# *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex (MAC) in potable and reuse water distribution pipelines

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## Statement of Authenticity

I certify that this thesis does not incorporate without acknowledgment and material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Harriet Whiley

20th August 2014

## Statement of Co-Authorship

The following people contributed to the publication of the work undertaken as part of this thesis. The co-authors are listed in the order that the co-authored publications appears in the thesis.

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All above listed contributions equated to no more that 25% of the work necessitated for publication of research manuscripts.

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# Publications

**Whiley, H**., Keegan, A., Giglio, S. & Bentham, R. (2012), Mycobacterium avium complex – the role of potable water in disease transmission, *Journal of Applied Microbiology*, 113**,** 223-232.

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**Whiley, H**., Keegan, A., Fallowfield, H., and Ross, K., (2014), Uncertainties associated with assessing the public health risk from *Legionella*, *Frontiers in Microbiology*, 5, doi: 10.3389/fmicb.2014.00501.

## Abstract

*Legionella* spp. and Mycobacterium avium complex (MAC) are opportunistic human pathogens of public health concern. The clinical manifestations of *Legionella* include Pontiac fever, an acute febrile illness, and Legionnaires' disease, a severe atypical pneumonia. *L. pneumophila* is the most common causative agent of Legionellosis. In Australia, MAC is not a nationally notifiable disease, but it is responsible for a wide spectrum of illness dependent on subspecies, route of infection and a patient's pre-existing conditions. This includes, but is not limited to, a range of respiratory, gastrointestinal and cutaneous infections. Evidence also suggests that MAC is a causative agent of Crohn's disease.

This study investigated the presence of *Legionella* spp., *L. pneumophila* and MAC along South Australian potable and reuse water distribution pipelines using qPCR. Two potable water distribution systems were chosen (one chlorine disinfected and the other chloramine disinfected) and two reuse water distribution systems (one utilising recycled wastewater treated with chlorine and UV disinfection and the other recycled wastewater combined with reclaimed stormwater treated with chlorine disinfection only). Samples were collected along each of the pipelines throughout the year, to determine any seasonal variation. Relationships between temperature, chlorine or chloramine residual, indicator bacteria, distance from treatment plant and concentration of *Legionella* and MAC was explored.

*Legionella* spp., *L. pneumophila* and MAC were detected in both potable water distribution systems throughout the year. Maximum concentrations detected were $10^3$, $10^3$ and $10^3$ copies/mL respectively in the chlorine disinfected system and $10^6$, $10^3$ and $10^4$ copies/mL respectively in the chloramine disinfected system. The

concentrations of these opportunistic pathogens were primarily controlled throughout the distribution network through the maintenance of disinfection residuals. At a dead-end where the disinfection residual was not maintained significant ($P<0.05$) increased numbers of *Legionella* spp., *L. pneumophila* and MAC were observed when compared to the concentration measured closest to the processing plant in the same pipeline and sampling period.

In the reuse water distribution systems *Legionella* spp., *L. pneumophila* and MAC were detected using qPCR 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 ($P<0.05$) increased 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.

This study demonstrates the ability of *Legionella* spp., *L. pneumophila* and MAC to survive the potable and reuse water disinfection process and highlights the need for greater understanding of *Legionella* ecology related to risk associated with point of use. Determining the potential public health risk presented by the presence of *Legionella* spp., *L. pneumophila* and MAC is difficult to quantify due to the uncertainties regarding *Legionella* and MAC epidemiology and detection. A comprehensive review comparing culture and qPCR method of *Legionella* detection from environmental samples was conducted. The uncertainties associated with *Legionella* risk assessment were also collated and discussed providing a useful tool for considering risk assessment data and important areas for future *Legionella* research were also identified.

## Aims

The aims of this investigate were:

- To determine whether opportunistic pathogens *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex (MAC) are present in South Australian potable and reuse water distribution pipelines.
- To identify factors that may influence the growth of these opportunistic pathogens along the potable and reuse water pipelines.

## Objectives

In order to achieve these aims, the objectives of this study were:

- To examine the literature and determine the potential role of potable water as a source of MAC infection.
- To evaluate the optimum method for *Legionella* spp., *L. pneumophila* and MAC enumeration in environmental sources.
- To examine the relationship between pipeline length, seasonality, water quality, disinfectant residual, indicator organisms and *Legionella* spp., *L. pneumophila* and MAC concentrations in the potable and reuse water distribution pipelines.
- To use information from this study to create a risk assessment for *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex (MAC) exposure from South Australian potable water.

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**This thesis is based on published manuscripts, therefore some repetition between chapters occurs.**

# Chapter 1: Introduction

## 1.1 Opportunistic pathogens

Opportunistic human pathogens are infectious microorganisms that are usually commensal, but may exploit a weakened immune system to cause disease. Some examples of opportunistic pathogens include: *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acanthamoeba* spp., *Legionella* spp. and Nontuberculous Mycobacterium (NTM), (Lehtola *et al.* 2007; Feazel *et al.* 2009; Wang *et al.* 2012).

This study investigated the presence of opportunistic pathogens, *Legionella* spp. and Mycobacterium avium complex (MAC), within South Australian potable and reuse water distribution systems.

## 1.2 Potable water

Potable water refers to water primarily intended for human consumption, either directly from the tap or indirectly in beverages, ice or food preparation. Potable water is also used for domestic purposes such as bathing. The Australian Drinking Water Guidelines (ADWG) provides methods to manage Australian drinking water. These are compiled to ensure maximum safety for human consumption, as well as aesthetic quality (NHMRC 2004). Despite undergoing water treatment and chlorine disinfection, potable water will still often contain low numbers of various

microorganisms (Anaissie *et al.* 2002; Pryor *et al.* 2004; Berry *et al.* 2006), which for the most part are harmless at these concentrations.

### 1.2.1 Contamination of potable water distribution systems

Microbial contamination of water distribution systems may occur when microorganisms survive disinfection treatment processes or by direct contamination of the supply system downstream of the processing plant (Propato 2004). Examples of deficiency or malfunctions of water distribution systems and that have resulted in outbreaks of waterborne infection include: failures in water treatment processes, dead-end or other deficiency in design which allow for biofilm accumulation (Hayes *et al.* 1989), cross-connection and back-siphonage of system pipes (Lahti and Hiisvirta 1995), contamination of water whilst in storage (Clark *et al.* 1996), contamination of system during construction or repair (Inglis 1999), and broken or leaking distribution pipes (Geldreich *et al.* 1992). Pressure transients, caused by an abrupt change in the velocity of water, may also result in leaks that provide a potential route for contaminated groundwater and soil to enter the system. These are caused by power outages or other pump shutdowns (LeChevallier *et al.* 2003).

### 1.2.2 Pathogenic organisms present in potable water

Contamination of potable water systems by pathogenic organisms constitutes a significant public health risk (Lehtola *et al.* 2007). This includes enteric viral, protozoan and bacterial pathogens such as norovirus, *Cryptosporidium* spp. and *Campylobacter* spp.; but also opportunistic pathogens such as *Legionella* spp.,

Nontuberculous mycobacterium (NTM), *Pseudomonas aerginose* and *Acanthamoeba* spp. (Szewzyk *et al.* 2000; Lehtola *et al.* 2007; Wang *et al.* 2012). Current public health guidelines primarily focus on the control of enteric pathogens and indicator organisms, which are used to monitor microbial water quality (Stevens *et al.* 2003). However, studies have demonstrated a lack of correlation between opportunistic pathogens and indicator organisms (Hsu *et al.* 1984; Hörman *et al.* 2004; Harwood *et al.* 2005), which is concerning as opportunistic pathogens are fast becoming the primary cause of waterborne diseases in developed countries (Wang *et al.* 2012). This thesis explores the potential health risks associated with the presence of microbial contaminates *Legionella* and MAC in potable water.

### 1.3 Water reuse

Worldwide there is increasing pressure being placed on available water resources (Daigger 2009); due to global population growth and urbanisation, compounded by drought and depletion or contamination of groundwater sources (Wade Miller 2006). Water reuse, including reclaiming of stormwater and recycling of wastewater or grey water, can potentially provide new sources of high quality water supplies (Casani *et al.* 2005).

Australia is the driest inhabited continent in the world and experiences the most variable rainfall (Apostolidis *et al.* 2011). A prolonged Australian drought, which commenced in 2002, prompted the development of the National Water Incentive program which has resulted in many new recycling projects across Australia. Currently, the implementation of water reuse programs are considered a potentially

significant tool for sustainable water management in Australia, with major ecological and economic benefits (Casani *et al.* 2005; Radcliffe 2010).

### 1.3.1 Potential health risks associated with water reuse

Potential health risks associated with reuse water include the presence of microbial contaminants (including viruses, bacteria, protozoa and helminths) and chemical contaminates (including endocrine disrupting chemicals and pharmaceutically active compounds) (Toze 2006b). The potential risk of water-borne infection associated with microbial contaminants is dependent on a range of factors including the species of pathogens present, pathogens numbers, infective dose, exposure route and the susceptibility of exposed population (Haas *et al.* 1999). This thesis explores the potential risks associated with the presence of microbial contaminants *Legionella* and MAC in reuse water.

## 1.4 Biofilms

The presence of biofilms in water distribution systems is one of the main determining factors for persistent microbial contamination. Bacterial biofilms are ubiquitous to all surface types and are present in both natural and artificial environments (Lindsay and Holy 2006). Biofilms can be defined as a community of microorganisms attached to a surface by extracellular polymeric substances (EPS) and can consist of a mixture of bacteria, yeast, fungi, algae, protozoa and viruses (Donlan 2002). Microorganisms within a biofilm display an altered phenotype when compared with planktonic cells, especially in regard to interactions with other

microorganisms. Bacterial cells within a biofilm can function in a coordinated manner and cells may communicate via quorum sensing, often imitating the behaviour of multi-cellular organisms (Donlan 2002; Hall-Stoodley *et al.* 2004; Lindsay and Holy 2006).

Biofilms are recognised as a major public health concern due to their role in certain infectious diseases. Pathogenic microorganisms are able to accumulate in large numbers within biofilms and are often protected from routine methods of disinfection, which is of particular concern for potable and reuse water distribution pipelines (Lindsay and Holy 2006). Bacterial cells within a biofilm display a much greater resistance to antibiotics and disinfectants compared with their unattached planktonic counterparts (Costerton *et al.* 1999; Stewart and Costerton 2001; Lindsay and Holy 2006). LeChevallier *et al.* (1988) found that microbes within a biofilm were 3000 times more resistant to hypochlorous acid (free chlorine, pH 7.0) compared with unattached cells.

### 1.4.1. Biofilm formation

Biofilm formation (shown in Figure 1) is a complex process that is dependent on the diverse characteristics of the growth medium, substratum, and cell surface (Donlan 2002). Surface conditioning is the first phase of biofilm formation and involves the adsorption of organic and inorganic nutrients by the surface which influences the subsequent bacterial attachment (Beveridge *et al.* 1997). The next phase is reversible attachment, which is considered the weakest stage of the biofilm formation. During this process adhering cells will be transported to a surface via a range of mechanisms. Bulk liquid containing bacteria is transported by convection currents

and motile bacteria will actively seek out the surface, while other microbial cells will rely on sedimentation and Brownian motion. Electrostatic and physical interactions between surface and microbial cells will also affect attachment (Lindsay and Holy 2006). Once bacterial cells are attached to a surface they will produce extracellular polymeric substances (EPS) which strengthens surface attachment and allows cell to cell binding, resulting in the biofilm formation (Costerton *et al.* 1999). The final stage is surface colonisation, which involves attached microorganisms growing and dividing whilst entrapping other planktonic cells within the EPS (Lindsay and Holy 2006).

Once attached, clumps of biofilm may detach or disseminate due to an increase in the size of the biofilm or turbulent shear forces. Dissemination of biofilms also occurs when cells located on the outside of a biofilm detach and return to the planktonic state in order to find new surfaces to colonise. It has been proposed that quorum sensing is involved in the organisation and dissemination processes of biofilms (Lindsay and Holy 2006).

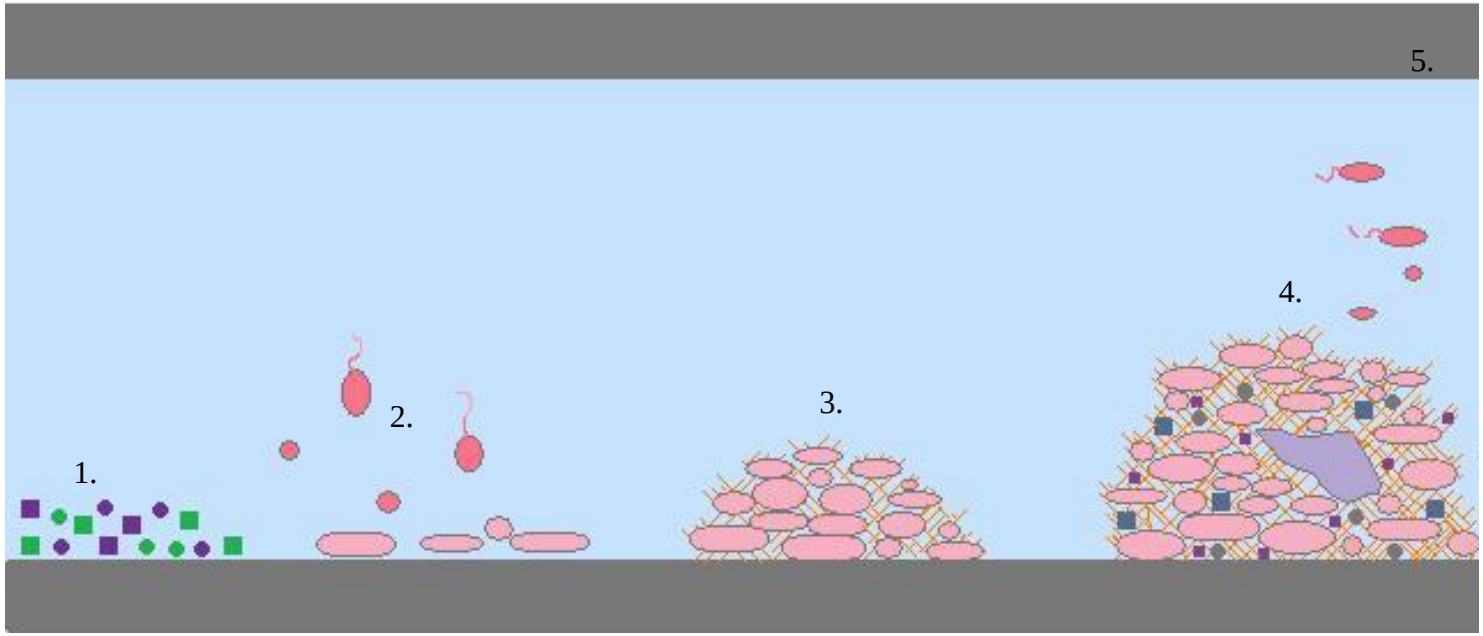


**Figure 1: Diagram displaying five stages of biofilm formation including: (1) surface conditioning, (2) reversible attachment, (3) production of EPS, (4) Surface colonisation, and (5) detachment (Lindsay and Holy, 2006).**

## 1.5 *Legionella* spp.

*Legionella* has been identified as a public health concern since 1976 and is recognised as the causative pathogen of Legionellosis (Buchbinder *et al.* 2002; Fields *et al.* 2002). The term Legionellosis refers collectively to the clinical syndromes resulting from *Legionella* infection which includes Legionnaires' disease, a *Legionella* derived pneumonic infection, and Pontiac fever, an acute febrile illness that has been linked serologically and by culture to *Legionella* spp. (Buchbinder *et al.* 2002). The people at highest risk are the elderly, smokers and patients with chronic lung disease or immunosuppressive conditions. Surgery, particularly transplant surgery, is also a major risk factor for nosocomial infection. The incidence of Legionnaires' disease in AIDS patients is low; however, the clinical manifestations of infection are more severe (Stout and Yu 1997).

The mortality rate of Legionellosis is dependent on the severity of disease, how it was acquired, timely diagnosis, the appropriateness and speed of which initial antimicrobial treatment is administered, and other patient risk factors including gender, age (men over 40 are more susceptible), smoking, alcohol abuse, diabetes, heart disease and other immunosuppression (Fields *et al.* 2002; WHO 2003; Bartram *et al.* 2007). Data from the USA and Australia reported the average mortality rate for nosocomial *Legionella* infections to be 14% and 5-10% for community acquired infections (Benin *et al.* 2002; Howden *et al.* 2003). However, the USA Center for Disease Control (CDC) has recorded nosocomial *Legionella* outbreaks to have mortality rates of up to 40% (Centers for Disease Control and Prevention 1997).

Across Europe in 2011, there were 4897 confirmed cases of Legionellosis which is a notification rate of 0.97 cases per 100,000 (European Centre for Disease Prevention

and Control 2013). In the USA from 2000-2009 the incidence of reported Legionellosis has almost tripled from 0.39 to 1.15 cases per 100,000 (Centers for Disease Control Prevention 2011). In 2013 Australia had 499 cases (2.2 per 100,000) of Legionellosis reported to the National Notifiable Disease Surveillance System. Of these, 67 cases were reported from South Australia, giving the state a notification rate of 3.7 per 100,000, which is higher than the country's average (Department of Health 2014).

### 1.5.1 Legionellosis and potable water

Numerous cases of Legionellosis have been linked to potable water as the source of infection (Craun *et al.* 2010). In USA between 2009-2010 57.6% of drinking water related outbreaks were due to *Legionella* spp. (Centers for Disease Control and Prevention 2013b). Outbreaks have been linked to contaminated shower heads (Hanrahan *et al.* 1987; Zmirou-Navier *et al.* 2007), spas (Jernigan *et al.* 1996; Benkel *et al.* 2000), baths (Sasaki *et al.* 2008), a hospital steam towel warmer (Higa *et al.* 2012), ice machines (Graman *et al.* 1997; Schuetz *et al.* 2009), decorative water fountains (Fleming *et al.* 2000; O'Loughlin *et al.* 2007; Haupt *et al.* 2012) and hospital water distribution systems (Tobin *et al.* 1981; Hanrahan *et al.* 1987).

Table 1 presents studies that have identified a range of *Legionella* spp. from potable water distribution supply systems. *Legionella* has been detected in potable water under varying environmental conditions using a range of detection methods. However, there is currently a lack of information about the presence of *Legionella* in Australian potable water systems.

It is thought that *Legionella* is able to persistently reside in potable water due to its resistance to chlorine (Kuchta 1985), intracellular parasitism of protozoan hosts (Taylor *et al.* 2009), biofilm formation (Rogers *et al.* 1994) and relative tolerance to low pH conditions for short periods of time (Bopp *et al.* 1981). Kuchta *et al.* (1983) demonstrated that agar grown *L. pneumophila* isolates had a greater resistance to chlorine than coliform bacteria. At 21 $^{o}$C, pH 7.6 and 0.1 mg/L of free chlorine, *L. pneumophila* were able to survive for 40 minutes before 99% were killed compared with *Escherichia coli* that survived less than one minute. Further studies demonstrated *L .pneumophila* strains maintained in potable water were even more resistant to chlorine than agar grown isolates (Kuchta 1985).

Although *Legionella* has been detected from numerous potable water sources (Table 1) this is the first study to enumerate *Legionella* at multiple points along potable water distribution pipelines. This provided an insight into the pathogens potential recovery post disinfection at the treatment plant, comparison of the effectiveness of chlorine and chloramine disinfection and identified areas along the distribution pipeline network which provided a growth niche for the organism.

**Table 1: Previous studies which have detected *Legionella* spp. in potable water distribution supply systems.**

| Country | Sample type | Temp. ($^{o}$C) | Disinfection residual | Detection method | Species | Concentration | Reference |
|---|---|---|---|---|---|---|---|
| United States | Potable water samples from distribution pipeline | 12 | 0.5-2.0mg/L free chlorine | Direct immunofluorescence | *Legionella* spp. | $<8x10^{3}$ to 2.2 x $10^{4}$ direct immunofluorescence positive cells per litre | (Tison and Seidler 1983) |
| United States | Potable water samples | | Chlorinated (concentration not measured) | Culture | *L. pneumophila* type 1<br>*L. jordanis*<br>*L. dumoffi* | | (Hsu *et al.* 1984) |
| United States | Potable water samples collected at point of use in residential homes | | | Culture direct fluorescent antibody testing | *L. pneumophila* (in 6.4% of residences tested) | | (Stout *et al.* 1992) |
| Germany | Biofilms from potable water distribution pipelines | | | PCR-Southern Blot hybridization, *in situ*-hybridization and EnviroAmp™ *Legionella* Kit (Perkin Elmer) | *Legionella* spp. (in 7% of samples) | | (Schwartz *et al.* 1998) |
| United States | Treated potable water sample from distribution pipeline<br>Biofilm samples from potable water pipeline | | 0.8-1.2mg/L free chlorine | PCR and Culture | *Legionella* spp. | | (Pryor *et al.* 2004) |

| Country | Sample type | Temp. ($^{o}$C) | Disinfection residual | Detection method | Species | Concentration | Reference |
|---|---|---|---|---|---|---|---|
| The Netherlands | Potable water samples | <15 | | PCR and 16S rRNA sequencing of PCR products | *L. worsleiensis*<br>*L. bozemanii*<br>*L. lytica*<br>*L. pneumophila*<br>*L. waltersii*<br>*L. quateirensis*<br>*L. donaldsonii*<br>*L. adelaidensis*<br>*L. dumoffii*<br>*L. londiniensis*<br>*L. anisa*<br>*L.f airfieldensis*<br>*L. fallonii*<br>*L. micadadei*<br>*L. stegerwaltii* | | (Wullings and van der Kooij 2006) |
| Caribbean | Potable water samples | 28-31.6 | | Culture | *Legionella* spp. | 41/49 samples were positive<br>Concentrations ranged from $2.5x10^{2} – 2.5x10^{5}$ CFU/mL | (Valster *et al.* 2011) |
| United States | Potable water samples collected at point of use in residential homes (Virginia) | 20.8 ± 2.8 | 2.02±0.63 mg/L total chlorine | qPCR | *Legionella* spp. (in 30% of samples)*L. pneumophila* (in 4.4% samples) | Average concentration 186.6±458.2 *Legionella* spp. copies/mL<br>Average concentration 9.8±4.4 *L. pneumophila* copies/mL | (Wang *et al.* 2012) |

| Country | Sample type | Temp. (°C) | Disinfection residual | Detection method | Species | Concentration | Reference |
|---|---|---|---|---|---|---|---|
| | potable water samples collected at point of use in residential homes (Florida) | 26.8 ±0.9 | 2.15±1.13mg/L total chlorine | | *Legionella* spp. (in 83.8% of samples)<br>*L. pneumophila* (in 5.6% samples) | Average concentration 100.8±184.2 *Legionella* spp. copies/mL<br>-Average concentration 90.4±111.9 *L. pneumophila* copies/mL | |

### 1.5.2 Legionellosis and reuse water

*Legionella* spp. has been identified as a potential pathogen of concern present in reuse water (Toze 2006a). Palmer *et al.* (1993) detected $10^3$ cells/mL *Legionella* spp. throughout a sewage treatment plant with no reduction in numbers observed post treatment. In London, Birks *et al.* (2004) detected *L. pneumophila* at concentration of 1500-1750 cells/mL of raw greywater sampled from The Thames Water recycling plant at the Millennium Dome. The presence of *Legionella* in reuse water is generally accepted; however, there is currently limited research investigating its ecology within reuse water and this is the first study to enumerate *Legionella* spp. and investigate its profile along the distribution pipelines of large scale reuse water systems.

### 1.5.3 Pathogenic species of *Legionella*

There are 19 species of *Legionella* which have been identified as human pathogens (Muder and Victor 2002). Worldwide, the most common causative agent of Legionellosis is *Legionella pneumophila* (Fields *et al.* 2002). However, in Australia *L. longbeachae* has also been identified as a major cause of Legionellosis (O'Connor *et al.* 2007) and recently, there has been a global increase in the number of reported cases (Whiley and Bentham 2011). *L. longbeachae* is associated with soils and potting mixes (Steele *et al.* 1990), whereas, *L. pneumophila* is endemic to aquatic environments. For this reason *L. pneumophila* was targeted in this study. Additionally, *Legionella* species was enumerated to account for potential

contamination of water sources with the other 19 potentially pathogenic species, including *L. longbeachae*.

### 1.5.4 *Legionella* pneumophila

*Legionella pneumophila* is responsible for approximately 80% of the notified cases of Legionellosis worldwide (Buchbinder *et al.* 2002). *L. pneumophila* is endemic to warm water environments such as cooling towers, shower heads and water fountains (Keleti *et al.* 1987) . It is transmitted from these environments via inhalation or aspiration of aerosolised *L. pneumophila* contaminated particles (Montanaro-Punzengruber *et al.* 1999).

### 1.5.5 Life cycle of *L. pneumophila*

The life cycle of *L. pneumophila* is shown in Figure 2. There are two main morphological forms of *L. pneumophila,* these are the mature intracellular form (MIF) and the replicative form (RF,) which both differentiate into each other via an intermediate form. MIFs, either free or packaged into vesicles, are the infective form of *L. pneumophila* which invade amoeba and human hosts. Amoeba can directly release vesicles containing MIFs or indirectly ciliates can release the MIFs through the addition of a packaging step (Garduño 2008).

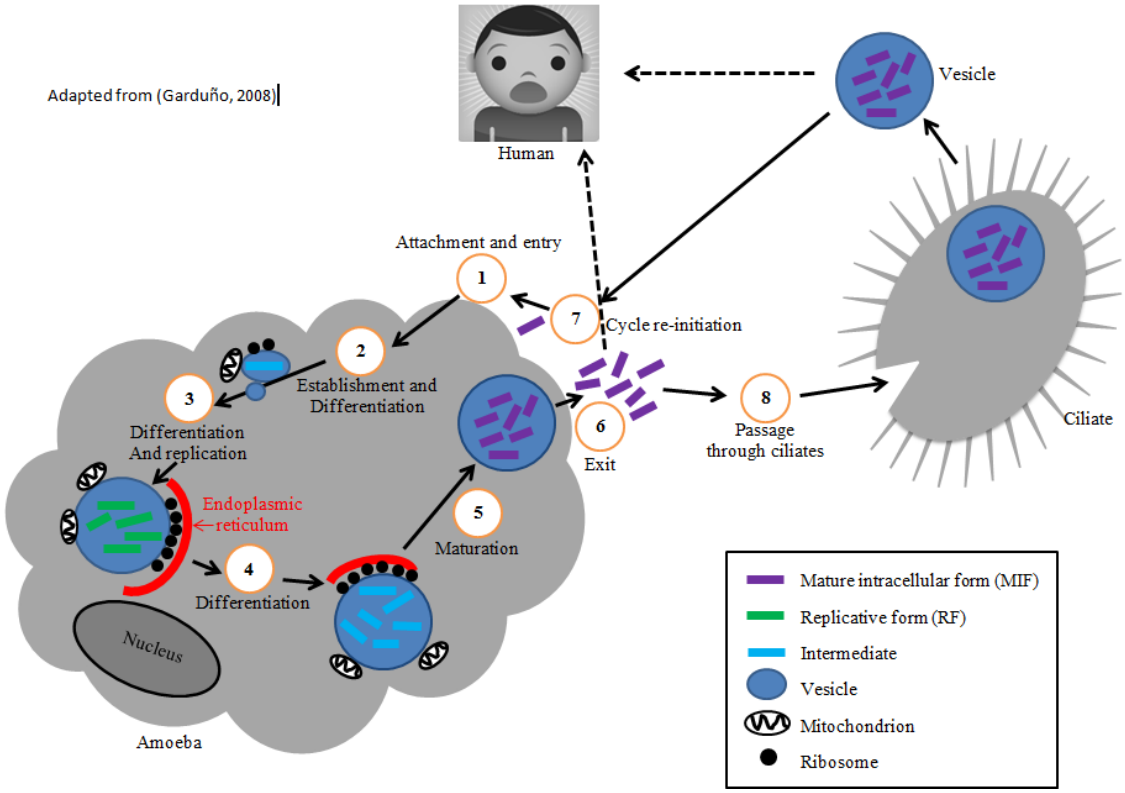


**Figure 2: The life cycle of *L. pneumophila* begins with attachment and entry into an amoeba host. This is followed by inhibition of the phagosome-lyosome fusion and alteration of organelle traffic. *L. pneumophila* replicates in a ribosome-decorated vacuole associated with the endoplasmic reticulum (ER) and acquires ER markers. After maturation is completed the mature intracellular forms (MIFs) exit the wasted amoeba and reinitiates the cycle. MIF laded vesicles may also be directly released by ciliated who are not wasted by the *L. pneumophila.***

### 1.5.6 Intracellular parasitism of protozoa by *L. pneumophila*

*L. pneumophila* is able to invade and replicate within a range of protozoan hosts (Abu Kwaik *et al.* 1998). This includes cyst forming species *Acanthamoeba* spp. (Rowbotham 1980; Tyndall and Domingue 1982; Anand *et al.* 1983; Holden *et al.* 1984; Miyamoto *et al.* 2003); *Hartmanella* spp. (Rowbotham 1986; Wadowsky *et al.* 1988); *Naegleria* spp. (Rowbotham 1980; Tyndall and Domingue 1982; Newsome *et al.* 1985); two species of the ciliate genus *Tetrahymena* (Barbaree *et al.* 1986; Fields *et al.* 1989; Kikuhara *et al.* 1994); *Balamuthia mandrillaris* (Shadrach *et al.* 2005); *Echinamoeba exundans* (Fields *et al.* 1989); *Vahlkampfia jugosa* (Rowbotham 1986); and the slime mould *Dictyostelium discoideum* (Hägele *et al.* 2000; Solomon *et al.* 2000). *L. pneumophila* has also been shown to colonise the intestinal tract of nematodes, resulting in worm death (Brassinga *et al.* 2010).

*Legionella* can infect and multiply within protozoan hosts in the absence of any other supporting nutrients (Taylor *et al.* 2009). Under adverse conditions protozoa may form cysts that are highly resistant to extreme temperatures and biocides (Aguilar-Díaz *et al.* 2011).

The presence of *L. pneumophila* within cysts contributes to the difficulties of its eradication from a contaminated water source (Barbaree *et al.* 1986; Winiecka-Krusnell and Linder 1999). Within a sewage treatment plant, where protozoan numbers are also high, the number of *Legionella* spp. was reported to be not significantly reduced by the primary and secondary treatment processes (Palmer *et al.* 1993). Studies have also demonstrated the ability of *L. pneumophila* to replicate within potable water contaminated with protozoa, with replication ceasing once the protozoa were removed (Wadowsky *et al.* 1988).

### 1.6 Nontuberculous mycobacterium (NTM)

Nontuberculous Mycobacterium (NTM) refers to Mycobacteria distinct from *Mycobacterium tuberculosis* complex (MTC, including *M. tuberculosis, Mycobacterium bovis* and *Mycobacterium africanum* and *Mycobacterium leprae*) (Schulze-Robbecke *et al.* 1992). NTM are opportunist human pathogens, causing a wide spectrum of diseases and are major public health concern, particularly in patients with reduced immune response (Covert *et al.* 1999; Falkinham *et al.* 2008).

#### 1.6.1 Mycobacterium avium complex (MAC)

The following manuscript (Whiley *et al.* 2012) was published in Journal of Applied Microbiology and details the wide range of MAC caused diseases and reviews the current literature identifying potable water sources of MAC infection.

**Citation:**

**Whiley, H., Keegan, A., Giglio, S. & Bentham, R. (2012). Mycobacterium avium complex – the role of potable water in disease transmission. *Journal of Applied Microbiology*, 113, 223-232.**

# Mycobacterium avium complex (MAC) - The role of potable water is disease transmission

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**Key Words:**

*Mycobacterium avium* complex (MAC); Nontuberculous Mycobacterium (NTM); potable water; hot tub lung

**Summary**

*Mycobacterium avium* complex (MAC) is a group of opportunistic pathogens of major public health concern. It is responsible for a wide spectrum of disease dependent on subspecies, route of infection and patients pre-existing conditions. Presently there is limited research on the incidence of MAC infection that considers both pulmonary and other clinical manifestations. MAC has been isolated from various terrestrial and aquatic environments including natural waters, engineered water systems and soils. Identifying the specific environmental sources responsible for human infection is essential in minimising disease prevalence.

This paper reviews current literature and case studies regarding the wide spectrum of disease caused by MAC and the role of potable water in disease transmission. Potable water was recognised as a putative pathway for MAC infection. Contaminated potable water sources associated with human infection included warm water distribution systems, showers, faucets, household drinking water, swimming pools and hot tub spas. MAC can maintain long term contamination of potable water sources through its high resistance to disinfectants, association with biofilms and intracellular parasitism of free living protozoa.

Further research is required to investigate the efficiency of water treatment processes against MAC and into construction and maintenance of warm water distribution systems and the role they play in MAC proliferation.

## Introduction

Nontuberculous Mycobacterium (NTM) refers to Mycobacteria distinct from *Mycobacterium tuberculosis* complex (MTC, including *M. tuberculosis, Mycobacterium bovis* and *Mycobacterium africanum* and *Mycobacterium leprae* ) (Schulze-Robbecke *et al.* 1992). More than 140 species of NTM have been described, with approximately 50 species identified as opportunistic human pathogens (Brown-Elliott *et al.* 2002). Pathogenic NTM cause a wide spectrum of disease and are a major public health concern, particularly in patients with a reduced immune response (Covert *et al.* 1999; Falkinham *et al.* 2008). *Mycobacterium avium* complex (MAC) is the most common cause of clinically significant NTM infections in developed countries including Australia (Haverkort 2003), Eastern Asia (Hong Kong, South Korea, Japan, Thailand, Singapore and Taiwan) (Simons *et al.* 2011), North America (Marras and Daley 2002) and The United Kingdom (Henry *et al.* 2004). MAC refers to *M. avium (M. avium* subspecies *avium* (MAA), *M. avium* subspecies *hominis* (MAH) and *M. avium* subspecies *paratuberculosis* (MAP)) and *Mycobacterium intracellulare.*

## Clinical presentations of MAC

### MAC infection in immunocompromised patients

Amongst immunocompromised patients, MAC is an opportunistic pathogen of major concern. In adult Human Immunodeficiency Virus (HIV) patient's living in developed countries, MAC infection is the most common opportunistic bacterial infection, with an annual frequency of 10-20% (Karakousis *et al.* 2004). Depending on patients pre-existing conditions, MAC can cause pulmonary infections,

gastrointestinal tract infections and cutaneous (skin) or soft tissue infections particularly in post surgery patients (Karakousis *et al.* 2004). Prior to the introduction of highly active antiretroviral therapy (HAART) in 1996, it was estimated that 40% of Acquired Immune Deficiency Syndrome **(**AIDS) patients infected by the organism developed disseminated infection (von Reyn *et al.* 1994). This was associated with significant mortality rates (Nightingale *et al.* 1992), as demonstrated by Kiehn *et al.* (1985) who found that from 1981 to 1984 at the Memorial Sloan-Kettering Cancer Centre in New York, 55 % (30/55) of deceased AIDS patients had disseminated MAC infections as determined through post mortem examination. In recent years the implementation of HAART has resulted in a decrease in the incidence of MAC infection. However, it still remains an important complication of AIDS, occurring in patients that do not have access to HAART or develop virological and immunological failure to HAART due to drug intolerance or the emergence of antiretroviral resistance (Wu *et al.* 2009). Not all MAC strains have the same virulence and in AIDS patients *M. avium* infection predominates over *M. intracellulare* (Guthertz *et al.* 1989). Dissemination of MAC infection can also occur in immune compromised patients without AIDS, including transplantation patients, chronic corticosteroids users and cancer patients (Wolinsky 1992).

**MAC infection in patients with no immune deficiency**

In previously healthy patients, without a history of lung disease or immunodeficiency, MAC has caused progressive lung disease leading to respiratory failure and even death (Prince *et al.* 1989). In non-AIDS patients *M. intracellulare* has been shown to have a higher pathogenicity than *M. avium* (Han *et al.* 2005), with

some studies finding *M. intracellulare* responsible for over 70% of MAC pulmonary infection in non-immunodeficient adults (Wallace *et al.* 1998; Wallace *et al.* 2002).

MAC fibrocavitary lung disease in patients with pre-existing lung conditions has occurred primarily in older men with a history of cigarette smoking and/or alcohol abuse (Field 2004). Whereas, MAC fibronodular bronchiectasis (otherwise known as "Lady Windermere's Syndrome), has been observed to occur predominately in thin elderly women, without history of lung disease or immunosuppression. Onset of bronchiectasis (permanent widening of the bronchi) is typically accompanied by presentation of multiple nodules in middle lobe and lingula lobe of the lung (Huang *et al.* 1999; Field 2004).

MAC pulmonary infection can also present as primary nodules simulating lung cancer (Gribetz *et al.* 1981; Davidoff *et al.* 2006; Teirstein 2008; Lakhanpal *et al.*). This can be a challenge for diagnosis as a solitary nodule due to MAC infection can be radiographically indistinguishable from lung cancer (Lakhanpal *et al.* 2011). There have also been reports of Pulmonary MAC infection with co-existing lung cancer (Sawai *et al.* 2008).

The severity of MAC pulmonary infection is greatly varied and the prognostic factors have not been clearly defined. A study conducted in Japan from 1999-2005 investigated 634 patients diagnosed with MAC pulmonary disease at the Saitama Cardiovascular and respiratory centre. 58.5% of patients were female, 41.5% were male, all were HIV negative and the mean age was 68.9 years. The overall 5- and 10-year mortality rates were 23.9% and 46.5% respectively. Of the deceased, 21.9% died from progression of the MAC pulmonary infection, 21.3% from pneumonia, 10.6% from lung cancer, 21.9% from non-pulmonary diseases and 13.1% from unknown causes. The specific 5- and 10-year mortality rates as a direct consequence

of the MAC pulmonary infection were 5.4% and 15.7% respectively (Hayashi *et al.* 2011). Another study conducted by The British Thoracic Society was investigating the effectiveness of different drug regimes on 170 patients diagnosed with MAC pulmonary infection. This study found the overall 5-year patient mortality rate to be 40%, but the specific 5-year mortality rate as a consequence of the MAC pulmonary infection was only 2.9% (Jenkins *et al.* 2008).

MAC hypersensitivity pneumonitis (inflammation of the alveoli within the lung caused by the body's immune response) has also been observed after inhalation of MAC contaminated aerosol, primarily from hot tubs. It was suggested that hypersensitivity to MAC, rather than infection was the likely mechanism for these respiratory illnesses (Embil *et al.* 1997; Rickman *et al.* 2002; Marras *et al.* 2005).

MAC has also been shown to cause cutaneous (skin) infections in healthy adults and children with no pre-exisiting health conditions (Kullavanijaya *et al.* 1997; Noguchi *et al.* 1998; Sugita 2000). A report by Sugita (2000) describes a healthy family of five in Japan, suffering inflammatory subcutaneous nodules and ulcerations as a result of MAC skin infections acquired from the family spa bath.

**MAC infection in children**

Cervical lymphadenitis (infection of lymph nodes) is the most common clinical manifestation of MAC infections in healthy children (Thegerstrom *et al.* 2008). The peak age of children with cervical lymphadenitis is 6 months to 2 years, which corresponds to the emergence of teeth. Soil and water have both been considered likely sources for children with MAC infection and the trauma to gums associated with erupting teeth is considered to be one of the main routes of infection

(Falkinham 2009). Treatment entails either surgical excision of affected lymph nodes or antibiotic therapy; however, surgical complications, post surgery infections and antibiotic resistance are of concern with these treatment regimes (Lindeboom *et al.* 2007). Patients treated with antibiotics may also experience reoccurrences of infection up to 7 years later (Wolinsky 1995).

A study conducted in the Netherlands from 2001-2003 estimated that the annual incidence of NTM infection in children was 77 cases per 100,000 with MAC responsible for 67% of these cases (Haverkamp *et al.* 2004). Another study conducted in Sweden reported that the average annual incidence of culture proven MAC lymphadenopathy in 1998-2003 was 4.5/100,000 children (Thegerstrom *et al.* 2008).

MAC infection is also a major concern for children with immune deficiencies and pre-existing conditions. It has been shown to cause pulmonary infection in children with cystic fibrosis (Olivier *et al.* 2003) and is a significant cause of mortality in children with AIDS. A study conducted in the United States from 1981-1995 found that out of 6778 children being treated for AIDS (as reported to the Centre for Disease Control and Prevention (CDC)), there were 441 (7.1%) confirmed cases of MAC infection. Of these cases 6% were less than 12 months old, 15% were 12-23 months, 33% were 24-71 months and 46% were 72 months or older. The MAC infection proved fatal in 338 (77%) of the 441 confirmed cases with an average of only 6 months between diagnosis and death (Lindegren *et al.* 1996).

