The presence of opportunistic pathogens, *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex, in South Australian reuse water distribution pipelines

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ABSTRACT

Water reuse has become increasingly important for sustainable water management. Currently, its application is primarily constrained by the potential health risks. Presently there is limited knowledge regarding the presence and fate of opportunistic pathogens along reuse water distribution pipelines. In this study opportunistic human pathogens *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex were detected using real-time polymerase chain reaction along two South Australian reuse water distribution pipelines at maximum concentrations of 10^5, 10^3 and 10^5 copies/mL, respectively. During the summer period of sampling the concentration of all three organisms significantly increased ($P < 0.05$) along the pipeline, suggesting multiplication and hence viability. No seasonality in the decrease in chlorine residual along the pipelines was observed. This suggests that the combination of reduced chlorine residual and increased water temperature promoted the presence of these opportunistic pathogens.

Key words | distribution pipelines, *Legionella* spp., *L. pneumophila*, Mycobacterium avium complex (MAC), public health, water reuse

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INTRODUCTION

Urbanisation and population growth have resulted in increased pressure on available water resources. Consequently, there is a need for more efficient use of water, both in urban and rural environments (Toze 2006). Water reuse is currently being considered as a potentially significant tool for sustainable water management and its implementation may have major ecological and economic benefits (Casani *et al.* 2005). The application of water reuse is predominately constrained by the potential public health risks (Chen *et al.* 2012). Water reuse guidelines currently focus on treatment processes that provide multiple barriers for pathogen control and include monitoring of indicator organisms such as faecal coliforms and intestinal nematodes (Harwood *et al.* 2005). However, there are few data regarding the presence and public health risk of opportunistic pathogens in reuse water (Maimon *et al.* 2010).

Legionella spp. and Mycobacterium avium complex (MAC) are opportunistic human pathogens that have been associated with potable water distribution systems as a source of infection (Kool *et al.* 1999; Zmirou-Navier *et al.* 2007; Falkinham *et al.* 2008; Nishiuchi 2009). They have also been detected in both wastewater and stormwater (Catalan *et al.* 1997; Pickup *et al.* 2006; Lampard *et al.* 2012) and hence have been identified as a potential public health risk associated with water reuse (Toze 2006).

Legionella spp. is a major public health concern. It is the causative agent of Legionellosis which includes Legionnaires' disease, an atypical pneumonic infection, and Pontiac fever, an acute febrile illness (Buchbinder *et al.* 2002). Worldwide, approximately 80% of Legionellosis is caused by *L. pneumophila* (Buchbinder *et al.* 2002). In the USA between 2009 and 2010, 57.6% of potable water-related disease outbreaks were due to *Legionella* spp. (Centers for Disease Control & Prevention 2013) and in 2011 across Europe there were 4,897 confirmed cases of

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Legionellosis (0.97 cases per 100,000) reported to the European Centre for Disease Control (European Centre for Disease Prevention & Control 2013). In 2013, Australia recorded 2.2 cases of Legionellosis per 100,000 (The Department of Health 2014). The true incidence of Legionellosis may be much higher as many community-acquired cases go unreported (Marston *et al.* 1997; Todd 2005).

It is difficult to determine the specific number of MAC cases as it is responsible for a wide spectrum of illness (Whiley *et al.* 2012). The clinical presentations include pulmonary infections (Huang *et al.* 1999; Field 2004; Marras *et al.* 2005; Lakhanpal *et al.* 2011), skin and soft tissue infections (Sugita 2000; Karakousis *et al.* 2004), lymph node infections (Thegerström *et al.* 2008), gastrointestinal infections (Nightingale *et al.* 1992) and debatably Crohn's disease (Naser *et al.* 2004). Recently an increase in the prevalence of MAC infection has been reported across the globe (Lai *et al.* 2010; Prevots *et al.* 2010; Al-Houqani *et al.* 2012).

This study used real-time polymerase chain reaction (qPCR) to investigate the presence and recovery post disinfection of opportunistic pathogens, *Legionella* spp., *L. pneumophila* and MAC, along the distribution pipelines of two South Australian reuse water systems. The two reuse water systems utilise different water resources and disinfection protocols. *Legionella* and MAC were chosen as pathogens of public health significance with mechanisms that may be enabling them to survive the disinfection protocols and unfavourable environmental conditions. Environmental strains of *Legionella* and MAC have been shown to be resistant to a range of disinfectants (George *et al.* 1980; Kuchta *et al.* 1985), associated with biofilms within water distribution pipelines (Schulze-Robbecke *et al.* 1992; Declerck 2010) and are opportunistic parasites of free living protozoa (Tyndall & Domingue 1982; Salah & Drancourt 2010). It has been suggested that these attributes enable them to persist in water distribution systems (Payment & Robertson 2004; Codony *et al.* 2011).

