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Article

Opportunistic premise plumbing pathogens. A potential health risk in water mist systems used as a cooling intervention.

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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 5 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 water temperature. Biofilms contained higher concentrations of *L. pneumophila* Sg 2 - 14 (1000 – 3000 CFU/ml) in comparison to water samples (0-100CFU/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*.

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, 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]. 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 to 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 <10μm 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 5 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 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 3 sampling events (February, May, and August) during 2019, representing the 3 climatic seasons of this region. Both culture and molecular (PCR) methods were used to detect the presence of 5 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 which was isolated from 5 (6%) of the total samples. Only 3 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.*

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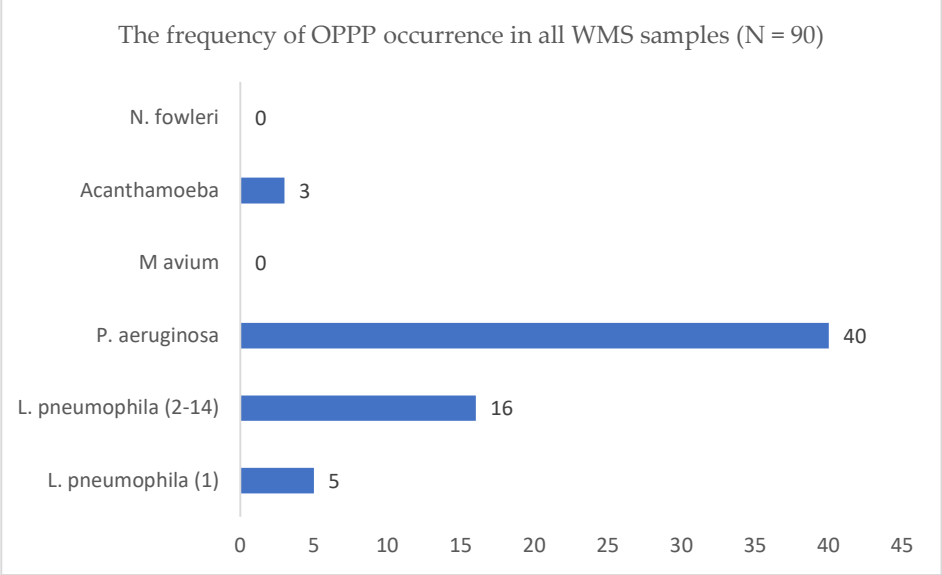

Figure 1. The frequency of opportunistic premise plumbing pathogens in all samples (n = 90)

2.2. The concentration of detected OPPPs

The results of this study as presented in Table 2, showed 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 biofilm being 30 x higher than in water. The biofilm concentration of L. pneumophila SG 2 - 14 was 3 times higher than that of water and *P. aeruginosa* in biofilm samples was 8 times higher than in water. The PCR Ct values for the abundance of the 16S rRNA gene copies of P. aeruginosa in the bioaerosols were measured at 30 – 37 indicating a moderate presence of the target RNA.

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 Ct values
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	30
	Highest	2000	350	37

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

2.3. The frequency and distribution of OPPPs differed by water mist system sample type

The frequency and distribution of OPPPs differed by the WMS sample type as shown in Figure 2. 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 -

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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 3 biofilm samples. *M. avium* and *N. fowleri* were not detected in any of the samples analyzed.