## Crohn's Disease

It has been suggested that *M. avium* subspecies *paratuberculosis* (MAP) is a causative agent of Crohn's disease (Mishina 1996; Hermon-Taylor 2000; Chamberlin *et al.* 2001; Naser *et al.* 2004; Sartor 2005; Pierce 2009). Similarities between mycobacterium infections and Crohn's disease have been noted since the emergence of the disease, but the role of MAP in the disease's occurrence, is highly controversial and widely debated (Chamberlin *et al.* 2001). Crohn's disease is an inflammatory bowel disease which resembles some aspects of tuberculosis and leprosy (Naser *et al.* 2004). Crohn's disease can affect any part of the gastrointestinal tract from the mouth to the anus and commonly presents with abdominal pain, loss of energy and loss of weight, night sweats, mouth ulcers and joint pain. Approximately 60% of patients suffer from diarrhoea containing mucous, pus and blood and about 40% of patients with colonic Crohn's disease will require an ileostomy or colostomy, resulting in the use of an abdominal bag to collect intestinal effluent. In children, Crohn's disease has been shown to retard or prevent growth and sexual maturation (Hermon-Taylor 2000). The economic burden of Crohn's disease is also great, this includes the direct cost of surgeries, drugs, hospital care and ambulatory care, and also the indirect costs such as sick leave and early retirement. A recent study by Peng Yu *et al.* (2008) estimated that in the total economic burden of Crohn's disease in the United States and in Europe was annually $10.9-15.5 billion and €2.1-16.7 billion respectively.

**Sources of MAP infection**

As the role of MAP as the causative agent of Crohn's disease is still unknown, so too is the organisms zoonotic potential (Grant 2005). In a variety of mammals including cattle, sheep, deer, bison, monkeys and chimpanzees, MAP is the causative agent of Johne's disease (an inflammatory bowel disease) (Chamberlin *et al.* 2001). Due to the long latency period, MAP may colonise animals for years without causing clinical disease. In Europe and North America the prevalence of MAP infection in dairy cows is estimated to be between 21-70% (Hermon-Taylor 2000). Dairy cows with clinical and subclinical Johne's disease will secrete MAP into their milk and excrete in faeces (Grant 2005). MAP has previously been cultured from milk taken from dairy cows carrying the bacteria (Sweeney *et al.* 1992) and it has been suggested that meat from old dairy cows used to make minced beef for human consumption may be another source of infection (Manning and Collins 2001). The shedding of MAP into feaces can also cause contamination of surface waters from agricultural run-off, which can then enter water supplies used for drinking water (Grant 2005). MAP has demonstrated to be highly resistant to water treatment processes and has been isolated from potable water sources (Glover 1994), although the role of water in transmitting MAP to humans has not been as extensively investigated (Pierce 2009). Pierce (2009) describes a cluster of 3 Crohn's diseases patients that were linked by the tap water pipes supplying their homes; however, the presence of MAP in the tap water was never confirmed.

## Sources of MAC (excluding MAP)

MAC other than MAP are also an important cause of disease in animals such as poultry and swine; however, the zoonotic ability of the organism is still unknown (Bono *et al.* 1995). Kunze *et al.* (1992) demonstrated using serological tests that different strains of MAC affect animals and humans, and animal-to-human transmission does not occur. Conversely, Bono *et al.* demonstrated that MAC strains isolated from pigs shared various genetic characteristics to those isolated from humans and suggested that this could be one source of infection. Human to human transmission of MAC has not been observed and it is generally accepted that environmental sources are the reservoir for most human infections (Falkinham 2009). MAC has been found in a range of environmental reservoirs including soil, house dust, natural waters, potable waters and water distribution systems (Schulze-Robbecke *et al.* 1992; Morris and Harrison 2003; Norton *et al.* 2004; Vaerewijck *et al.* 2005b).

## MAC contamination of potable water

MAC has been isolated from potable water in Australia, Netherlands, USA, Finland, Zaire, Germany, United Kingdom, Canada and Japan (Tuffley and Holbeche 1980; Havelaar *et al.* 1985; von Reyn *et al.* 1993; Eaton *et al.* 1995; Peters *et al.* 1995; Hunter *et al.* 2001; Marras *et al.* 2005; Nishiuchi 2009). Studies have shown that potable water systems can have up to $10^3$ colony forming units (CFU) of planktonic MAC per 100 mL of water (Norton *et al.* 2004) and biofilm samples taken straight from water distribution pipes have demonstrated MAC counts up to $10^6$ CFU/cm$^2$ (Schulze-Robbecke *et al.* 1992).

MAC are not transient contaminants of potable water systems; they are able to grow and persist in plumbing of the distribution system. Falkinham *et al.* (2001) demonstrated this by reporting an increase in MAC numbers throughout a distribution pipelines as the distance from the treatment plant increased. This does not preclude the possibility of contamination from other sources such as dirt and soil through cracks, seals or pipeline leaks (Pedley *et al.* 2004). Previous research has also demonstrated that MAC can grow in water samples to which no additional nutrients have been added. MAC can grow in temperatures ranging from 15-45 $^{o}$C and salinities from 0-2 % Sodium Chloride (NaCl) (George *et al.* 1980). DNA fingerprinting studies have shown that single unique strains of MAC can persist for up to 41 months in a water distribution system (von Reyn *et al.* 1994; Norton *et al.* 2004).

A study conducted in 1993 used culture techniques to demonstrate the presence of MAC in 3/14 of potable water samples collected from USA, 5/11 from Finland and 1/5 from Zaire. MAC was not present in 14 of the potable water samples collected from Kenya; however, it was found in 2/17 of natural water samples (von Reyn *et al.* 1993). Eaton *et al.* (1995) also used culture methods in Uganda to determine that 3 of 7 natural water samples tested contained MAC. In the United Kingdom, MAC has been cultured from drinking water treatment and distribution systems, with 3 out of 170 (1.7%) samples testing positive for the organism (Hunter *et al.* 2001). In Australia, a study conducted from 1973-1974 isolated MAC from 32 of 141 (23%) rainwater tanks in central Queensland (Tuffley and Holbeche 1980).

**MAC infection associated with potable hot water distribution systems within buildings**

When microbes enter water distribution systems, particularly in large buildings such as hospitals, they may be incorporated within biofilms and replicate to hazardous levels (Anaissie *et al.* 2002). This process is assisted by the use of thermostatic mixing values which prevent the hot water from exceeding 45$^{o}$C at the outlet and ultimately promotes bacterial growth and biofilm formation (Cavagnino 1992).

In 1994 a study conducted in the USA investigated a hospitals potable hot water distribution system as a potential source of MAC infection in AIDS patients. Pulsed field gel electrophoresis (PFGE) of clinical isolates from 36 AIDS patients with MAC infection found that three patients were infected by the same strain that had also been isolated from the hospital hot water system where they had been treated. The three patients had no other common potential exposure sources and the MAC strain had been isolated repetitively from the hospital hot water supply for 41 months, suggesting that the hospitals potable water was the likely cause of the MAC infections (von Reyn *et al.* 1994). In two similar studies conducted in the USA, large-restriction-fragment (LRF) pattern analyses and PFGE were used to compare clinical isolate to isolates from the hospital water distribution system. A strong similarity was found with MAC isolated from patients and those isolates recovered from hospital water supplies, where it was concluded that the hospital water supply was the most likely source of the nosocomial MAC infection (Aronson 1999; Tobin-D'Angelo *et al.* 2004).

Falkinham (2011) investigated the presence of NTM within the household plumbing of patients with NTM infections. In this study both *M. intracellulare* and *M. avium*

were identified among both the clinical isolates and the environmental isolates found in patient homes. Of the 37 households sampled, 17 contain the same NTM species as was infecting the patient and 7 of these strains were determined to have the same DNA fingerprint as the respective clinical isolate. Table 1 presents the incidences of MAC infection that have been linked to the potable water source.

**MAC infection associated with showers**

It has also been postulated that an increase in shower usage over baths is responsible for the increase in NTM pulmonary infections as showers provide an ideal niche for bacterial biofilm formation. Bacteria present in biofilm are dispersed into the passing water and aerosolised by the outlet (shower head or faucet). Shower/faucet aerosols are small enough to sufficiently carry bacteria deep into the respiratory system (Zhou 2007). In 2002 MAC was isolated from a showerhead in the home of an American women infected with MAC. The woman had no known risk factors for MAC infection. DNA fingerprinting demonstrated that the MAC isolated from the showerhead biofilm had a clonal relationship with the patient's MAC isolate (Falkinham *et al.* 2008). In 2007 a study was conducted in Japan investigating the presence of MAC in the homes of MAC infected out-patients. Of the 29 residential bathrooms of patients with pulmonary MAC infection, 15 (52 %) were positive for MAC, including 14 samples from bathtub inlets and 3 from showerheads. Out of the 15 positive MAC isolates, 7 (47 %) had identical PFGE profiles when compared to their respective clinical isolates (Nishiuchi 2009).

**MAC infection associated with Spa Baths/Hot tubs**

Hot tubs or Spas provide an optimum potable water environment for the growth and proliferation of MAC (Embil *et al.* 1997; Kahana *et al.* 1997; Rickman *et al.* 2002; Tobin-D'Angelo *et al.* 2004; Marras *et al.* 2005; Thomson *et al.* 2008) as steam and bubbles produce contaminated aerosols that facilitate the inhalation of MAC (Rickman *et al.* 2002) . The warm temperature of the environment promotes growth and inhibits disinfection (at temperatures greater than 29$^{o}$C the solubility of chlorine in water is reduced, resulting in a decrease in disinfectant properties) (Pelletier *et al.* 1988). The contamination of spas/hot tubs is often exacerbated by owners frequently not cleaning or changes filters as often as is recommended (Rickman *et al.* 2002).

Predominately MAC contamination of hot tubs/spa, resulting in illness, has occurred indoors within households/private residences (Tsuchiya 2008). Although, Lumb *et al.* (2004) reported four cases in Australia of MAC related lung disorders associated with two poorly maintained outdoor spas. Pulmonary illness as a result of exposure to MAC contaminated hot tubs or spas is often referred to as “hot tub lung” and includes pulmonary infection and hypersensitivity pneumonitis (Embil *et al.* 1997). There are also reports of MAC contamination of hot tubs/spas causing cutaneous MAC infection (Sugita 2000).

**MAC infection associated with swimming pools**

Contamination of swimming pools is another potential source of MAC infection. Koschel *et al.* (2006) describe a patient in Germany with MAC hypersensitivity pneumonitis as a result of cleaning an indoor swimming pool. MAC was identified from the swimming pool water and by ceasing exposure to the swimming pool, the

patient recovered within 3 months without any pharmacological treatment. Population based studies in the United States have also identified that Lifeguards who have a long-term exposure to swimming pool aerosols have an increased rate of hypersensitivity pneumonitis (Lynch *et al.* 1992; Rose *et al.* 1998).

Previous studies conducted in Finland and Italy found NTM to be present in 71% (5/7 pools sampled) (Iivanainen *et al.* 1999) and 88.2% (60/66 samples taken from 12 pools) (Rose *et al.* 1998) respectively. In these studies MAC was not detected; however, both relied on culture techniques, which may not account for the presence of viable non-culture MAC cells (Archuleta *et al.* 2002).

**MAC resistance to disinfectants**

MAC has a high resistance to both chemical disinfectants and Ultra Violet (UV) irradiation, which allows for continuous contamination of water sources (von Reyn *et al.* 1994; Lee *et al.* 2010). Taylor *et al.* (2000) demonstrated that the Chlorine $CT_{99.9\%}$ (calculated disinfection = disinfectant concentration in ppm x contact time in minutes that results in 99.9% inactivation) for *M. avium* strains were 580-2,300 times greater than those for *Escherichia. coli* Similarly they found the $CT_{99.9\%}$ values of chlorine dioxide and ozone for *M. avium* were respectively 50-fold and 100-fold greater than those for *E. coli.* A recent study also demonstrated that MAC is highly resistant to both low pressure and medium pressure UV irradiation. The UV dose to achieve a 1 $\log_{10}$ inactivation of *M. avium* was much higher than required to achieve the same 1 $\log_{10}$ inactivation of *E.coli* (UV doses of 6 $mJ/cm^2$ and 1.5$mJ/cm^2$ respectively). In fact, *M. avium* has a greater resistance to UV that most protozoan

parasites and is equally resistant as many pathogenic viruses (except rotaviruses and adenoviruses) (Shin *et al.* 2008).

**Intracellular parasitism of protozoa by MAC**

There has been cumulative evidence to indicate that MAC is able to reside in potable water within free-living amoeba (Salah *et al.* 2009). Experimentally MAC have been shown to replicate within the amoeba *Acanthamoeba castellanii* (Cirillo 1997), *Dictoystelium discoideum* (Skriwan and Skriwan 2002) and *Acanthamoeba polyphaga* (Salah and Drancourt 2010) providing a level of protection to the bacteria.

MAC that has replicated within protozoa display an enhanced resistance to antibacterial agents and an increased virulence when compared to culture grown isolates in both chicken (Falkinham *et al.* 2001) and mouse (Cirillo 1997) models of infection. It has also been demonstrated that MAC can include their presence during protozoan cyst formation, which would enable persistent contamination of potable water (Steinert *et al.* 1998). Protozoan cysts are a shielded, encapsulated form of protozoa, which are able to survive against unfavourable environmental conditions including water treatments and low nutrients (Taylor *et al.* 2009). MAC encapsulated within cysts are shielded from these damaging external factors until the environmental conditions become more favourable. Salah and Drancourt (2010) demonstrated that the preferential localisation of MAC within an amoebal cyst is within the exocyst. It has been suggested that the position of MAC within the exocyst is advantageous as it allows for a rapid escape from the cyst in favourable environmental conditions.

## MAC Detection methods

A limitation of reviewing the current literature on the presence of MAC in potable water is the absence of standardised detections methods. The detection of MAC from environmental samples using traditional culture techniques is hampered by their specific culture requirement and long incubation time (12-16 weeks) (Fang *et al.* 2002). The decontamination of water samples is vital to prevent plates from becoming overgrown by faster growing organisms; however, these same decontamination processes have been shown to impair viability. Another limitation is that the use of molecular methods, such as gene sequencing, are required for identification of NTM species (Hussein *et al.* 2009).

Molecular based techniques such as polymerase chain reaction (PCR) and quantitative real time PCR (qPCR) are often used as an alternative to traditional culture Methods. These methods are advantageous as they have a faster turnaround time and higher specificity (Hussein *et al.* 2009). The disadvantage of PCR-based methods is their inability to discriminate between live and dead cells; however, recent studies using pre-treatment's with propidium monoazide or ethidium monoazide have shown promising results in removing non-viable cells prior to DNA extraction ensuring only viable cells are detected (Kralik *et al.* 2010).

## Conclusion

The clinical manifestations of MAC infection are broad and complex depending on a patient's pre-existing conditions. Clinical presentations include fibrocavitary lung disease (Field 2004), fibronodular bronchiectasis (Huang *et al.* 1999), pulmonary

nodules simulating lung cancer (Lakhanpal *et al.* 2011), hypersensitivity pneumonitis (Marras *et al.* 2005), cutaneous skin (Sugita 2000) and soft tissue infection particularly in post surgery patients (Karakousis *et al.* 2004), cervical lymphadenitis in children (Thegerström *et al.* 2008), gastrointestinal tract and disseminated infection in immune compromised patients (Nightingale *et al.* 1992), and debatably Crohn's disease (Naser *et al.* 2004). Currently, there is limited research on the incidence of MAC infection that considers all clinical manifestations of the bacteria. This suggests that globally the incidence of illness as a result of exposure to MAC could be much higher and henceforth its impact on public health may be much greater than currently considered.

Potable water has been identified as a putative pathway of MAC infection (Bono *et al.* 1995; Tobin-D'Angelo *et al.* 2004; Marras *et al.* 2005; Falkinham *et al.* 2008). MAC is highly resistant to disinfection processes and can maintain long term contamination of potable water systems. Contamination of potable water with MAC is augmented by the use of hot water distribution systems which allow the bacteria to proliferate. This is particularly of concern in hospitals where immune compromised patients may be exposed to the contaminated water (Tobin-D'Angelo *et al.* 2004). It is advisable that high risk patients use commercially available point of use filters on all taps and showers to prevent this pathway of MAC infection (Sheffer *et al.* 2005). Other than water distribution systems, showers systems were also identified as a source of MAC infection. In these instances MAC was able to proliferate within the showerhead biofilm (Falkinham *et al.* 2008). Self purging showers are commercially available and are used to prevent biofilm build up within showerheads; the effectiveness of flushing showers against MAC biofilm formation still needs investigation.

*In situ* testing of water distribution systems would be beneficial in determining efficiency of the current water treatment processes and disinfecting agents against MAC. Further research is also required to identify and determine the effect that different environmental factors within water distribution systems have on the proliferation of MAC; this includes plumbing materials, temperature and water flow rate. Additionally, the ability of MAC to replicate intracellularly within protozoan hosts needs to be investigated as a possible mechanism enabling survival of disinfection processes and long term contamination of potable water systems. The species of protozoan hosts would have to be identified and disinfection processes optimised to ensure their removal.

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**Declarations of interest**

The authors report no declarations of interest.

**Table 2: (Manuscript Table 1) Incidences of MAC clinical cases which have been linked to a potable water source.**

| Year | Country | Source of MAC | Linked to clinical disease | Extra info | Reference |
|---|---|---|---|---|---|
| 1980 | Australia | Rainwater tanks | - | Serotypes isolated have been associated with pulmonary disease | (Tuffley and Holbeche 1980) |
| 1985 | Netherlands | public whirlpools and swimming pools | - | MAC was found in water samples taken from pools in hotels, recreational parks and camping grounds and whirlpools in sauna institutes, fitness clubs and recreational parks | (Havelaar *et al.* 1985) |
| 1988 | USA | Water samples from dialysis centres | - | NTM (including MAC) were detected in water from 95/115 randomly selected dialysis centres (83%) | (Carson *et al.* 1988) |
| 1988 | USA | Hospital drinking water (sampled from both hot and cold water taps) | - | 14/34 (44%) (11 hot water sample and 3 cold water) of water sample taken from 2 vacant hospital floor tested positive for MAC | (du Moulin *et al.* 1988) |
| 1988 | USA | Potable water from diagnostics laboratory and four hospital wards | - | Possibly caused contamination of phenol red solution used for processing urine specimens and the deionized tap water used to make mycobacteria diagnostic reagent, resulting in false positive patient results. | (Graham *et al.* 1988) |
| 1993 | USA, Finland, Zaire, | Drinking water (including piped municipal and private drinking water) | - | Water supply samples were collected from wells, hot and cold municipal water supplies, showers, and standpipes. | (von Reyn *et al.* 1993) |
| 1994 | USA | Hospital hot water | 36 AIDS patients with disseminated MAC | PFGE identified same strains from hospital | (von Reyn *et al.* |

| Year | Country | Source of MAC | Linked to clinical disease | Extra info | Reference |
|---|---|---|---|---|---|
| | | system | infection | water distribution system and patient clinical isolates | 1994) |
| 1994 | USA | Reservoir water, hospital drinking water and residential drinking water | | MAC was identified from 2/13 water reservoirs, 5/58 residentials drinking water and 7/10 hospitals drinking water | (Glover 1994) |
| 1995 | Germany | Drinking water | | MAC was isolated from both hot and cold drinking water from 1/ 2 hospitals tested and 0/4 residential home | (Peters *et al.* 1995) |
| 1995 | USA | Potable water from homes of HIV patients | - | 4/528 (0.76%) water samples tested positive using PCR for MAC | (Yajko *et al.* 1995b) |
| 1997 | Canada | Residential Hot tub | MAC pulmonary disease in a healthy 20 yr old female | RFLP anaylsis identified same strain from hot tub and patient | (Kahana *et al.* 1997) |
| 1997 | Canada | Hot tub | 5 cases of hypersensitivity pneumonitis | Was not confirmed by identification, but MAC was the suspected causative organism | (Embil *et al.* 1997) |
| 1999 | USA | Potable water from 3 homes, 2 commercial buildings and 8 hospitals | Compared to Clinical isolates of AIDS and non-AIDS patients with MAC infection | Large-restriction-fragment (LRF) environmental isolates had various degrees of genetic relatedness to clinical isolates recovered from 17 patients. | (Aronson 1999) |
| 2000 | Japan | Residential hot tub | Cutaneous MAC infection in 5 healthy family members | MAC isolated from family hot tub's filter | (Sugita 2000) |
| 2000 | Japan | Residential Showerhead and bathtub inlets of MAC | MAC pulmonary disease | PFGE identified same MAC strain in showerhead and bathtub inlets as clinical isolate | (Nishiuchi 2009) |

| Year | Country | Source of MAC | Linked to clinical disease | Extra info | Reference |
|---|---|---|---|---|---|
| | | pulmonary infection patients | | | |
| 2000 | Japan | 24hr circulating and filtering hot bath | 3 cases of cutaneous MAC infection | 5/ 32 (15.6%) water samples, 22/29 (75.8%) sponge filters and 20/32 (62.5%) filter samples tested positive by culture for *M.avium* | (Saito *et al.* 2000) |
| 2001 | Japan | 24hr circulating and filtering hot bath | 1 case of pulmonary MAC infection in a 21yr old HIV negative woman | PFGE identified same MAC strain in hot bath as clinical isolate | (Watando *et al.* 2001) |
| 2001 | United Kingdom | Potable water treatment and distribution systems | - | 3/170 (1.7%) tested positive for MAC using culture | (Hunter *et al.* 2001) |
| 2001 | USA | 8 Potable water distribution systems | - | 8/351(2.3%) water samples taken from 8 distribution systems and 16/55 biofilm samples taken from the pipes tested positive for MAC | (Falkinham *et al.* 2001) |
| 2002 | USA | Hot Tub | hypersensitivity pneumonitis in 2 patients | MAC isolated from patients and hot tub. Discontinuation of hot tub use, without antimycobacterial therapy, led to prompt improvement in symptoms, pulmonary function, and radiographic abnormalities, | (Rickman *et al.* 2002) |
| 2003 | Japan | 24hr circulating and filtering hot bath | 1 case of pulmonary MAC infection in an immunecompetent 65 year old woman | RLFP identified same MAC strain in hot tub as clinical isolate | (Takahara *et al.* 2003) |
| 2004 | USA | Hospital water distribution system | 35 Patients with confirmed MAC pulmonary infection | PFGE identified same strains from hospital water distribution system and patient clinical isolates | (Tobin-D'Angelo *et al.* 2004) |
| 2004 | USA | Home spa pool | Three cases of MAC related lung disorders | MAC was isolated from 2 home spa pool water | (Lumb *et al.* 2004) |

| Year | Country | Source of MAC | Linked to clinical disease | Extra info | Reference |
|---|---|---|---|---|---|
| | | | were associated with the 2 contaminated home spa pools | and filters. Inadequate disinfection had allowed MAC to accumulate to concentrations of up to 4.3 x $10^4$ CFU/mL | |
| 2005 | Canada | Patients residential shower | hypersensitivity pneumonitis | Identical strain from shower and clinical isolate were identified using PFGE analysis | (Marras *et al.* 2005) |
| 2005 | Japan | 24hr circulating and filtering hot bath | 1 case of hypersensitivity pneumonitis | MAC cultured from hot bath | (Kenmotsu *et al.* 2005) |
| 2006 | Germany | Residential Swimming Pool | 1 case of hypersensitivity pneumonitis | MAC isolated from swimming pool water | (Koschel *et al.* 2006) |
| 2006 | USA | Drinking water sampled from cold water taps in medium sized public or commercial buildings | - | MAC was identified from 30/139 samples of cold tap water and was persistent for up to 26 months | (Hilborn *et al.* 2006) |
| 2008 | Japan | Residential Hot tub | 1 case of hypersensitivity pneumonitis | MAC cultured from hot tub | (Tsuchiya 2008) |
| 2008 | USA | Residential showerhead of MAC patient | 1 patient with MAC pulmonary infection | DNA fingerprinted demonstrated same strain as clinical | (Falkinham *et al.* 2008) |
| 2009 | USA | Drinking water from patient homes | Potential cause of a cluster of Crohn's disease | A cluster of Crohn's disease was identified by a common tap water pipe supplying patient homes | (Pierce 2009) |
| 2009 | USA | Potable water | A cluster of 3 patients with Crohn's disease. Linked by drinking water | Was not confirmed by identification by MAP was the suspected causative organism | (Pierce 2009) |
| 2010 | USA | Drinking water | - | 88% (29/33) water samples and 76% (25/33) | (Beumer *et al.* 2010) |

| Year | Country | Source of MAC | Linked to clinical disease | Extra info | Reference |
|---|---|---|---|---|---|
| | | sampled from homes and commercial buildings | | biofilm samples (swabbed from tap faucet) were positive for MAP | |
| 2010 | USA | Hospital water collected from the bronchoscopy preparation Laboratory | Several *M. avium* and *M. intracellulare* isolates from water samples in the bronchoscopy laboratory had rep-PCR patterns matching those of patient bronchoscopy isolates. Five of the 22 (23 %) *M. avium* patient bronchoscopy isolates and 42 of the 56 (75 %) *M. intracellulare* patient bronchoscopy isolates could have been due to contamination from the water supply. | Bronchoscopes and the filters used for washing them, water and biofilm samples collected from bronchoscopy preparation laboratory tested positive for MAC | (Falkinham 2010) |

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### 1.6.2 MAC and reuse water

NTM has been identified as a potential pathogen of concern present in reuse water (Toze 2006b; Castillo-Rodal *et al.* 2012). However, there is currently limited knowledge regarding the presence of MAC in reuse water and this is the first study to investigate the profile of MAC at multiple points along large scale reuse water distribution pipelines.

## 1.7 Detection methods

### 1.7.1 *Legionella* spp. and *L. pneumophila* enumeration

For this study, real time Polymerase Chain Reaction (qPCR) was the method chosen for *Legionella* spp. and *L. pneumophila* enumeration. The rationale for this is explained in the following publication (Whiley and Taylor 2014) which was published in Critical Reviews in Microbiology.

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# *Legionella* detection by culture and qPCR: comparing apples and oranges

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**Keywords:**

*Legionella* spp.; *L. pneumophila*; public health; risk assessment; environmental samples; culture; qPCR; PCR

**Abstract**

*Legionella* spp. are the causative agent of Legionnaire's disease and an opportunistic pathogen of significant public health concern. Identification and quantification from environmental sources is crucial for identifying outbreak origins and providing sufficient information for risk assessment and disease prevention. Currently there are a range of methods for *Legionella* spp. quantification from environmental sources, but the two most widely used and accepted are culture and real-time polymerase chain reaction (qPCR). This paper provides a review of these two methods and outlines their advantages and limitations. Studies from the last 10 years which have concurrently used culture and qPCR to quantify *Legionella* spp. from environmental sources have been compiled. 26/28 studies detected *Legionella* at a higher rate using qPCR compared to culture, whilst only 1 study detected equivalent levels of *Legionella* spp. using both qPCR and culture. Aggregating the environmental samples from all 28 studies, 2856/3967 (72%) tested positive for the presence of *Legionella* spp. using qPCR and 1331/3967 (34%) using culture. The lack of correlation between methods highlights the need to develop an acceptable standardised method for quantification that is sufficient for risk assessment and management of this human pathogen.

**Introduction**

*Legionella* is a genus of opportunistic pathogens of significant public health concern (Borges *et al.* 2012). It is the causative agent of Legionellosis, which collectively refers to Legionnaires' disease and Pontiac fever (Fields *et al.* 2002). Legionnaires' disease is a serious atypical bacterial pneumonia; whereas Pontiac fever is a self-

limiting febrile illness (Neil and Berkelman 2008). There have been no reports of human to human transmission of *Legionella* spp. (Khweek *et al.* 2013) and inhalation or aspiration of contaminated aerosols is the most commonly accepted mechanism of infection (Cianciotto 2001). Community and nosocomial cases of Legionellosis are typically associated with cooling towers (Nhu Nguyen *et al.* 2006), hot water systems (Leoni *et al.* 2005, Goetz *et al.* 1998), potable water (Stout 1992), spa pools (Benkel *et al.* 2000), decorative water fountains (Haupt *et al.* 2012) and potting mix (O'Connor *et al.* 2007).

*Legionella* spp. are difficult to control in environmental sources due to their resistance to disinfectants (Kim *et al.* 2002), association with biofilms (Murga *et al.* 2001) and parasitism of protozoan hosts (Thomas *et al.* 2004). Environmental surveillance and monitoring of *Legionella* spp. is crucial for evaluating risk and identifying control strategies (Cristino *et al.* 2012). This requires a quick and accurate method for detecting and enumerating *Legionella* spp. in environmental sources (Declerck *et al.* 2006).

Currently there are several methods for *Legionella* spp. detection and enumeration including: culture (Bopp *et al.* 1981); PCR (Mahbubani *et al.* 1990); qPCR (Behets *et al.* 2007); Fluorescent *in situ* hybridisation (FISH) (Deloge Abarkan *et al.* 2007); solid phase cytometry (Aurell *et al.* 2004); optical wavelight spectroscopy (Cooper *et al.* 2009); Enzyme-Amplified Electrochemical Detection with DNA probe (Miranda-Castro *et al.* 2007) and Surface plasmon resonance immunosensor (Oh *et al.* 2003). Many national bodies have adopted guidelines which use culture methods as the standard; however, the development of more rapid techniques highlights the need for reassessment of guidelines. This review will compare culture and qPCR,

which are the two methods which have gained prominence as the most widely used and accepted by analytical labs (Lee *et al.* 2011, Krøjgaard *et al.* 2011).

**Detection of *Legionella* by culture methods**

Due to the microbial complexity of environmental samples, isolating *Legionella* spp. by culture methods presents a range of challenges, which have been addressed by the development of specific agar formulations and sample treatments (Bopp *et al.* 1981). Sample collection protocols, sampling location and storage will not be specifically addressed in this manuscript; however, in its own right the sampling method used may exert a significant impact upon the likelihood of detection *Legionella spp*. in the environment (Asadi *et al.* 2011, CDC 2005).

The requirement for sample treatment is generally contingent on the properties of the environmental source. Potable water and water from hot water reservoirs often requires less destructive sampling techniques than samples collected from cooling towers and potting mix as they are generally less microbially complex (Joly *et al.* 2006, Steele *et al.* 1990). For potable water, it may be sufficient to simply filter concentrate 1 L of water to ensure that sufficient microbial flora is present before plating (Fiume *et al.* 2005). Samples collected from chlorine treated water sources should be dosed with 0.5 mL of 0.1 N sodium thiosulfate per 1 L to neutralize residual disinfectants (CDC 2005).

Samples from cooling towers, potting mix, reuse water distribution systems and non-potable sources generally require either heat or acid treatment to reduce the microbial content of the sample before plating (Bopp *et al.* 1981). As *Legionella* is thermally tolerant up to 63 °C (Fliermans *et al.* 1981) samples may be heat treated to

reduce the content of competitive bacteria and fungi in a sample. Commonly this treatment comprises 30 minute exposure to 50 °C (Leoni and Legnani 2001, Roberts *et al.* 1987). However, increasing exposure time or temperature further may reduce *Legionella* cultivability, particularly at temperatures above 60 °C (Rogers *et al.* 1994).

More commonly, water samples are acid treated using an adaptation of methods developed by Bopp *et al.* (1981). In this procedure water samples are either filter concentrated, or centrifuged and resuspended to produce a concentrate of the original sample. This concentrate is then diluted in a HCl-KCl buffer of pH 2.2 and incubated for ~15 minutes before plating. To further reduce interfering microbial growth, plates may be incubated under a microaerophillic in a candle jar or under a 2.5% $CO_2$ atmosphere at 35 °C (CDC 2005).

For laboratory culture, *Legionella* spp. requires relatively complex culture media in order to multiply. This requires specific additions to standard nutrient media, including L-cycseine, arginine, isoleucine, leucine, threonine, valine, methionine, phenylalanine, tyrosine, and serine (Pine *et al.* 1979). The addition of trace elements iron, calcium, cobalt, copper, magnesium, manganese, molybdenum, nickel, vanadium, and zinc has also been shown to stimulate the growth of *Legionella* species in culture (Warren and Miller 1979, Reeves *et al.* 1981).

Several agar formulations exist, with slight differences in selectivity and growth characteristics for different *Legionella* species. Buffered charcoal yeast extract agar (BCYE) is the most commonly used for general growth and maintenance of *Legionella* spp. and contains 0.1% α-ketoglutarate and a range of supportive amino acids and micro-nutrients (Feeley *et al.* 1979, Pendland *et al.* 1997, Roberts *et al.* 1987, CDC 2005, Ta *et al.* 1995). A modification of this agar containing 1% albumin

(ABCYE) has been shown to slightly enhance the recovery and growth of *L. micdadei* and *L. bozemanii* (Morrill *et al.* 1990).

A range of antimicrobial compounds may be added to the BCYE agar base, which are designed to reduce the growth of competing bacteria and fungi without altering the growth of *Legionella* spp. This range of compounds incorporates vancomycin, polymyxin B and cycloheximide and will often include glycine to reduce the growth of glycine sensitive Gram negative bacteria (Wadowsky and Yee 1981). Treated environmental samples may also be plated onto selective media deficient in L-cysteine to serve as a negative control for *Legionella* spp. growth.

The most significant barriers to quantitative, reproducible enumeration of *Legionella* spp. using culture arises from two distinct problems; the growth of unwanted microorganisms which obscure identification (Bopp *et al.* 1981) and the presence of viable but non-culturable (VBNC) *Legionella* spp. (Shih and Lin 2006). *Legionella* which have replicated intracellularly (within marcophagic hosts) are morphologically distinct from other *Legionella* cells (Al-Bana *et al.* 2013). These cells have thickened outer membranes, greater resistance to environmental and chemical stresses, lower metabolic rates and readily enter a VBNC state in water. Hence the number of *Legionella* cells detected using culture immediately post-parasitisation of amoebic hosts may be distinctly lower than *Legionella* in different life-cycle stages. Chang *et al.* (2007) also demonstrated that *L. pneumophila* became VBNC after starvation in nutrient free water for 33–40 days. They also demonstrated that heat disinfection at temperatures 60$^{o}$C or higher for between 5 and 30 minutes caused *L. pneumophila* to become completely unculturable but a large number of cells remained viable as determined by LIVE/DEAD BacLight bacterial viability kit (Molecular Probe, Eugene, OR, USA). The disinfection efficiency of heat treatment

and chlorine was also significantly reduced the longer a cell had undergone starvation. A similar study by Alleron *et al.* (2008) demonstrated that *L. pneumophila* treated with 1-10 mg/L of monochloramine became unculturable on BCYE; however, 28.8-29.4% of cells were viable as determined by the LIVE/DEAD BacLight kit. *Legionella* recovery from environmental sources has been shown to be enhanced by passage through amoebic hosts (La Scola *et al.* 2001, Rowbotham 1983). It has also been demonstrated that non cultureable *L. pneumophila* can be resuscitated by coculture with *Acanthamoeba polyphaga* (García *et al.* 2007) and *Acanthamoeba castellanii* (Steinert *et al.* 1997). However this adds further time to the isolation process and at best only allows for a qualitative presence/absence assessment of *Legionella* presence in a sample. Sample holding time also exerts a significant impact upon *Legionella* recovery by culture, with enumerated *Legionella* changing by up to 50% within 6 hours and up to 2 $\log_{10}$ difference after 24 hours (McCoy *et al.* 2012).

If culturable *Legionella* is present its slow growth rate often leads to plate overgrowth by competing organisms with more rapid generation times (Steele 1990, Bopp *et al.* 1981, Alary and Joly 1992). Plates often require at least 5-7 days before *Legionella* colonies become visible, at which point the density of competing organisms often either renders enumeration infeasible or completely obscures the surface of culture plates (Bopp *et al.* 1981, Leoni and Legnani 2001). Once *Legionella* colonies are visible, their positive identification is often primarily visual and/or confirmed by latex agglutination (Sathapatayavongs *et al.* 1983). However these methods in their own right present limitations, as noted by Carvalho *et al.* (2007) who demonstrated that from 20 colonies presenting the characteristic *Legionella* "ground glass" appearance on BYCE GVPC agar, all 20 were negative

when tested using latex agglutination and fluorescent antibody assays but were confirmed to be *Legionella* when sequencing of 16S rDNA was carried out.

As *Legionella* is a pathogen of public health concern, the consequences of reporting false negatives or underreporting the concentration of *Legionella* may be serious. The potential harm caused by the failure to detect and treat systems containing *Legionella* may be ultimately deemed greater than the cost of presumptively treating/cleaning systems where results are equivocal. As culture results tend to underestimate the presence of *Legionella* in water systems, it may be better suited to use culture as an adjunct to molecular detection rather than an alternative.

**qPCR enumeration**

qPCR is an alternative method for rapid *Legionella* spp. enumeration from environmental samples (Joly *et al.* 2006). It simultaneously amplifies and quantifies a target DNA sequence (Templeton *et al.* 2003), giving the number of genome units (GU) per litre. An equivalence with the number of CFU has not been established and the results obtained are highly dependent upon the method used and the sample composition (Wellinghausen *et al.* 2001).

The rapid turn-around time and sensitivity of qPCR is advantageous when compared to traditional culture methods (Yaradou *et al.* 2007). The main limitation is a tendency to overestimate due to the amplification of non-viable or "dead" cells (Delgado-Viscogliosi *et al.* 2009). DNA within environmental samples can be very stable and may persist for extended lengths of time (Nocker *et al.* 2007). Josephson *et al.* (1993) demonstrated that in biofilm DNA from non-viable cells persisted from days to weeks depending on the microbial consortium present. Some studies have

shown that pre-treatments with ethidium monoazide (EMA) and propidium monoazide (PMA) prior to DNA extractions enable amplification of viable cells only (Chang *et al.* 2009, Delgado-Viscogliosi *et al.* 2009, Chen and Chang 2010, Qin *et al.* 2012). When exposed to light EMA and PMA bind to DNA that is not protected by a cell membrane and prevents its amplification and hence enumeration by qPCR. These methods have not been optimised for differing sample types and their reliability and accuracy of these results is still debated (Hein *et al.* 2006). Pisz *et al.* (2007) demonstrated that EMA was not effective in preventing the amplification of non-viable cells within biofilm samples, and suggested that the presence of extracellular polymeric substances could interfere with either the DNA binding or the photo-activation of EMA. Other studies have shown EMA to penetrate intact cells, with the extent of EMA uptake dependant on bacterial species and EMA concentration (Flekna *et al.* 2007, Kobayashi *et al.* 2009). Conversely if the concentration of EMA or PMA is too low insufficient free DNA will be bound resulting in further confounding unknowns (Fittipaldi *et al.* 2011).

Another difficultly with qPCR is the presence of environmental compounds inhibiting the qPCR reaction (Brooks *et al.* 2004). However, conducting 1:10 dilutions of DNA extracts has been shown to be effective at reducing inhibitors and enabling quantification of target DNA (Ballard *et al.* 2000). There are also a range of commercially available kits which contain components that may aid in the removal of qPCR inhibitors from DNA extracted from target samples (Wilson 1997).

**Comparison of current literature**

Publications from the last 10 years which concurrently used culture and qPCR to detect *Legionella* spp. from environmental samples were collated in Table 1. Papers published in English and between the years 2003-2013 were included. Clinical and *in situ* experiments, including artificially spiked environmental samples were not included in this review.

Some studies which compared PCR (not qPCR) and culture were included in Table 1 if they provide a particular point of interest; however, for the basis of comparison the authors will only use the results obtained from the 28 studies which specifically enumerated *Legionella* using both culture and qPCR concurrently.

When the results of these studies are aggregated it becomes apparent that culture is more likely to underreport the presence of *Legionella* in water samples, with only 1/28 studies reporting higher detectable *Legionella* using culture and qPCR. In contrast 25/28 studies reported higher detectable levels of *Legionella* using qPCR and 1 study reported equivalent results using both methods. On a sample per sample basis, samples analysed concurrently by qPCR and culture were approximately 50% more likely to return a positive result by qPCR, with 2856/39673 (72%) of all samples positive by qPCR and 1331/3967 (34%) of samples positive by culture.

The one study by Levi *et al.* (2003) which reported higher detectable *Legionella* using culture compared to qPCR could be explained by the high limit of detection (800 CFU/L) of the qPCR method used in this study. Four of the studies noted the presence of qPCR inhibitors (Behets *et al.* 2007, Brooks *et al.* 2004, Parthuisot *et al.* 2010, Yaradou *et al.* 2007), which either couldn't be removed or required additional sample processing to allow for qPCR detection. Whilst one study noted complete

disparity between results, reporting 5 samples as positive by culture, 5 samples positive by qPCR, but no samples returning a positive result by both culture and qPCR (Hsu *et al*. 2009).

## Conclusion

*Legionella spp*. continues to exist as a public health concern; an ongoing risk assessment focus and an obstacle for cooling tower operators and facility managers. The discrepancies between testing procedures highlights the requirement for adopting a standard method for *Legionella spp*. detection in environmental samples. This review identifies the numerous inconsistencies between culture and qPCR enumeration, with studies from the last decade reporting a 50% difference between methods. International consensus is required to develop a universality accepted testing protocol to ensure consistency of results for both research purposes and risk assessment and management legislation.

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## Declarations of interest

The authors report no declarations of interest.