The two reuse water systems investigated in this study have currently been approved for irrigation purposes, domestic non-potable purposes (toilet flushing) and industrial purposes (toilet flush and cooling towers in large buildings). Current reuse regulatory guidelines enforce protocols to reduce potential public exposure. This includes irrigation at night for parklands, withholding times and restricted use around the home (South Australia Department for Health & Ageing 2012).

METHODS

Sampling

Samples were collected four times during 2012, once during each of the seasons, from two South Australian reuse water distribution pipelines. South Australia has a Mediterranean climate with warm summers and cold winters and sampling occurred in February (summer), May (autumn), August (winter) and November (spring). Distribution system 1 (DS1) utilised recycled wastewater, whereas distribution system 2 (DS2) utilised recycled wastewater combined with reclaimed stormwater. Samples were collected from multiple points along each of the distribution pipelines at varying distances from the water treatment plant and are described in Table 1. At each sampling point the total and free chlorine was measured using the standard method (American Public Health Association 2005) before three 500 mL water samples were collected aseptically, using the

Table 1 | Description of distribution systems and distance from processing plants of the sampling points

Sample	Distance from processing plant (km)	Water source	Treatment
Distribution system 1 (DS1)			
A	0	Recycled wastewater	Screening, grit removal, activated sludge, clarifier, then pre-filtration chlorination, filtration, UV and chlorine disinfection
B	11		
C	14		
Distribution system 2 (DS2)			
A	0	Recycled wastewater and reclaimed stormwater	Screening, grit removal, activated sludge, clarifier, lagooning, dissolved air flotation filtration and chlorine disinfection filtration and chlorine disinfection
B	1		
C	1		
D	2		

AS/NZS 5667 standard method for water quality sampling, and the chlorine quenched with sodium thiosulphate. Samples were stored at 4 °C and biological analysis and DNA extraction occurred within 12 hours.

Water temperature

Water temperature data were mined from water utilities' routine monitoring data records. Unfortunately temperature data were only available for DS2 and not for DS1. The average temperature measured from multiple points along the pipeline during the month of sampling is shown in Table 3. Both DS1 and DS2 pipelines are below ground and are situated <20 km away from each other. The average monthly water temperatures of DS2 were compared to the monthly mean ambient temperature for this location provided by the Australian Bureau of Meteorology (www.bom.gov.au/). The resulting r^2 value was 0.8816 ($P < 0.0001$), indicating almost 90% of the variation in water temperature can be attributed to the variation in the ambient temperature. Taking this into consideration we would expect to observe similar seasonality trends in water temperature in DS1 as in DS2.

Enumeration of coliforms

Escherichia coli and total coliforms were enumerated with Colilert™ trays (IDEXX Laboratories, NSW, Australia) using the standard method.

qPCR enumeration of *Legionella* spp., *L. pneumophila* and MAC

DNA was extracted for qPCR analysis from 450 mL of the sampled water using the BIO-RAD Aquadien™ Kit following manufacturer's instructions giving a final volume of 100 μL of DNA extract (Bio-Rad Laboratories, Inc., NSW, Australia). Triplicate qPCR was then performed for the enumeration of *Legionella* spp., *L. pneumophila* and MAC.

Legionella spp. qPCR was performed as previously described (Giglio *et al.* 2005). The 25 μL reaction volume contained 1 X PCR buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), 2.5 mM SYTO9 fluorescent dye (Invitrogen), 0.2 mM deoxynucleoside triphosphate mix (Invitrogen), I U platinum Taq DNA polymerase (Invitrogen), 0.3 μM JFP primer (5′-AGGGTTGATAGGTTAAGAGC-3′), 0.3 μM JRP primer (5′-CCAACAGCTAGTTGACATCG-3′) and 5 μL of template DNA. The cycling conditions included an initial hold at 95 °C for 5 min, followed by 45 cycles consisting of 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s.