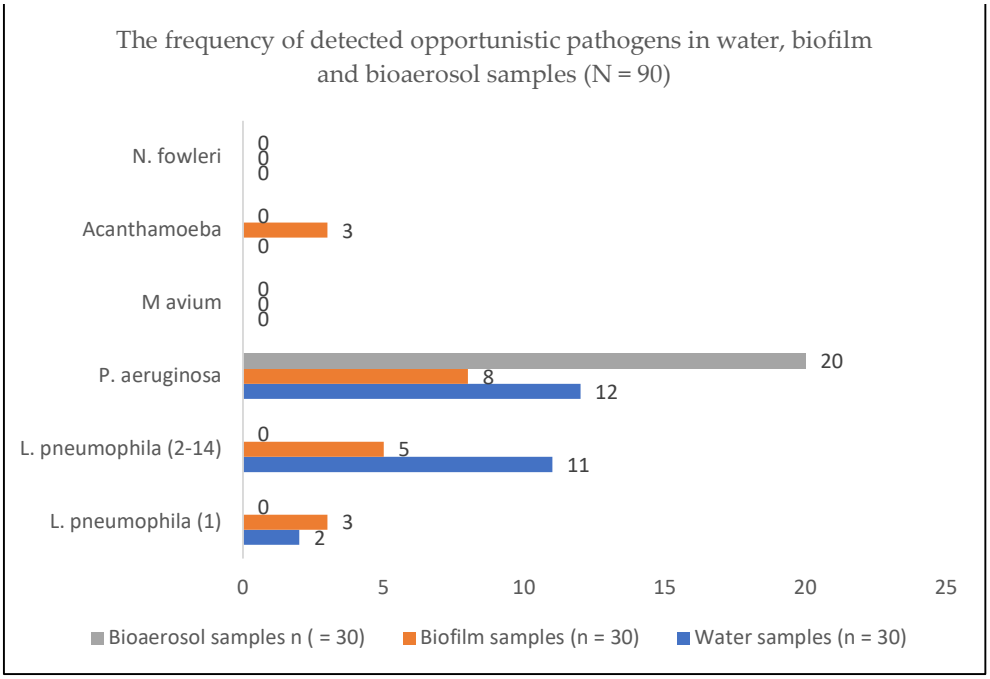

Figure 2. The distribution of OPPP occurrence by sample type (n = 90)

2.4. Opportunistic premise plumbing pathogen occurrence by water source

The occurrence of OPPPs in WMS samples differed by the source of water used as shown in Figure 3. The percentage occurrence of *L. pneumophila* Sg 2 - 14 in bore water samples was 4 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*. *Legionella pneumophila* Sg 1 was only detected in 5 bore water samples.

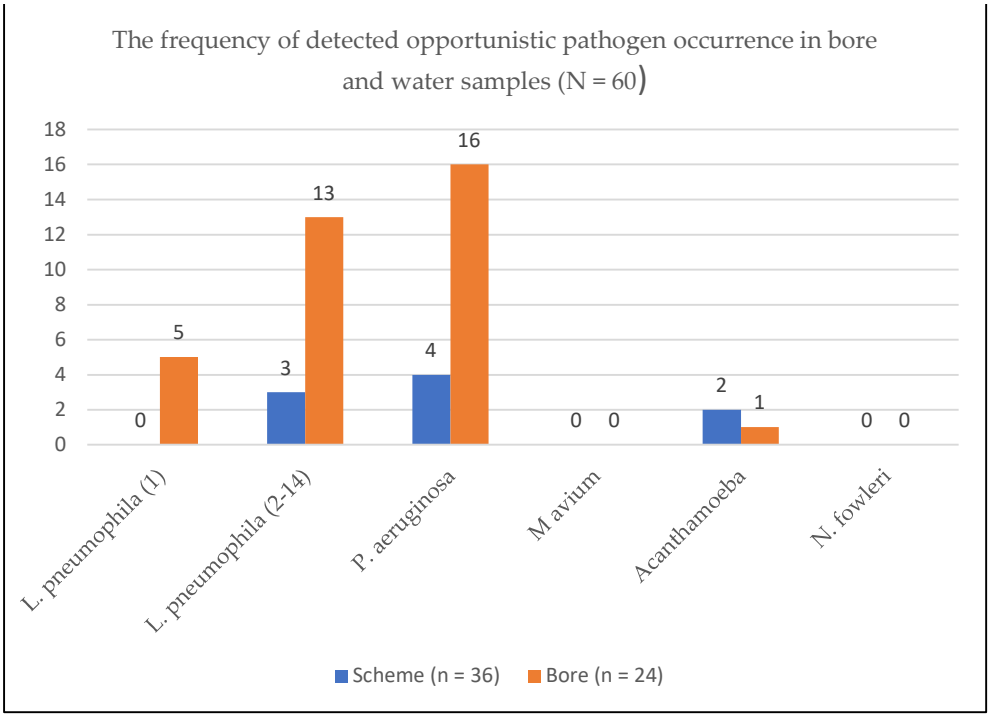

Figure 3. The frequency of OPPP occurrence by the type of water source (Bore, n = 24; Scheme, n = 36)

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, 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 3 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 $rs = 0.31$, $p < 0.05$, however, a weak, positive, and insignificant 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

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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 3 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 1st 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 3-sampling events $H(2) = 5.33$, $P = 0.07$.

2.9. Free chlorine residual

The concentration of free chlorine residual measured across the 3 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 3 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 3-sampling events $H(2) = 0.26$, $P = 0.88$.