**Table 3: (Manuscript Table 1) Comparison of published studies from 2003-2013 using qPCR and culture enumeration of *Legionella* spp. from environmental samples.**

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| United Kingdom<br>100 environmental samples (68 from various hospitals, 32 from private domestic water supplies and 4 from external sites)<br><br>20 hospital and environmental water samples known to be *L. pneumophila* culture-positive. | 4/100 (4%) were positive for *L. pneumophila.*<br><br>14/20 (70%) were positive for *L. pneumophila.* | 14/100 (70%) were positive for *L. pneumophila.*<br><br>All previously tested positive for *L. pneumophila* (however, this was not done concurrently). | All 10 samples positive for *L. pneumophila* by culture and not qPCR contained ≤200CFU/L. Also one culture negative sample was repeatedly qPCR-positive. | (Levi *et al.* 2003) |
| USA and Canada<br>114 Water and biofilm samples from both warm and colder groundwater | | 29/87 (33.3%) were positive for *Legionella* spp. | 40/87 (46.0%) were positive for *Legionella* spp. using PCR. 61/87 (70.6%) samples were observed to contain PCR inhibitors. | (Brooks *et al.* 2004) |
| Belgium<br>46 water samples (25 shower water, 12 industrial water, 4 natural water, 5 tap water) | | 19/46 (41%) were positive for *Legionella* spp. | 26/46 (56%) and 45/46 (98%) of samples were positive for *Legionella* spp. using PCR with LEG 225-LEG 858 and JFP-JRP primer respectively. | (Devos *et al.* 2005) |
| Italy<br>124 tap water samples from hospitals and private residence | 111/124 (90%) were positive for *Legionella* spp. using TaqMan qPCR method. | 77/124 (62%) were positive for *Legionella* spp. | 88/124 (71%) were positive for *Legionella* spp. using nested PCR method. | (Fiume *et al.* 2005) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| Spain<br>water samples<br>40 water samples (spas, hotels, hospitals, residential tap water.) | 17/40 (43%) were positive for *Legionella* spp. using qPCR.<br>15/40 (38%) were positive for *Legionella* spp. using PMA-qPCR. | 12/40 (30%) were positive for *Legionella* spp. | qPCR and PMA-qPCR (propidium monoazide pre-treatment) compared. | (Yáñez *et al.* 2005) |
| France<br>Hot water system<br>128 samples tested in laboratory1 | 117/128 (91.4%) were positive for *Legionella* spp. and of these 89/128 (69.5%) were quantifiable. 94/122 (77%) were positive for *L. pneumophila* and 55/122 (45.1%) were quantifiable. | 55/128 (43%) were positive for *Legionella* spp. and 27/128 (21.1%) of these had ≥250 CFU/L. | | |
| 92 samples tested in laboratory 2 | 76/92 (82.6%) were positive for *Legionella* spp. and of these 56/92 (60.9%) were quantifiable. 57/91 (62.6%) were positive for *L. pneumophila* and of these 31/91 (34.1%) were quantifiable. | 41/92 (44.6%) were positive for *Legionella* spp. and 24/92 (26.1%) of these had ≥250 CFU/L. | | (Joly *et al.* 2006) |
| 36 Cooling tower samples | 36/36 (100%) were positive for *Legionella* spp. and 35/36 (97.2%) of these were quantifiable. 31/33 (93.9%) were positive for *L. pneumophila* and 19/33 (57.6%) were quantifiable. | 9/36 were positive for *Legionella* spp. (25%) and of these 8/36 had ≥250 CFU/L (22.2%). | | |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| Netherlands<br>68 commercial bottled mineral waters | | 0/68 were positive for *L. pneumophila.* | 6/68 (9%) were positive for *L. pneumophila* using PCR. | (Klont *et al.* 2006) |
| Netherlands<br>16 surface water samples<br><br>97 treated surface and ground water samples | 14/16 (86%) were positive for *Legionella* spp.<br><br>97/97 (100%) were positive for *Legionella* spp. | 0/16 were positive for *Legionella* spp.<br><br>0/97 were positive for *Legionella* spp. | | (Wullings and van der Kooij 2006) |
| Belgium<br>30 tap water samples (showers taps, eyewash stations, fire sprinklers and recirculation loops) | 14/30 (46.7%) were positive for *L. pneumophila* at concentrations ranging from $4.4 \times 10^3$ to $3.1 \times 10^5$ GU/L. | 10/30 (33%) were positive for *L. pneumophila* at concentrations ranging from $4.0 \times 10^2$ to $9.0 \times 10^3$ CFU/L. | 12/30 (40%) contained qPCR inhibitors. | (Behets *et al.* 2007) |
| Brazil<br>River water | | *Legionella* spp. was not identified by culture. | 24 sequences were identified as more closely related to *Legionella* spp. than any other genera.<br>Some colonies present on *Legionella* selective medium had the typical "ground glass" appearance of *Legionella* but the results of the latex test kit and fluorescent antibody assay were negative for *Legionella* spp. | (Carvalho *et al.* 2007) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| Netherlands<br>357 water samples from 250 public buildings | 311/357 (87.1%) were positive for *Legionella* spp. | 8/357 (2.2%) were positive for *Legionella* spp. | | (Diederen *et al.* 2007) |
| Spain<br>25 potable water<br><br>30 cooling tower samples | 21/25 (84%) were positive for *Legionella* spp.<br><br>28/30 (93%) positive for *Legionella* spp. | 13/25 (52%) were positive for *Legionella* spp.<br><br>22/30 (73%) positive for *Legionella* spp. | 19/25 (76%) of potable water samples and 30/30 (100%) cooling tower samples were positive for *Legionella* spp. using PCR. | (Yáñez *et al.* 2007) |
| France<br>136 hot water system samples collected form 55 sites.<br><br>46 cooling tower water samples collected from 20 sites. | 87/132 (65.9%) *L. pneumophila* positive and of these 50/132 (37.9%) were quantifiable (>25 GU/reaction).<br><br>28/46 (60.9%) *L. pneumophila* positive and of these 20/46 (43.5%) were quantifiable (>25 GU/reaction). | 59/132 (44.7%) were positive for *L. pneumophila* and of these 40/132 (30.3%) were quantifiable (>250 CFU/L).<br><br>15/46 (32.6%) were positive for *L. pneumophila* and of these 9/46 (19.6%) were quantifiable (>250 CFU/L). | 4 hot water system samples and 3 cooling tower samples contained inhibitors which would not be removed using dilution and were not used for the study. | (Yaradou *et al.* 2007) |
| Japan<br>130 Hot water samples from 40 public buildings (hotels, offices, schools, stores, assembly halls) | 24/130 (18%) were positive for *Legionella* spp.<br>In 21 of these samples concentrations of *Legionella* ranged from $1.7 \times 10^5$ – $2.6 \times 10^{11}$ GU/L. | 5/130 (4%) were positive for *Legionella* spp.<br>Concentration of *Legionella* ranged from $1.8 \times 10^2$ – $8.3 \times 10^3$ CFU/L. | 17 of the 40 sites were positive by qPCR. | (Edagawa *et al.* 2008) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| Kuwait<br>263 swabs from hospital faucets and showerheads.<br><br>20 water samples from hospital water storage facility | | 61/263 (23%) were positive for *Legionella* spp.<br><br>0/20 (0%) were positive for *Legionella* spp. | 61/263 (23%) were positive for *Legionella* spp. using PCR.<br><br>6/20 (30%) were positive for *Legionella* spp. using PCR. | (Qasem *et al.* 2008) |
| France<br>120 Hot water samples collected a hospital (including hot water tanks, showers and taps) | 57/120 (47.5%) were positive for *L. pneumophila* (only 35/120 (29.1%) were quantifiable). | 31/120 (25.8%) were positive for *L. pneumophila.* | Samples taken from 6 different distribution systems within the hospital. | (Morio *et al.* 2008) |
| USA<br>276 environmental water samples | 138/276 (50%) tested positive for *L. pneumophila.* | 99/276 (35.8%) tested positive for *L. pneumophila.* | 97/140 (69.3%) positive samples tested both culture and qPCR positive. 41/140 (29.3%) tested qPCR-positive only. 2/140 (1.4%) tested culture-positive only. | (Nazarian *et al.* 2008) |
| Switzerland<br>46 brands of potting mix | 41/46 (89.1%) tested positive for *Legionella* spp. | 21/46 (45.7%) tested positive for *Legionella* spp. | | (Casati *et al.* 2009) |
| Japan<br>25 water samples (9 public spas and 16 from model spa systems) | 16/25 (64%) tested positive for *Legionella* spp.<br><br>10/25 (40%) tested positive for *Legionella* spp. after pre-treatment | 14/25 (56%) were positive for *Legionella* spp. | Results with pre-treatment of 1, 5, and 10µg/mL of Ethidium monoazide (EMA) are also presented. | (Chang *et al.* 2009) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| | with 20µg/mL of Ethidium monoazide (EMA). | | | |
| Taiwan<br>34 samples from 13 spring resorts (source water, facility water and waste water) | 5/34 (15%) tested positive for *Legionella* spp. | 5/34 (15%) tested positive for *Legionella* spp. | No samples test both qPCR and culture positive. Therefore a total of 10/34 samples were positive by any method. | (Hsu *et al.* 2009) |
| Italian<br>76 water samples cold water tap, boiler room, shoers, hot water recycling) from 19 hotels. | 56/76 (74%) were positive for *Legionella* spp.<br>37/76 (49%) were positive for *L. pneumophila.* | 32/76 (42%) were positive for *Legionella* spp.<br>19/76 (25%) were positive for *L. pneumophila.* | | (Bonetta *et al.* 2010) |
| Spain<br>20 cooling tower water samples<br>30 hot water system samples | 10/20 (50%) were positive for *L. pneumophila* using *mip* Primers.<br>9/20 (45%) were positive for *L. pneumophila* using *dot* primers.<br>19/30 63%) were positive for *L. pneumophila* using *mip* primers.<br>20/30 (67%) were positive for *L. pneumophila* using *dot* primers. | 8/20 (40%) were positive for *L. pneumophila.*<br>14/30 (46.7%) were positive for *L. pneumophila.* | | (Fittipaldi *et al.* 2010) |
| Canada<br>101 samples from 95 whirlpool spas | 72 /101 (72%) were positive for *Legionella* spp. | 27/101 (27%) were positive for *Legionella* spp. | | (Guillemet *et al.* 2010) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| in semi-public establishments. | 42 /101 (42%) could be enumerated with concentrations ranging from 1000 (limit of detection) to 6.1 x $10^7$ GU/L. | 14/101 (14%) could be enumerated with concentrations ranging from 250 to 3.5 x $10^5$ CFU/L. | | |
| France<br>Water samples from the Tech River | 72/72 (100%) were positive for *Legionella* spp. | 15/72 (20.8%) were positive for *Legionella* spp. | qPCR inhibitors were present in all non-diluted DNA extracts. | (Parthuisot *et al.* 2010) |
| Indonesia<br>9 cooling tower water samples (from 9 cooling towers) | 7/9 (78%) were positive for *Legionella* spp. | 0/9 were positive for *Legionella* spp. | | (Yasmon *et al.* 2010) |
| China<br>51 samples from recreation spring facilities (source water, waste water and facility water). | | 3/51 (5.9%) were positive for *Legionella* spp. | 5/51 (10%) were positive for *Legionella* spp. using PCR.<br>11/51 (22%) were positive using free living amoeba (FLA) culture method. | (Huang *et al.* 2011a) |
| Taiwan<br>47 Carbonate springs<br>17 Mud springs<br>4 Sodium bicarbonate spring | | 7/47 (15%) were positive for *Legionella* spp.<br>0/17 was positive for *Legionella* spp.<br>1/4 (25%) were positive for *Legionella* spp. | 10/47 (21%) were positive for *Legionella* spp.<br>5/17 (29%) were positive for *Legionella* spp.<br>1/4 (25%) were positive for *Legionella* spp.<br>Using PCR method. | (Huang *et al.* 2011b) |
| Denmark | 84/84 (100%) were positive for *Legionella* spp. | 43/84 (51%) were positive for *Legionella* spp. | | (Krøjgaard *et al.* 2011) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| 84 Hot water samples | 75/84 (89%) were positive for *L. pneumophila.* | | | |
| 6 European Countries<br>232 cooling tower water samples<br><br>506 hot and cold water samples | 221/232 (95%) were positive for *Legionella* spp. and 114/232 (49%) were positive for *L. pneumophila*.<br><br>495/506 (98%) were positive for *Legionella* spp. and 417/506 (82%) were positive for *L. pneumophila*. | 73/232 (31%) were positive for *Legionella* spp. and 62/232 (27%) were positive for *L. pneumophila*.<br><br>278/506 (55%) were positive for *Legionella* spp. and 249/506 (49%) were positive for *L. pneumophila*. | 7 laboratories from 6 countries participated in this study. This study also discussed the potential of qPCR results predicting culture results. | (Lee *et al.* 2011) |
| France<br>185 water samples from 9 cooling water systems at 4 different sites. | 181/185 (98%) were positive for *Legionella* spp. and 111/185 (60%) were positive for *L. pneumophila*. | 102/165 (62%) were positive for *Legionella* spp. and 101/164 (63%) were positive for *L. pneumophila*. | | (Touron-Bodilis *et al.* 2011) |
| Spain<br>25 samples from hotel potable hot water systems.<br><br>35 cooling tower water samples. | 15/25 (60%) were positive for *Legionella* spp.<br><br>28/35 (80%) were positive for *Legionella* spp. | 13/25 (52%) were positive for *Legionella* spp.<br><br>22/35 (63%) were positive for *Legionella* spp. | Regression analysis showed that average concentration of *Legionella* spp. determined by qPCR were 20-fold higher that the culture determined concentrations. | (Yáñez *et al.* 2011) |
| Netherlands<br>Two un-chlorinated drinking water supplies (water and Biofilm samples) | *Legionella* spp. detected at ranges of $7.6 \times 10^{1}$ to $3.9 \times 10^{2}$ GU/L. | No *Legionella* spp. were detected from culture in any of the water or biofilm samples. | | (Wullings *et al.* 2011) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| Kuwait<br>Domestic water samples<br>82 samples from bathroom faucets and showerheads.<br><br>51 from kitchen taps<br><br>71 from hot/cold water tanks | 45/82 (55%) were positive for *Legionella* spp.<br><br>19/51 (37%) were positive for *Legionella* spp.<br><br>21/71 (30%) were positive for *Legionella* spp. | 27/82 (33%) were positive for *Legionella* spp.<br><br>15/51 (29%) were positive for *Legionella* spp.<br><br>6/71 (8.5%) were positive for *Legionella* spp. | | (Al-Matawah *et al.* 2012) |
| China<br>216 cooling tower water<br><br>132 piped water samples<br><br>90 hot spring water samples | 67/216 (31%) were positive for *Legionella* spp.<br><br>32/132 (24%) were positive for *Legionella* spp.<br><br>84/90 (93%) were positive for *Legionella* spp. | 57/216 (26%) were positive for *Legionella* spp.<br><br>25/132 (19%) were positive for *Legionella* spp.<br><br>49/90 (93%) were positive for *Legionella* spp. | 43/216 (20%) cooling tower samples, 27/132 (20%) piped water and 64/90 (71%) of hot spring samples were positive for *Legionella* spp. using PCR. | (Qin *et al.* 2012) |
| USA<br>Chloraminated potable water distribution systems | 27/90 (30%) of water samples were positive for *Legionella* spp. at average concentration of 186.6 ± 458.2 GU/L and 4/90 (4%) were positive for *L. pneumophila* at average concentrations of 9.8 ± 4.4 | 1/56 (2%) were positive for *Legionella* spp. at density of 2 CFU/mL. | | (Wang *et al.* 2012) |

| Source | Detection Method | | Comments | Reference |
|---|---|---|---|---|
| | qPCR | Culture | | |
| | GU/mL. | | | |
| Taiwan<br>Puzih River water samples | 59/150 (39%) were positive for *Legionella* spp. and 3/150 (2%) were positive for *L. pneumophila*. | 14/150 (9%) were positive for *Legionella* spp. and 14/150 (9%) were positive for *L. pneumophila*. | | (Tung *et al.* 2013) |

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### 1.7.2 Fluorescent *in situ* hybridisation (FISH)

Fluorescent *in situ* hybridisation (FISH) with rRNA targeted oligonucleotide probes has been frequently used to examine bacterial communities within environmental samples (Zarda *et al.* 1997; Daims *et al.* 2001). It is also an alternative method for *Legionella* detection and enumeration (Buchbinder *et al.* 2002). Free rRNA may degrade faster than DNA and the detection of rRNA has been previously used as an indicator of viability (Garcia-Armisen and Servais 2004; García-Hernández *et al.* 2012). However, there is growing evidence that demonstrates the long term persistence of RNA under certain conditions, which discounts its value as an indicator of viability (Tolker-Nielsen *et al.* 1997; McKillip *et al.* 1998; Fordyce *et al.* 2013). Additionally the use of FISH for enumeration purposes is also time consuming due to the time required to manually count organisms or the rigorous validation requirements of using microscope software programs for counting, making it unsuitable for routine enumeration. A greater number of published studies use qPCR compared to FISH for *Legionella* enumeration in environmental samples. Ultimately in this study qPCR was chosen for *Legionella* enumeration, due to the high specificity, fast turnaround time and it ability to enumerate viable non-culturable cells.

### 1.7.3 MAC detection

Culture based methods of MAC enumeration from environmental samples are time consuming as it can take up to 6 weeks for growth on agar plates (Eaton *et al.* 1995). Mycobacterium species identification is then done using Ziehl-Neelson staining method to visualise the acid-fast colonies and mycobacterium morphology (Chen *et*

*al.* 2012). Identification of specific mycobacterium species can be then completed using molecular techniques such as PCR (Wilton and Cousins 1992) or 16s rRNA sequencing (Han *et al.* 2005). Due to the time requirements of culture based methods (and since it was chosen for *Legionella* enumeration), qPCR was chosen as the method for MAC enumeration.

# Chapter 2: General methods

This chapter describes the general methods and apparatus used throughout this project including: maintenance of stock cultures, real time PCR quantification of *Legionella* spp., *L. pneumophila* and MAC cloning of PCR products into plasmid, coliform quantification and general statistical tests used for analysis of results. Where appropriate, the rational for a particular method is discussed. The characteristics of each distribution system and sampling procedures are described in subsequent chapters.

## 2.1 Media Composition

### 2.1.1 Reasoner´s 2A agar (R2A)

R2A Agar (Oxoid) was prepared according to manufacturer's instructions.

Briefly 18.1 g R2A Agar was dissolved in 1 L Milli-Q® Water and sterilised by autoclaving at 121$^{o}$C for 30 min. Agar was then aseptically poured into sterile petri dishes and stored at 4$^{o}$C.

### 2.1.2 *Legionella* BCYE GVPC Media

*Legionella* Charcoal Yeast Extract Agar **(**CYE) (Oxoid) was prepared according to manufacturer's instructions with the addition of the BCYE *Legionella* growth supplement (Oxoid), containing ACES buffer/potassium hydroxide, ferric

pyrophosphate, L-cysteine HCl and α-ketoglutarate, and the *Legionella* GVPC selective supplement (Oxoid), containing glycine, vancomycin hydrochloride, polymyxin B sulphate and cycloheximide.

Briefly 14.5 g CYE agar base was dissolved in 450 mL Milli-Q® Water and sterilised by autoclaving at 121$^{o}$C for 30 min. Agar was allowed to cool in a water bath to 50$^{o}$C and then 1 vial of BCYE *Legionella* growth supplement and 1 vial of *Legionella* GVPC selective were reconstituted in sterile water and aseptically added to the medium, which was then gently mixed. The pH was adjusted to 6.9 ± 0.1 with 0.2 M potassium hydroxide (KOH) and agar was aseptically poured into sterile petri dishes and stored at 4$^{o}$C.

### 2.1.3 LB broth with 100 µg/mL ampicillin

LB Broth (Oxoid) was prepared according to manufacturer's instructions with the addition of 100 µg/mL ampicillin. Briefly 25.0 g LB Broth was dissolved in 1 L Milli-Q® Water and sterilised by autoclaving at 121$^{o}$C for 30 min. Broth was allowed to cool to <50$^{o}$C prior to the addition of 100 µg/mL ampicillin, then stored at 4$^{o}$C.

### 2.1.4 LB agar plates with 100 µg/mL ampicillin and 100 mM IPTG and 40 mg/mL X-Gal

LB Agar (Oxoid) was prepared according to manufacturer's instructions with the addition of 100 µg/mL ampicillin. Briefly 40.0 g LB Agar was dissolved in 1 L Milli-Q® Water and sterilised by autoclaving at 121$^{o}$C for 30 min. Agar was

allowed to cool in a water bath to 50$^{o}$C prior to the addition of 100 µg/mL ampicillin. Agar was then aseptically poured into sterile petri dishes and stored at 4$^{o}$C. Immediately prior to use with *E. coli* containing targeted plasmid, 40 µL 100 mM IPTG and 40 mg/mL X-Gal (Invitrogen) were spread onto the LB agar plates.

## 2.2 Bacterial cultures used as controls in this study

### 2.2.1 Mycobacterium avium

A clinical isolate of M. avium (Figure 3) was provided by SA Pathology (Adelaide, Australia) and streaked onto R2A Agar and incubated at 37oC under aerobic conditions for 3-4 weeks. Prior to incubation plates were sealed with parafilm to prevent drying out.

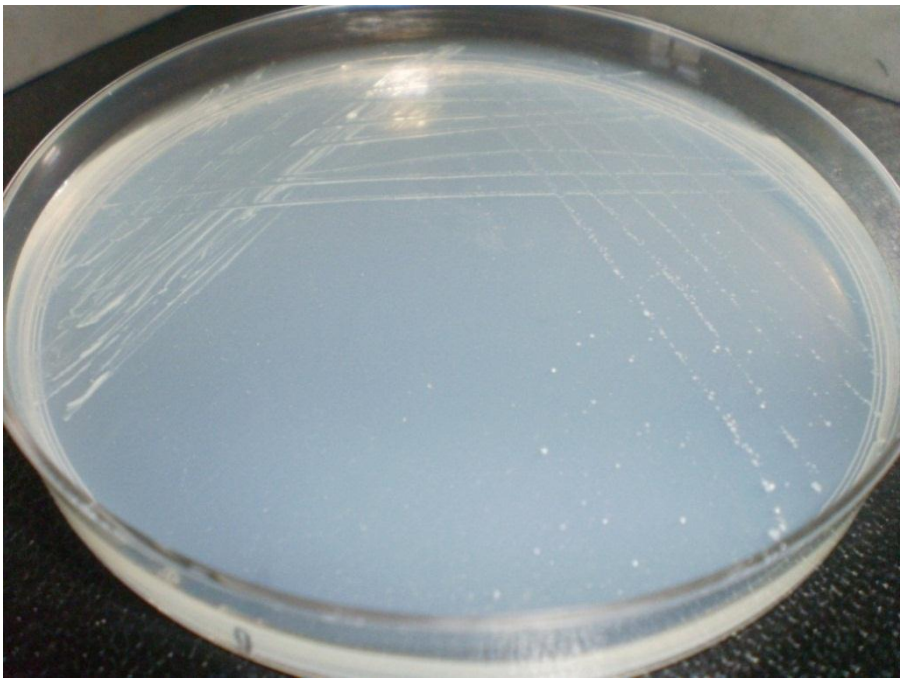


**Figure 3: Image of Mycobacterium avium on R2A agar after 21 days of incubation at 37$^{o}$C. Colonies can be seen after 10 days of incubation and appear as very small, fine white colonies.**

### 2.2.2 *Legionella* pneumophila

A bronchial isolate of *L. pneumophila* SG1 (Figure 4) was provided by SA Pathology (Adelaide, Australia) and streaked onto *Legionella* BCYE GVPC agar and incubated at 37$^{o}$C under aerobic conditions for 5-7 days.

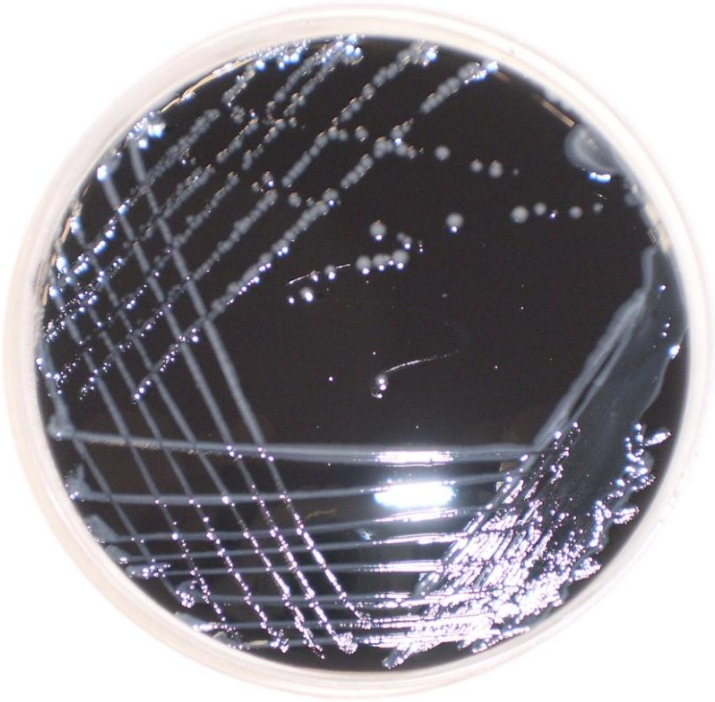


**Figure 4: Image of *Legionella pneumophila* SG1 on BCYE GVPC Agar after incubation at 37$^{o}$C for 5 days. Colonies appear opalescent and when viewed under a dissecting microscope they are seen to be convex with an internal ground-glass appearance.**

### 2.2.3 Long term storage of bacterial cultures

Aliquots of 1 mL 50:50 glycerol:water solutions were pipetted into 1.5 mL glass GC vials and autoclaved at 121$^{o}$C for 30 min. Individual bacterial colonies, taken from agar plates, were suspended in the sterile glycerol solution and stored at -80$^{o}$C. When required, cultures were removed from storage and the surface of the frozen

culture was aseptically scraped using a sterile loop and streaked onto a relevant agar plate.

### 2.3 Total coliforms and *Escherichia coli* enumeration

*E. coli* and total coliforms were quantified using the defined substrate method of Colilert™ (IDEXX Laboratories, Maine, USA). The standard method was followed and 100 mL of sample water and Colilert™ substrate was added to a sterile 100 mL vessel and swirled until the substrate dissolved. The whole sample was then decanted into a Quanti-tray/2000®. Trays were heat sealed with a Quanti-tray sealer and incubated for 24 hr at 35ºC. The most probable number (MPN) of total coliforms /100 mL was then determined by counting wells in which the sample had turned yellow and referring to the Quanti-tray/2000 MPN table. The most probable number (MPN) of *E. coli* /100 mL was determined by counting fluorescing wells under a UV lamp (365 nm) and referring to the Quanti-tray/2000 MPN table.

### 2.6 DNA extraction

DNA extraction was conducted using the BIO-RAD Aquadien$^{TM}$ Kit following manufacturer's instructions.

Briefly, 350 mL sample water (unless otherwise specified) was vacuum filtered onto a 0.4 µm polycarbonate membrane filter. The membrane filter was then carefully folded into a cone using sterile tweezers and placed into a cryotube containing 2 mL R1 (included in the BioRad DNA extraction kit). The cryotube was vortexed for 20 s prior to incubation in a 95$^{o}$C water bath for 15 min. The cryotube was vortexed again

for 20 s then the membrane filter was carefully removed using sterile tweezers and thrown away. The sample was left at room temperature for 20 min to allow the R1 solution to pellet. A 500 µL aliquot of the supernatant was then pipetted into a purification column and centrifuged for 10 min at 6000 g. The liquid in the collector vial was discarded and another 500 µL of the supernatant was transferred into a purification column and centrifuged for 10 min at 6000 g. A 100 µL aliquot of R2 solution (included in the BioRad DNA extraction kit) was added to the purification column. The collector vial was then discarded and the purification column was covered with a new collector vial and both the purification column and collector vial were turned upside down and centrifuged for 3 min at 1000 g. The purification column was then discarded and the collector vial containing 100 µL of extracted DNA and was stored at -20$^{o}$C.

### 2.7 Real time polymerase chain reaction (qPCR)

All qPCR reactions were conducted on a Corbett Research RotorGene Thermal Cycler 6000. For quality control, every qPCR run contained a positive control (containing 20 µL of the respective mastermix and 5 µL of the relevant plasmid containing purified PCR product) and a non-template control (containing 20 µL of respective mastermix and 5 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen)). For the results of a run to be included the positive control had to be present with the correct melt peak and the non-template control had to be negative. A positive non-template control was due to contamination and results from this run were not included and the samples were rerun.

### 2.7.1 *Legionella* spp. qPCR

*Legionella* spp. were enumerated by a qPCR method previously described (Giglio *et al.* 2005) using SYTO9 as the intercalating fluorescent dye and JFP and JRP primers shown in Table 4. The reagents added to each qPCR reaction are presented in Table 5 and the cycling conditions in Table 6. For each reaction the melt curve was analysed and a positive *Legionella* spp. was confirmed with a $T_m$ of 88±1$^oC$ (see Figure 5). A standard curve was created using purified PCR product which had been cloned into a plasmid to prevent deterioration (2.7.4 qPCR positive controls). The concentration of the DNA plasmid was determined (see 2.7.7 Calculating DNA concentration) and then a one in ten series of dilutions were created using a Corbett Research Liquid Handling System®.

**Table 4: Primer sequences used in this study**

| Name | Target organism | Sequence | Reference |
|---|---|---|---|
| JFP | *Legionella* spp. | 5'-AGGGTTGATAGGTTAAGAGC-3' | Giglio *et al.* 2005 |
| JRP | *Legionella* spp. | 5'-CCAACAGCTAGTTGACATCG-3' | Giglio *et al.* 2005 |
| Mip99F | *L. pneumophila* | 5'-TGTCTTATAGCATTGGTGCC-3' | Giglio *et al.* 2005 |
| Mip213R | *L. pneumophila* | 5'-CAATTGAGCGCCACTCATAG-3' | Giglio *et al.* 2005 |
| MACF | MAC | 5'-CCCTGAGACAACACTCGGTC -3' | Park *et al.* 2000 |
| MACR | MAC | 5'-ATTACACATTTCGATGAACGC-3' | Park *et al.* 2000 |

**Table 5: Concentrations of reagents used for *Legionella* qPCR assays and amount of working stock added to each 25 µL reaction in order to achieve this concentration**

| Reagent | Final concentration | Concentration of working stock | volume added to 25 µL reaction (µL) |
|---|---|---|---|
| Water | - | - | 12.55 |
| $MgCl_2$ (Invitrogen) | 2.5 mM | 50 mM | 1.25 |
| PCR Buffer (Invitrogen) | 1X | 10X | 2.5 |
| dNTPs (Invitrogen) | 0.2 mM | 10 mM | 0.5 |
| SYTO9 (BioRad) | 2.5 µM | 25 µM | 2.5 |
| Platinum Taq DNA polymerase (Invitrogen) | 1 U | 5 U/µL | 0.2 |
| DNA sample | - | - | 5 |
| *JFP* primer | 0.3 µM | 10 µM | 0.75 |
| *JRP* primer | 0.3 µM | 10 µM | 0.75 |

**Table 6: Cycling conditions for *Legionella spp*. qPCR conducted on Corbett Rotor Gene Thermal Cycler 6000.**

| Hold | Cycling 40X | | | Melt |
|---|---|---|---|---|
| | **Step 1** | **Step 2** | **Step 3** | Ramping rate of 1°C/60 s from 75°C-95°C |
| 95°C | 94°C | 60°C | 72°C | |
| 5.00 min | 10 sec | 20 sec | 20 sec | |

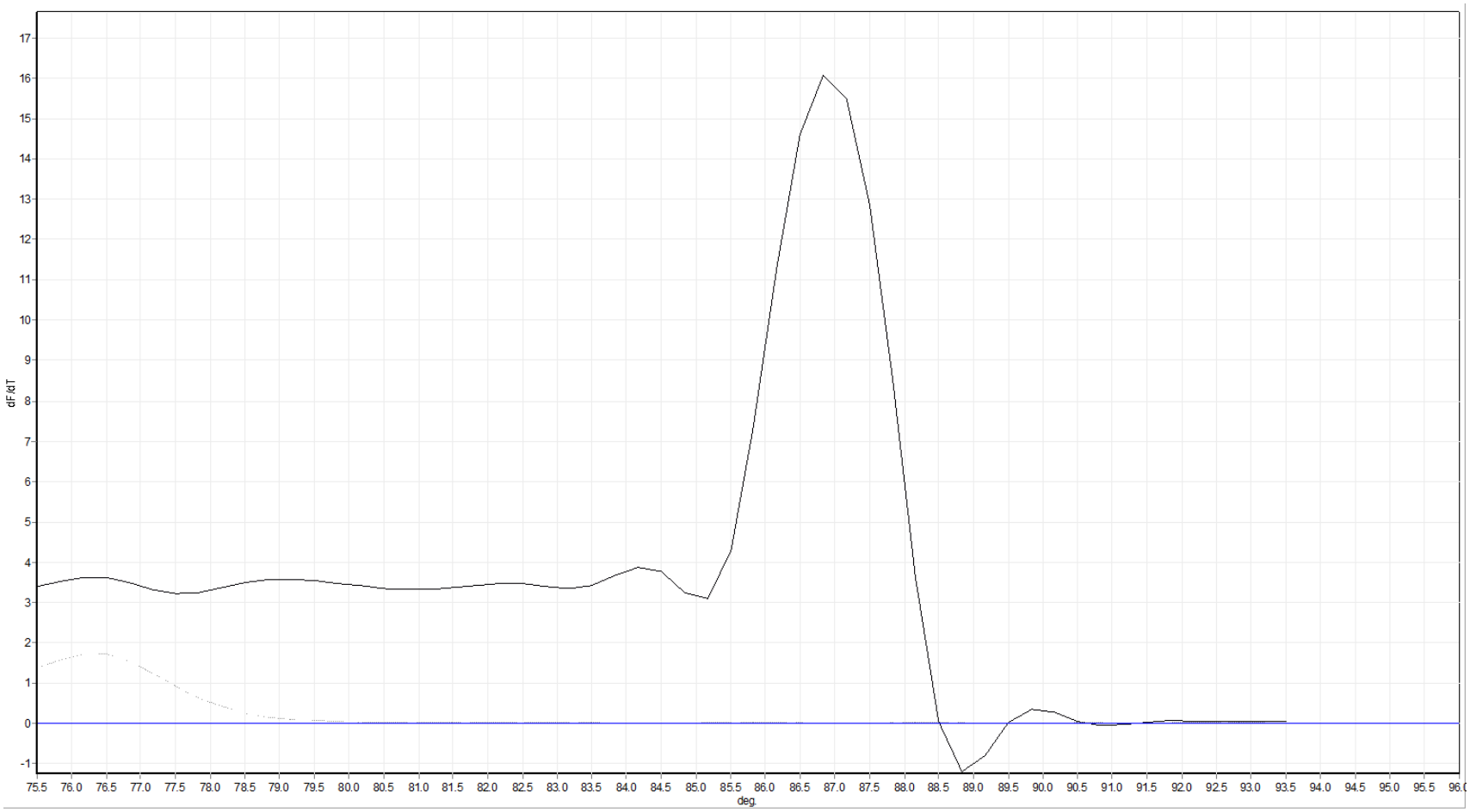



**Figure 5: *Legionella* qPCR melt curve. *Legionella spp*. positive shown by a Tm of 88±1°C (solid line) and Non-template control (dashed line).**

### 2.7.2 qPCR quantification of *Legionella pneumophila*

*L. pneumophila* were enumerated by a qPCR method previously described (Giglio *et al.* 2005) using SYTO9 as the intercalating fluorescent dye and mip 99F and mip 213R primers shown in Table 4. The reagents added to each qPCR reaction are presented in Table 7 and the cycling conditions in Table 8. For each reaction the melt curve was analysed and a positive *L. pneumophila* was confirmed with a $T_m$ of 82.5±1$^{o}$C. A standard curve was created using purified PCR product which had been cloned into a plasmid to prevent deterioration (see 2.7.4 qPCR positive controls). The concentration of the DNA plasmid was determined (see 2.7.4 Calculating DNA concentration) and then a one in ten series of dilutions were created using a Corbett Research Liquid Handling System®.

**Table 7: Concentrations of reagents used for *L. pneumophila* qPCR assays and amount of working stock added to each 25 µL reaction in order to achieve this concentration**

| Reagent | Final concentration | Concentration of working stock | Volume added to 25 µL reaction (µL) |
|---|---|---|---|
| Water | - | - | |
| $MgCl_2$ (Invitrogen) | 2.5 mM | 50 mM | 1.25 |
| PCR Buffer (Invitrogen) | 1X | 10X | 2.5 |
| dNTPs (Invitrogen) | 0.2 mM | 10 mM | 0.5 |
| SYTO9 (BioRad) | 2.5 µM | 25 µM | 2.5 |
| Platinum Taq DNA polymerase (Invitrogen) | 1 U | 5 U/µL | 0.2 |
| DNA sample | - | - | 5 |
| Mip99F primer | 0.5 µM | 10 µM | 1.25 |
| MIP213R primer | 0.5 µM | 10 µM | 1.25 |

**Table 8: Cycling conditions for *L. pneumophila* qPCR conducted on Corbett Rotor Gene Thermal Cycler 6000.**

| Hold | Cycling 40X | | | Melt |
|---|---|---|---|---|
| | **Step 1** | **Step 2** | **Step 3** | Ramping rate of 1°C/60 s from 75°C-95°C |
| 95°C | 94°C | 60°C | 72°C | |
| 5.00 min | 20 sec | 20 sec | 25 sec | |

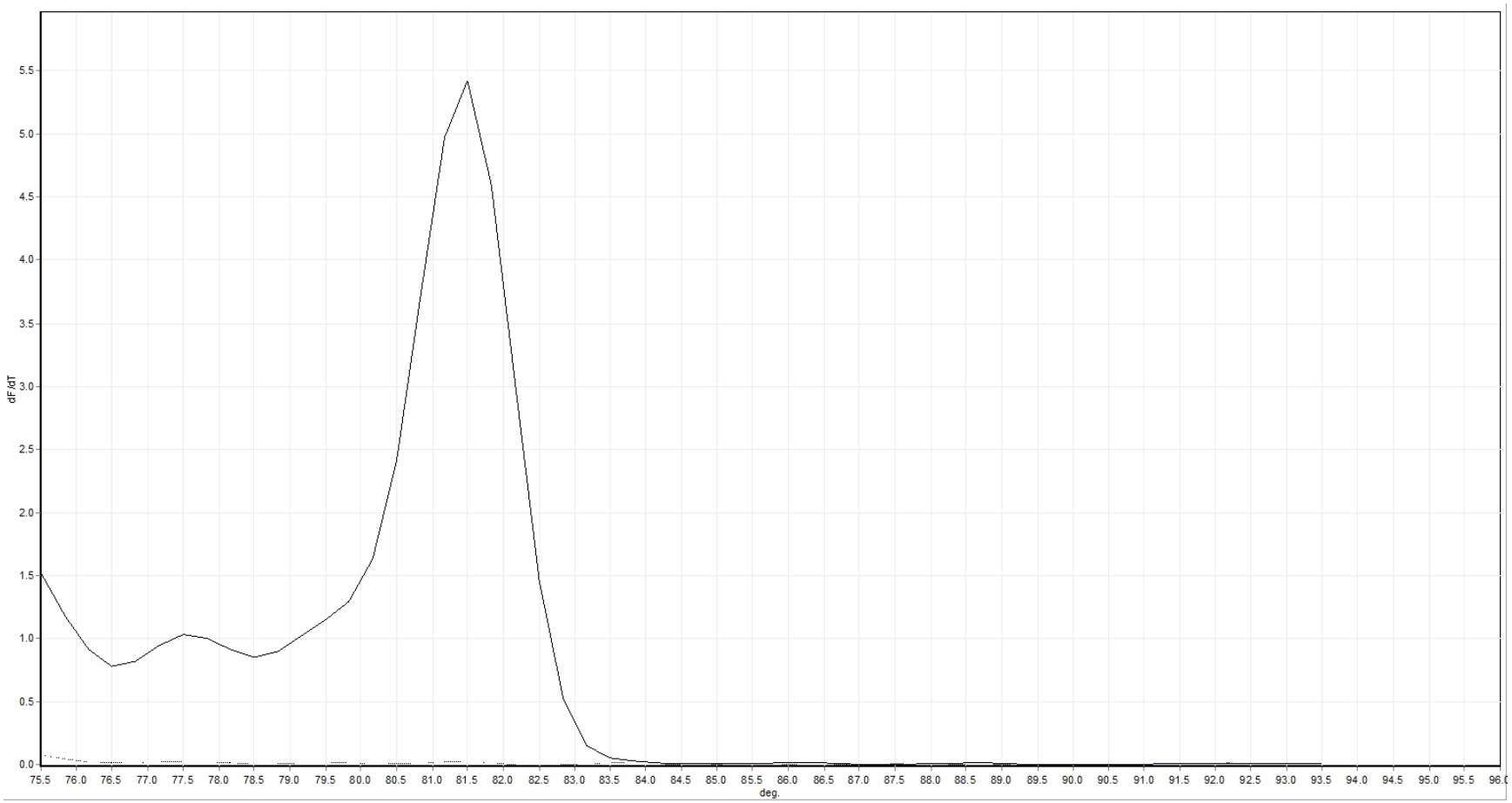



**Figure 6: *Legionella pneumophila* qPCR melt curve. *L. pneumophila* positive shown by a Tm of 82.5±1$^{o}$C (solid line) and Non-template control (dashed line).**

### 2.7.3 Development of MAC qPCR assay

MAC real time PCR quantification assay was developed using previously described primers (Park *et al.* 2000) shown in Table 4 and applying the assay onto a real-time platform using SYTO9 as the intercalating fluorescent dye. The reagents added to each qPCR reaction are presented in Table 9 and the cycling conditions in Table 10. To optimise the qPCR a range of final primer concentrations (10 pmol, 30 pmol and 50 pmol) were compared at annealing temperatures of 50$^{o}$C and 60$^{o}$C. All primer concentrations were tested in triplicate with a standard concentration ($10^4$ copies/mL) of MAC DNA. The $C_T$ value of each reaction was compared by allocating an arbitrary threshold value of 0.2 (see Figure 8 and Figure 9) (personal communication, Giglio 2010). PCR products generated under each of the conditions were also compared by gel electrophoresis which is shown in Figure 12 (see method 2.7.3). Initial comparison of $T_m$ (see Figure 8 and Figure 9) and the gel electrophoresis images (Figure 12) showed there to be little difference between the two annealing temperatures. An annealing temperature of 50$^{o}$C was chosen and 50 pmol primer concentration as it resulted in a more defined melt curve and a higher concentration of MAC product.