Legionella pneumophila qPCR was performed as previously described (Giglio *et al.* 2003). The reaction volume was 25 μL and included 1 X PCR buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), 2.5 mM SYTO9 fluorescent dye (Invitrogen), 0.2 mM deoxynucleoside triphosphate mix (Invitrogen), I U platinum Taq DNA polymerase (Invitrogen), 0.5 μM mip99F primer (5′TGTCTTATAGCATTGGTGCC3′), 0.5 μM mip213R primer (5′CAATTGAGCGCCACTCATAG3′) and 5 μL of template DNA. The cycling conditions included an initial hold at 95 °C for 5 min, followed by 40 cycles consisting of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 25 s.

MAC qPCR was performed using previously described primers MACF primer (5′-CCCTGAGACAACACTCGGTC-3′) and MACR primer (5′-ATTACACATTTCGATGAACGC-3′) (Park *et al.* 2000). The 25 μL reaction volume contained 1 X PCR buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), 2.5 mM SYTO9 fluorescent dye (Invitrogen), 0.2 mM deoxynucleoside triphosphate mix (Invitrogen), IU platinum Taq DNA polymerase (Invitrogen), 0.3 μM MACF primer, 0.3 μM MACR primer and 5 μL of template DNA. The cycling conditions included an initial hold at 95 °C for 5 min, followed by 45 cycles consisting of 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 20 s.

All qPCR reactions were carried out in a RotorGene 3000 (Corbett Research, NSW, Australia) with data acquisition at 72 °C on the 6-carboxyfluorescein channel (excitation at 470 nm, detection at 510 nm) at a gain of 5. Melt curve data were also acquired on this channel at gains of 2 and 5 using a ramping rate of 1 °C/60 s from 75 to 95 °C. For each reaction the melt curve was analysed and the presence of *Legionella* spp., *L. penumophila* and MAC was confirmed with melting temperatures (T_m) of 88 ± 1 °C, 82.5 ± 1 °C and 85 ± 1 °C, respectively.

To determine the presence of environmental inhibitors in the extracted DNA, the qPCR reactions were conducted in triplicate for both neat DNA extract and 1/10 dilution of the same sample into nuclease-free water (Invitrogen). If the cycle threshold (C_T) value for the 1/10 dilution of DNA extract was less than approximately 3.3 (representing an approximately 1-$\log_{10}$ concentration value) (Livak 2001)

of the pure DNA extract, then it was assumed that environmental inhibitors were present. When inhibition was present in the undiluted DNA extract and the 1/10 dilution had the correct T*m*, this was used to calculate copies/mL.

Standard curves were created using positive PCR product purified using a Montage PCR Centrigual Filter Device (Millipore, VIC, Australia) following the manufacturer's instructions. The concentration of purified DNA was calculated by reading the absorbance using a spectrophometer at 260 and 280 nm. The number of copies of PCR product was determined using the URI Genomics & Sequencing Center calculator for determining the number of copies of a template, available at http://cels.uri.edu/gsc/cndna.html (Staroscik 2004). A 1 in 10 series of dilutions (ranging from 10^9 to 10^0 copies) was created using the Corbett Research liquid handling system (Corbett Research, Sydney, Australia). This was used to determine both the limit of detection of each assay and the calculated copies. The limits of detection for the PCRs were 2.5, 2.5 and 25 copies/reaction for *Legionella* spp., *L. pneumophila* and MAC, respectively. If amplification was not detected or the melt curve was incorrect the sample was allocated a value of half the limit of detection. If a sample contained multiple melt peaks with a $>1\,^{\circ}C$ shift from the expected T*m*, this value was not included (Giglio *et al.* 2005).

Statistical analysis

Statistical analysis of results was conducted using Graph Pad™ Prism 5.0 (Graph Pad Software Inc., CA, USA). Comparisons of the average calculated copies were performed using one-way analysis of variance (ANOVA) with a Bonferroni post hoc test; statistical significance was accepted at $P < 0.05$.

RESULTS

Legionella spp., *L. pneumophila* and MAC were detected using qPCR in all distribution systems during each season of sampling with maximum concentrations of 10^5, 10^3 and 10^6 copies/mL, respectively. The average concentration and standard deviation detected for each organism is shown in Table 2 for DS1 and Table 3 for DS2. The concentrations highlighted indicate where a significantly ($P < 0.05$) higher concentration of an organism was detected compared to the concentration measured at sample point A for the same sampling time period. Order of magnitude increases of concentration which are not statistically significant are also highlighted.