2.10. Total organic carbon (TOC)

Seventy percent (21 out of 30) 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 (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 1 presents the Spearman rho correlation results among the water profile parameters. A weak negative monotonic correlation was observed between free chlorine residual concentration and water temperature, rs (30) = - 0.185, p > 0.05. 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

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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* (weak positive monotonic correlation, *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)

In this study, a weak positive monotonic correlation between water temperature and the occurrence of *L. pneumophila* Sg 1 and Sg 2 - 14 was established. A weak positive monotonic correlation between water temperature and *P. aeruginosa* was established, *rs* (30) = 0.31, $p < 0.05$. The measured total dissolved solids in the WMS had a moderately weak, and positive monotonic correlation with the occurrence of all OPPPs except with *Acanthamoeba* (weak negative monotonic correlation, *rs* (30) = - 0.06, $p > 0.05$), however, a

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significant moderate and positive correlation between *P. aeruginosa* and TDS concentration was determined. This study established that TOC had a moderate positive monotonic correlation with *L. pneumophila* Sg 1 and *L. pneumophila* Sg 2-14 and a weak positive monotonic correlation with *P. aeruginosa* and *Acanthamoeba.*

3.0. Discussion

Several opportunistic premise plumbing pathogens including *L. pneumophila, M. avium, P. aeruginosa, Acanthamoeba* and *N. fowleri* have been found growing in premise plumbing systems such as drinking water distribution networks [28], building hot water systems [29], showers [5] and hospital tap water systems [30]. Several studies have investigated the occurrence of some of these pathogens and determined incidences of 38% for *L. pneumophila* in 108 water taps located in houses and offices across 31 states in the United States of America [31], 29% for *P. aeruginosa* in foot operated faucets in hospitals [32] and 67% for *M. avium* in 64 domestic water samples in the USA [33]. However, 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.

Several studies of OPPPs have established that their distribution and concentration differ by target media of colonization within different water systems [34-36]. Additionally, biofilms and water are favorable growth media for OPPPs in water systems [14]. In a molecular survey of OPPPs in a drinking water system, *L. pneumophila* occurred at a rate of 34.6% and 83.3% in water and biofilm samples respectively, *M. avium* at a rate of 84.6% and 98.1% and *P. aeruginosa* at a rate of 3.8% and 5.6% [28]. 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 [37, 38]. 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 [27]. However, 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 [39]. 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 [40].

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* [41] and *Acanthamoeba* [42].

The inhalation of aerosols contaminated with OPPPs including *M. avium*, *P. aeruginosa* and *L. pneumophila* has been linked to various pneumonic infections [10, 18, 21, 28]. In our 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

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inhalation of *P. aeruginosa* [43]. This high detection of *P. aeruginosa* can be attributed to its ability to adapt and thrive in various environments [44].

Legionella pneumophila species are the OPPPs widely associated with the outbreaks of a pneumonic infection called Legionellosis in people exposed to contaminated aerosols released by various water features [18, 45, 46]. In this study, both *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. The 18 % occurrence of *L. pneumophila* Sg 2-14 detected in this study is higher than the 11% isolated from premise plumbing in Greek hotels [47]. However, the 6 % occurrence of *L. pneumophila* Sg 1 detected in our study is lower than the detection rates of the same pathogen in several studies worldwide [31, 47].

According to existing knowledge, contamination of domestic water storage systems with *Acanthamoeba* is a potential source for *Acanthamoeba* related infections including amoebic keratitis and granulomatous amoebic meningoencephalitis [26, 48, 49]. Free living amoebic species of *Acanthamoeba* were detected in a study investigating OPPP occurrence in domestic tap water systems in China, and their presence being positively correlated with *L. pneumophila, M. avium* and *P. aeruginosa* [27]. In this study, we detected a 3% (n = 90) occurrence of *Acanthamoeba* in WMS water and biofilm samples, with this occurrence being positively correlated with free chlorine residual, water temperature and TDS. The positive detection of *Acanthamoeba* in these WMS presents a health risk, 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 [50].

Several studies that have investigated the occurrence of OPPPs in premise plumbing systems such as domestic water taps, hospital faucets and showers have reported varying results ranging from zero detection of *M. avium* and *N. fowleri* in domestic tap water [27], to 15 % (n = 134) occurrence of *N. fowleri* in roof harvested rainwater tanks in Australia [51] and 13.5 % (n = 656) mean abundance of *Mycobacterium* species in showerhead biofilm samples from Europe and the USA [52]. Our investigation did not detect any *M. avium* nor *N fowleri* in all samples, water (30), biofilms (30) and bioaerosols (30). Although not isolated in any samples, the potential presence of *M. avium* and *N. fowleri* in WMS cannot be completely ruled out. 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 liter have previously been used to successfully detect these pathogens from water samples [53], hence higher sample volumes will 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 [54]. However, our study failed to demonstrate a statistically significant difference across seasons, a result which could be attributed to a loss in statistical power due to the smaller sample size [55]. Furthermore, water temperatures > 25 °C are one of the common and critical growth factors for most of the OPPPs investigated in this study [56], however, the mean ambient water temperature measured in the WMS across the 3 sampling events (29.9 °C) was optimum for the growth of all the targeted OPPPs and could have influenced this result. Further research to understand the seasonal variation of *Acanthamoeba* and other OPPPs' occurrence in WMS is warranted.