For each reaction the melt curve was analysed and a positive MAC was confirmed with a $T_m$ of 85±1$^{o}$C. This $T_m$ (Figure 11) was predicted by POLAND algorithm (Rasmussen *et al.* 2007) based on the sequence from *M. avium* (X74494.1). A standard curve was created using purified PCR product which had been cloned into a plasmid to prevent deterioration (2.7.4 qPCR positive controls). The concentration of the DNA plasmid was determined (see 2.7.7 Calculating DNA concentration) and then a one in ten series of dilutions were created using a Corbett Research Liquid Handling System®.

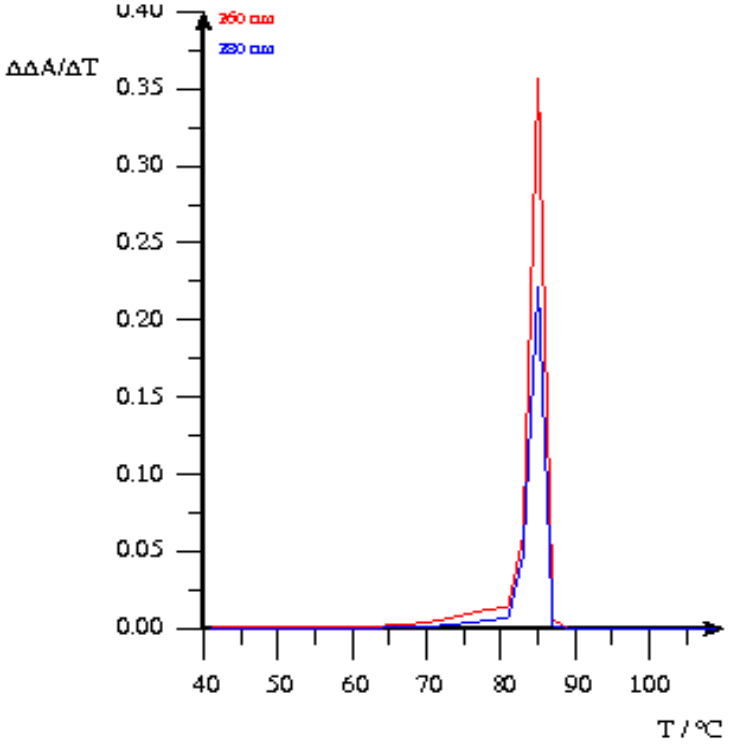



**Figure 7: Predicted Melt curve (Tm) of MAC PCR product (available at http://www.biophys.uni-duesseldorf.de/local/POLAND/).**

**Table 9: Concentrations of reagents used for MAC qPCR assays and amount of working stock added to each 25 µL reaction**

| Reagent | Final concentration | Concentration of working stock | Volume added to 25 µL reaction (µL) |
|---|---|---|---|
| Water | | | |
| Final primer concentration of 10 pmol | - | - | 12.55 |
| Final primer concentration of 30 pmol | | | 11.55 |
| Final primer concentration of 50 pmol | | | 10.55 |
| $MgCl_2$ (Invitrogen) | 2.5 mM | 50 mM | 1.25 |
| PCR Buffer (Invitrogen) | 1X | 10X | 2.5 |
| dNTPs (Invitrogen) | 0.2 mM | 10 mM | 0.5 |
| Syto9 (BioRad) | 2.5 µM | 25 µM | 2.5 |
| Platinum Taq DNA polymerase(Invitrogen) | 1 U | 5 U/µL | 0.2 |
| DNA sample | - | - | 5 |
| MAC forward primer | | | |
| Final primer concentration of 10 pmol | 0.1 µM | 10 µM | 0.25 |
| Final primer concentration of 30 pmol | 0.3 µM | | 0.75 |
| Final primer concentration of 50 pmol | 0.5 µM | | 1.25 |
| MAC reverse primer | | | |
| Final primer concentration of 10 pmol | 0.1 µM | 10 µM | 0.25 |
| Final primer concentration of 30 pmol | 0.3 µM | | 0.75 |
| Final primer concentration of 50 pmol | 0.5 µM | | 1.25 |

**Table 10: Cycling conditions for MAC qPCR conducted on Corbett Rotor Gene Thermal Cycler 6000.**

| Hold | Cycling 45X | | | Melt |
|---|---|---|---|---|
| | **Step 1** | **Step 2** | **Step 3** | Ramping rate of 1°C/60 s from 75° C-95°C |
| 95°C | 95°C | 50°C or 60°C | 72°C | |
| 5 min | 15 sec | 30 sec | 20 sec | |

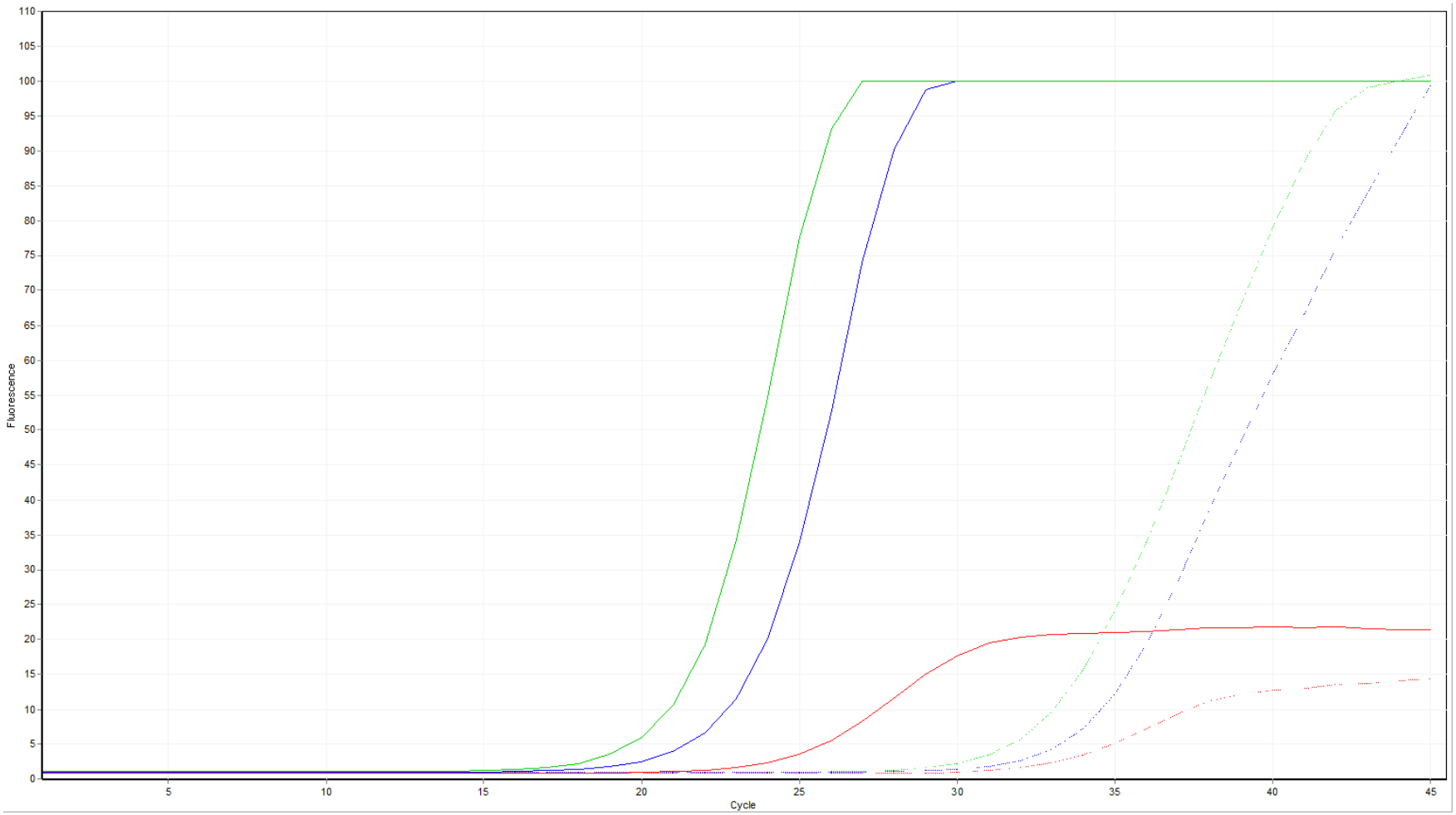



**Figure 8: MAC qPCR cycling curve with 104 copies/µL of MAC DNA and NTC (dashed line) with 10pmol (red line), 30pmol (blue line) and 50pmol (green line) primer concentration with annealing temperature of 50oC.**

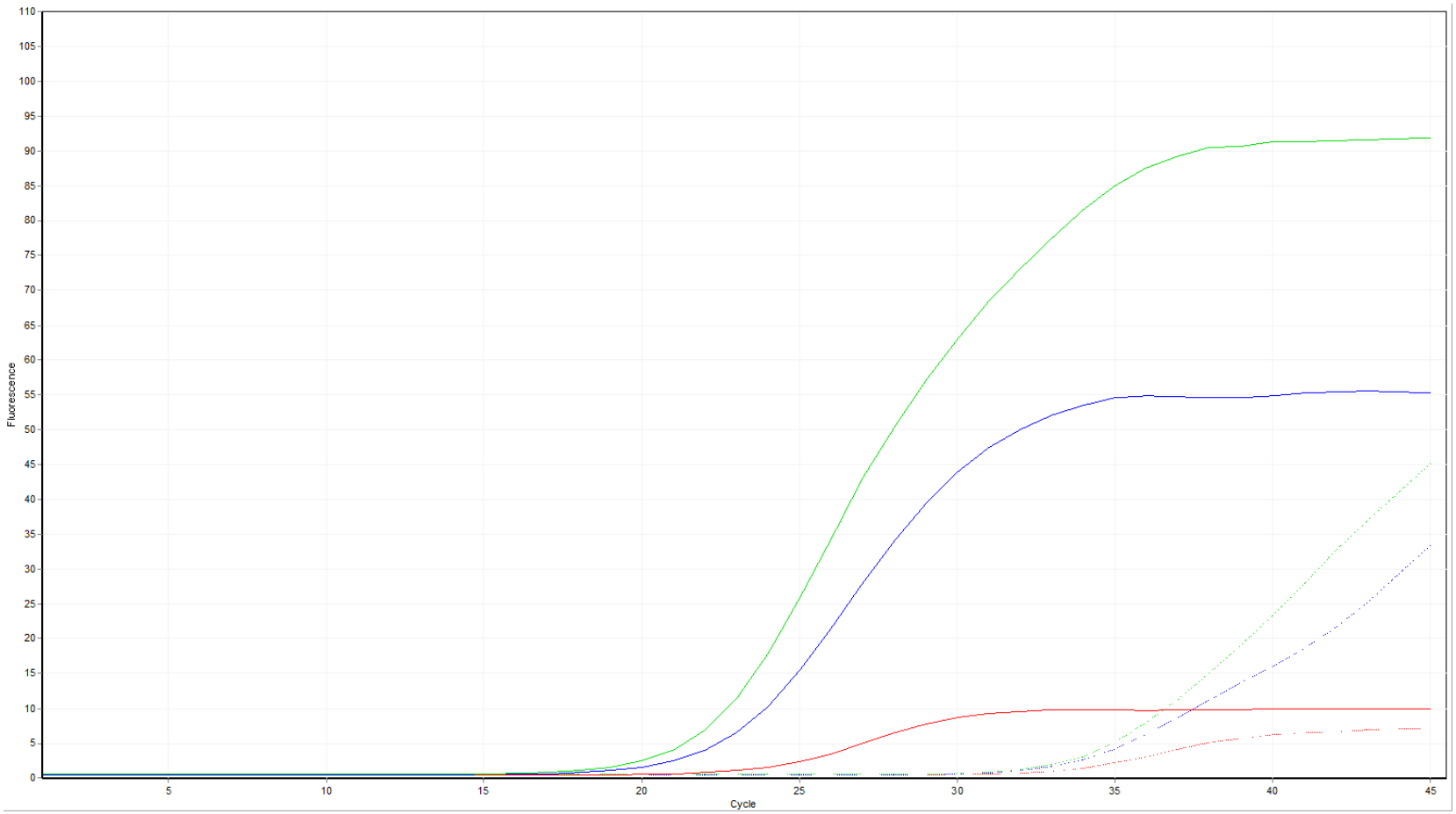



**Figure 9: MAC qPCR cycling curve with $10^4$ copies/µL of MAC DNA and NTC (dashed line) with 10pmol (red line), 30pmol (blue line) and 50pmol (green line) primer concentration with annealing temperature of 60$^{o}$C.**

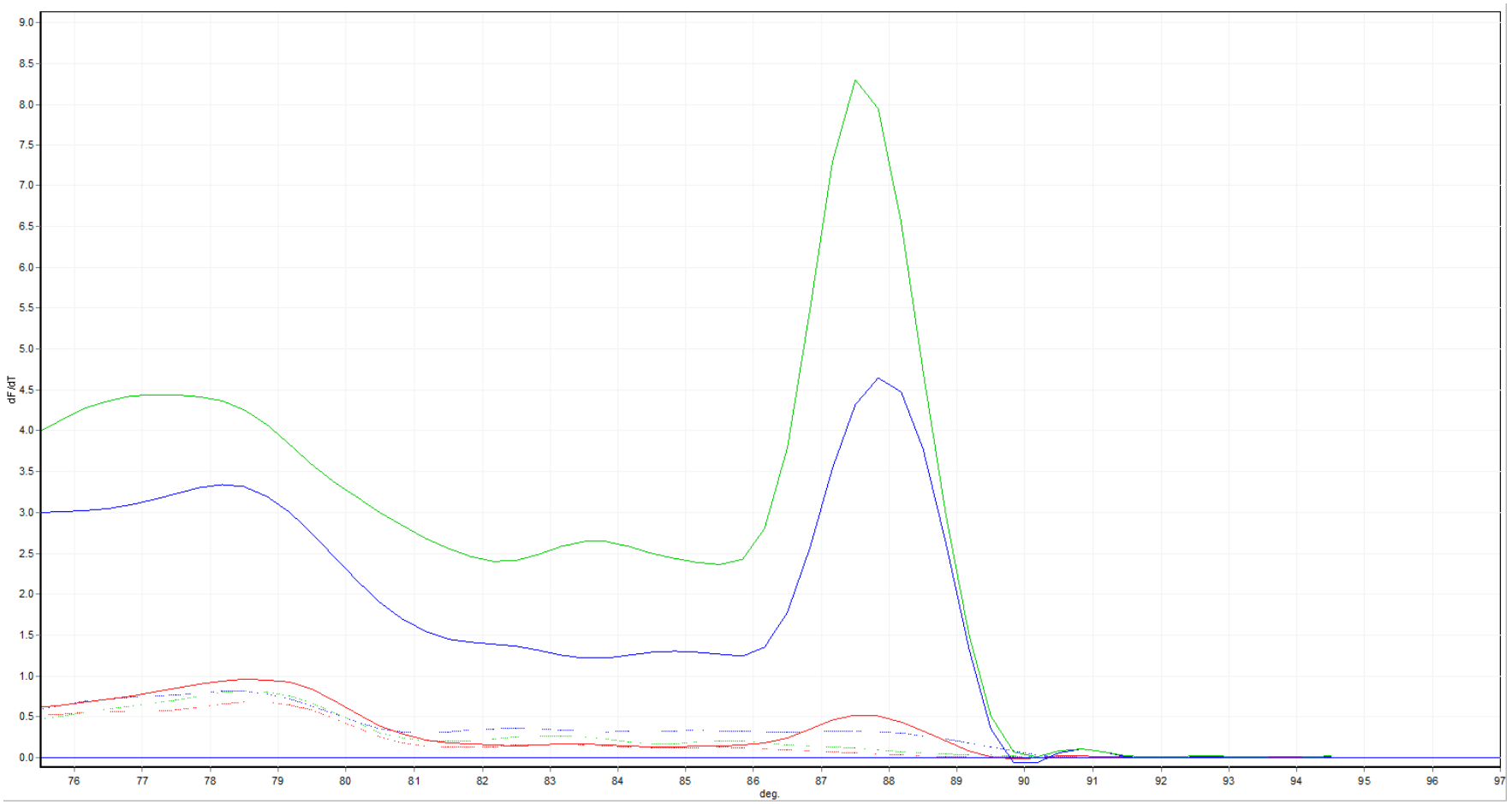



**Figure 10: MAC qPCR Melt curve with $10^4$copies/µL of MAC DNA and NTC (Dashed line) with 10pmol (red line), 30pmol (blue line) and 50pmol (green line) primer concentration with an annealing temperature of $50^oC$.**

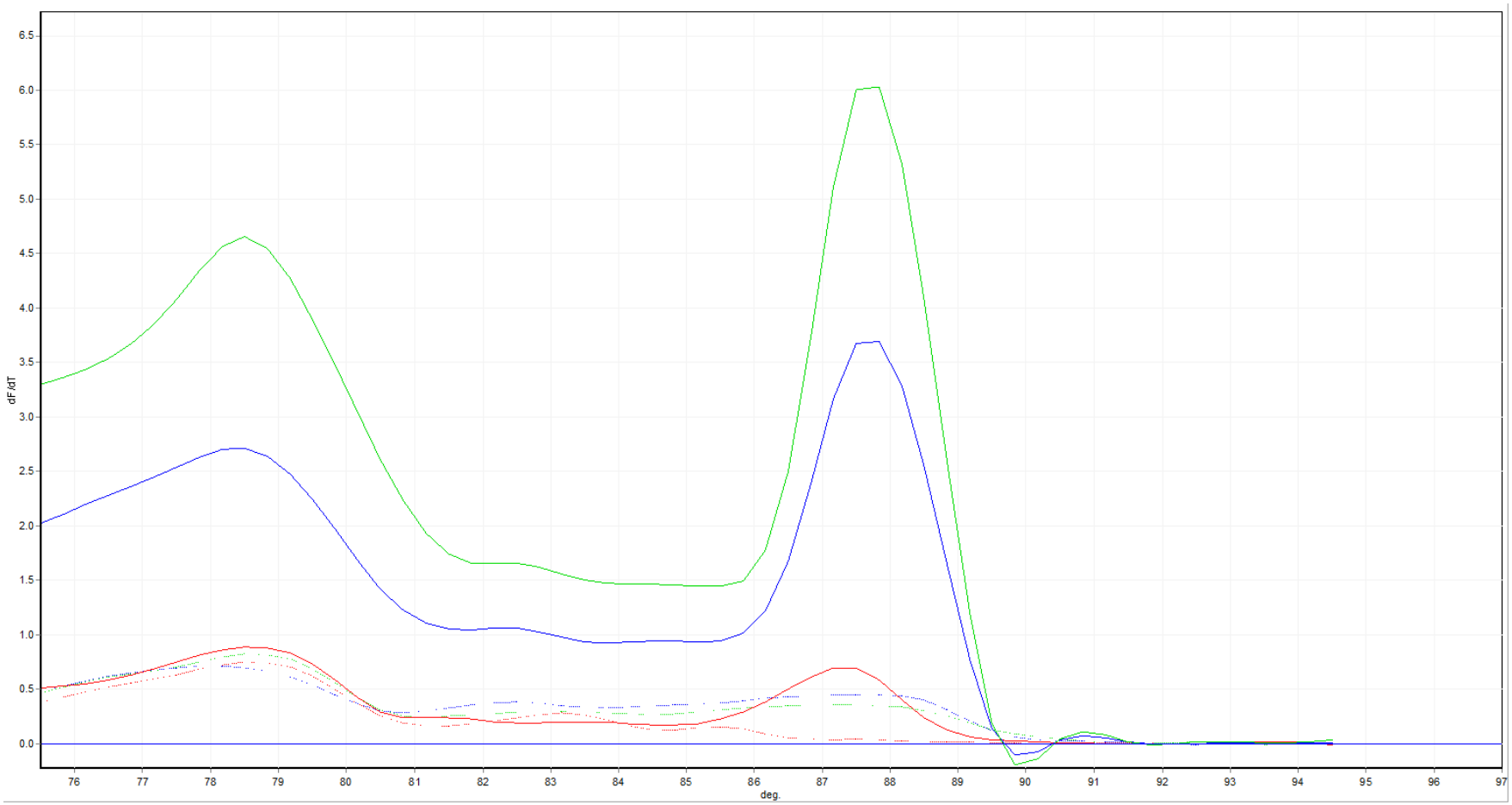
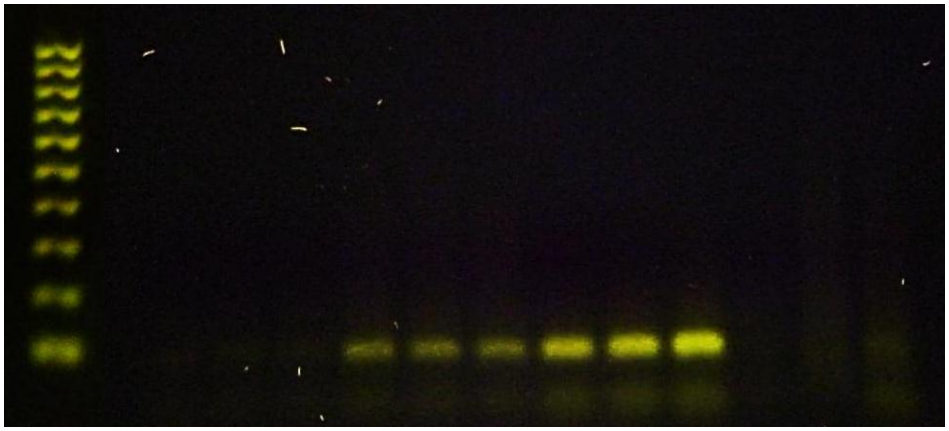


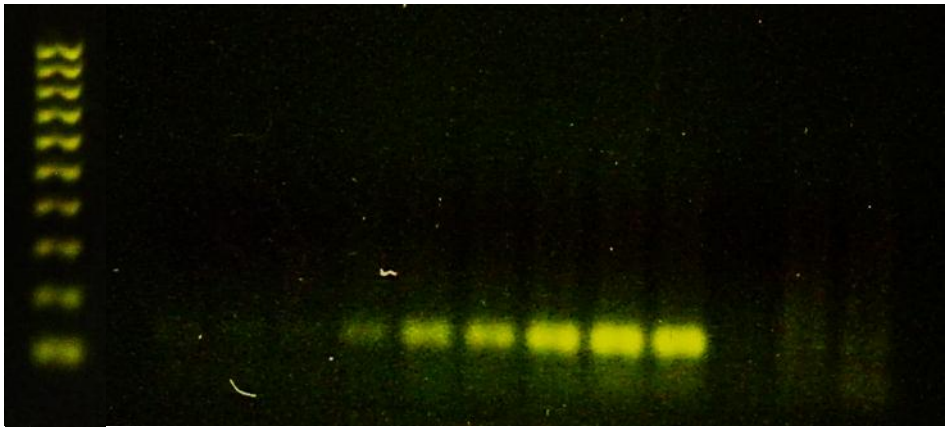

**Figure 11: MAC qPCR Melt curve with $10^4$copies/µL of MAC DNA and NTC (Dashed line) with 10pmol (red line), 30pmol (blue line) and 50pmol (green line) primer concentration with an annealing temperature of 60$^o$C.**

**A**



1 2 3 4 5 6 7 8 9 10 11 12 13 14

**B**



1 2 3 4 5 6 7 8 9 10 11 12 13 14

| Legend | |
|---|---|
| Lane 1: 100 bp ladder | Lane 9-11: 50 pmol primer |
| Lane 2: blank | Lane 12: 10 pmol primer NTC |
| Lane 3-5:10 pmol primer | Lane 13: 30 pmol primer NTC |
| Lane 6-8: 30 pmol primer | Lane 14: 50 pmol primer NTC |

**Figure 12: Gel Electrophoresis of MAC qPCR with $10^4$ copies/µL of MAC DNA and NTC with 10 pmol, 30 pmol and 50 pmol primer concentration and at 50°C (A) and 60°C (B).**

### 2.7.3 Gel electrophoresis to confirm PCR products

PCR products were visualised by gel electrophoresis using 1% agarose gels supplemented with 10 µL Sybr Safe DNA Gel Stain (Invitrogen). To each well 9 µL of PCR product and 1 µL of 10 X loading buffer (Invitrogen) was added. Gels were run in TBE Buffer at 100 volts and a 100 bp ladder (Geneworks) was used to compare the generated amplicon size against the standards included in the ladder. A positive MAC result was indicated by the presence of a DNA band at 141 bp.

### 2.7.4 qPCR positive controls

For qPCR quantification and confirmation purposes, standard curves and positive controls were created using the relevant purified PCR 16s product which had been cloned into a pCR®2.1 plasmid.

### 2.7.5 PCR product purification

Fresh qPCR product was obtained and run on a gel to confirm correct amplification and to determine whether further purification was required. Purification was achieved using the Montage PCR Centrigual Filter Device (Millipore) following manufacturer's instructions.

### 2.7.6 Cloning purified PCR product into a plasmid

Purified PCR product (see 2.7.5) was cloned into a plasmid using the TA cloning kit (Invitrogen) following manufacturer's instructions. Briefly the ligation reaction

contained 1 µL fresh PCR product, 1 µL 10X PCR ligation buffer, 2 µL of PCR 2.1 vector 25 ng/µL, 5 µL of nuclease free water, 1 µL T4 DNA ligase (4.0 Weiss units) and was incubated overnight in the dark at 14$^{o}$C. The next day 2 µL of ligation reaction were added to 5 µL ® INVαF´ chemically competent E. *coli* culture and incubated on ice for 30 mins. Cells were then heat shocked for 30 s at 42$^{o}$C in a water bath without shaking. Next 250 µL of room temperature S.O.C medium (Invitrogen) was added to the cells prior to shaking at 37$^{o}$C for 1 hr at 225 rpm in a shaking incubator. Then 20 µL and 200 µL of each transformation product was spread plated onto an LB agar plates containing Xgal, IPTG and ampicillin (see method 2.1.4) and incubated overnight at 37$^{o}$C.

After 24 hr the plates contained both blue (*E. coli* not containing plasmid) and white colonies (*E. coli* containing plasmid). Of these, 5-10 white colonies were chosen and grown up by incubating at 37$^{o}$C overnight in LB broth with 100 mg/mL ampicillin. Then qPCR of selected *E. coli* colonies containing plasmid was run and the melt curves were checked to confirm that the correct plasmid had been inserted. The plasmids were extracted from the *E. coli* using the QIAprep spin miniprep kit (Qiagen) following manufacturer's instructions and stored at -20$^{o}$C.

### 2.7.7 Calculating DNA concentration

The concentration of plasmid was calculated by reading the absorbance spectrophometerically at 260 nm 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).

Once an *E. coli* culture containing the correct plasmid was identified -80$^{o}$C stock solutions were created. These stock solutions contained 850 µL culture and 150 µL glycerol (Invitrogen) mixed within a cryotube and were frozen by placing samples in liquid nitrogen for 30 s prior to storage at -80$^{o}$C.

### 2.7.8 Creating a standard curve

A 1:10 series of dilutions of *Legionella spp.*, *L. pneumophila* and MAC qPCR product plasmids (ranging from $10^{9}$-$10^{0}$ copies) into UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) were 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 standard curve to calculate the number of copies (see 2.6 Calculating total copies/mL from qPCR results). The corresponding $R^2$ values (calculated using the Corbett Research RotorGene Thermal Cycler 6000 software) for *Legionella spp.*, *L. pneumophila* and MAC standard curves, was 0.99167 ($P<0.05$) (Figure 13), 0.93295 ($P<0.05$) (Figure 14) and 0.98177 ($P<0.05$) (Figure 15) respectively. The high $R^2$ values indicate that the qPCR methods have a high level of precision when applied to replicate qPCR analysis from the same sample preparation.

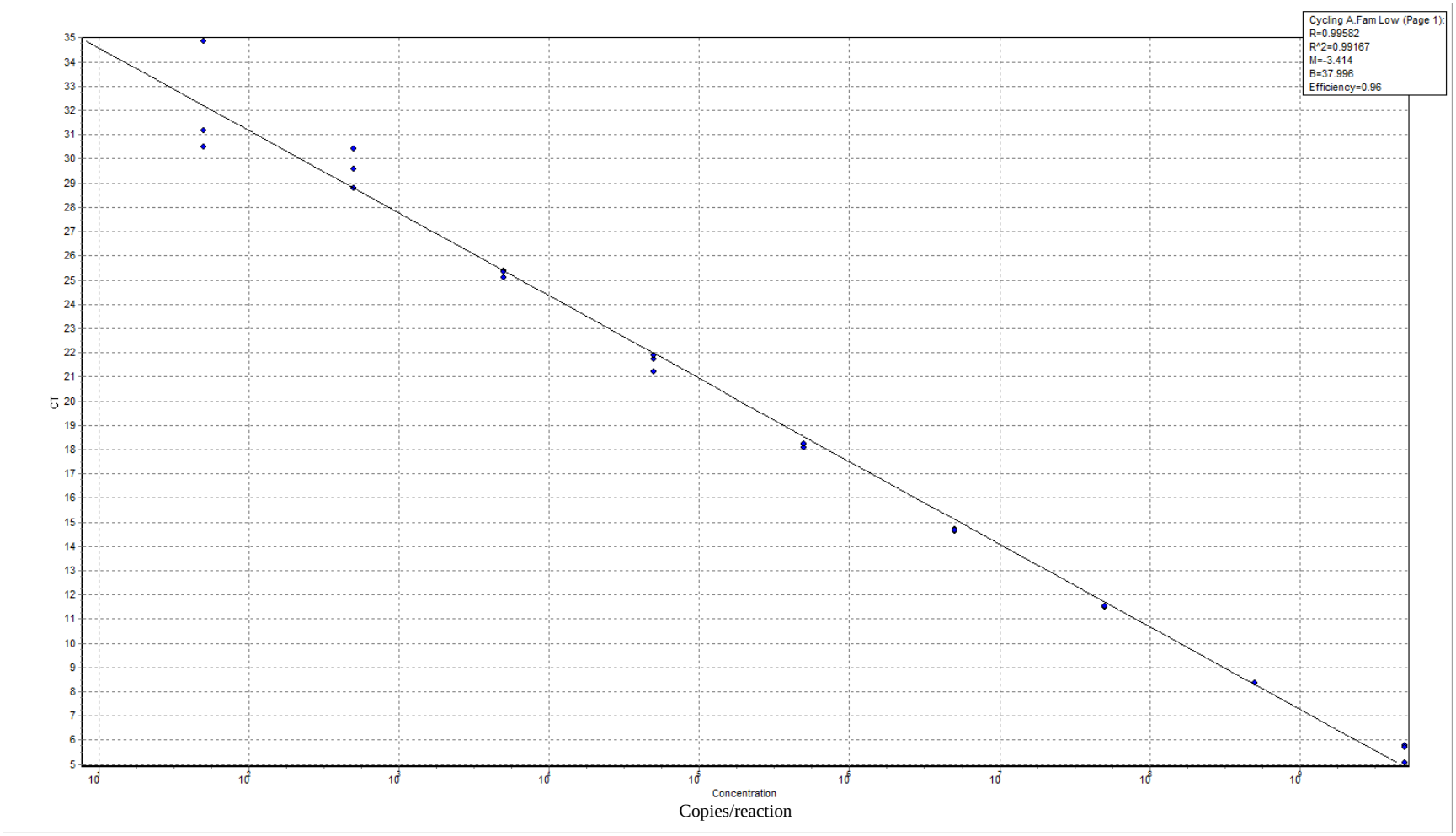



**Figure 13: *Legionella spp*. qPCR standard curve ($R^2$ value of 0.99167).**

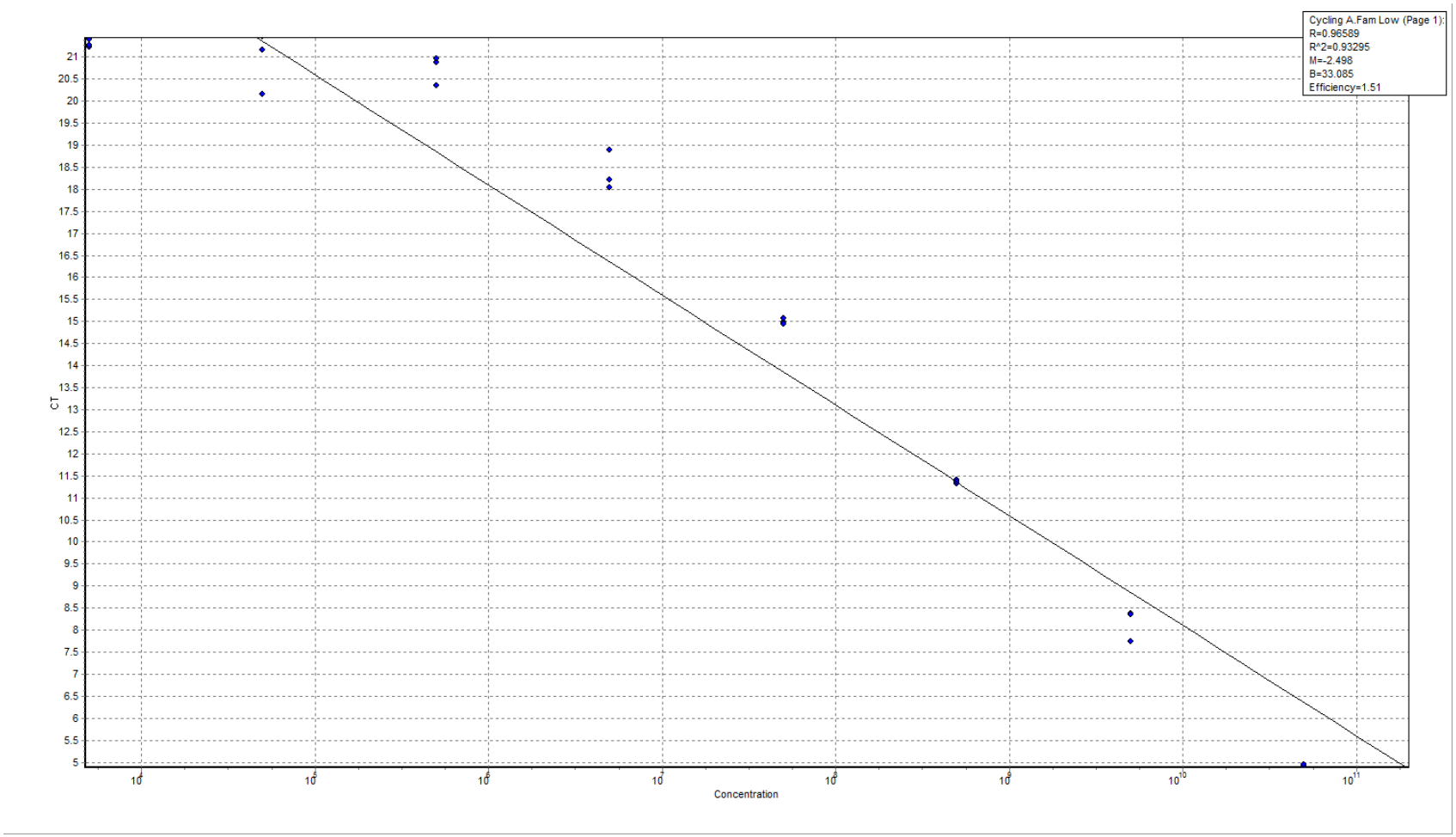



**Figure 14: *L. pneumophila* qPCR standard curve ($R^2$ value of 0.93295).**

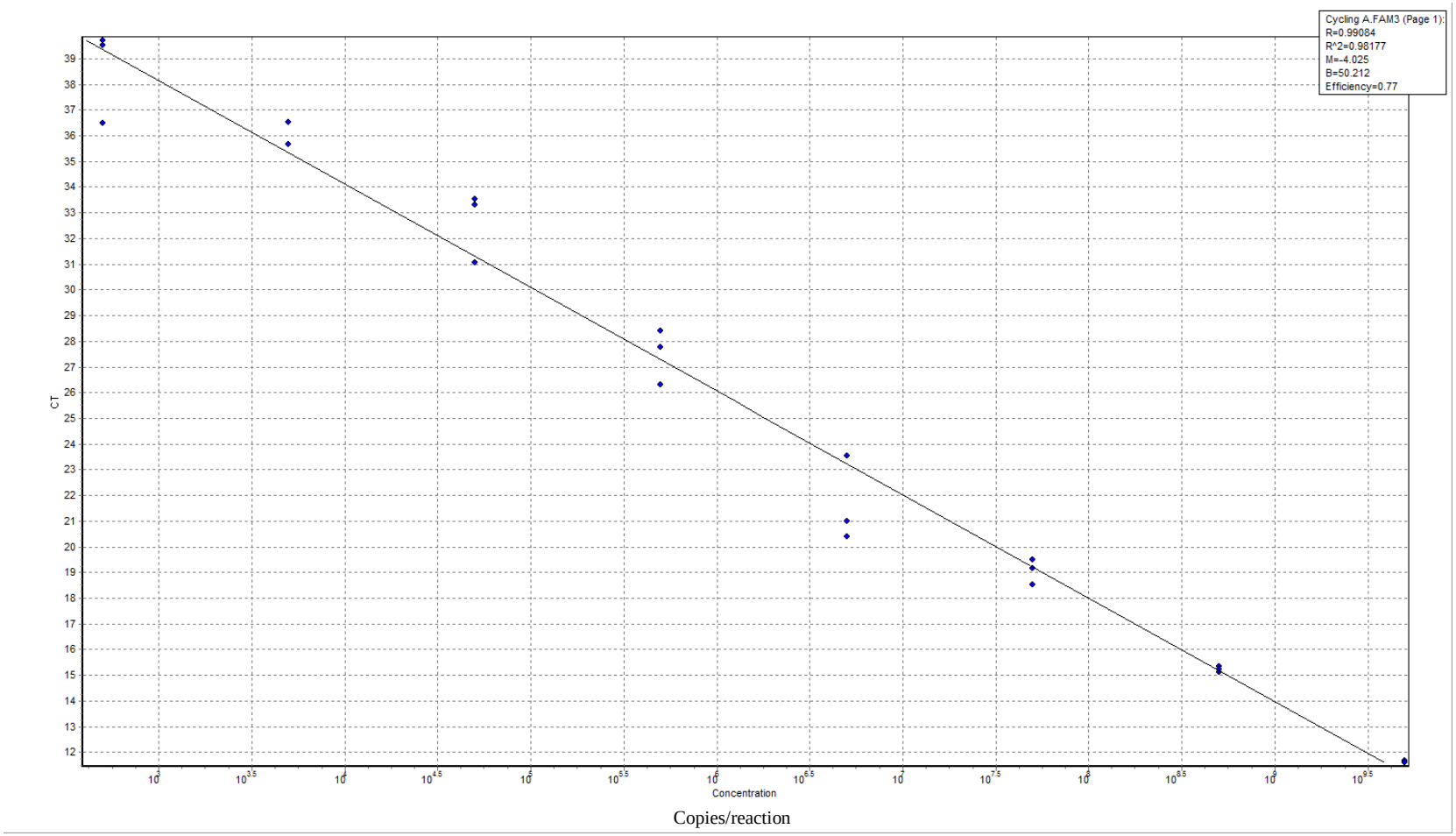



**Figure 15: MAC qPCR standard curve ($R^2$ value of 0.98177).**

### 2.7.9 Environmental inhibitors

To determine the presence of environmental inhibitors in the extracted DNA, the qPCR reactions were conducted in triplicate for both undiluted DNA extract and 1:10 dilution UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) of the same sample. If the cycle threshold ($C_T$) value for the 1:10 dilution of DNA extract was less than approximately 3.3 (representing approximately 1-$\log_{10}$ concentration value) (Livak 2001) than the pure DNA extract then it was assumed that environmental inhibitors were present. When inhibitors were present in the undiluted DNA extract and the 1:10 dilution had the correct T*m* the 1:10 dilution was used to calculate copies/mL.

### 2.7.10 Calculating total copies/mL from qPCR results

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, even if it included the correct one this value was not included. The qPCR calculated copies/ reaction were generated by comparing values to the standard curve. The total copies/mL were then calculated using Table 11.

The relationship between *Legionella* CFU/mL and *Legionella* copies/mL has previously been explored. Joly *et al.* (2006) demonstrated that an increase in *Legionella* CFU/mL (determined by growth on GVPC agar) caused an increase in *Legionella* copies/mL (determined by qPCR), which is shown by Figure 16 ($R^2$= 0.985). According to this relationship 1 CFU = 4.4326 copies, which means there is less than a 1 $\log_{10}$ difference between the number of *Legionella* detected by culture and the number of copies detected by qPCR.