During the summer month of sampling, a significant ($P < 0.05$) increase in *Legionella* spp., MAC and total coliforms was detected along both DS1 and DS2 and a significant increase in *L. pneumophila* was detected in DS1. In DS1 and DS2 for each sampling period a total of five water samples were collected (not including sample point A); for each of these, four organisms (*Legionella* spp., *L. pneumophila*, MAC and total coliforms) were enumerated. A statistically significant increase in an organism's concentration when compared with sample point A was observed 10/20 in summer (plus three non-statistically significant increases in magnitude), 1/20 in autumn (plus five non-statistically significant increases in magnitude), 5/20 in winter (plus three non-statistically significant increases in magnitude) and 0 times in spring (plus four non-statistically significant increases in magnitude).

During each sampling period the concentrations of *Legionella* spp., *L. pneumophila* or MAC measured leaving the processing plant were not significantly different between the two distribution systems ($P < 0.05$). The free and total chlorine residual decreased along both pipelines for all sampling periods to a concentration of <0.1 and ≤ 0.2 mg/L, respectively, as shown in Tables 2 and 3. No seasonality in the reduction of chlorine residual was observed. Coliforms were detected when total chlorine decreased to a level <0.3 mg/L and significantly increased during summer in DS1 and DS2 and winter in DS1.

DISCUSSION

This study used qPCR over culture methods for *Legionella* and MAC enumeration as traditional culture techniques are tedious and can be inaccurate (Hussong *et al.* 1987). The slow growth rate of *Legionella* (5–7 days) (Steele *et al.* 1990) and MAC (10–14 days) (Falkinham *et al.* 2008) makes their isolation time consuming and allows for plates to become overgrown by faster growing organisms. Also,

Table 2 | Average concentration of *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex (mean ± standard deviation copies/mL) measured at each sampling point of reuse in distribution system 1 using real-time polymerase chain reaction. Total and free chlorine (mg/L) measured when samples were collected are also shown. The sampling points where a significant increase ($P < 0.05$) in the concentration of an organism compared to the concentration measured at sample point A within the same sampling period are also highlighted[a]

	Sample point	A	B	C
Season sampled	**Distance from treatment plant (km)**	**0**	**11**	**14**
Summer	Total chlorine (mg/L)	2.2	≤0.1	≤0.1
	Free chlorine (mg/L)	1.3	≤0.1	≤0.1
	Average *Legionella* spp. (copies/mL)	1,946 ± 123	19,460 ± 1,317[b]	345,332 ± 65,451[a]
	Average *L. pneumophila* (copies/mL)	1,665 ± 1,314	825 ± 238	4,897 ± 621[a]
	Average MAC (copies/mL)	3,406 ± 1,370	992,525 ± 305,265[a]	779,822 ± 359,538[a]
	Average total coliforms (MPN/100 mL)	0 ± 0	*175 ± 55	*308 ± 68
Autumn	Total chlorine (mg/L)	3.6	≤0.1	≤0.1
	Free chlorine (mg/L)	1.9	≤0.1	≤0.1
	Average *Legionella* spp. (copies/mL)	3 ±	585,030 ± 34,282[a]	143,918 ± 25,069[b]
	Average *L. pneumophila* (copies/mL)	18 ± 6	298 ± 271	447 ± 19
	Average MAC (copies/mL)	63 ± 47	4,480 ± 1,431[b]	15,943 ± 3,843[b]
	Average total coliforms (MPN/100 mL)	0 ± 0	45 ± 3	9 ± 1
Winter	Total chlorine (mg/L)	4.6	≤0.1	≤0.1
	Free chlorine (mg/L)	2.8	≤0.1	≤0.1
	Average *Legionella* spp. (copies/mL)	18 ± 13	237.404 ± 23,732[a]	265,010 ± 215,332[a]
	Average *L. pneumophila* (copies/mL)	62 ± 45	4,847 ± 1,034[a]	1,093 ± 663
	Average MAC (copies/mL)	149 ± 72	10,397 ± 4,736[b]	24.366 ± 3,533[b]
	Average total coliforms (MPN/100 mL)	0 ± 0	*1,817 ± 127	*329 ± 41
Spring	Total chlorine (mg/L)	1.6	0.3	0.2
	Free chlorine (mg/L)	0.6	0.3	≤0.1
	Average *Legionella* spp. (copies/mL)	62 ± 49	26,679 ± 9,432[b]	73,096 ± 6,946[b]
	Average *L. pneumophila* (copies/mL)	80 ± 55	7 ± 6	134 ± 161
	Average MAC (copies/mL)	936 ± 808	23,029 ± 3,490[b]	36,711 ± 3,346[b]
	Average total coliforms (MPN/100 mL)	0 ± 0	2 ± 1	21 ± 1

MPN: most probable number.