Recent studies have acknowledged the important part played by water quality parameters such as residual free chlorine concentration, temperature, pH, TDS, and TOC in promoting the regrowth of OPPPs [12, 13, 56]. 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 from where this water is drawn from [57]. Typically, bore water sources in Northern Australia tend to have a higher level of dissolved minerals such as iron and this promotes the colonization of plumbing systems by iron eating bacteria as established by recent research which found that these conditions promoted the formation of biofilms and regrowth of opportunistic pathogens including *P. aeruginosa and L. pneumophila* [58]. Based on current knowledge, water pH that is outside the recommended zone of 7.2 – 7.8 can cause corrosion of premise plumbing materials, resulting in the leaching of wastes that can promote the formation of biofilms and growth of OPPPs including *L. pneumophila* [59]. Our research found no significant difference in the water pH measured across the 3 sampling events. This finding could be attributed to the fact that the primary source of all the water used in the WMS is a shallow aquifer system where the water quality is influenced by infiltration from surface waters [57], therefore, it is assumed that the water chemistry used in all WMS would be similar.

Existing research on water supplies has established that its quality is heavily influenced by the TDS concentration in the source water, with groundwater supplies often containing higher TDS levels because of the chemical composition of the geological bedrocks that leach various salts [60]. 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, $H\,(1) = 16.78$, $P < 0.05$.

The conventional treatment that the scheme water goes through, a process involving the reduction of TDS to meet the standard of 500 mg/L set by the Australian Drinking Water Guidelines [61] could be the reason for this difference. The TDS composition of water in premise plumbing could include some inorganic salts and dissolved traces of organic matter as well as nutrients that leach from the internal surfaces of plumbing materials [62], and often promote the formation of biofilms on which premise plumbing pathogens grow, and when present in water supplies can serve as an energy source for microorganisms [63, 64]. However, the effect of plumbing materials on the regrowth of opportunistic pathogens in WMS was not the focus of this study. Although TDS, measured as specific conductivity, is a water quality parameter often used to measure the concentration of dissolved ions in water [65], it can also play a key role in the internal corrosion of plumbing materials, leading to disinfectant decay and biofilm growth [66] and increased survival of OPPPs such as *L. pneumophila, M. avium, P. aeruginosa and Acanthamoeba* [67]. The positive relationship between the formed biofilms and occurrence of *L. pneumophila* observed in this study is consistent with other studies [56, 68], 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 [69].

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, 56, 70]. Our study demonstrated a positive correlation between water temperature and the occurrence of all detected OPPPs, with a significant correlation existing with *Acanthamoeba*. These results are like those of a study by Lu, Buse [71], which established out that the occurrence of *Legionella*, *Mycobacterium*, *Pseudomonas,* and other pathogenic species in bathroom tap and shower waters tended to increase with water temperature. Furthermore, the significant positive correlation observed in this study between *P. aeruginosa* and water temperature may be explained in terms of this pathogen's ability to adapt to various environmental conditions including surviving temperatures ranging from 10 °C - 42 °C and its microbial competition and antagonism against other OPPPs [44]. 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 3 sampling episodes conducted

during our study, and with the higher winter temperatures being typical of the tropics where this study area is located [72].

Additionally, many water mist systems are situated outdoors and are reticulated by uninsulated pipework which can easily absorb elevated levels of radiant heat, resulting in elevated water temperatures in these systems. Elevated water temperatures in similar premise plumbing systems are a critical factor for the selective regrowth of OPPPs [71]. 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 [56], making it that the measured narrow temperature range may have failed to exhibit the linear growth curve.