**Table 11: Formula for calculating organism concentration (copies/mL) from qPCR results. An example of a qPCR result of 20 copies / reaction is given.**

| Description | Formula | Example |
|---|---|---|
| qPCR calculated copies/ reaction | α | 20 copies/reaction |
| The step is for all samples which underwent a 1:10 dilution | α x 10 | 200 |
| Only 5 µL of the final 100 µL DNA extract was analysed during qPCR reaction | α x 10 x 20 | 4000 |
| Only 62.5% of the DNA is extracted from a sample using the BioRad DNA Extraction kit | α x 10 x 20 x1.6 | 6400 |
| Divided by the number of mL DNA was extracted from (initial sample volume) | α x 10 x 20 x 1.6 /450 | 14 copies/mL |

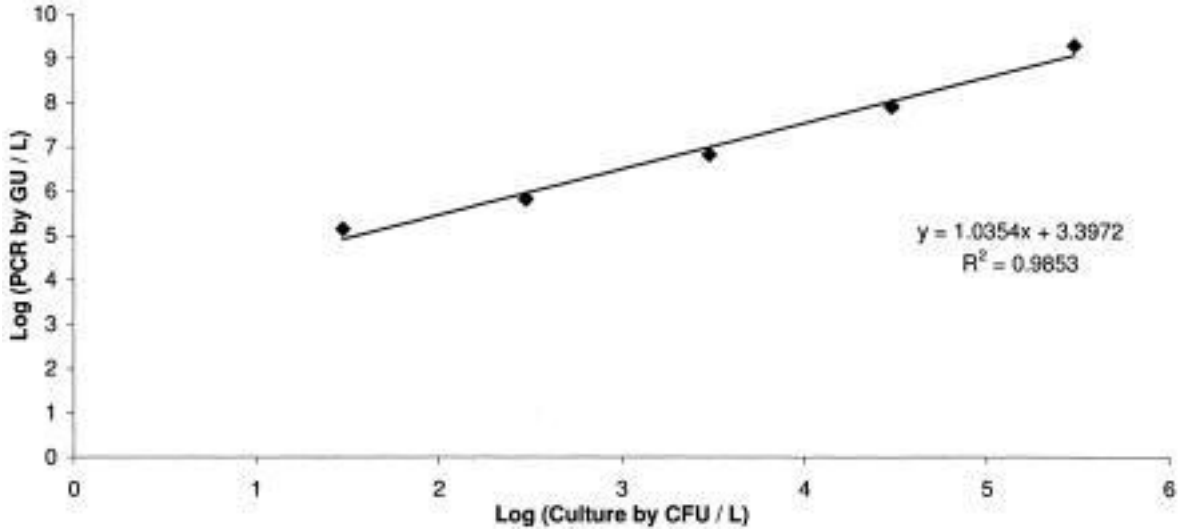



**Figure 16: Relationship between *Legionella* CFU/mL (determined by growth on GVPC agar) and *Legionella* copies/mL (determined by qPCR) from Joly *et al.* (2006).**

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## 2.8 Statistical analyses

Statistical analysis of results was conducted using Graph Pad$^{TM}$ prism 5.0 (Graph Pad Software Inc. USA). Graphs were created using Excel 2010 (Microsoft corporation, USA). Normality was tested using the Kolmogorov–Smirnov test. Statistical significance was identified by a P value of $<0.05$. More detailed information regarding statistical analyses is presented in the relevant chapters.

# Chapter 3: Potable water

This chapter describes the use of qPCR to investigate the fate of opportunistic human pathogens *Legionella* spp. *L. pneumophila* and MAC throughout two South Australian potable water distribution pipelines. One system utilised chlorine disinfection and the other chloramine disinfection. The effect of seasonality, distance from processing plant and disinfectant residual on these organisms was explored. The relationship with total coliforms and *Legionella* spp. *L. pneumophila* and MAC was also addressed. A risk assessment of potential exposure to these pathogens through potable water is described in Chapter 5.

## 3.1 Potable water Distribution System 1 (DS1)

Potable water DS1 provides chlorine disinfected potable water across a metropolitan area. All the pipes for this distribution system are below ground. Table 12 shows the pipe diameter, pipe material and distance from processing plant of each sampling location chosen for this study.

## 3.2 Potable water Distribution System 2 (DS2)

Potable water DS2 provides chloramine disinfected potable water across a rural area. The majority of the pipes for this distribution system are below ground; however, some sections are above ground. Table 13 shows the pipe diameter, pipe material and distance from processing plant of each sampling location chosen for this study.

**Table 12: Distance from processing plant, pipe diameter and pipe material for each sampling location chosen along potable water DS1.**

| Sampling point | Distance from processing plant (km) | Pipe diameter (mm) | Pipe material |
|---|---|---|---|
| A | 5 | 1050 | Mild steel cement lined |
| B | 7 | 200 | Cast iron |
| C | 10 | 100 | Polyvinyl chloride (PVC) |
| D | 18 | 100 | Asbestos cement |
| E | 22 | 100 | Asbestos cement |

**Table 13: Distance from processing plant, pipe diameter and pipe material for each sampling location chosen along potable water DS2.**

| Sampling point | Distance from processing plant (km) | Pipe diameter (mm) | Pipe material |
|---|---|---|---|
| A | 1 | 750 | Mild steel cement lined |
| B | 4 | 600 | Mild steel cement lined |
| C | 54 | 600 | Mild steel cement lined |
| D | 137 | 100 | Asbestos cement |
| E (this sampling point is at a dead end and chloramine residual is not maintained) | 54 | unknown | unknown |

### 3.3 Water quality data

To further characterise the potable water systems and provide information regarding the effect of seasonality in the water quality, data was accessed from the water utility's routine sampling data records. Information regarding seasonality of water temperature and disinfection residual along the pipelines was targeted as these are widely recognized as the primary factors influencing bacterial growth (LeChevallier 2003). Once all the data available was collated statistical consultation was sought from a statistician at Flinders University. Unfortunately due to gaps in the data and limited replications this information could not be used for statistical analysis or modelling purposes. However, monthly averages of the available data have been included (see appendix) to provide an overview of the water quality of the potable water systems and for comparisons to other studies in the future. The monthly averages demonstrated the variation in water quality parameters over the year sampled and throughout the pipelines.

#### 3.3.1 Water quality of potable DS1

The chlorine residual measured at the outlet leaving potable treatment plant of DS1 appeared to decrease over the year (Figure 17) with the chlorine residual measured in January higher than in December. This slight decline in chlorine residual throughout the year does not appear to be related to the seasonal changes in water temperature (Figure 27) and was most likely due to variation in system processes rather than seasonal variations of environmental factors.

The turbidity measured at the outlet leaving the potable water treatment plant of DS1 treatment plant was consistent throughout the year with the exception of two spikes in turbidity, one observed in January and one in July (Figure 18). Increases in turbidity can be caused by runoff from watersheds particularly from areas that are disturbed or eroding, algae or aquatic weeds and their breakdown products, humic acids and other organic compounds caused by decaying plant materials and high iron concentration (NHMRC 2011). The spike in turbidity observed in January occurred during summer months when the water temperature was the warmest and could be attributed to an increase in algae entering the potable water treatment plant. The spike in July occurred in winter and could be attributed to a heavy rainfall causing greater runoff and hence turbidity.

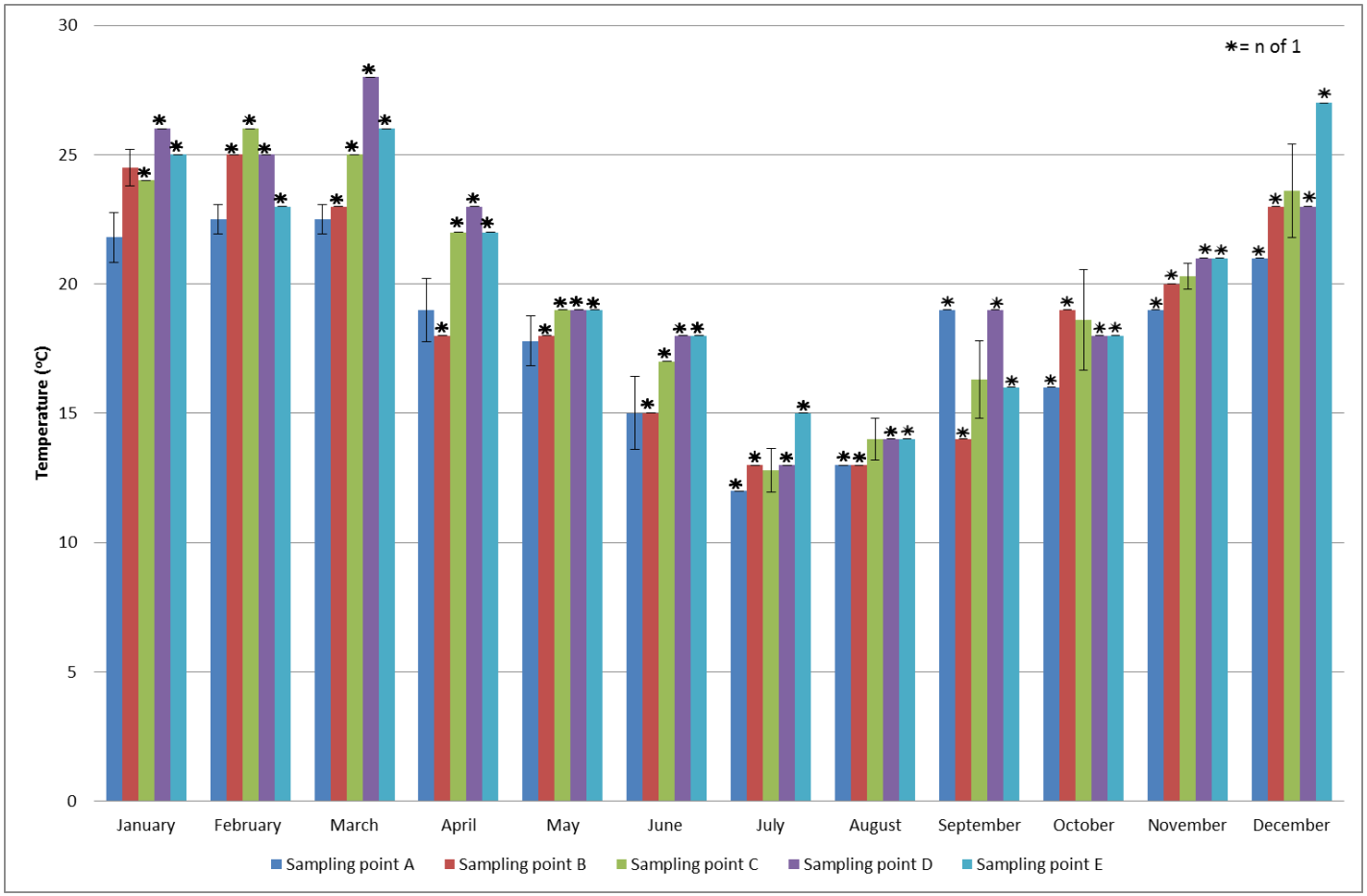


**Figure 17: Monthly averages (±SD) of free chlorine (mg/L) and total chlorine (mg/L) measured in water leaving potable water DS1 treatment plant throughout the year to show seasonality**.

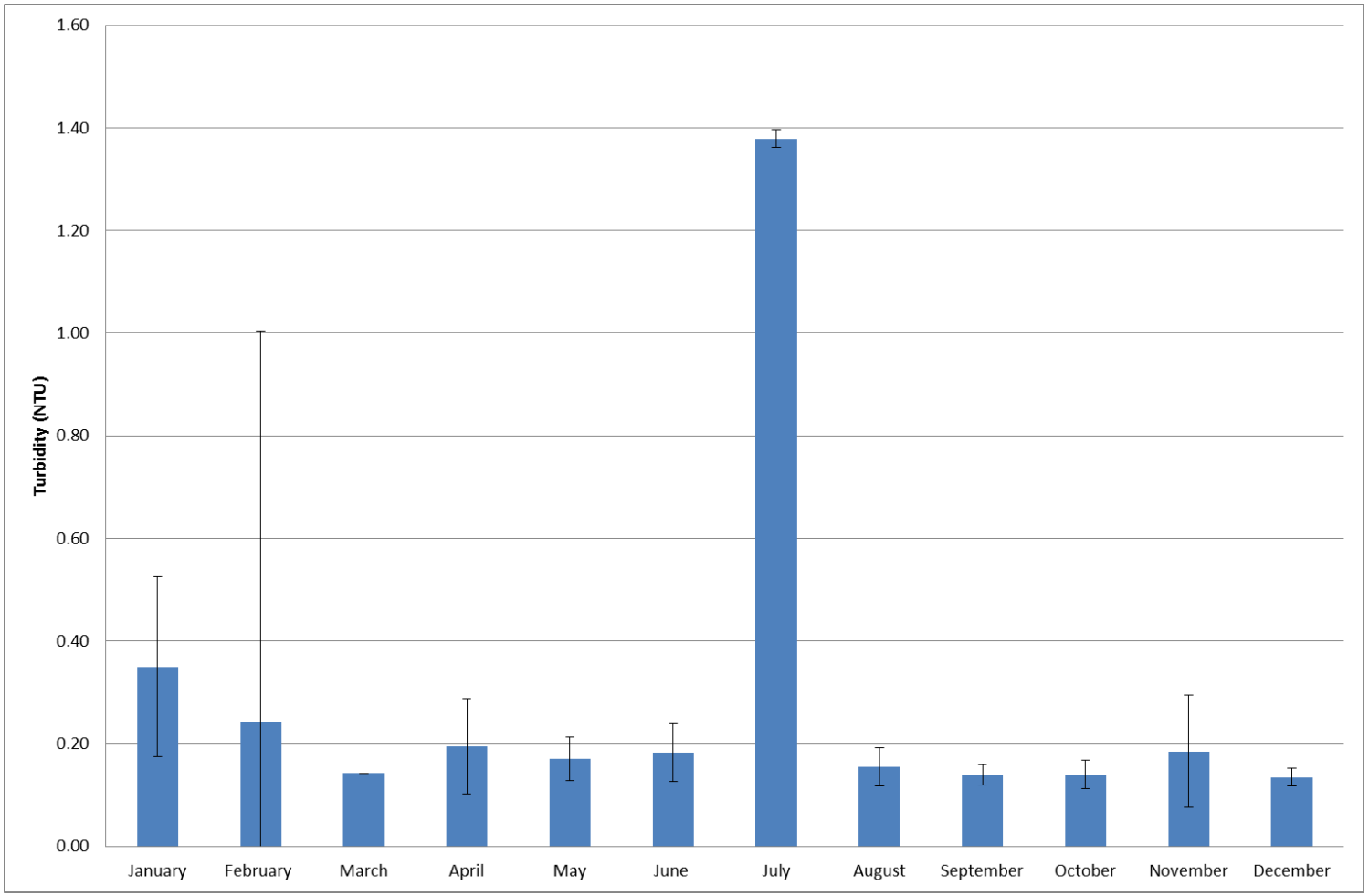


**Figure 18: Monthly averages (±SD) of Turbidity (NTU) in water leaving potable water DS1 treatment plant throughout the year to show seasonality.**

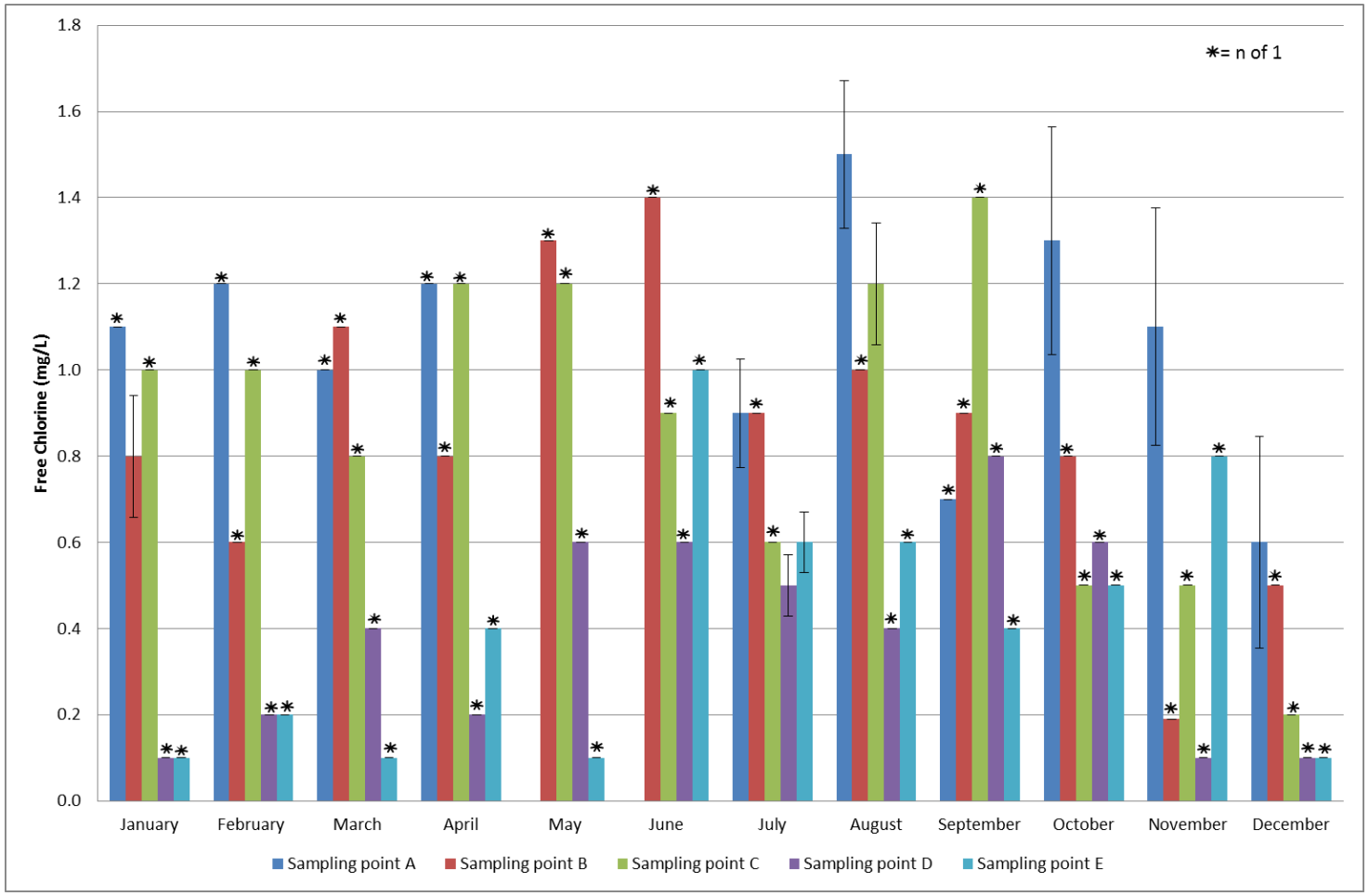


**Figure 19: Monthly averages (±SD) of free chlorine (mg/L) measured along potable water DS1, throughout the year to show seasonality.**

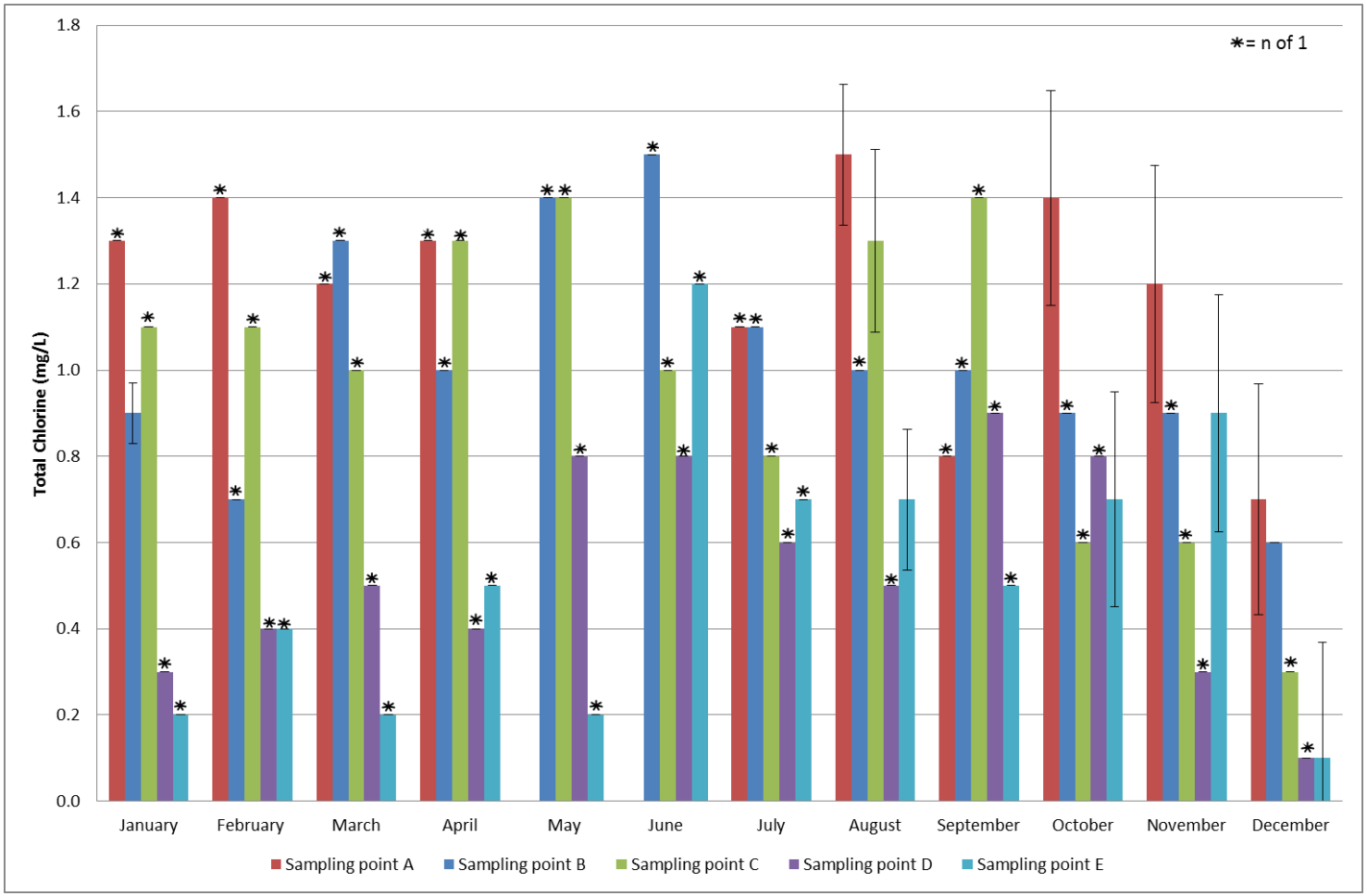


**Figure 20: Monthly averages (±SD) of total chlorine (mg/L) measure along potable water DS1throughout the year to show seasonality.**

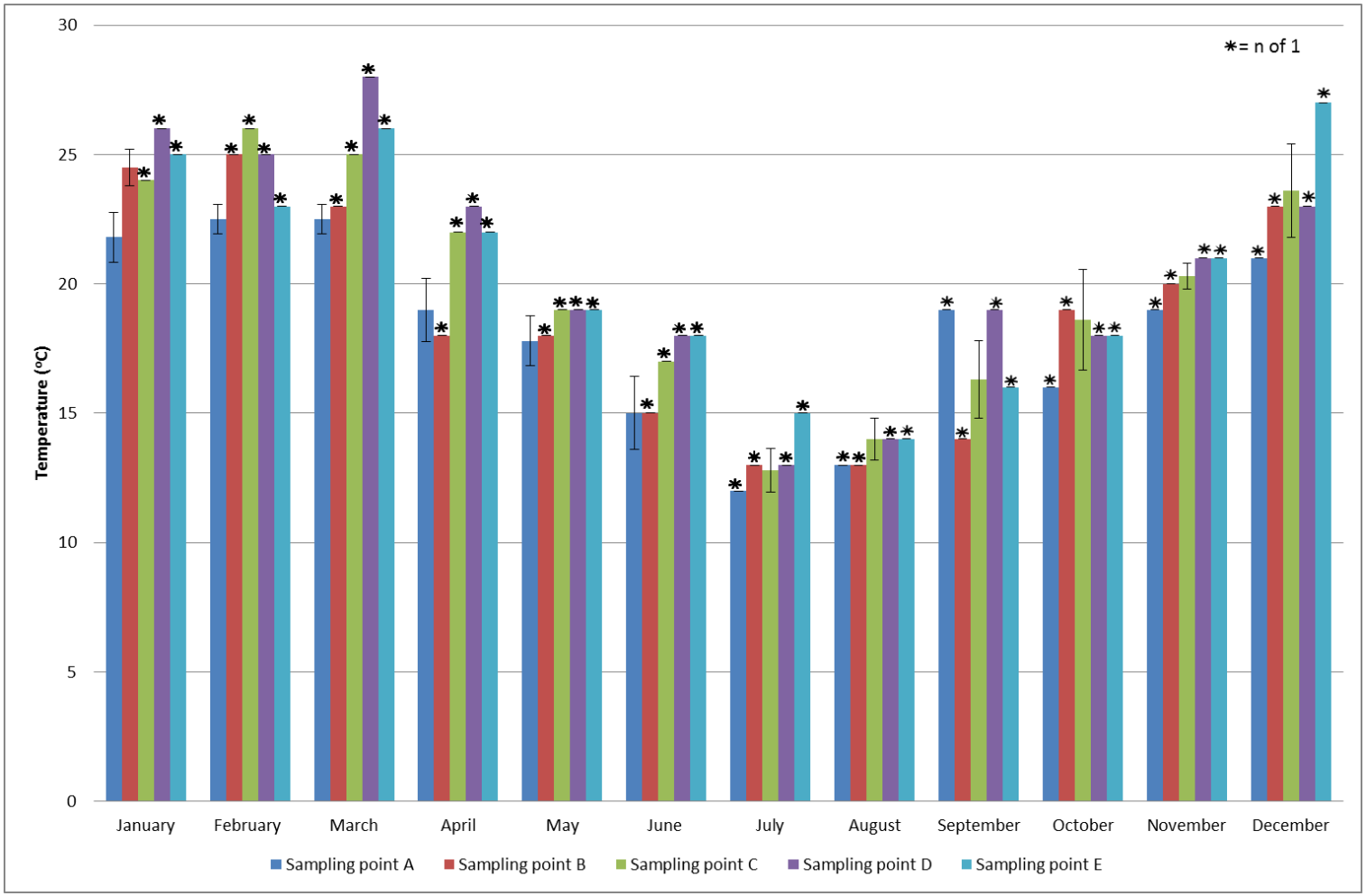


**Figure 21: Monthly averages (±SD) of water temperature ($^{o}$C) along potable water DS1 throughout the year to show seasonality.**

### 3.3.2 Water quality of potable DS2

In the raw water entering the treatment plant of potable water DS2 the total coliforms/100 mL (Figure 22) and total *E. coli*/100 mL (Figure 23) were consistent throughout the year with an a spike in the total coliforms observed in March/April and a spike in *E. coli* observed in November/December. No relationship between coliforms and *E. coli* was observed. There was seasonal trends observed in the turbidity (Figure 24) and the dissolved organic carbon (Figure 25) measured in the raw water entering DS2 treatment plant. An increase in turbidity was observed in March and an increase in dissolved organic carbon was observed March through to April. These increases were most likely due to early rainfalls which would wash run off built up over summer into the water.

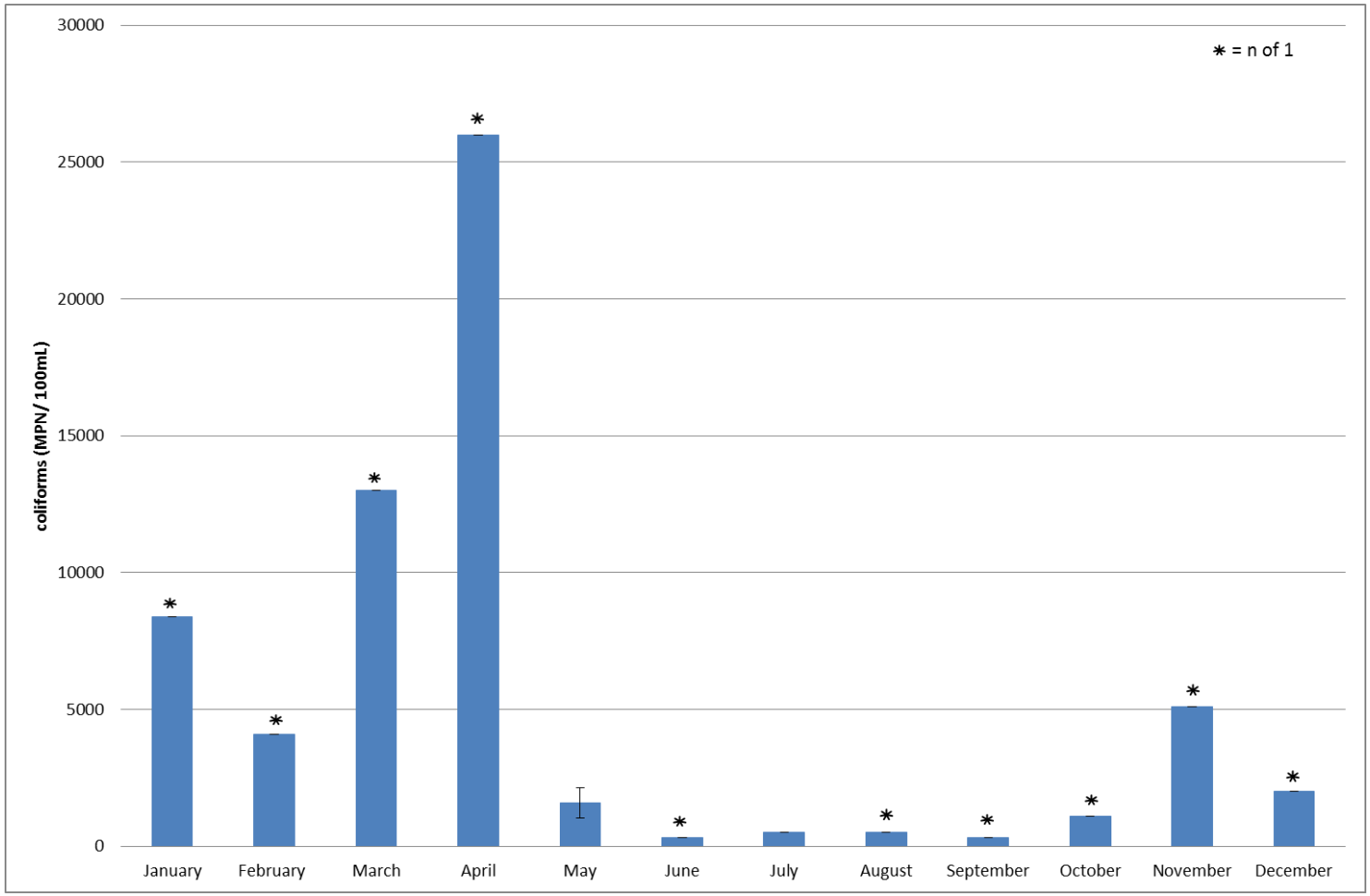


**Figure 22: Monthly averages (±SD) of total coliforms (MPN/100 mL) present in raw water entering potable water DS2 treatment plant throughout the year.**

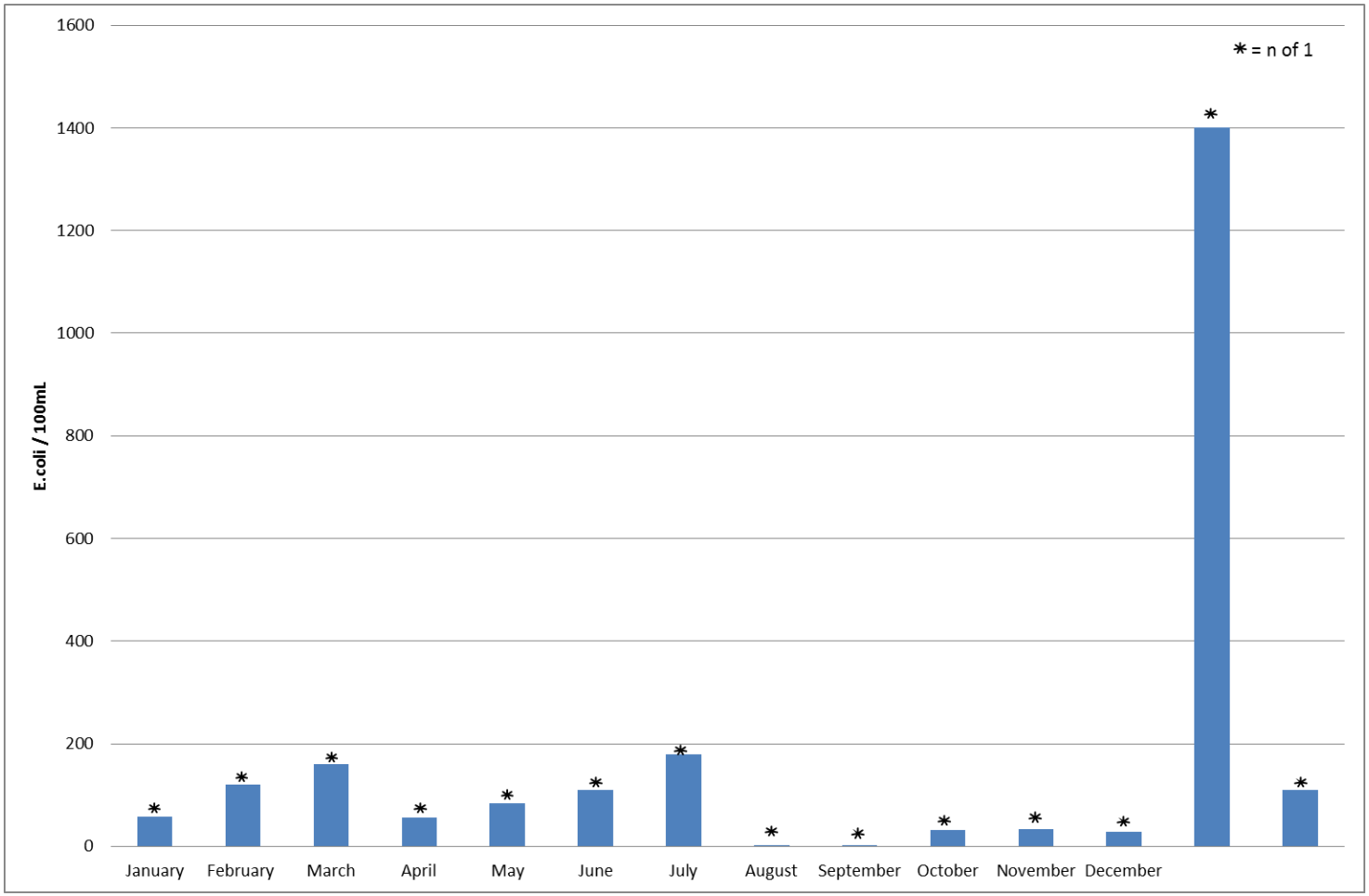


**Figure 23: Monthly averages (±SD) of *E. coli* (MPN/100 mL) present in raw water entering potable water DS2 treatment plant throughout the year.**

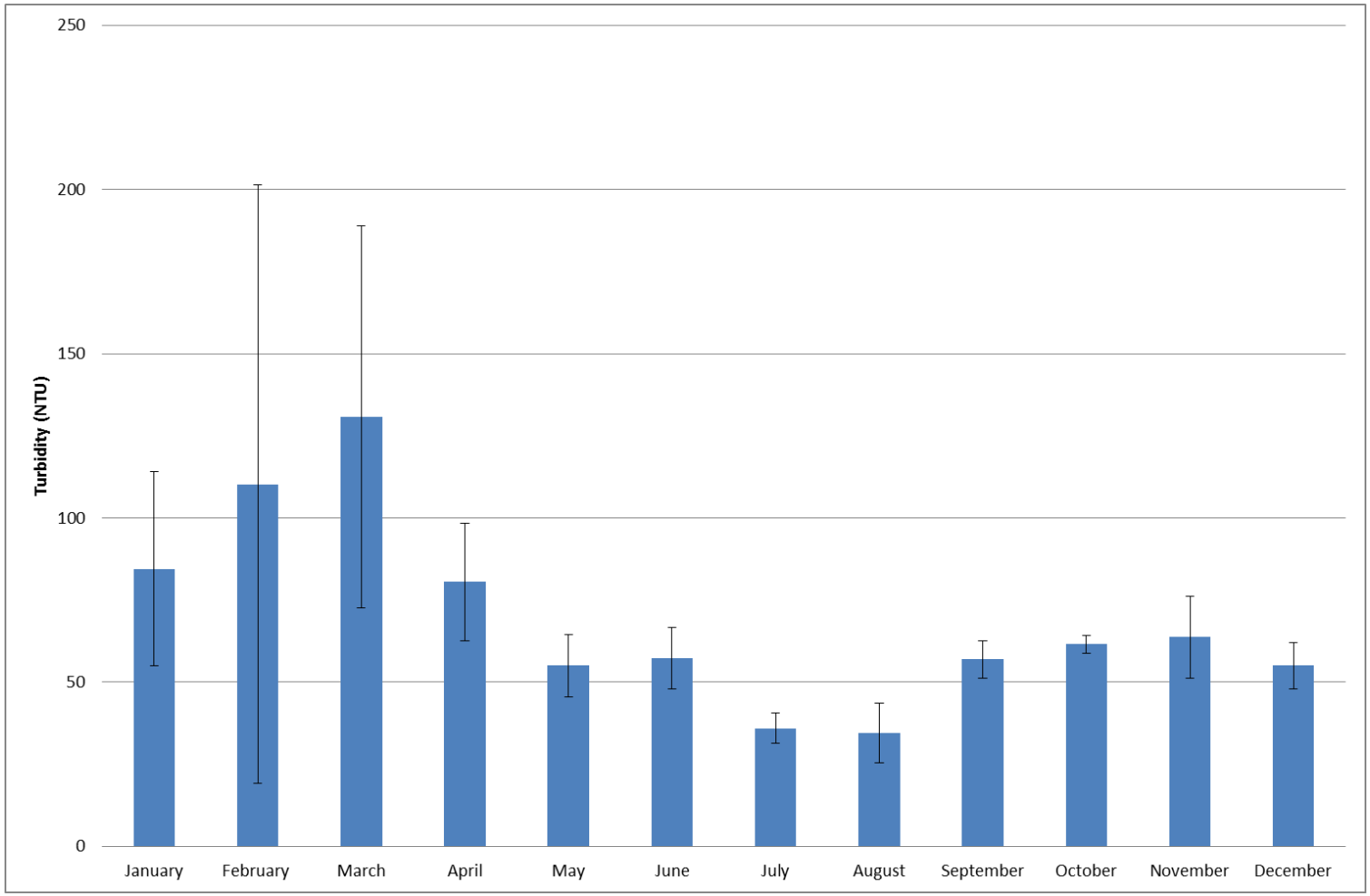


**Figure 24: Monthly averages (±SD) of turbidity (NTU) present in raw water entering potable water DS2 treatment plant throughout the year.**

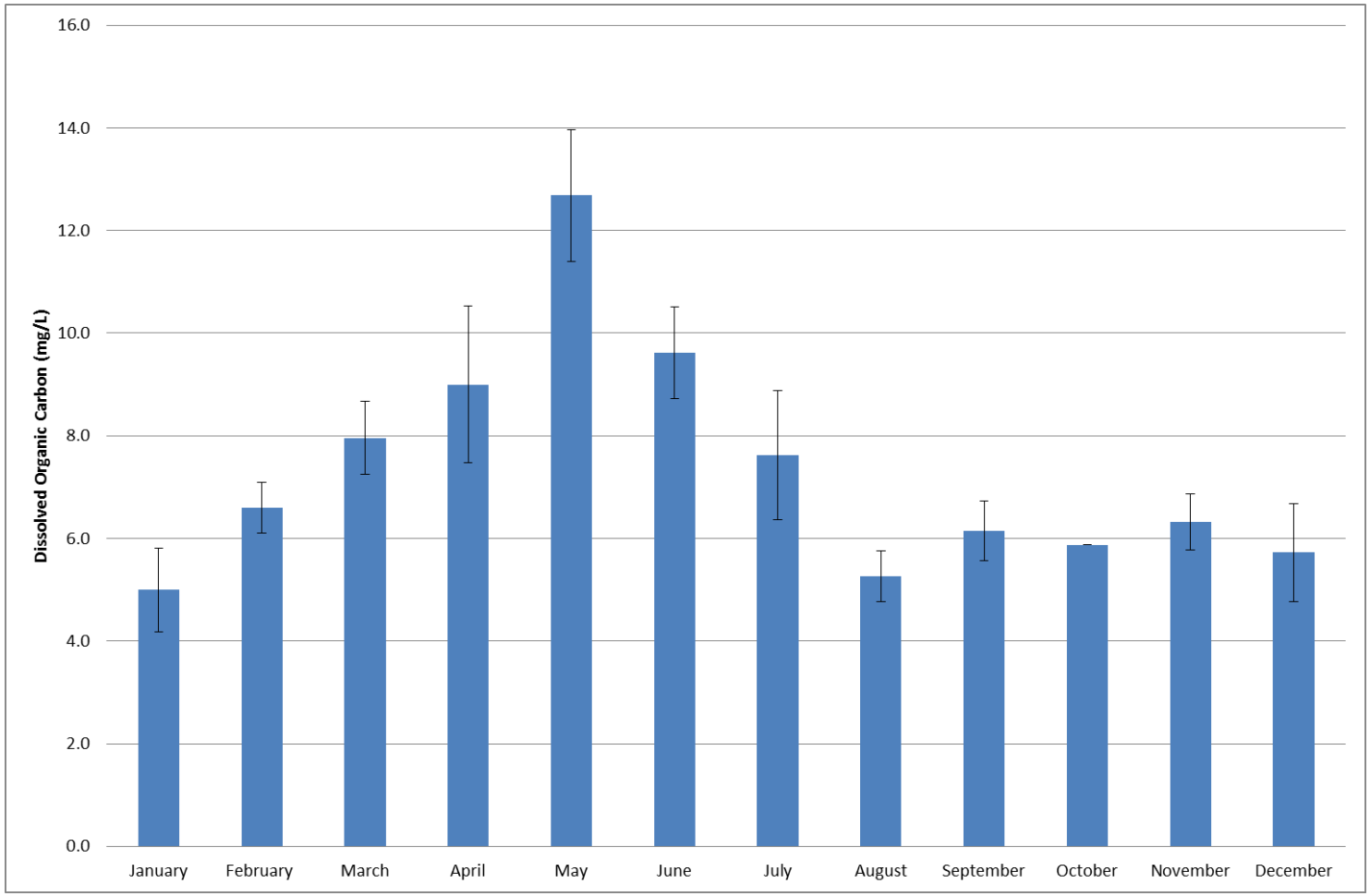


**Figure 25: Monthly averages (±SD) of dissolved organic carbon (mg/L) present in raw water entering potable water DS2 treatment plant throughout the year.**