[a]Statistically significant increase.

[b]An increase of concentration by an order of magnitude. The lack of statistical significance ($P > 0.05$) is possibly due to the large variance in environmental samples shown by the standard deviation.

*$P < 0.05$.

culture does not account for the presence of viable but non-culturable organisms (Shih & Lin 2006; Chang *et al.* 2007; Radomski *et al.* 2010). qPCR was chosen for its rapid turn-around time and high sensitivity (Yaradou *et al.* 2007). The main disadvantage of qPCR is that it enumerates the DNA present in a sample and does not differentiate between live and dead cells (Delgado-Viscogliosi *et al.* 2009).

The average concentrations of each organism, the season that the sample was collected, the distance from the processing plant, average water temperature (for DS2 only) and the total and free chlorine residuals are shown in Tables 2 and 3. The concentrations that show a statistically significant increase compared to the concentration measured at sample point A for the sampling period are highlighted. When an order of magnitude increase was observed but was not statistically significant, this was also highlighted. The lack of statistical significance could be explained by the small sample size and the variability (shown by the standard deviation) due to environmental samples. The significance of the *P* value should not detract from the public health significance of a magnitude of increase in *Legionella* concentration (Nuzzo 2014).

The significant increase in copies/mL detected along a distribution pipeline (Tables 2 and 3) could be due to contamination of the pipeline or detached biofilm fragments causing higher cell counts. However, it was inferred that it was most likely due to multiplication and indicated the presence of viable organisms. This was supported by the fact that

Table 3 | Average concentration of *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex (mean ± standard deviation copies/mL) measured at each sampling point of reuse in distribution system 2 using real-time polymerase chain reaction. Total and free chlorine (mg/L) measured when samples were collected are also shown as well as the average water temperature for the month during which the sample was taken. The sampling points where a significant increase ($P < 0.05$) in the concentration of an organism compared to the concentration measured at sample point A within the same sampling period are also highlighted[a]

Season sampled and average water temperature	Sample point	A	B	C	D
	Distance from treatment plant (km)	0	1	1	2
Summer 26.1 °C ($n = 21$)	Total chlorine (mg/L)	0.3	0.2	0.3	0.2
	Free chlorine (mg/L)	≤0.1	≤0.1	≤0.1	≤0.1
	Average *Legionella* spp. (copies/mL)	789 ± 370	29,694 ± 7,694	16,690 ± 2,196[b]	734,073 ± 71,060[a]
	Average *L. pneumophila* (copies/mL)	824 ± 256	1,632 ± 814	1,492 ± 528	1,587 ± 298
	Average MAC (copies/mL)	810 ± 928	1,514 ± 1,525	4,917 ± 2,675	63,785 ± 1,712[a]
	Average total coliforms (MPN/100 mL)	0 ± 0	183 ± 22[a]	1 ± 1	283 ± 113[a]
Autumn 18.9 °C ($n = 21$)	Total chlorine (mg/L)	1.0	0.3	0.7	≤0.1
	Free chlorine (mg/L)	0.8	≤0.1	0.6	≤0.1
	Average *Legionella* spp. (copies/mL)	2,721 ± 752	2,250 ± 1,329	1,201 ± 990	7,346 ± 1,949
	Average *L. pneumophila* (copies/mL)	27 ± 16	38 ± 9	116 ± 62	48 ± 25
	Average MAC (copies/mL)	237 ± 195	344 ± 129	4,115 ± 1,641[b]	6,143 ± 247[b]
	Average total coliforms (MPN/100 mL)	0 ± 0	0 ± 0	0 ±	52 ±
Winter 15.2 °C ($n = 21$)	Total chlorine (mg/L)	0.9	0.5	0.5	≤0.1
	Free chlorine (mg/L)	0.7	0.3	0.3	≤0.1
	Average *Legionella* spp. (copies/mL)	10 ± 14	7 ± 5	9 ± 6	16,490 ± 15,964[b]
	Average *L. pneumophila* (copies/mL)	20 ± 26	3 ± 0	20 ± 26	85 ± 95
	Average MAC (copies/mL)	25 ± 0	208 ± 269	25 ±	25 ± 0
	Average total coliforms (MPN/100 mL)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Spring 22 °C ($n = 21$)	Total chlorine (mg/L)	0.9	0.5	0.5	≤0.1
	Free chlorine (mg/L)	0.7	0.3	0.3	≤0.1
	Average *Legionella* spp. (copies/mL)	53 ± 64	46 ± 23	7 ± 7	40 ± 43
	Average *L. pneumophila* (copies/mL)	3 ± 0	3 ± 0	3 ± 0	3 ± 0
	Average MAC (copies/mL)	875 ± 1,202	371 ± 305	261 ± 380	742 ± 715
	Average total coliforms (MPN/100 mL)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

MPN: most probable number.