Existing research has determined the critical role played by an adequate level of free residual chlorine in controlling the occurrence of OPPPs such as *L. pneumophila, P. aeruginosa and M. avium* [12, 41, 62, 73, 74]. Chlorine disinfectant decay precipitated by premise plumbing factors such as water age, stagnation, temperature, and pipe materials has been found to correlate with increased OPPP growth in these systems [75, 76]. This study determined a significant correlation between free residual chlorine concentration and the occurrence of all detected OPPPs except *Acanthamoeba*, highlighting the effectiveness of the monochloramine disinfectant used in the WMS over other forms of chlorine disinfectants as demonstrated in a previous study [42]. The observed negative correlation of residual chlorine and *Acanthamoeba* in premise plumbing is not entirely new, with similar findings having been established by Liu, Xing [27] in a one-year survey of these pathogens in premise plumbing. 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 demonstrated in a previous study [77]. Furthermore, the phagocytic nature of this free-living amoeba on other OPPPs may have resulted in their reduced numbers in the WMS [78]. Additionally, this study determined a significant difference in the free residual chlorine concentration of bore water and scheme water, due to the absence of effective water quality management systems for bore water supplied WMS, contrary to the scheme water supplied systems. Scheme water production and supply is licensed and regulated, ensuring that drinking water quality management plans are developed and implemented [79]. However, the effectiveness of drinking water quality management plans in ensuring the maintenance of an adequate level of free chlorine residual in WMS was not the focus of this study.

Low organic carbon or oligotrophic conditions have been established as a critical factor that enable selective regrowth of opportunistic pathogens in premise plumbing systems [17]. Some of the pathogens that have been found to thrive in these conditions include *L. pneumophila* [63], *N. fowleri* [80] and *Pseudomonas* species [81]. *Mycobacterium avium* can thrive in plumbing waters with organic carbon concentration < 5 µg/L [82]. This study determined that the TOC concentration in the WMS water samples were exceptionally low, with 70 % (n=30) being lower than the detection limit of < 1mg/L. The observed low concentration of TOC in the WMS is typical of premise plumbing waters [56], due to targeted control of this parameter of natural organic matter origin at the water treatment stage through flocculation and coagulation [83]. The observed difference in the TOC concentration of bore and scheme water may be due to the absence of a water treatment process to reduce TOC in the bore water supplies.

4.0. 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 north- western part of Australia over 3 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.

4.1 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 [84]. 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 minutes 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 quantitative polymerase chain reaction (qPCR) for *M. avium, P. aeruginosa, and N. fowleri*, and polymerase chain reaction (PCR) for *Legionella* species (including *L. pneumophila*), and *Acanthamoeba*.

4.1.1.. Bioaerosol sample processing

The inside of both 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 being used to sterilize the forceps after each filter transfer. This solution (with the filter paper) was incubated at 60 °C for 30 minutes to achieve lysis, 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 as a *Ct* value that corresponds to the concentration of the bacterial species genotype isolated [85].

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*" [86]. 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. 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"* [87]. 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 [88]. A calibrated industrial HM Digital TDS and water temperature thermometer with a measuring range of 0 ˚C – 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. free chlorine residual disinfectant level, pH, and temperature profile of the water samples.

4.3.1. 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)"* [89]. 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"* [90]. Confirmation of *L. pneumophila* from the observed colonies was done by an in-house PCR multiplex method (EDP-312).

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"* [91]. Confirmation of *P. aeruginosa* was determined by a modified laboratory inhouse method (AS 4276.13 EDP – 306).

4.4.3. Detection and measurement of Acanthamoeba and Naegleria fowleri

An 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 a nalidixic acid (NNA) agar plate and incubated at 42 °C for 48 hours for *Naegleria*, and at 25 °C for 3 days for *Acanthamoeba*, and the presence of amoeba was confirmed using microscopy. Any plaques were then picked for confirmation of *Naegleria* sp. by PCR, and then for *N. fowleri* and *Acanthamoeba* by qPCR and PCR, respectively.

4.4.4. Detection and measurement of Mycobacteria avium

The detection of *M. avium* was done using PCR. 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 minutes, and then 400. µL was extracted using the QIAsymphony instrument. The extract was analyzed on a Rotorgene instrument for the detection of *M. avium* using qPCR.

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 *Ct* counts.

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 [92]. Using this method, all the non-detect values were assigned a value of -1 respectively before the application of the Kruskal-Wallis hypothesis test of significance [93]. 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 [92].

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 $p < .05$ was used to accept or reject the null hypothesis. The Minitab version 18 statistical package was used for all statistical analysis.

5.0. 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, water temperature, pH, 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.

Author contributions

Conceptualization, E.M.; methodology, E.M, S.R, J.O and M.D.; software, E.M.; validation, E.M, S.R, J.O, and M.D., formal analysis, E.M..; resources, S.R and J.O.; data curation, E.M, S.R and J.O.; writing—original draft preparation, E.M writing—review and editing, S.R, J.O and M.D and E.M.; visualization, E.M, S.R and J.O; supervision, S.R, J.O. and M.D. project administration, E.M, S.R; J.O. and M.D funding acquisition, S.R and J.O. All authors have read and agreed to the published version of the manuscript."

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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.

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Conflicts of interest

All the authors declare no conflict of interest.

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