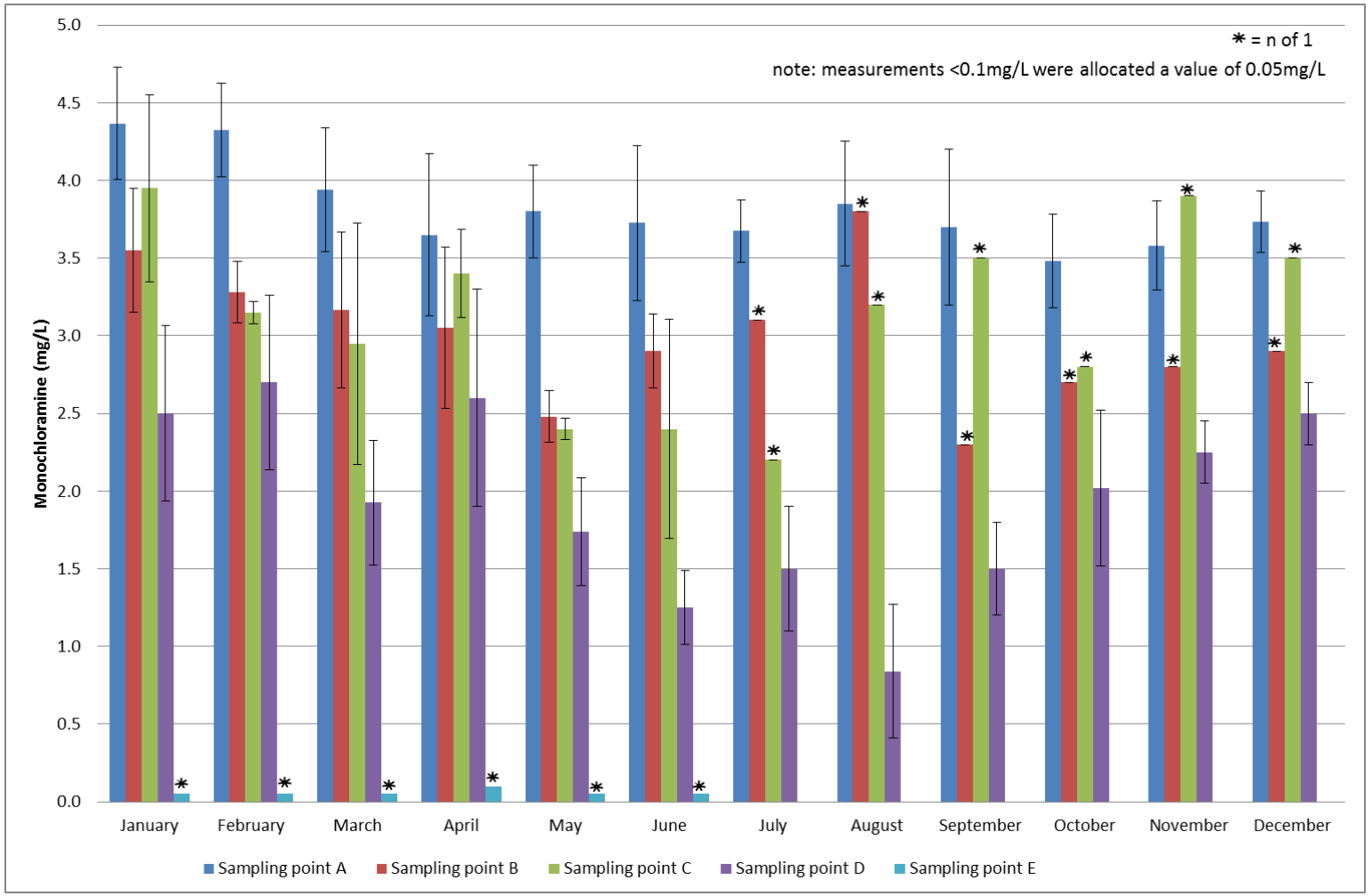


**Figure 26: Monthly average (±SD) Monochloramine (mg/L) residual measured along potable water DS2 throughout the year.**

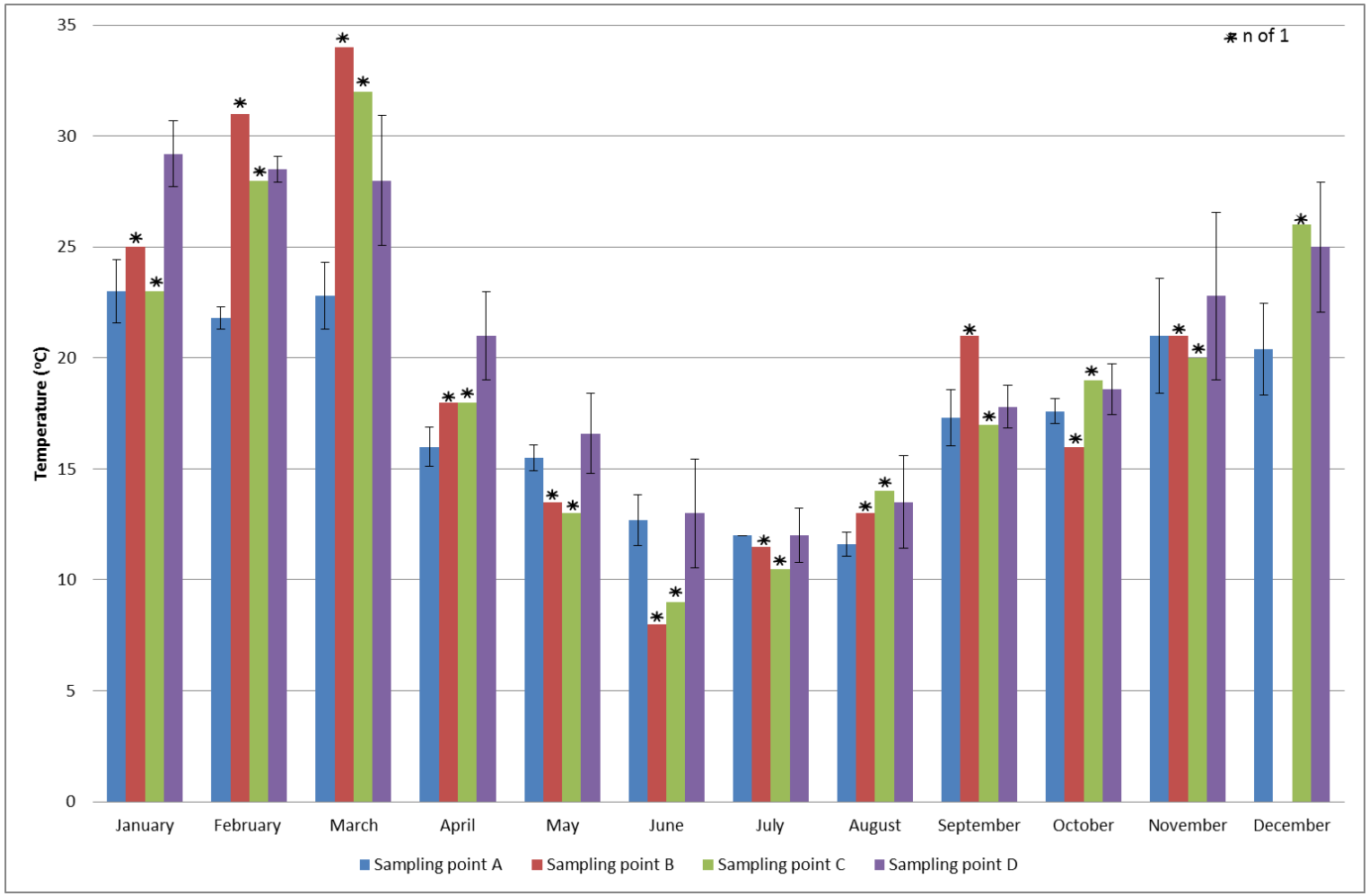


**Figure 27: Monthly average (±SD) water temperature ($^{o}$C) measured along potable water DS2 throughout the year.**

### 3.3.3 Comparison of water quality

It is well documented that variations in temperature and rainfall due to seasonal changes affects the microbial quality of raw water entering potable water treatment plants (NHMRC 2011). Also that increased temperature to >15$^{o}$C and total organic carbon levels >2.4 mg/L promote the recovery and growth of coliforms along potable water distribution systems (LeChevallier *et al.* 1991). The total organic carbon levels measure in DS2 (Tables 20-25) were >2.4 mg/L throughout the year.

In both potable water DS1 and DS2 the water temperature was seasonal (Figure 21 and Figure 27) and despite section of DS2 pipeline being above ground there was very little difference between the average water temperature in the two systems (Figure 28). There was no seasonality observed in the chlorine residual measured along DS1 (Figure 20) or the chloramine residual measured along DS2 (Figure 26) and both measurements were quite variable. In DS2 the decrease in chloramine residual observed along the pipeline at the different sampling points was quite clear where as the decrease in chlorine residual measured at the different sampling points of DS1 was more variable. No coliforms or *E. coli* was observed at any point along either distribution system.

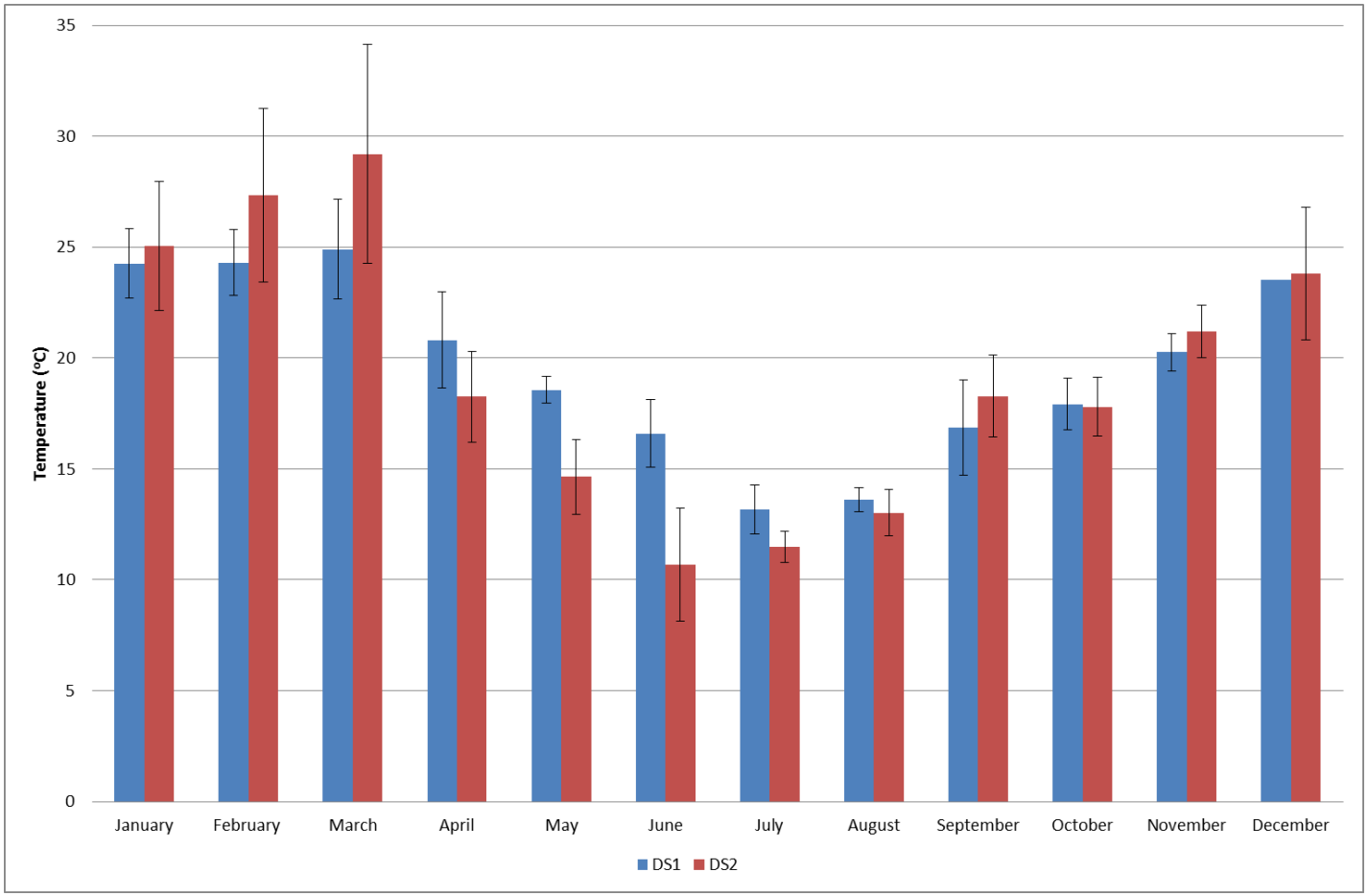


**Figure 28: Comparison of the monthly average (±SD) water temperature ($^{o}$C) along potable water DS1 and potable water DS2 throughout the year.**

**Citation:**

**Whiley, H., Keegan, A., Fallowfield, H. and Bentham, R (2014). *Legionella , L. pneumophila* and Mycobacterium avium complex (MAC) detected in South Australian potable water distribution systems, *International Journal of Environmental Research and Public Health*, 11 (7), 7393-7405; doi:10.3390/ijerph110707393**

# *Legionella, L. pneumophila* and Mycobacterium avium complex (MAC) detected in South Australian potable water distribution systems

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**Keywords:**

*Legionella* spp.; *L. pneumophila*; public health; risk assessment; environmental samples; culture; qPCR; PCR

**Abstract**

Inhalation of potable water presents a potential route of exposure to opportunistic pathogens and hence warrants significant public health concern. This study used qPCR to detect opportunistic pathogens *Legionella spp.*, *L. pneumophila* and MAC at multiple points along two potable water distribution pipelines. One used chlorine disinfection and the other chloramine disinfection. Samples were collected four times over the year to provide seasonal variation and the chlorine or chloramine residual was measured during collection. *Legionella spp.*, *L. pneumophila* and MAC were detected in both distribution systems throughout the year and were all detected at a maximum concentration of 103 copies/mL in the chlorine disinfected system and 106, 103 and 104 copies/mL respectively in the chloramine disinfected system. The concentrations of these opportunistic pathogens were primarily controlled throughout the distribution network through the maintenance of disinfection residuals. At a dead-end and when the disinfection residual was not maintained significant ($P<0.05$) increases in concentration were observed when compared to the concentration measured closest to the processing plant in the same pipeline and sampling period. Total coliforms were not present in any water sample collected. This study demonstrates the ability of *Legionella spp.*, *L. pneumophila* and MAC to survive the potable water disinfection process and highlights the need for greater measures to control these organisms along the distribution pipeline and at point of use.

## Introduction

The presence of pathogenic organisms in potable water constitutes a significant public health risk (Lehtola *et al.* 2007). This includes not only enteric pathogens such as noroviruses, *Cryptosporidium* spp. and *Campylobacter* spp.; but also opportunistic pathogens such as *Legionella* spp., Nontuberculous Mycobacterium (NTM), *Pseudomonas aeruginosa* and *Acanthamoeba* spp. (Szewzyk *et al.* 2000; Lehtola *et al.* 2007; Wang *et al.* 2012). Current public health guidelines primarily focus on the control of enteric pathogens and indicator organisms to monitor microbial water quality (Stevens *et al.* 2003). Consistently, studies have demonstrated a lack of correlation between opportunistic pathogens and indicator organisms (Hsu *et al.* 1984; Hörman *et al.* 2004; Harwood *et al.* 2005). This is of concern as opportunistic pathogens are fast becoming the primary cause of waterborne disease in developed countries (Wang *et al.* 2012).

*Legionella* spp. are the causative agent of Legionelloses, including Legionnaire's disease a serious atypical pneumonia infection, and Pontiac fever, an acute febrile illness (Buchbinder *et al.* 2002). Legionellosis is primarily caused by inhalation of contaminated aerosols and was first associated with potable water in 1980 when *Legionella* isolates from patients in a renal graft unit were identified as similar to strains isolated from shower-bath mixers in the same unit (Tobin *et al.* 1980). Recently a significant increase in the incidence of Legionellosis has been observed in the United States (Neil and Berkelman 2008) and across Europe (GIDEON Global infectious disease and epidemiology network, 2012). In the USA from 2009-2010 *Legionella* spp. were responsible for 58% of USA drinking water related disease outbreaks reported to the CDC (Centers for Disease Control and Prevention 2013c).

NTM refers to Mycobacteria distinct from the *Mycobacterium tuberculosis* complex (Schulze-Robbecke *et al.* 1992). NTM have been identified in drinking water systems, hospital distribution systems and domestic tap water (Thomson *et al.* 2013). In recent years an increase in the incidence of NTM pulmonary disease has been reported in many parts of the world (Thomson 2010). One of the most common NTM associated with human disease is Mycobacterium avium complex (MAC) which includes *M. avium (M. avium* subspecies *avium* (MAA), *M. avium* subspecies *hominis* (MAH), *M. avium* subspecies *paratuberculosis* (MAP)) and *Mycobacterium intracellulare* (Shin *et al.* 2010). In Australia 74% of all non-AIDS related NTM cases are due to MAC (O'Brien D. P. *et al.* 2000).The routes of MAC infection are via inhalation or ingestion of MAC contaminated materials. The complex is responsible for a wide range of illnesses including fibrocavitary lung disease (Field 2004), fibronodular bronchiectasis (Huang *et al.* 1999), pulmonary nodules simulating lung cancer (Lakhanpal *et al.* 2011), hypersensitivity pneumonitis (Marras *et al.* 2005), cutaneous skin (Sugita 2000) and soft tissue infection (Karakousis *et al.* 2004), cervical lymphadenitis in children (Thegerstrom *et al.* 2008), gastrointestinal tract and disseminated infection in immune compromised patients (Nightingale *et al.* 1992), and putatively Crohn's disease (Naser *et al.* 2004), cited in Whiley *et al.* (2012).

Numerous studies have linked MAC infections to potable water sources including hospital water distribution systems (von Reyn *et al.* 1994; Aronson 1999; Tobin-D'Angelo *et al.* 2004) and domestic hot water systems (Falkinham 2011). Notwithstanding, there is a paucity of studies addressing the presence of MAC in Australian potable water and routine monitoring/testing for *Mycobacteria* and *Legionella* in potable water is not mandated by Australian public health guidelines

(NHMRC 2011; Thomson *et al.* 2013). In this paper qPCR was used to investigate and compare the presence of *Legionella* spp., *L. pneumophila* and MAC along two South Australian potable water distribution pipelines. One system utilising chlorine disinfection and the other chloramine disinfection. The influence of temperature, chlorine or chloramine disinfection residual and distance along the pipeline from the water treatment plant on the concentrations of MAC and *Legionella* were assessed. The results of this investigation may permit a preliminary assessment of the possible exposure of the population to these organisms via the potable water supply.

**Experimental Section**

Samples were collected aseptically, using the AS/NZS 5667 standard method for water quality sampling, from multiple points along two South Australian potable water distribution networks, shown in Figure 1. It is important to note distribution system 2 is significantly longer than distribution system 1. Sampling was repeated four times over the year, once during summer (February), autumn (May), winter (August) and spring (November) (South Australia has a Mediterranean climate with warm summers and cool winters). The water temperature during each sampling periods was measured and provided by the water utility company. Water within Distribution System 1 (DS1) was treated with coagulation, flocculation, sedimentation, full-flow micro-filtration and disinfection with chlorine whereas water within Distribution System 2 (DS2) was treated with coagulation, flocculation, sedimentation, sand filtration, disinfection with ultra violet light and chloramine. At each sampling point the total and free chlorine or monochloramine residual was measured and 500 mL water samples collected in triplicate. The chlorine or

monochloramine present in the samples was quenched with excess sodium thiosulphate and samples stored at 4$^{o}$C for up to 12 hours before biological analysis and DNA extraction. *Escherichia coli* and total coliforms were enumerated with Colilert™ trays (IDEXX Laboratories) using the standard method.

DNA was extracted for qPCR analysis from 450mL of the sampled water using the BIO-RAD Aquadien$^{TM}$ Kit following manufacturer's instructions giving a final volume of 100 µ of DNA extract (Bio-Rad Laboratories, Inc.). Triplicate qPCR was then performed for the enumerations 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 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.

*L. pneumophila* qPCR was also performed as previously described (Giglio *et al.* 2003). The reaction volume was 25 µL and included 1 X PCR buffer (Invitrogen), 2.5 mM MgCl2 (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′ TGTCTTATAGCATTGGTGCC 3′), 0.5 µM mip213R primer (5′ CAATTGAGCGCCACTCATAG 3′) 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), 2.5mM $MgCl_2$ (Invitrogen), 2.5mM SYTO9 fluorescent dye (Invitrogen), 0.2mM deoxynucleoside triphosphate mix (Invitrogen), IU platinum Taq DNA polymerase (Invitrogen), 0.3µM MACF primer, 0.3µM 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, Sydney, Australia) with data acquisition at 72$^{o}$C on the 6-carboxyfluorescein channel (excitation at 470 nm, detection at 510 nm) at a gain of 5. Melt curve data was also acquired on this channel at gains of 2 and 5 using a ramping rate of 1°C/60 s from 75°C to 95 °C. Each qPCR run included a positive control and a non-template control of nuclease free water. For each reaction the melt curve was analysed and a positive *Legionella* spp., *L. pneumophila* and MAC was confirmed with a melting temperature ($T_m$) of 88±1$^{o}$C, 82.5±1$^{o}$C, 85±1$^{o}$C respectively.

To determine the presence of environmental inhibitors in the extracted DNA, the qPCR reactions were conducted in triplicate for both undiluted DNA extract and 1:10 dilution UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) of the same sample. If the cycle threshold ($C_T$) value for the 1:10 dilution of DNA extract was less than approximately 3.3 (representing approximately 1-$\log_{10}$ concentration value) (Livak 2001) than 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) following the manufacturer's instructions. The concentration of purified DNA was calculated by reading the absorbance spectrophotometrically at 260 nm 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). Then a 1:10 series of dilutions (ranging from $10^9$-$10^0$ copies) were 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. 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, that included the correct one this value was not included.

Statistical analysis of average calculated copies/mL of each organism at the different sampling points, time periods and distribution systems was conducted using Graph Pad$^{TM}$ prism 5.0 (Graph Pad software Inc. U.S.A.). Comparisons of the average calculated copies were performed using a one-way ANOVA with Bonferroni post hoc test, statistical significance was accepted at $P<0.05$.

**Results**

*E.coli* and total coliforms were not detected at any time throughout either distribution system. Using qPCR, *Legionella , L. pneumophila* and MAC were detected in both distribution systems throughout the year. Within each season, the concentration of *Legionella , L. pneumophila* and MAC measured at the sampling points A, closest to each respective water treatment plant as shown in Figure 1, were not statistically significantly different ($P<0.05$) between the two distribution systems. Within the pipelines, *Legionella , L. pneumophila* and MAC were all detected at a maximum concentration of $10^3$ copies/mL for the chlorine disinfected DS1 and $10^6$, $10^3$ and $10^4$ copies/mL respectively for the chloramine disinfected DS2.

The average concentrations of each organism, the season that the sample was collected, the distance from the processing plant, average water temperature and chlorine or monochloramine residual are shown in Table 1 for DS1 and Table 2 for DS2. The samples highlighted indicate where a significantly ($P<0.05$) higher concentration of an organism was detected compared to the concentration of the organism measured at sample point A for the same sampling time period. In DS1 and DS2 throughout the year there are a total of 16 water samples collected (not including sample point A) for each of these, 3 organisms were enumerated. A statistically significant increase in an organism's concentration when compared with sample point A was observed twice in DS1 and four times for DS2 and a non statistically significant increase in magnitude was observed three times in DS1 and once in DS2.

**Table 14: (Manuscript Table 1) Average concentration of *Legionella spp. L. pneumophila* and MAC (mean ± standard deviation copies/mL) measured at each sampling point of Distribution system 1 (DS1) using qPCR. Total and free chlorine (mg/L) measured when samples were collected is 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 (*).**

| Season sampled and average water temperature | Sample point | A | B | C | D | E |
|---|---|---|---|---|---|---|
| | Distance from treatment plant (km) | 5 | 7 | 10 | 18 | 22 |
| Summer | Total Chlorine (mg/L) | 1.4 | 0.7 | 1.1 | 0.4 | 0.4 |
| 24.3°C (n=8) | Free chlorine (mg/L) | 1.2 | 0.6 | 0.1 | 0.2 | 0.2 |
| | Average *Legionella spp*. (copies/mL) | 37 ±53 | 9 ± 4 | 3 ±0 | 187 ±22 | +1238 ±47 |
| | Average *L. pneumophila* (copies/mL) | 10 ±8 | 3 ±0 | 3 ±0 | 375 ±305 | *1981 ±298 |
| | Average MAC (copies/mL) | 36 ±19 | 42 ±19 | *31813 ±17017 | 116 ±118 | +4395 ±2176 |
| Autumn | Total chlorine (mg/L) | 1.5 | N/A | 1.0 | 0.8 | 1.1 |
| 18.6°C (n=8) | Free chlorine (mg/L) | 1.3 | N/A | 0.8 | 0.6 | 0.9 |
| | Average *Legionella spp*. (copies/mL) | 5 ±4 | N/A | 41 ±21 | 47 ±13 | 46 ±17 |
| | Average *L. pneumophila* (copies/mL) | 3 ±0 | N/A | 3 ±0 | 46 ±68 | +487 ±406 |
| | Average MAC (copies/mL) | 25 ±0 | N/A | 25 ±0 | 200 ±157 | 25 ±0 |
| Winter | Total chlorine (mg/L) | 1.2 | N/A | 1.3 | 0.4 | 0.7 |
| 13.6°C (n=8) | Free chlorine (mg/L) | 1.1 | N/A | 1.3 | 0.3 | 0.6 |
| | Average *Legionella spp*. (copies/mL) | 22 ±31 | N/A | 3 ±0 | 5 ±1 | 3 ±0 |
| | Average *L. pneumophila* (copies/mL) | 81 ±134 | N/A | 3 ±1 | 3 ±0 | 3 ±0 |
| | Average MAC (copies/mL) | 25 ±0 | N/A | 25 ±0 | 25 ±0 | 25 ±0 |
| Spring | Total chlorine (mg/L) | 1.0 | 0.7 | 0.3 | 0.4 | 0.7 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 20.3°C (n=8) | Free chlorine (mg/L) | 0.9 | 0.6 | 0.2 | 0.3 | 0.6 |
| | Average *Legionella spp*. (copies/mL) | 43 ±69 | 120 ±151 | 8 ±3 | 93 ±139 | 6 ±6 |
| | Average *L. pneumophila* (copies/mL) | 36 ±34 | 15 ±12 | 9 ±1 | 166 ±122 | 3 ±0 |
| | Average MAC (copies/mL) | 2468 ±317 | 2224 ±2342 | 1112 ±328 | 294 ±58 | 101 ±69 |

N/A sample was not available to be collected at this time.
*statistically significant increase
+an increase of concentration by an order of magnitude. The lack of statistical significance (P>0.05) is possible due to the large variance in environmental samples shown by the standard deviation.

**Table 15: (Manuscript Table 2) Average concentration of *Legionella spp. L. pneumophila* and MAC (mean ± standard deviation copies/mL) measured at each sampling point of Distribution system 2 (DS2) using qPCR. Total monochloramine (mg/L) measured when samples were collected is 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 (*).**

| Season sampled and average water temperature | Sample point | A | B | C | D | E |
|---|---|---|---|---|---|---|
| | Distance from treatment plant (km) | 1 | 40 | 54 | 137 | 54 |
| Summer | Monochloramine (mg/L) | 3.6 | 3.0 | 3.6 | 2.7 | <0.05 |
| 27.3°C (n=10) | Average *Legionella spp.* (copies/mL) | 444 ±96 | 161 ±19 | 134 ±94 | 423 ±399 | *316956 ±1698982 |
| | Average *L. pneumophila* (copies/mL) | 105 ±8 | 712 ±158 | 479 ±177 | 12 ±16 | 941 ±154 |
| | Average MAC (copies/mL) | 9755 ±7808 | 6910 ±6128 | 2803 ±584 | 1739 ±539 | 5362 ±1612 |
| Autumn | Monochloramine (mg/L) | 3.8 | 2.3 | 2.4 | 1.5 | <0.05 |
| 14.7°C (n=13) | Average *Legionella spp.* (copies/mL) | #24238 ±2918 | 260 ±10 | 24 ±19 | 1597 ±600 | 1094 ±284 |
| | Average *L. pneumophila* (copies/mL) | 238 ±232 | 87 ±14 | 26 ±40 | 666 ±73 | 333 ±86 |
| | Average MAC (copies/mL) | 542 ±103 | 663 ±325 | 4068 ±1193 | 586 ±0 | +1424 ±482 |
| Winter | Monochloramine (mg/L) | 3.6 | 3.7 | 2.3 | 0.8 | 0.2 |
| 13.0°C (n=9) | Average *Legionella spp.* (copies/mL) | 2016 ±60 | 883 ±143 | 303 ±34 | 197 ±99 | *177727 ±102437 |
| | Average *L. pneumophila* (copies/mL) | 248 ±31 | 566 ±220 | 573 ±133 | 281 ±185 | *3176 ±1950 |
| | Average MAC (copies/mL) | 367 ±395 | 277 ±144 | 4228 ±3607 | 433 ±271 | +9526 ±3271 |
| Spring | Monochloramine (mg/L) | 2.0 | 2.8 | 3.9 | 2.1 | <0.05 |
| 21.2°C (n=10) | Average *Legionella spp.* (copies/mL) | 913 ±88 | 1780 ±251 | 914 ±48 | 1111 ±1359 | *1289587 ±53042 |
| | Average *L. pneumophila* (copies/mL) | 10 ±2 | 3 ±0 | 4 ±2 | 38 ±31 | 19 ±17 |

| | | | | | |
|---|---|---|---|---|---|
| Average MAC (copies/mL) | 5184 ±1464 | 2577 ±483 | 2507 ±1615 | 2039 ±475 | 11445 ±3478 |

N/A sample was not available to be collected at this time.
*statistically significant increase
+an increase of concentration by an order of magnitude. The lack of statistical significance (P>0.05) is possible due to the large variance in environmental samples shown by the standard deviation.
# magnitude higher, assumed to be due to biofilm fragment

Table 1 shows that in the chlorine disinfected DS1 a statistically significant ($P<0.05$) increase in *L. pneumophila* and MAC and a decrease in chlorine residual was observed during the summer time point of sampling. Although the concentration of *Legionella* spp. detected at sampling point E was not significantly different ($P>0.05$) to that measured a point A there was still a magnitude increase. The lack of statistical significance was possibly due to the small sample size and the variability (shown by the standard deviation) due to environmental samples and should not detract from the public health significance of a magnitude of increase in *Legionella* concentration given the logarithmic action approach to these organisms adopted by most guidelines(Bartram *et al.* 2007). Bearing this in mind the significance of the p value should not negate the significance of an effect in terms of human health and regulation (Nuzzo 2014). Although a similar decrease in the chlorine residual was observed during the winter and spring sampling periods there were no increases in *Legionella* , *L. pneumophila* or MAC concentrations. This suggests that the combination of low chlorine residual and warmer water at the summer time point may have resulted in the increased detections of *L. pneumophila* and MAC observed.

In Distribution system 2, sample point E, is the location of a dead-end where the monochloramine residual was not maintained. Table 2 shows that *Legionella spp*. and *L. pneumophila* were shown to significantly increase ($P<0.05$) only at this sampling point. An increase in magnitude of MAC concentration was also observed at sample point E during the sampling that occurred in autumn and winter. A significant increase in organism concentrations did not occur at sample point C which is also 54km from processing plant but not a dead-end. This suggests that the environmental conditions occurring at the dead-end are promoting growth. The significant increases in *Legionella* , *L. pneumophila* or MAC concentrations at

sample point E occurred during summer, winter and spring suggesting that water temperature was not the dictating factor for these increases. Sample point E is 54 km away from the processing plant; however, no increase in *Legionella* spp. was observed at sample point D which is 137 km from the processing plant. The monochloramine residual was maintained along the pipeline prior to sample point D; however, it was not maintained prior to sample point E.

**Discussion**

This study detected *Legionella, L. pneumophila* and MAC in two South Australian potable water distribution systems, but failed to detected *E.coli* or total coliforms. The absence of these indicator organisms further supports previous studies which suggest that monitoring of these organisms alone is not sufficient for determining the presence or absence of potential public health risks (Stevens *et al.* 2003).

This study used qPCR to detect *Legionella , L. pneumophila* and MAC due to the difficulties with culturing *Legionella* spp., specifically with the possible presence of viable but non-culturable cells (VBNC) (Whiley and Taylor 2014). *Legionella* spp. have been shown to become VBNC in low nutrient environments and in the presence of chlorine (Chang *et al.* 2007) or monochloramine (Alleron *et al.* 2008). However, a challenge with qPCR is that it enumerates both viable and killed intact cells. The discrepancies in results obtained between different *Legionella* detection methods make risk assessment extremely difficult. This is because of a lack of comparability in *Legionella* detection during different studies (Whiley and Taylor 2014). Some studies have demonstrated that qPCR coupled with ethidium monoazide (EMA) or propidium monoazide (PMA) pre-treatment enables quantification of only viable

cells (Chang *et al.* 2009; Delgado-Viscogliosi *et al.* 2009; Yáñez *et al.* 2011; Qin *et al.* 2012). EMA and PMA are light activated compounds which bind to free DNA not protected by a cell wall and hence allows only DNA contained within intact cells to be amplified by qPCR. The difficulty with these methods is that the concentration of EMA or PMA has to be optimised for the total DNA present in a sample which is unfeasible for environmental samples of unknown concentration. Studies have shown if the concentration of EMA is too high it may penetrate intact cell, potentially resulting in false negatives (Flekna *et al.* 2007; Kobayashi *et al.* 2009). Alternatively, insufficient concentration may result in free DNA remaining unbound, causing false positive results (Fittipaldi *et al.* 2011).

A statistically significant increase in the concentration of *Legionella, L. pneumophila* or along the pipeline was observed 2/16 times in DS1 and 4/16 for DS2. This suggests that although there is a potential for amplification of these organisms within the system it is not commonplace. Statistically significantly increases in an organisms' concentrations occurred sporadically, with low disinfectant residual the only reoccurring accompanying trend. The detected and demonstrable increases in concentrations of an organism at certain points along the distribution network could be due to a number of factors. These might include: the inclusion of a biofilm fragment in the water sample; the accumulation of organisms due to a dead-end; contamination through breaks cracks and joints in the pipeline, or multiplication of organisms (Robertson *et al.* 2003). An inclusion of a biofilm fragment could explain the significant increase observed at DS1 point C during summer, as the increase was not observed further down the distribution line at point D. This is supported by the large standard deviation would could be cause by variation in biofilm fragments within water samples. A $\log_{10}$ decrease in MAC copies/mL was also observed from

point C to D in DS2 during autumn and winter; however, this was not statistically significant due to the large variation in samples and could also be attributed to the inherent variation observed with environmental water samples and the possible addition of biofilm fragments.

The correlation of increases in microbial concentration with low disinfectant residual was expected due to replication. Further research may be required to confirm this opinion. Significant increases due to the inclusion of a biofilm fragment, still represents a result of public health significance. In this instance the multiplication within the biofilm indicates viability and persistence, and the sporadic release of concentrations of public health concern. Sample point E in DS2 was a dead-end, the increased concentrations of *Legionella* observed here demonstrated that dead-ends are clearly of concern. The increase in detected *Legionella* copies may be due to low flow rate, low turbulence resulting in the quenching of disinfectant residual by biofilm and organic matter within the dead-end. Further investigation may determine the relative influence of these factors.

The concentration of *Legionella* spp. measured at sample point A in DS2 during the autumn sampling period has been highlighted in Table 2 as it was a magnitude higher than the concentrations measured at the sampling points further down the pipeline. This increase was assumed likely to be due to the inclusion of a biofilm fragment in the water sample. It highlights the difficulty of measuring the concentration of an organism in environmental water sample which may include biofilm fragments.

The presence of *Legionella* and MAC in South Australian potable water supports the work by Wang *et al.* (2012) who used qPCR to detect *Legionella* spp. and

*Mycobacterium* spp. from point of use domestic potable water taps in the USA. The highest concentrations of *Legionella, L. pneumophila* and *Mycobacterium* spp. detected by Wang *et al.* (2012) was $10^3$, $10^1$ and $10^5$ copies/mL respectively, which was comparable with the highest concentrations found in these two distribution pipelines.

**Conclusions**

This study confirms the presence of opportunistic pathogens *Legionella spp., L. pneumophila* and MAC in both a chlorine and a chloramine disinfected potable water distribution system. The concentrations of these opportunistic pathogens were primarily controlled throughout the distribution network through maintenance of disinfection residuals. However, at a dead-end and when the disinfection residual was not maintained, the pathogens were able to significantly increase in concentration. The potential for dead-ends in pipes to promote growth warrants more attention in efforts to control *Legionella, L. pneumophila* and MAC within these environments. The public health significance of these increases in *Legionella, L. pneumophila* and MAC is challenging to assess due to the difficulties with interpreting qPCR results. Improved detection methods will result in a better understanding of the environmental factors influencing colonisation of these systems. The increases in concentrations also demonstrated that these opportunistic pathogens have the potential to survive both disinfection processes. This may have important implications for control along the distribution network and at point of use, particularly in large buildings that may require water to be stored or piped a considerable distance prior to usage.

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**Conflicts of Interest**

The authors declare no conflict of interest

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# Chapter 4: Water Reuse

The reuse of water is becoming increasing important for sustainable water management. There is limited knowledge on the presence and fate of opportunistic pathogens and the occurrence of microbial regrowth along reuse water distribution pipelines. This chapter describes the use of qPCR to detected opportunistic human pathogens, *Legionella* spp., *L. pneumophila* and MAC along two South Australian reuse water distribution systems. This is the first study to detect *Legionella* spp., *L. pneumophila* and MAC in South Australian reuse water. The effect of seasonality, and disinfection residual on *Legionella* spp., *L. pneumophila,* MAC and the relationship with coliforms are also explored. The risk assessment for the potential exposure to these opportunistic pathogens through reuse water is explored in Chapter 5.

## 4.1 Reuse water distribution system 1 (DS1)

Reuse water DS1 reticulates recycled wastewater. The water quality is classified as suitable for "unrestricted municipal use for irrigation", this includes open spaces, sports grounds and golf courses. The reuse water treatment plant disinfects secondary effluent from wastewater with micro-screening, ultra filtration, ultraviolet light and chorine disinfection processes. Water samples were collected along the pipeline and the distance away from the reuse water treatment plant, pipe diameter and pipe material of each sample location is shown in Table 16.

**Table 16: Description of sampling points along reuse water DS1.**

| Sample | Approx. distance from WWTP (km) | Description | |
|---|---|---|---|
| | | Pipe diameter (mm) | Pipe material |
| A | 0 | Within the recycled water treatment plant Outlet to chlorine | |
| B | 11 | 900 | Mild steel cement lined |
| C | 14 | 150 | Polyvinyl chloride (PVC) |

### 4.2 Reuse water distribution system 2 (DS2)

Reuse water DS2 is comprises of recycled wastewater and reclaimed stormwater. The reclaimed stormwater is filtered through wetlands and reuse water is treated with chlorine disinfection. The water is utilised for flushing toilets, watering gardens including fruit trees and vegetable gardens, washing cars, washing pets and for evaporative coolers and air conditioners. Figure 29 shows a schematic of how the reuse water system is utilised within the residential community. Water samples were collected along the pipeline and the distance away from the reuse water treatment plant of each sample location is shown in Table 17.