[a]Statistically significant increase.

[b]An increase of concentration by an order of magnitude. The lack of statistical significance ($P > 0.05$) is possibly due to the large variance in environmental samples shown by the standard deviation.

the majority of increases in organisms along the pipelines were primarily observed in the summer, presumably because the warmer weather encouraged growth. Also typically, increases in Legionellosis cases are observed during the summer months (Diederen 2008). However, if the significant increases were due to the inclusion of a biofilm fragment, this still represents a result of potential public health significance if the water was used for toilet flushing or cooling towers which may facilitate the production of aerosols (Ishimatsu *et al.* 2001; Barker *et al.* 2005; Morawska *et al.* 2006). The multiplication within the biofilm indicates viability and persistence, and the sporadic release of concentrations of public health concern. The increase along the pipeline observed during winter could be explained by increased rainfall (Australian Bureau of Meteorology 2014), which has been shown to also coincide with an increase in Legionellosis cases (Hicks *et al.* 2007).

Although the chlorine residual decreased along the pipeline, there was no seasonal relationship with the decrease and hence the decline in chlorine residual does not singularly explain the increase in *Legionella, L. pneumophila* or MAC. This was supported by the observation that, for each sampling period, the chlorine residual in the water leaving the processing plants was significantly different between systems, but the concentrations of *Legionella* spp. and *L. pneumophila* was not significantly different ($P > 0.05$).

Coliforms were not detected at any time leaving either processing plant, but were later detected along the pipeline. This could be due to contamination of the pipeline or recovery of the coliforms. Previous studies have demonstrated

recovery and growth of coliforms in the presence of chlorine residual (Wierenga 1985; LeChevallier 1987; LeChevallier *et al.* 1996). Biofilms have been identified as one of the main sources of coliforms (LeChevallier *et al.* 1988). LeChevallier concluded that no one factor could account for coliform occurrences in distribution systems and coliform recovery is dependent on interactions between a range of chemical, physical and operational parameters (LeChevallier *et al*. 1996).

Total coliforms were only detected when the total chlorine residual decreased to <0.3 mg/L and may be an adequate indicator of overall system health. However, correlation between the total coliforms and the opportunistic pathogens was not observed. This supports the work by Harwood *et al*. (2005) who found no strong correlation between indicator organisms and human pathogens in recycled water systems.

CONCLUSIONS

Using qPCR, this study found *Legionella* spp., *L. pneumophila* and MAC to be present in two South Australian reuse water distribution pipelines. During each sampling period the concentration of each pathogen leaving either processing plant was not significantly different. Although qPCR cannot differentiate between live and killed cells, during the summer period of sampling the concentration of *Legionella* spp., *L. pneumophila* and MAC significantly increased along both distribution pipelines, which could be indicative of viable and multiplying organisms. Although these increases could also be explained through contamination of the pipeline or detachment of biofilm fragments, these scenarios still warrant addressing due to the public health significance of these opportunistic pathogens. There was no seasonality in the reduction of chlorine residual; however, seasonality in the increases of the opportunistic pathogens was observed. This suggests that the combined effect of warmer temperatures and low chlorine residual could be responsible for the observed increases in concentrations of *Legionella* spp., *L. pneumophila* and MAC.

The number of total coliforms was not representative of the number of opportunistic pathogens. The presence of potentially viable opportunistic human pathogens in reuse water distribution pipelines is a potential public health concern if the reuse water is used for applications that produce aerosols. This study highlighted the need for a better understanding of how water quality parameters, disinfection protocols and environmental factors (plumbing materials, temperature, flow rate, frequency of use, chlorine residual and organic content) influence opportunistic pathogen growth along these systems. There is also a need for accurate risk assessments regarding the different applications of this recycled water that specifically account for the potential presence of *Legionella* and MAC.

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