT CGT TTG CAT TTA TAT TTA – 3')* *Lp-mip-F2 (5' – TTG TCT TAT AGC ATT GGT GCC G – 3')* *Lp-mip-R (5' – CCA ATT GAG CGC CAC TCA TAG – 3')* *Lp-wzm-F(5' – TGC CTC TGG CTT AGC AGT TA – 3')* *Lp-wzm-R(5' – CAC ACA GGC ACA GCA GAA ACA -3')*
P. aeruginosa	*1. 1 denaturation cycle at 95 °C for 3 min* *2. 35 cycles with each one made up of:* *- 1m at 94 °C, 68 °C for 90s, 72 °C for 1 min* *3. Extension cycle of 10 min at 72 °C*	***qPCR*** ***Khan & Cerniglia, 1994*** *F: (ETA1: 5'-GAC AAC GCC CTC AGC ATC ACC AGC-3')* *R: (ETA2: 5'-CGC TGG CCC ATT CGC TCC AGC GCT-3')*
M. avium	*1. 1 denaturation cycle at 95 °C for 8 minutes* *2. 29 amplification cycles made up of:* *3. Denaturation for 60s at 95 °C* *4. Annealing for 60s at 40 °C* *5. Extension for 35s at 72 °C* *6. Last extension cycle for 10 minutes at 72 °C*	***qPCR*** ***Uppal et. al., 2002*** *F: (5'ATAAGCCTGGGAAACTGGGT3')* *R:(5'CACGCTCACAGTTAAGCCGT3')* *F:(5' GCGTGAGGCTCTGTGGTGAA3')* *R:(5'ATGACGACCGCTTGGGAGAC3')*
Acanthamoeba	*1. 1 cycle for initial denaturation at 95 °C for 5min* *2. 40 cycles with each made up of: -* *denaturation at 95 °C for 30s* *annealing at 56 °C for 30s* *extension at 72 °C for 1min* *3. 1 cycle for holding at 72 °C for 7 min*	***PCR*** ***Schroeder et al., 2001*** *JDPI F: (5'-GGCCCAGATCGTTTACCGTGAA)* *JDP2 R:(5'-TCTCACAAGCTGCTAGGGAGTCA)*
N. fowelri	*1. 1 cycle for initial activation at 95 °C for 5 min* *2. 60 cycles for denaturation at 95 °C for 10s* *3. 60 cycles of annealing and extension at 95 °C for 45s*	***PCR*** ***Puzon et al., 2009*** *F: (5'-GAA CCT GCG TAG GGA TCA TTT)* *R:(5' -TTT CTT TTC CTC CCC TTA TTA -3')* *F:(5' GTG AAA ACC TTT TTT CCA TTT -3')* *R:(5' TTT CTT TTC CTC CCC TTA TTA -3')*