**Table 17: Description of sampling points along reuse water DS2.**

| Sample | Approx. distance from WWTP (km) | Description | |
|---|---|---|---|
| | | Pipe diameter (mm) | Pipe material |
| A | 0 | N/A | N/A |
| B | 1 | N/A | N/A |
| C | 1 | N/A | N/A |
| D | 2 | N/A | N/A |

*N/A = data not available

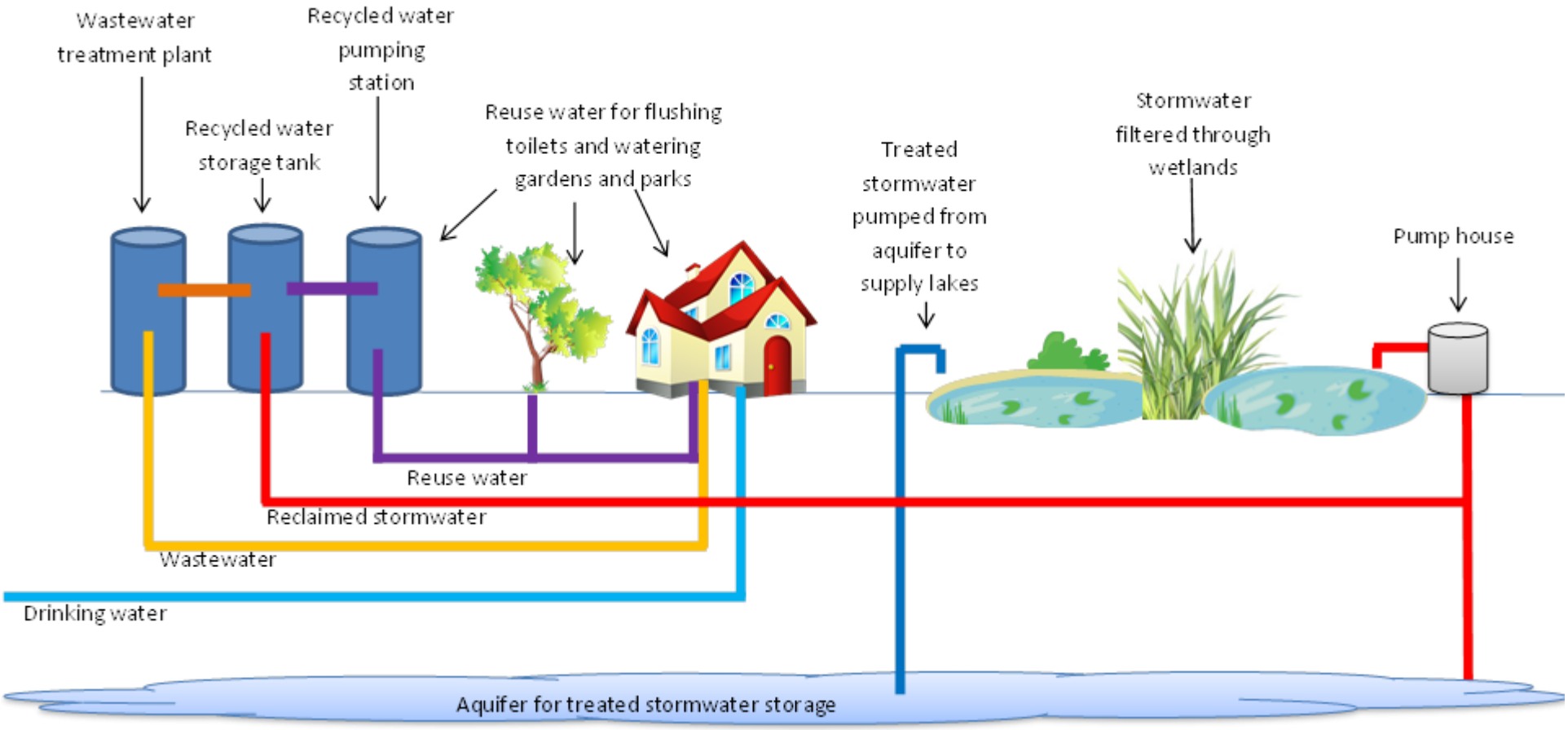


**Figure 29: schematic of how reuse water DS2 functions within the residential community.**

### 4.3 Water quality data

To further characterise the reuse water systems and provide information regarding the effect of seasonality in the water quality, data was accessed from the water utility's routine sampling data records. Information regarding seasonality of water temperature and chlorine residual was targeted as these are widely recognized as the primary factors factors influencing bacterial growth (LeChevallier 2003). Data for key physicochemical water quality parameters such as Biochemical Oxygen Demand (BOD) (the amount of oxygen utilised by micro-organisms through decomposition of organic material over a period of 5 days at 20$^{o}$C), turbidity and suspend solids (insoluble solid matter suspended in water) were also targeted. These parameters are often used as indicators of wastewater quality and effectiveness of the treatment process (Greenberg *et al.* 1992). Information was gathered from the wastewater prior to treatment and also at each sampling location. The seasonality of water usage volumes and flow rates was also targeted, but due to commercial confidentiality these values could not be included. Typically highly flow rates and usage volumes were observed during summer when there was a greater demand for reuse water utilised for irrigation purposes. Once all the data available was collated statistical consultation was sought from a statistician at Flinders University. Unfortunately due to gaps in the data and limited replications this information could not be used for statistical analysis or modelling purposes. However, monthly averages of the available data have been included (see appendix) to provide an overview of the water quality of the reuse systems and for comparisons to other studies in the future. The monthly averages demonstrated the variation in water quality parameters over the year sampled and throughout the pipelines.

In both DS1 and DS2 there is little seasonal variation in the BOD and suspended solids present in the sewage entering the two water treatment plants. There was a small decrease in DS2 during July which could be attributed to increased rainfall during winter. No seasonal trends in BOD are observed in either pipeline, although there is some variation throughout the year in DS2. There is no variation in total dissolved solids (Figure 35 and Figure 38) between different sample points of the same pipeline during a particular time period, this suggest that it is unlikely that there any cracks or breaks in the pipeline allowing contaminants to enter into the system . In both distribution systems the free chlorine was higher during the summer months and there were small variations between sampling points along the pipeline which was expected as the chlorine residual would decrease further down the pipeline. There was no seasonality observed in the total coliforms/100mL in DS2 and no relationship between levels observed at the two sampling points.

### 4.4.1 Temperature data

For the year sampled water temperature data was only available for DS2 (Figure 31). Both DS1 and DS2 pipelines run below ground and are situated <20km away from each other. The average monthly water temperatures of DS2 were compared to the monthly mean ambient temperatures for this location provide by the Australian Bureau of Meteorology (BOM) (Australian Bureau of Meteorology 2014). The resulting $r^2$ value (shown in figure 30) for this relationship was 0.8816 ($P<0.0001$), which means 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 (shown in Figure 31).

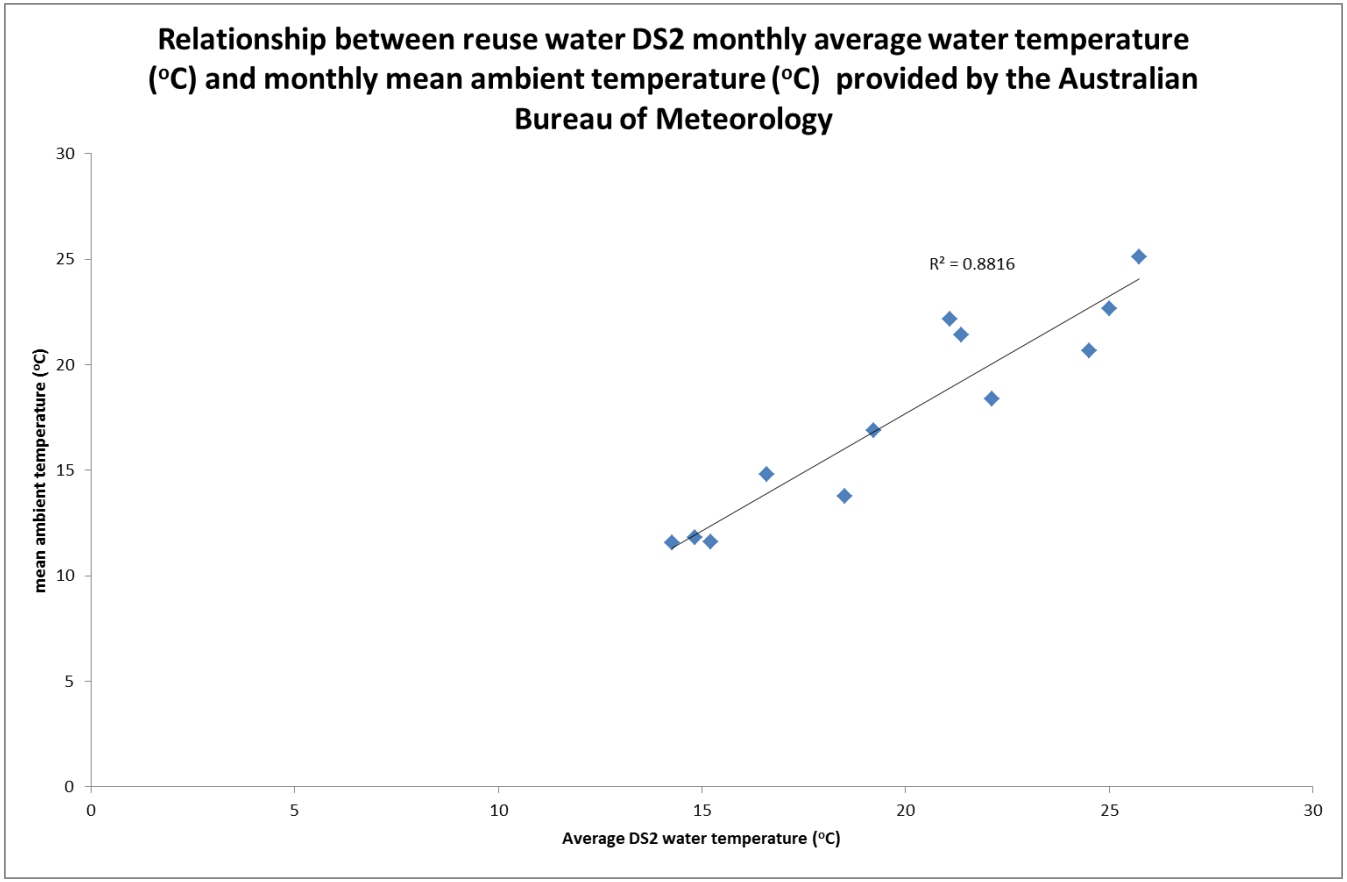


**Figure 30: Relationship between reuse water DS2 monthly average water temperature ($^{o}$C) and monthly mean ambient temperature ($^{o}$C) provided by the Australian Bureau of Meteorology (r2 = 0.8816).**

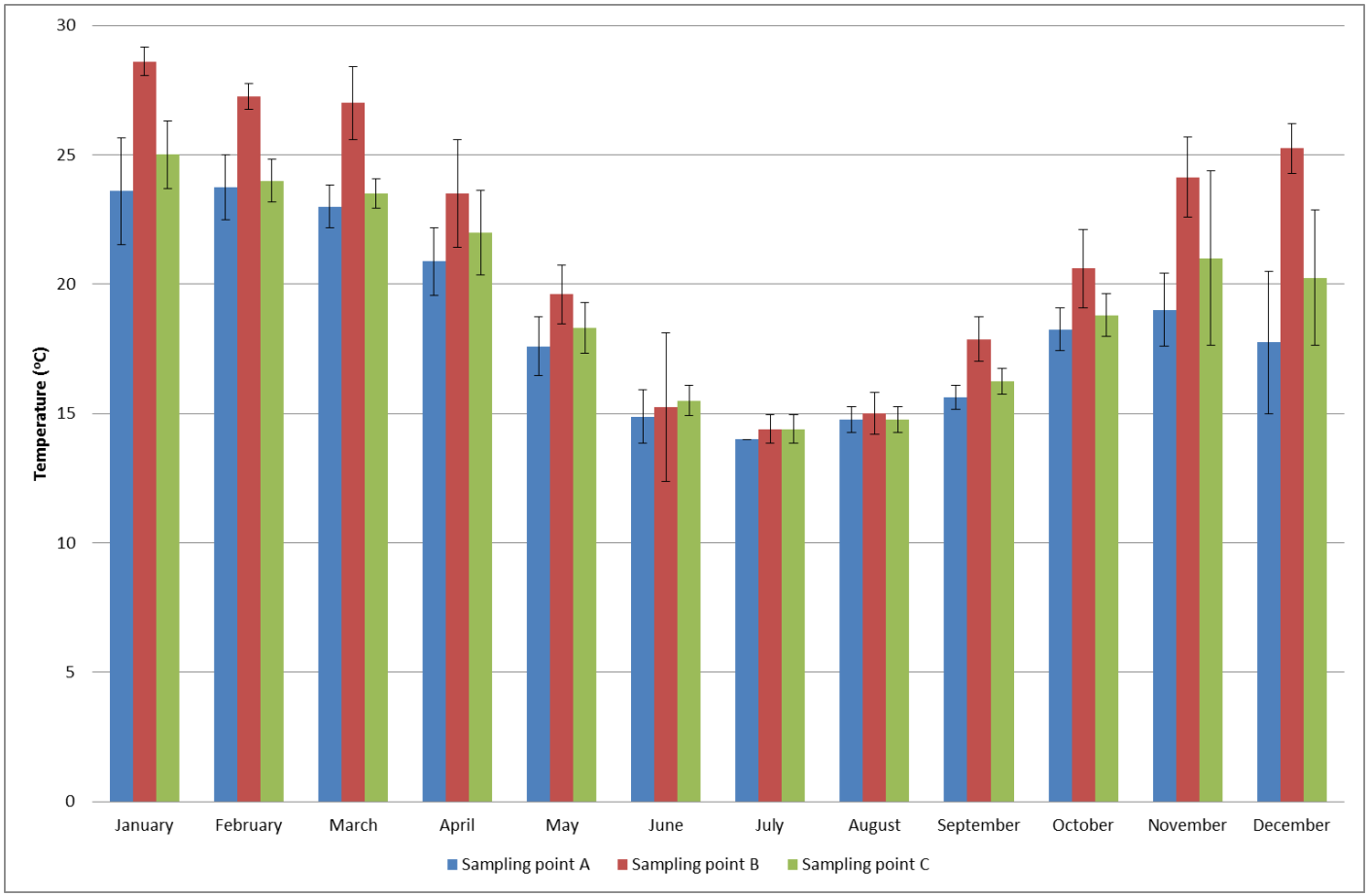


**Figure 31: Monthly averages (±SD) of water temperature ($^{o}$C) measured at sampling point A, B and C of reuse DS2 throughout the year.**

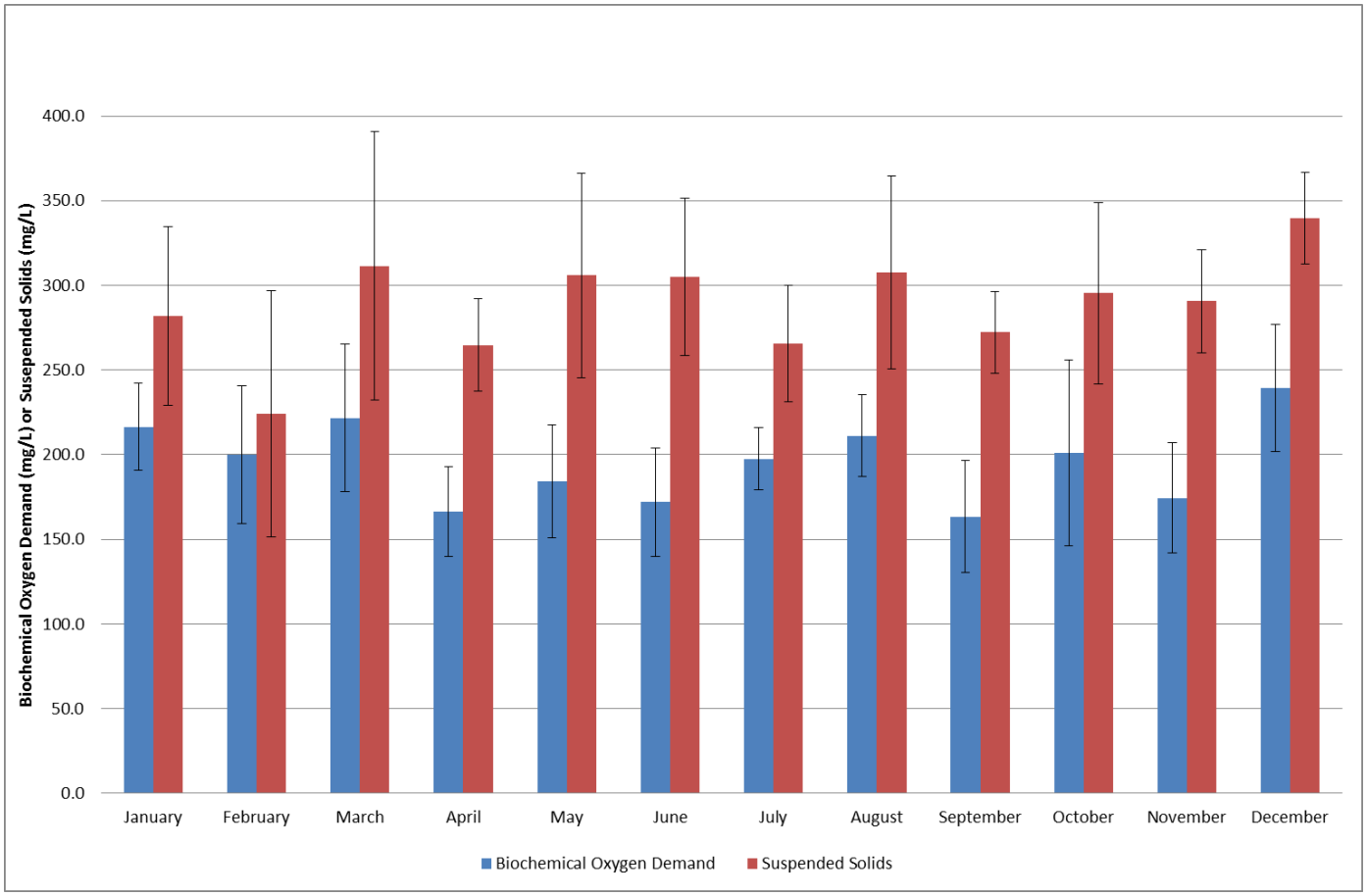


**Figure 32: Monthly average (±SD) of Biochemical Oxygen Demand (mg/L) and Suspended Solids (mg/L) present in sewage entering treatment plant of reuse DS1 throughout the year.**

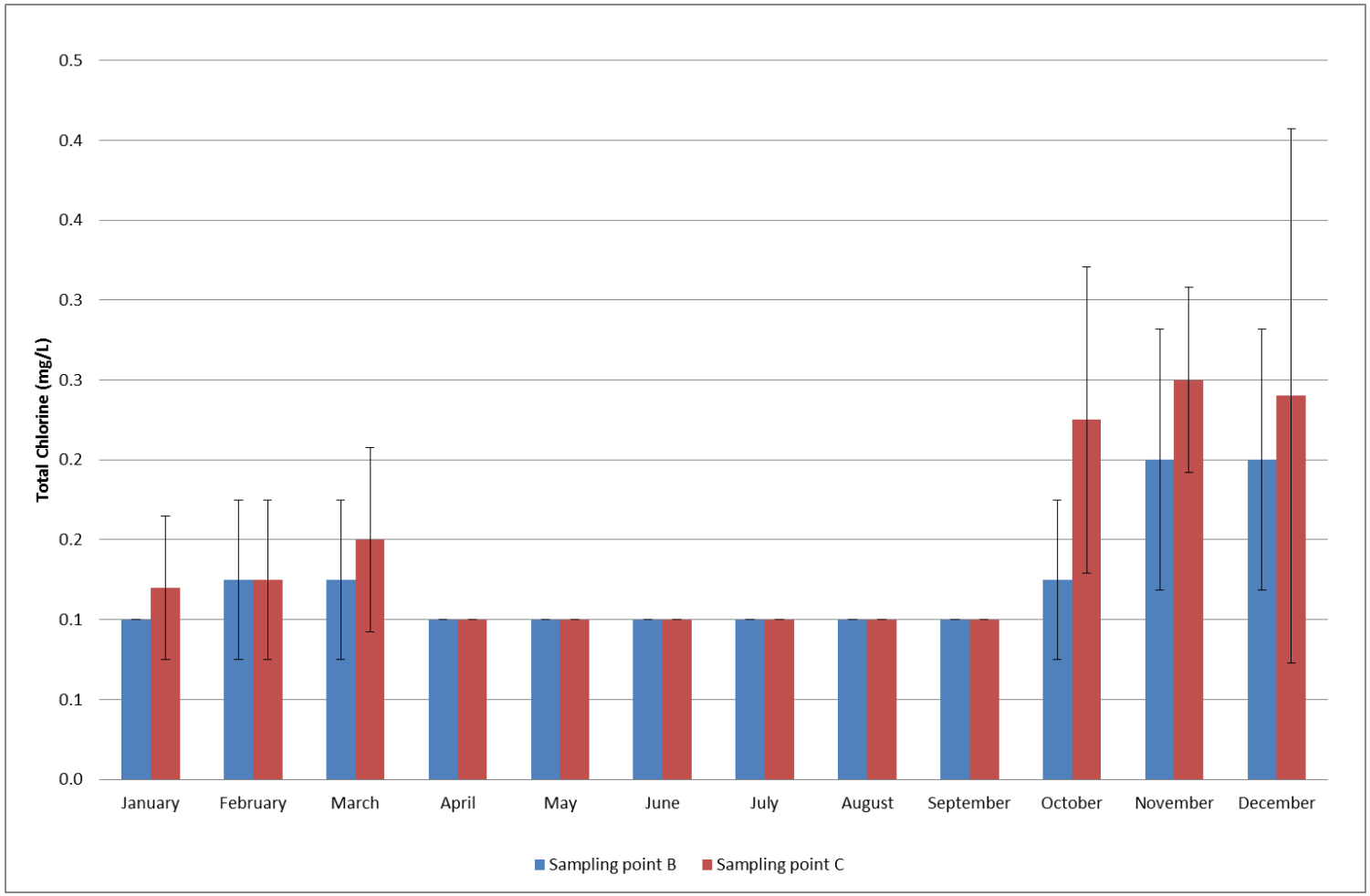


**Figure 33: Monthly averages (±SD) of total chlorine (mg/L) measured at sampling points B and C of reuse DS1 throughout the year to show seasonality.**

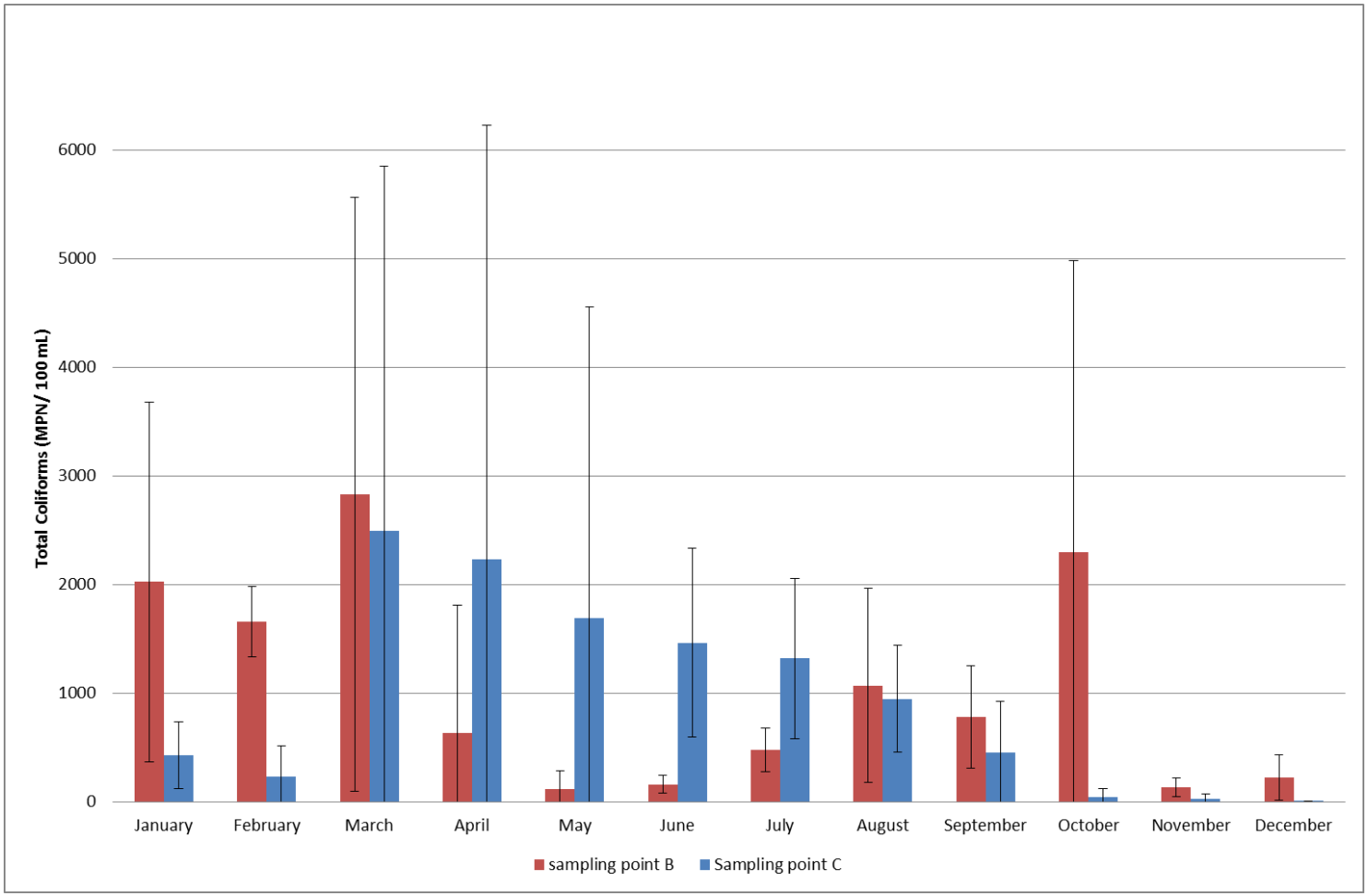


**Figure 34: Monthly averages (±SD) of total coliforms/100mL measured at sampling points B and C of reuse DS1 throughout the year to show seasonality.**

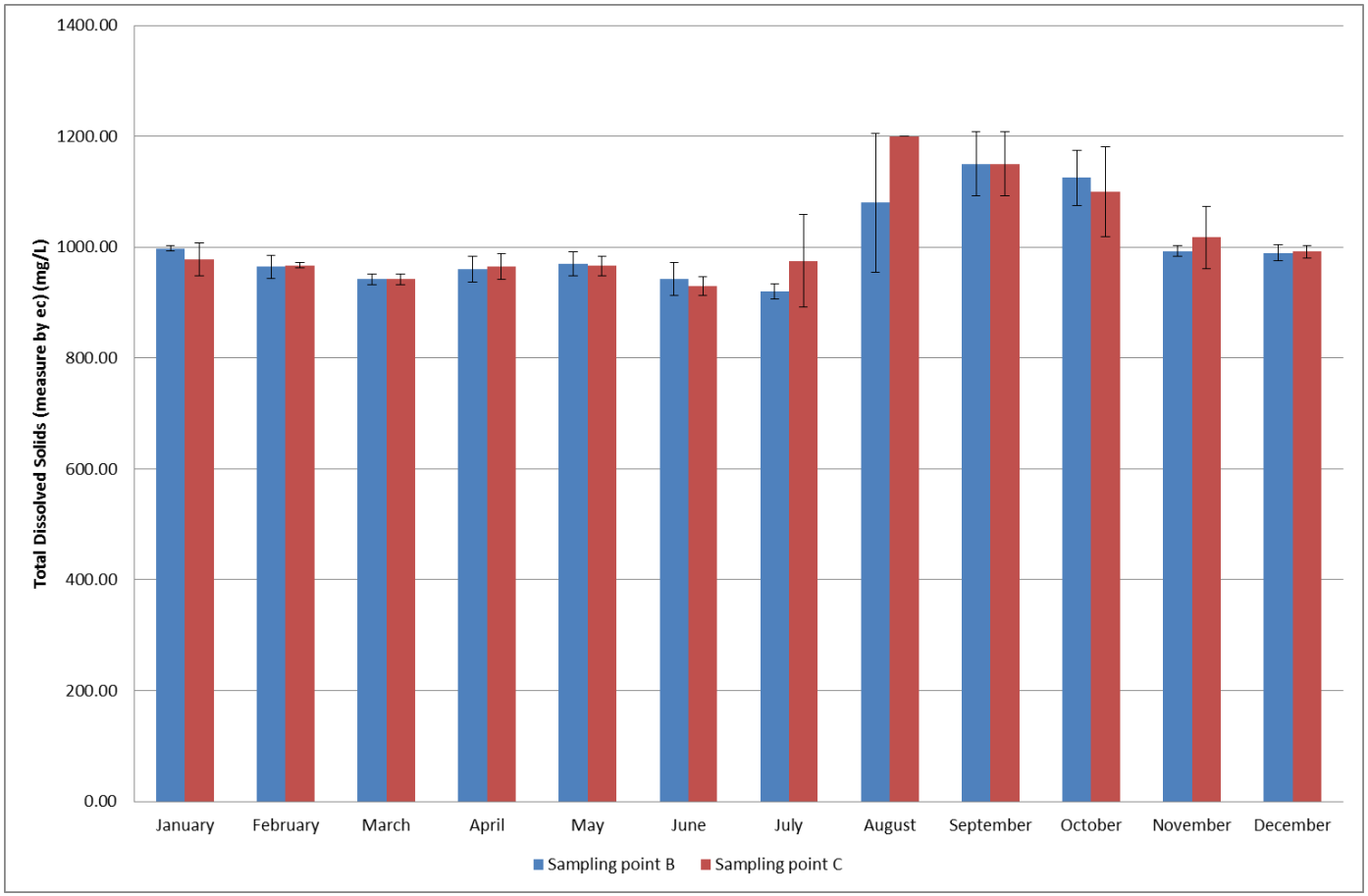


**Figure 35: Monthly averages (±SD) of total dissolved solids (mg/L) measured at sampling points B and C of reuse DS1 throughout the year.**

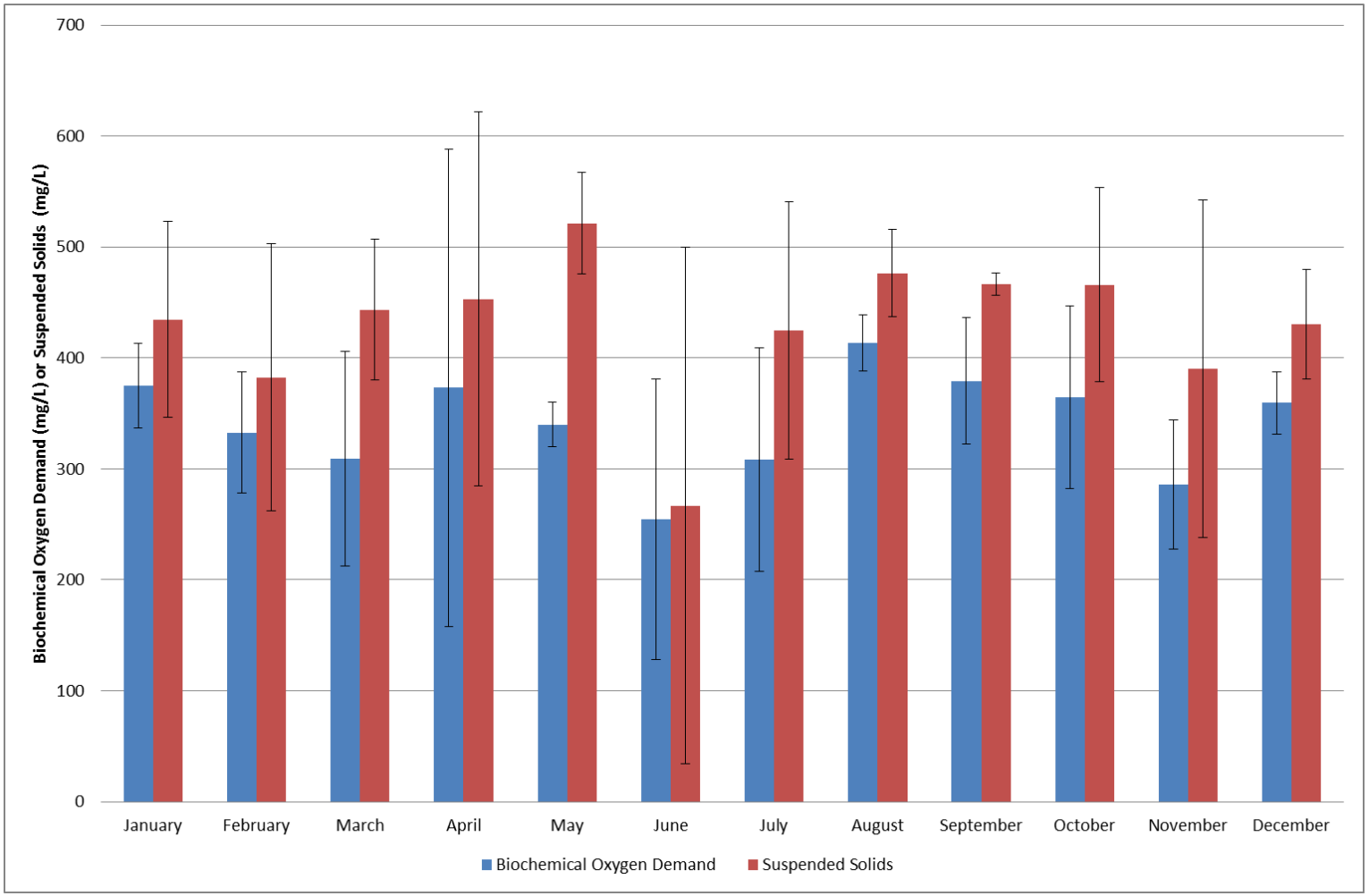


**Figure 36: Monthly averages (±SD) of biochemical oxygen demand (mg/L) and suspended solids (mg/L) present in sewage entering DS2 water treatment plant throughout the year.**

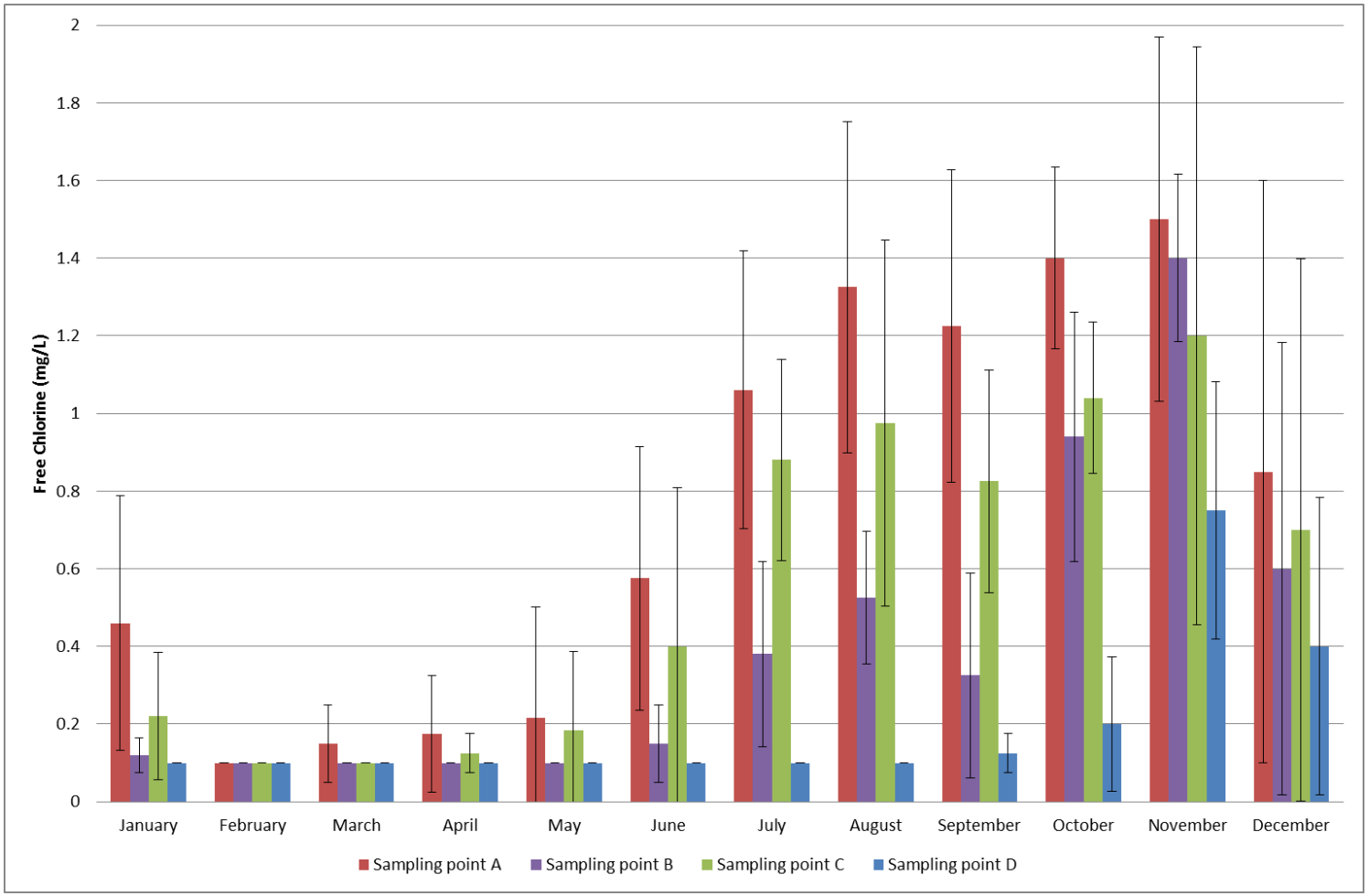


**Figure 37: Monthly averages (±SD) of free chlorine (mg/L) measured at each sampling point along reuse DS2 throughout the year.**

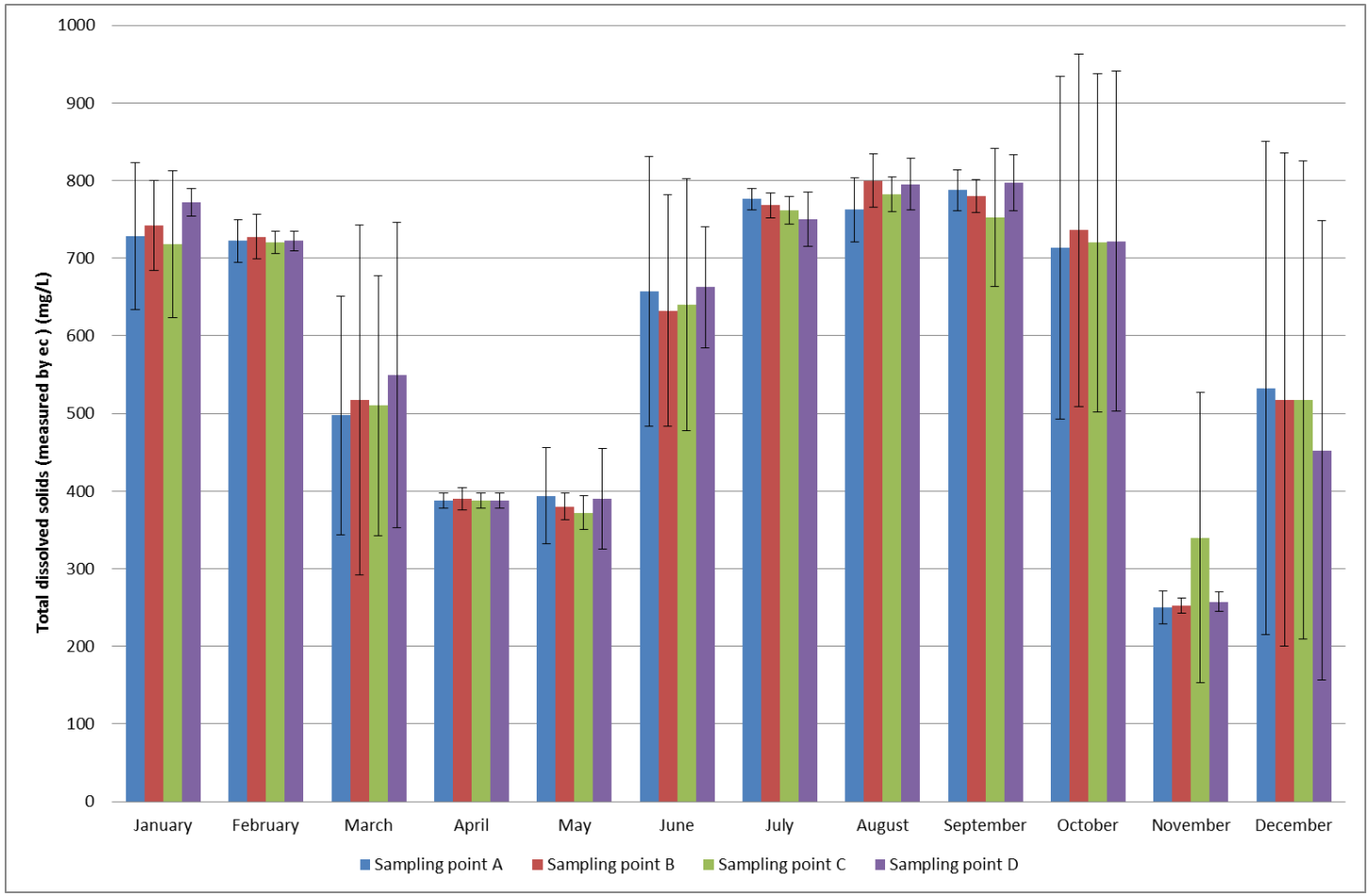


**Figure 38: Monthly averages (±SD) of total dissolved solids (mg/L) measured at each sampling point along reuse DS2 throughout the year.**

**Citation:**

**Whiley, H., Keegan, A., Fallowfield, H., and Bentham, R., (2014), The presence of opportunistic pathogens, *Legionella* spp., *L. pneumophila* and Mycobacterium avium complex (MAC), in South Australian reuse water distribution pipelines, *Journal of Water and Health*, doi:10.2166/wh.2014.317.**

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

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**Running title:** *Legionella* and MAC in reuse water

**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 MAC were detected using qPCR 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 ($P<0.05$) increased 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.

## Introduction

Urbanisation and population growth has 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 is little 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 (Falkinham *et al*., 2008, Kool *et al*., 1999, Nishiuchi, 2009, Zmirou-Navier *et al*., 2007). They have also been detected in both wastewater and stormwater (Catalan *et al*., 1997, Lampard *et al*., 2012, Pickup *et al*., 2006) 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, Trebesius and Heesemann, 2002). In USA between 2009-2010, 57.6%

of potable water related disease outbreaks were due to *Legionella* spp. (Centers for Disease Control and Prevention, 2013) and in 2011 across Europe there were 4897 confirmed cases of Legionellosis (0.97 cases per 100,000) reported to the European Centre for Disease Control (European Centre for Disease Prevention and 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 (Field, 2004, Huang *et al*., 1999, Lakhanpal *et al*., 2011, Marras *et al*., 2005), skin and soft tissue infections (Karakousis *et al*., 2004, Sugita, 2000), 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 (Al-Houqani *et al*., 2012, Lai *et al*., 2010, Prevots *et al*., 2010).

This study used 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 (Declerck, 2010, Schulze-Robbecke *et al*., 1992) and are opportunistic parasites of free living protozoa (Salah and Drancourt, 2010, Tyndall and Domingue, 1982). It has been suggested that these attributes enable them to persist in water distribution systems (Codony *et al*., 2011, Payment and Robertson, 2004).

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

**Water temperature**

Water temperature data was data mined from water utilities routine monitoring data records. Unfortunately temperature data was 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 <20km 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 (BOM) (http://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) using the standard method.

**Real time polymerase chain reaction (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$^{TM}$ Kit following manufacturer's instructions giving a final volume of 100 µL of DNA extract (Bio-Rad Laboratories, Inc.). 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.

*L. 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′ TGTCTTATAGCATTGGTGCC 3′), 0.5 µM mip213R primer (5′ CAATTGAGCGCCACTCATAG 3′) 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), 2.5mM $MgCl_2$ (Invitrogen), 2.5mM SYTO9 fluorescent dye (Invitrogen), 0.2mM deoxynucleoside triphosphate mix (Invitrogen), IU platinum Taq DNA polymerase (Invitrogen), 0.3µM MACF primer, 0.3µM 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) with data acquisition at 72$^{o}$C on the 6-carboxyfluorescein channel (excitation at 470 nm, detection at 510 nm) at a gain of 5. Melt curve data was also acquired on this channel at gains of 2 and 5 using a ramping rate of 1°C/60 s from 75°C to 95 °C. For each reaction the melt curve was analysed and a positive *Legionella* spp., *L. penumophila* and MAC was confirmed with a melting temperature ($T_m$) of 88±1$^{o}$C, 82.5±1$^{o}$C, 85±1$^{o}$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 approximately 1-$\log_{10}$ concentration value) (Livak, 2001) than 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) following the manufacturer's instructions. The concentration of purified DNA was calculated by reading the absorbance using a spectrophometer at 260 nm 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 $10^9$-$10^0$ copies) were 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 $>1^oC$ 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$^{TM}$ Prism 5.0 (Graph Pad Software Inc. U.S.A.). Comparisons of the average calculated copies were performed using a one-way ANOVA with Bonferroni post hoc test, statistical significance was accepted at $P<0.05$.

**Table 18: (Manuscript Table 1) Description of distribution systems and distance from processing plants of the sampling points.**

<table>
<tr><th>sample</th><th>Distance from processing plant (km)</th><th>Water source</th><th>Treatment</th></tr>
<tr><td colspan="4">Distribution System 1 (DS1)</td></tr>
<tr><td>A</td><td>0</td><td rowspan="3">Recycled wastewater</td><td rowspan="3">screening, grit removal, activated sludge, clarifier, then pre filtration chlorination, filtration, UV and chlorine disinfection</td></tr>
<tr><td>B</td><td>11</td></tr>
<tr><td>C</td><td>14</td></tr>
<tr><td colspan="4">Distribution System 2 (DS2)</td></tr>
<tr><td>A</td><td>0</td><td rowspan="4">Recycled wastewater<br><br>and<br><br>Reclaimed stormwater</td><td rowspan="3">screening, grit removal, activated sludge, clarifier, lagooning, dissolved air flotation filtration and chlorine disinfection</td></tr>
<tr><td>B</td><td>1</td></tr>
<tr><td>C</td><td>1</td></tr>
<tr><td>D</td><td>2</td><td>filtration and chlorine disinfection</td></tr>
</table>

**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 there is a total of 5 water samples collected (not including sample point A) for each of these, 4 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 3 non statistically significant increases in magnitude), 1/20 in Autumn (plus 5 non statistically significant increases in magnitude), 5/20 in Winter (plus 3 non statistically significant increases in magnitude) and 0 times in spring (plus 4 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

mg/L and ≤ 0.2 mg/L respectively as shown in Table 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.

**Table 19: (Manuscript Table 2) Average concentration of *Legionella spp. L. pneumophila* and MAC (mean ± standard deviation copies/mL) measured at each sampling point of Reuse Distribution system 1 (DS1) using qPCR. Total and free chlorine (mg/L) measured when samples were colle collected is 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 (*).**

| Season sampled | Sample point | A | B | C |
|---|---|---|---|---|
| | 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 | *345,332 ±65,451 |
| | Average *L. pneumophila* (copies/mL) | 1,665 ±1314 | 825 ±238 | *4,897 ±621 |
| | Average MAC (copies/mL) | 3,406 ±1,370 | *992,525 ±305,265 | *779,822 ±359,538 |
| | Average total coliforms (MPN/100mL) | 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 | +143,918 ±25,069 |
| | Average *L. pneumophila* (copies/mL) | 18 ±6 | 298 ±271 | 447 ±19 |
| | Average MAC (copies/mL) | 63 ±47 | +4,480 ±1,431 | +15,943 ±3,843 |
| | Average total coliforms (MPN/100mL) | 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 | *265,010 ±215,332 |
| | Average *L. pneumophila* (copies/mL) | 62 ±45 | *4,847 ±1,034 | 1,093 ±663 |

| | | | | |
|---|---|---|---|---|
| | Average MAC (copies/mL) | 149<br>±72 | +10,397<br>±4,736 | +24.366<br>±3,533 |
| | Average total coliforms (MPN/100mL) | 0<br>±0 | *1817<br>±127 | *329<br>±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<br>±49 | +26,679<br>±9,432 | +73,096<br>±6,946 |
| | Average *L. pneumophila* (copies/mL) | 80<br>±55 | 7<br>±6 | 134<br>±161 |
| | Average MAC (copies/mL) | 936<br>±808 | +23,029<br>±3,490 | +36,711<br>±3,346 |
| | Average total coliforms (MPN/100mL) | 0<br>±0 | 2<br>±1 | 21<br>±1 |

*****statistically significant increase

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

**Table 20: (Manuscript Table 3) Average concentration of *Legionella spp. L. pneumophila* and MAC (mean ± standard deviation copies/mL) measured at each sampling point of Reuse Distribution system 2 (DS2) using qPCR. Total and free chlorine (mg/L) measured when samples were collected is 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 (*).**

| 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 | *734,073 ±71,060 |
| | 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 |
| | Average total coliforms (MPN/100mL) | 0 ±0 | *183 ±22 | 1 ±1 | *283 ±113 |
| 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 | +6,143 ±247 |
| | Average total coliforms (MPN/100mL) | 0 ±0 | 0 ±0 | 0 ± | 5 2± |
| 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 |
| | Average *L. pneumophila* (copies/mL) | 20 ±26 | 3 ±0 | 20 ±26 | 85 ±95 |

| Season sampled and average water temperature | Sample point | A | B | C | D |
|---|---|---|---|---|---|
| | **Distance from treatment plant (km)** | **0** | **1** | **1** | **2** |
| | Average MAC (copies/mL) | 25 ±0 | 208 ±269 | 25 ± | 25 ±0 |
| | Average total coliforms (MPN/100mL) | 0 ±0 | 0 ±0 | 0 ±0 | 0 ±0 |
| Spring 22.7 °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/100mL) | 0 ±0 | 0 ±0 | 0 ±0 | 0 ±0 |

*****statistically significant increase

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

**Discussion**

This study used qPCR over culture methods for *Legionella* and MAC enumeration as traditional culture technique 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, Iseman, Haas and Soolingen, 2008) makes their isolated time consuming and allows for plates to become overgrown by faster growing organisms. Also culture does not account for the presence of viable but non-culturable (VBNC) organisms (Chang *et al*., 2007, Radomski *et al*., 2010, Shih and Lin, 2006). 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 Table 2 and 3. The concentrations which 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 (Table 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 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 residual chorine 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 (LeChevallier, 1987, LeChevallier *et al*., 1996, Wierenga, 1985). 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, Welch and Smith, 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 increase along both distribution pipelines, which could be indicative of viable and multiplying organisms. Although

these increases could also be explain through contamination of the pipeline of 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 a 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 which 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, organic content) influence opportunistic pathogen growth along these systems. There is also a need for accurate risk assessments regarding the different application of this recycled water which specifically which account for the potential presence of *Legionella* and MAC.

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**Declarations of interest**

The authors report no declarations of interest.

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# Chapter 5: Uncertainties associated with assessing the public health risk from *Legionella* and MAC

This is the first study to identify *Legionella* spp., *L. pneumophila* and MAC in South Australian potable and reuse water. This chapter covers the uncertainties with *Legionella* and MAC risk assessment and highlights areas requiring future research to better inform risk management.

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# Uncertainties associated with assessing the public health risk from *Legionella*

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**Running title:** Uncertainties of *Legionella* RA

## Introduction

*Legionella* spp. is the causative agent of Legionellosis and has been identified as a public health concern since 1976 (Fields *et al.* 2002; Bartram *et al.* 2007; Berger 2012). Currently, government bodies rely on risk assessment models to inform the development of regulatory tools for the control of Legionellosis (Cooper *et al.* 2004). Current *Legionella* risk assessments may be compromised by uncertainties in *Legionella* detection methods, strain infectivity and infectious dose. This paper follows the EnHealth Risk Assessment Framework (Figure 1) (Priestly *et al.* 2012) developed in Australia to review current knowledge of *Legionella* risk and discuss the uncertainties and assumptions made. These uncertainties associated with each component of the risk assessment framework are collated in Figure 2 and provide a useful tool when evaluating data used for *Legionella* risk assessment.

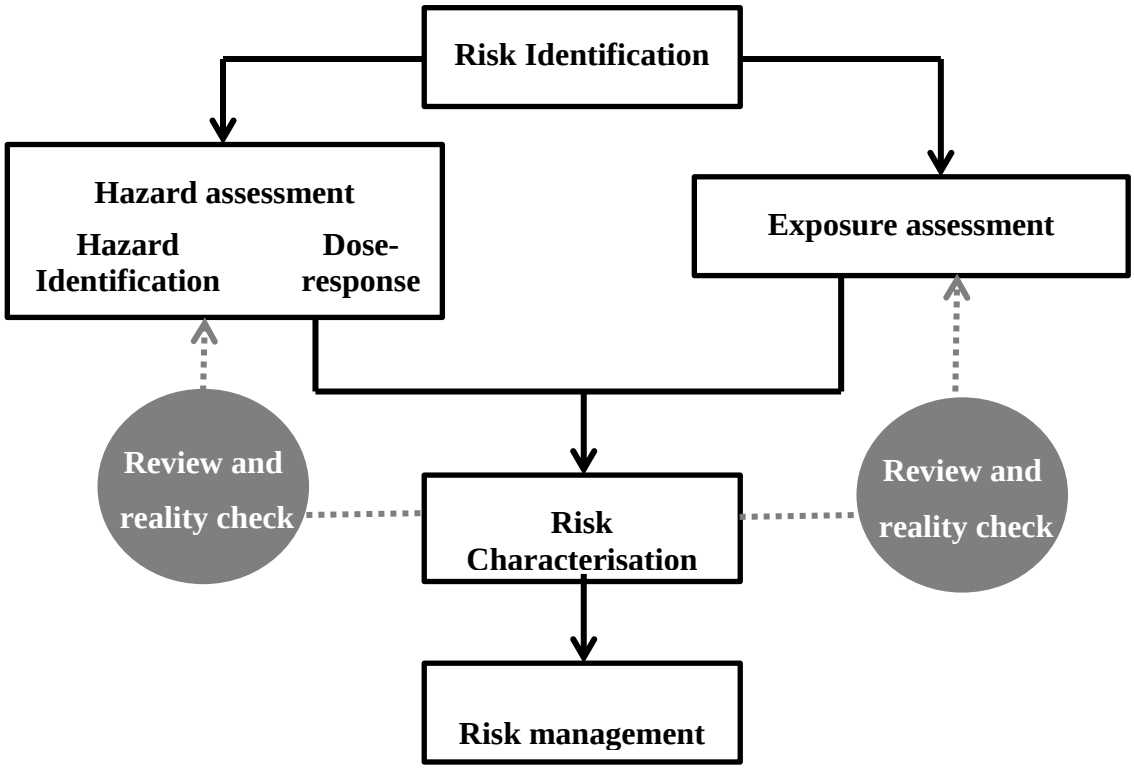


**Figure 39: (Manuscript Figure 1) EnHealth risk assessment framework adapted from Priestly *et al.* 2012.**

**Risk Identification**

Worldwide, *L. pneumophila* is the most common causative agent of Legionellosis (Buchbinder *et al.* 2002). Recently, a global increase in the incidence of reported Legionellosis has been observed (Centers for Disease Control Prevention 2011; Beauté *et al.* 2013). In 2011, there were 4897 confirmed Legionellosis cases across Europe (incidence rate of 0.97 cases per 100,000) (European Centre for Disease Prevention and Control, 2013) and 4,202 cases across the United States (incidence rate of 1.36 cases per 100,000) (Centers for Disease Control and Prevention 2013a). In 2013, Australia recorded 2.2 cases of Legionellosis per 100,000 (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).

Legionellosis outbreaks are primarily associated with artificial aquatic environments (Fields *et al.* 2002). Hence, the risk assessment for *Legionella* is especially important for public health officials and managers responsible for maintenance of water distribution systems and cooling towers within industrial or public buildings (Cooper *et al.* 2004). Risk identification is the first component of the risk assessment framework, for *Legionella* this is limited as the true incidence of Legionellosis is unknown and it has been estimated that the true incidence of Legionellosis could be 20 times greater than the currently reported incidence (Marston *et al.* 1997). Many Legionellosis community acquired cases go unreported, which places the focus of nosocomial infection and makes assumptions regarding disease epidemiology within the whole community difficult.

**Hazard Assessment**

Legionellosis collectively refers to clinical syndromes as a consequence of *Legionella* infection (Fields *et al.* 2002). This includes Pontiac fever, a self-limiting febrile illness and Legionnaires' diseases, a severe multisystem illness involving atypical pneumonia (Buchbinder *et al.* 2002; Fields *et al.* 2002; Bartram *et al.* 2007). The mortality rates of Legionellosis are highly variable and can range from 1% to 80%, depending on the underlying health of a patient, promptness of diagnosis and treatment and whether the disease is nosocomial, sporadic or part of an outbreak (Bartram *et al.* 2007; Diederen 2008). Currently, there is no consensus as to why exposures to *L. pneumophila* may result in either Pontiac fever or Legionnaires' disease (Diederen 2008). Occasionally simultaneous outbreaks of Pontiac fever and Legionnaires' disease from the same source have been observed (Bartram *et al.* 2007; Euser *et al.* 2010). In 2007 the overall case fatality rate for reported cases of Legionellosis across Europe was 6.6% (Joseph and Ricketts 2010) and from 2005-2009 the case fatality rate was 8% across the United States (Centers for Disease Control Prevention 2011). The annual cost of hospitalisations due to Legionellosis in the United States is estimated to exceed US$716 million (Giambrone 2013).

There are limited data regarding human dose response for *L. pneumophila* and the concentration of *Legionella* required to result in an outbreak is unknown (O'Brien and Bhopal 1993; Armstrong and Haas 2007a). The organism is ubiquitous to many natural and artificial environments which suggests people are frequently exposed to low concentration of the organism with no consequence or asymptomatic production of *Legionella* antibodies (Bartram *et al.* 2007). This was demonstrated by Boshuizen *et al.* (2001) who investigated an outbreak of Legionnaires' disease caused by an display whirlpool spa at floral trade show and found that 742 exhibitors without

Legionnaires' disease had higher average antibody levels than the general population. The exhibitors were surveyed regarding their whereabouts during the fair and those who ventured closer to the whirlpool spa had higher antibody levels. The data from animal models for *Legionella* dose response have been used for quantitative microbial risk assessment (QMRA) purposes. *In vitro* inhalation exposure data for *L. pneumophila* is available for guinea pigs (Davis *et al.* 1982; Breiman and Horwitz 1987), mice (Wright Jr *et al.* 2003), rats (Davis *et al.* 1982), marmosets (Baskerville *et al.* 1983) and monkeys (Kishimoto *et al.* 1979; Baskerville *et al.* 1983). However the infectious dose ($LD_{50\%}$) across these animal models range from 1200 to 1000000 CFU (Armstrong and Haas 2007a). Guinea pigs models have been generally accepted as the most appropriate representation of human dose response for *L. pneumophila*, primarily because *in vitro* studies show similarities for *Legionella* uptake, survival and replication within guinea pigs and human macrophages (Rechnitzer *et al.* 1992; Armstrong and Haas 2008). Armstrong and Haas (2007b) used guinea pig $ID_{50\%}$ (12 CFU) to a to create a QMRA model for *Legionella* exposure, (Armstrong and Haas 2007a) the justification for using this guinea pig model was also published (Armstrong and Haas 2008). This study used composite data from animal dose response models, average environmental concentrations from previous studies and exposure data from three outbreaks, one associated with one whirlpool spa and two hot spring spas. From this QMRA model the predicted infectious dose from the whirlpool spa was a mean of 10CFU and had a 95% range for 1.3-34 CFU, and the predicted infectious dose for the two hot spring spas was a mean of 47 CFU with a 95% range of 24-84 and for the other a mean of 2.3 CFU with a 95% range of 1.1 to 4.1 CFU.. Although the models acknowledges uncertainties associated with the QMRA model, the final predicted infectious dose

values calculated for the specific outbreaks are significantly lower compared to the concentrations of *Legionella* detected from environmental sources not associated with infection reported in numerous published studies (Buchbinder *et al.* 2002; Valster *et al.* 2011; Wang *et al.* 2012).

Uncertainties with *Legionella* dose response data also arise due to the large variation in virulence of environmental *Legionella* strains (Bollin *et al.* 1985b; Alli *et al.* 2003). Several studies have demonstrated that variation in growth temperature affect the virulence of *L. pneumophila* (Edelstein *et al.* 1987; Mauchline *et al.* 1994). However even these studies are conflicting, Edelstein *et al.* (1987) reported *L. pneumophila* grown at 25$^{o}$C were more virulent compared to those grown at 41$^{o}$C; whereas Mauchline *et al.* (1994) reported that *L. pneumophila* grown at 37$^{o}$C were more virulent than those grown at 24$^{o}$C. Increased virulence of *L. pneumophila* is also associated with flagellation which is life cycle dependent and genetically associated to the expression of a virulent phenotype (Heuner and Steinert 2003). Cirillo *et al.* (1999) also reported that *L. pneumophila* grown intracellular within an amoeba host has greater virulence than culture grown strains.

The disparity between Legionnaires' disease and Pontiac fever further confounds *L. pneumophila* infectious dose data. Currently there is no consensus for an epidemiological definition of Pontiac fever (Tossa *et al.* 2006). Furthermore, some experts believe that Pontiac fever is caused by exposure to a mixture of live and dead microorganisms including endotoxins made by non-*Legionella* bacteria plus low doses of live or dead *Legionella* which are unable to cause pneumonia in the infected host. However, more research is required to confirm this assumption (Burnsed *et al.* 2007; Edelstein 2007; Diederen 2008). Legionnaires' disease and Pontiac fever vary in regards to patients risk factors and disease outcomes (Diederen 2008). The

incubation period for Legionnaires' disease is 2-10 days (WHO 2003); whereas Pontiac fever has an incubation period of 30-90 h (Pancer and Stypułkowska-Misiurewicz 2002).

**Exposure Assessment**

Men aged 40 years and over with underlying health issues including; smoking; alcohol abuse; diabetes; heart disease and other immunosuppression are the most susceptible population for community acquired or travel associated Legionnaires' disease. Susceptible patients for nosocomial Legionnaires' disease include transplant recipients; other immunosuppression; surgery; cancer; diabetes; treatment with respiratory devices; chronic heart or lung disease; smoking and alcohol abuse, which are also associated with higher mortality rates (Fields *et al.* 2002; Bartram *et al.* 2007). However, Pontiac fever preferentially affects the younger population and the median age range from several outbreaks was reported to be 29-32 years (Tossa *et al.* 2006). Age, gender and smoking have not been observed to be risk factors for Pontiac fever (Friedman *et al.* 1987).

*Legionella* is present in a range of aquatic environments and human infection occurs through the inhalation of contaminated aerosol or aspiration of contaminated water (Bartram *et al.* 2007). Incidences of Legionellosis have been linked to contaminated shower heads (Hanrahan *et al.* 1987; Zmirou-Navier *et al.* 2007), spas (Jernigan *et al.* 1996; Benkel *et al.* 2000), baths (Sasaki *et al.* 2008) a hospital steam towel warmer (Higa *et al.* 2012), ice machines (Graman *et al.* 1997; Schuetz *et al.* 2009), mist generators (Mahoney *et al.* 1992), decorative water fountains (Fleming *et al.* 2000; O'Loughlin *et al.* 2007; Haupt *et al.* 2012), hospital water distribution systems

(Tobin *et al.* 1981; Hanrahan *et al.* 1987) dental units (Reinthaler *et al.* 1988; Atlas *et al.* 1995) and cooling towers (Isozumi *et al.* 2005; Nguyen *et al.* 2006). *L. pneumophila* has also been detected in potable water and in 2011, 57.6% of all potable water related disease outbreaks in the United States were due to *Legionella* spp. (Centers for Disease Control and Prevention 2013b). A recent study also used qPCR to detect *Legionella* spp. and *L. pneumophila* ubiquitously through South Australian potable and reuse water distribution pipelines. Within the potable water distribution system *Legionella* spp. and *L. pneumophila* was detected at maximum concentrations of $10^6$ and $10^3$ copies/mL respectively (Whiley *et al.* 2014). Human to human transmission of *Legionella* has not been observed (Albert-Weissenberger *et al.* 2007).

There have been numerous studies which have investigated the production, size and spread on *Legionella* contaminated aerosols (Bollin *et al.* 1985a; Ishimatsu *et al.* 2001; Nguyen *et al.* 2006; Dutil *et al.* 2007; Zmirou-Navier *et al.* 2007; Chang *et al.* 2010). The ability of *Legionella* to access the human respiratory tract is governed primarily by the size of the aerosol. Aerosols >10 µm in diameter get captured within the nose and throat, between 5-10µm and aerosols can reach the upper and lower respiratory tract and between 2-5 µm they can reach the lungs and conducting airways (Cox and Wathes 1995). In 1985 Bollin *et al.* (1985a) demonstrated that 90% showerhead aerosol contaminated with *L. pneumophila* sampled above a shower door were between 1-5µm in diameter and 50% of *Legionella* contaminated aerosols from facets were 1-8 µm in diameter. These aerosols are small enough to efficiently transport the *L. pneumophila* into the lower respiratory system. The production of aerosols also provides *Legionella* a method to further spread contamination. This is particularly important for cooling towers. Nguyen *et al.*

(2006) demonstrated that contaminated aerosols from a cooling tower identified as the source of an outbreak of Legionnaires' disease spread up to 6 km from the cooling tower. Dennis and Lee (1988) demonstrated that virulent strains of *L. pneumophila* survived longer within aerosols compared to avirulent strains, which is important to consider when determining the potential spread of contaminated aerosols.

This difference in susceptible population for Legionnaires' disease and Pontiac fever is a significant limitation for *Legionella* risk assessment. The potential for contaminated aerosols to spread considerable distances makes it challenging to identify the origin of the aerosol and limits knowledge regarding sources of Legionellosis (Nguyen *et al.* 2006). Variation in the size of aerosols also affects the infectivity, which makes it difficult to determine the infectious dose and what environmental concentrations are considered acceptable.

In order to quantify the risk of Legionellosis, enumeration of *Legionella* from a source is required. Many regulatory guidelines are based on the detection of *Legionella.* For example, in Australia each state has different cooling tower legislation regarding *Legionella.* In South Australia, Queensland and The Australian Capital Territory detection of ≥1000 *Legionella* CFU/mL from a cooling tower water sample requires mandatory reporting to the relevant health department (Australian Capital Territory Department of Health 2005; Workplace Health and Safety Queensland 2008; South Australian Department of Health and Aging 2013). Whereas, in Victoria mandatory reporting is required if there are three consecutive detections of *Legionella* ≥10 CFU/mL (Public Health and Wellbeing Victoria 2009). The problem with this legislation is the inherent difficultly regarding the detection of

*Legionella* from environmental samples (Hussong *et al.* 1987; Centers for Disease Control and Prevention 2005; Whiley and Taylor 2014).

Currently, culture is considered the 'gold standard' for *L. pneumophila* detection (Reischl *et al.* 2002). However, the slow growth rate of *L. pneumophila* makes the method tedious and can be inaccurate due to plate being overgrown from faster growing organism (Bopp *et al.* 1981; Hussong *et al.* 1987). Further inaccuracies occur with variation of sample holding time prior to culturing. McCoy *et al.* (2012) demonstrated that sample holding time significantly impacted *Legionella* recovery by culture, with enumerated *Legionella* changing by up to 50% within 6 hours and up to 2 $\log_{10}$ difference after 24 hours. In Australia the standard holding time for NATA (National Association of Testing Authorities) accredited laboratories is <8hrs (McCoy *et al.* 2012). Inaccuracies with culture enumeration may also occur if final confirmation of all *Legionella* isolates are not performed using an alternative method such as 16sRNA sequencing, PCR, latex agglutination test or immunofluorescence antibody test. Borge *et al.* (2012) used the standard *Legionella* culturing method and found that 40 isolates from natural and artificial water samples grew on GVPC selective *Legionella* agar, had the same morphological 'ground glass' appearance of *Legionella* , and when restreaked onto blood agar isolates did not grow. However, 16sRNA sequencing confirmed that the isolates were not *Legionella* and in fact were from the Chitinophagaceae family. Although not an issue in accredited laboratories which would complete final confirmation tests, it does present the possibility of false positives when culturing Legionella, a concept that should be considered when reading past studies relying on culture for detection.

A significant limitation of culture detection is that it does not account for the presence of viable but non-culturable (VBNC) organisms (Chang *et al.* 2009).

Studies have shown that *Legionella* becomes VBNC during starvation, when exposed to high temperatures and monochloramine disinfection (Chang *et al.* 2007; Alleron *et al.* 2008). The problem with the presence of VBNC *Legionella* is that using the viable culture method of detection a negative result does not necessarily mean that *Legionella* is not present. This has serious ramifications for public health protection using routine sampling.

*Legionella* detection using qPCR is becoming a popular alternative to culture methods as it has a quick turnaround time and high specificity. The main problem with qPCR is that it enumerates both live cells and intact killed cells (Delgado-Viscogliosi *et al.* 2009). This means there is a significant discrepancy between detection of *Legionella* using either culture or qPCR. A review of studies which detected *Legionella* from environmental samples with culture and qPCR simultaneously found that from a total of 28 studies, 2856/3967 (72%) samples tested positive for *Legionella* spp. using qPCR and 1331/3967 (34%) using culture (Whiley and Taylor 2014). This discrepancy highlights the limitation of both the current detection methods and potential concerns with relying on these results for risk assessment purposes.

Another difficulty of detection from environmental sources is the ability of *Legionella* to opportunistically parasitise free living protozoa (Walser *et al.* 2014). Berk *et al.* (1998) demonstrated that vesicles expelled from amoeba may contain 20-200 *Legionella ;* however, only one colony forming unit (CFU) was detected using culture. This study also demonstrated that over 90% of vesicles containing *L. pneumophila* expelled from *Acanthamoeba polyphaga* and *Acanthamoeba castellanii* were 2.1 to 6.4 μm in diameter which is within the respirable size range. A single *A. polyphaga* was able to expel 25 *L. pneumophila* filled vesicles over a 24 h period.

Buse and Ashbolt (2012) demonstrated that under conditions representative of a drinking water system the maximum number of *L. pneumophila* release from *A. polyphaga* and *Naegleria fowleri* was respectively 1,348 and 385 CFU per trophozoite. Comparison of these concentrations to a guinea pig aerosol infection model (Berendt *et al.* 1980) demonstrated that as few as 1 to 75 infected amoebae within aerosols may contain enough pathogenic *L. pneumophila* to cause human infection.

The significant discrepancies between infectious dose models and detection methods has resulted in published studies giving *Legionella* counts which are potentially meaningless for risk assessment purposes. Armstrong and Hass (2007a) extrapolated animal modelling and data from 3 outbreaks of *Legionellosis* for QMRA modelling and predicted infectious doses or *Legionella* ranging from 1.3-47 CFU. The South Australia, Queensland and Australian Capital Territory government require mandatory reporting if *Legionella* is detected at a concentration ≥1000 *Legionella* CFU/mL from a cooling tower water (Australian Capital Territory Department of Health 2005; Workplace Health and Safety Queensland 2008; South Australian Department of Health and Aging 2013). Wang *et al.* (2012) used qPCR to detect *Legionella* in potable water from point of use at maximum concentrations of $2.3x10^3 \pm 9. x10^2$ copies/mL. Whiley *et al.* (2014) used qPCR to detect *Legionella* at a dead-end of a potable water distribution system at a maximum concentration of $10^6$ copies/mL. The inconsistencies of these values highlight the biggest flaw with current *Legionella* risk assessment and question the value of routine sampling. The discrepancies between *Legionella* concentrations measured using the different detection methods also make it difficult to compare findings from published studies. This makes it challenging to identify environmental sources of potential public

health significance and to compare the effectiveness of different control measures and protocols.

**Risk Characterisation**

Presently there are risk assessments models available for *Legionella* (Bentham 2003; Mouchtouri *et al.* 2010; Torrisi *et al.* 2012). This include QMRA models for *Legionella* exposure from spas (Armstrong and Haas 2007b), distributed water (Storey *et al.* 2004) and rainwater (Ahmed *et al.* 2010). These risk assessments characterise the nature and magnitude of risk associated with environmental sources of Legionellosis using the information currently available. However, often results of these risk assessments are not consistent or considerate of the literature regarding *Legionella* in the environment and its ubiquitous nature in aquatic environments. When utilising risk assessments for the purpose of regulatory tools the realities of the limitation and assumptions made must be taken into consideration, particularly when considering potential cases of liability.

**Risk Communication and Management**

Currently in most developed countries there are many models of risk communication regarding Legionellosis including: training and education programs, management procedures and established documentation and communication procedures (Cooper *et al.* 2004; Bartram *et al.* 2007). Current risk management strategies for *Legionella* in built water systems are focused on maintaining overall system health to control biofilm formation. This can be achieved by maintaining water temperature at $<20^{o}C$ or $> 50^{o}C$, periodical flushing of the system with hot water, or disinfection with

biocides, copper-silver ionisation, anodic oxidation or ultra violet light (Sidari III *et al.* 2004; Bartram *et al.* 2007). The uncertainties associated with *Legionella* risk assessment presented in this paper also highlight areas requiring greater research in the future.

Routine testing for *Legionella* is required by most regulatory bodies. This is aimed at monitoring the effectiveness of treatment and management protocols, but also is a result of political expediency. Politicians and government officials often require routing testing for *Legionella* to demonstrate that the public health risk is being managed, despite the uncertainties of current detection methods. The main danger of this is the false sense of security gained from a negative *Legionella* test result, as there is little correlation between a positive *Legionella* test results using culture and human health risks (Kool *et al.* 1999). Communicating this concept to the public proves a challenging proposition, especially considering the fear association with public perception regarding Legionellosis (Irie *et al.* 2004; Laws *et al.* 2006).

In Japan, from 1997-2000 there was a significant decrease in sales of 24 hour hot water baths due the public fear of Legionellosis after the 1996 detection of *L. pneumophila* a public bath (Irie *et al.* 2004). In Australia, the largest outbreak of Legionellosis occurred in April 2000 and was caused by the Melbourne aquarium cooling towers. This outbreak resulted in 2 deaths and 111 identified cases of Legionellosis. The public fear in response to this outbreak was devastating to Melbourne's tourism, with significant trading losses and legal claims exceeding than $35 million (Laws *et al.* 2006).

One difficulty with communicating risk occurs when a situation is highly publicised and raises significant 'public outrage', for example a cooling tower testing positive

for *Legionella* . This causes the potential risk level to be perceived to be much higher than an actual calculated risk level. This is something which must be considered when completing *Legionella* risk assessments as the implementation of risk decisions has a much greater chance of success when supported by the public (Finucane 2004).

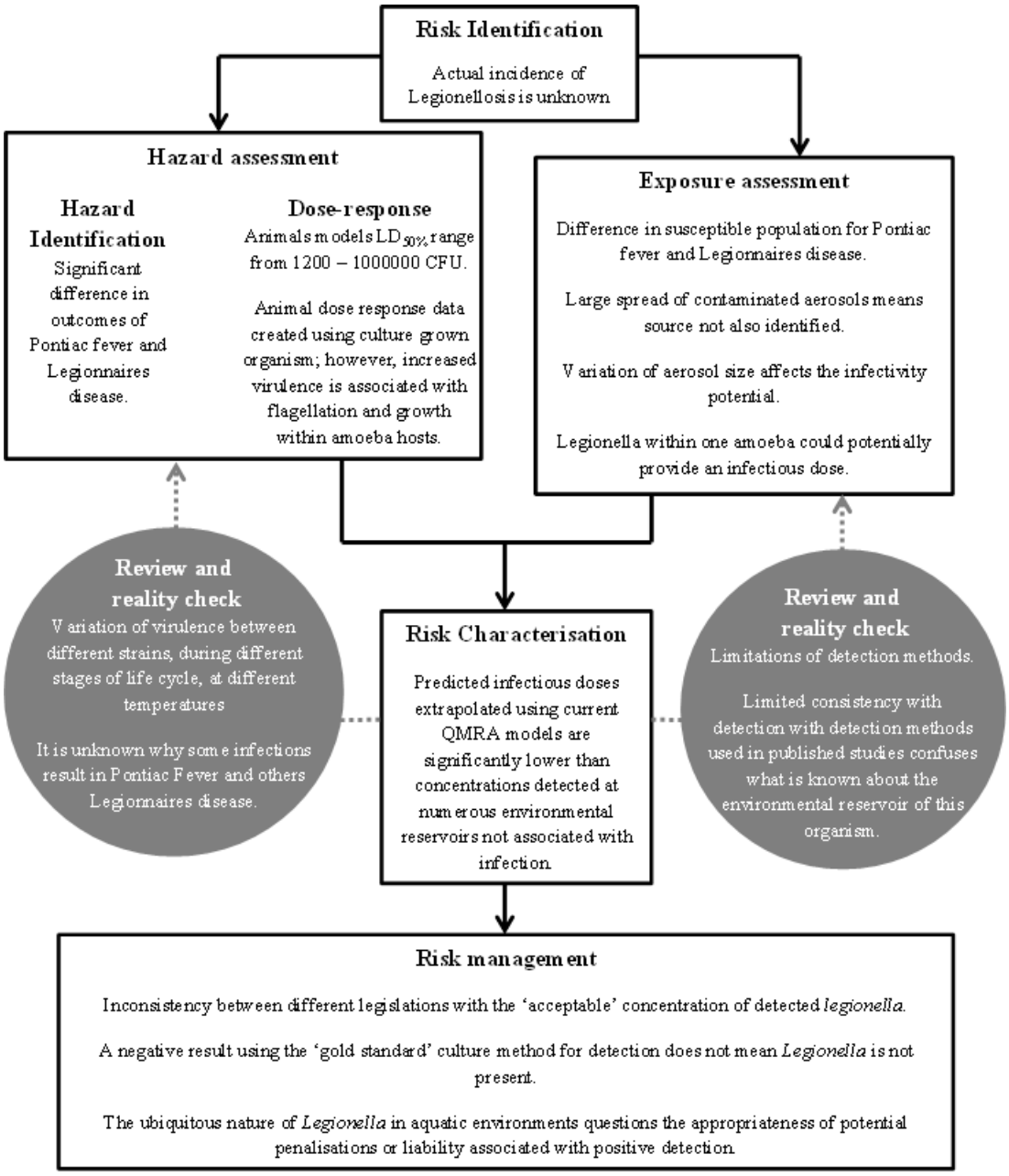


**Figure 40: (Manuscript Figure 2) Uncertainties of *Legionella* risk assessment highlighted through each step of the EnHealth risk assessment frame work.**

## Conclusion

Present regulatory models manage the risk of *Legionella* through strategies maintaining good system health, disinfection residuals and minimising exposure routes. These regulatory guidelines are informed by *Legionella* risk assessment models which best use the information currently available. The uncertainties associated with each components of *Legionella* risk assessment have been highlighted in this paper. Minimising these uncertainties will result in improved management protocols. The effectiveness of these management protocols is an important public health issue. Underestimating the risk of *Legionella* may have serious public health consequences; however, overestimating the risk may result in significant economic costs. The paper provides a tool for understanding the uncertainties associated with *Legionella* risk assessment and also provide an overview of the areas that require future research.

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## Declarations of interest

The authors report no declarations of interest.

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## 5.1 *Legionella* risk management

These uncertainties associated with *Legionella* risk assessment were discussed with a South Australian *Legionella* risk manager. The key points of this discussion and highlighted in Figure 41 (see appendix) describe a risk manager's perspective on current public health risk posed by *Legionella.*

The potentially ubiquitous nature of *Legionella* raises another issues regarding liability. There have been numerous cases where owners and managers of apartment buildings, hotels, and hospitals have faced legal action due to Legionellosis. Reports of settlements range from US$255,000 to US$5.2 million. The reported values of settlements are difficult to find as most agreements include stipulations that payout amounts remain confidential (Smith and Bernier 2014). Many of these cases are centred around the organism being detected within the water system or cooling tower of a premise; however, this practice is arguably questionable given the uncertainties associated with *Legionella* detection methods examined in this study. If the organism is present in potable water and it is recognised that the presence of the organism only infrequently results in an outbreak; should business holders be held accountable?

## 5.2 Uncertainties associated with accessing the public health risk from MAC

The EnHealth Risk Assessment framework (Priestly *et al.* 2012) was followed to review the current uncertainties associated with MAC risk assessment.

### 5.2.1 Issue identification

MAC has been identified as a Nontuberculous Mycobacteria (NTM) of public health concern (Marras and Daley 2002; Haverkort 2003; Falkinham 2011). It is difficult to determine prevalence of MAC in Australia as it is not a nationally notifiable disease (Australian National Notifiable Diseases Surveillance System 2014). A study conducted by the Australian Mycobacterium Reference Laboratory Network (AMRLN) in 2000 collated data from clinical specimens that were culture positive for NTM across Australia. The resulting annual incidence rate for NTM was 1.8 per 100,000 and MAC was the most predominately identified. All respiratory and lymphatic NTM specimens from South Australia were MAC isolates (Haverkort 2003). Overseas, there have been studies which have investigated the incidence rate of a specific clinical presentation of MAC. From 2001-2003 a study conducted in the Netherlands estimated the annual incidence of MAC lymphadenitis was 52 per 100,000 children (Haverkamp *et al.* 2004) and in 2003 a study in Sweden reported the annual incidence of culture confirmed MAC lymphadenitis in children was 4.5 per 100,000 (Thegerström *et al.* 2008). Due to the wide spectrum of illnesses caused by MAC and the varying patient outcomes and mortalities for each clinical presentation (which has been covered in Chapter 1) the true impact of MAC is unknown.

### 5.2.2 Hazard assessment

The clinical presentations of MAC are covered in Chapter 1 and summarised in Table 21. Each clinical manifestation has different potential outcomes varying in severity. Currently there is limited knowledge regarding MAC dose response in humans. *M. avium* subspecies *paratuberculosis* (MAP) is responsible for Johne's disease in animals and due to the consequences of Johnes disease in livestock there are a range of animal dose response studies available. Some of these studies were collated by Craven and Morgan (2000) and are presented in Table 22. These studies were representative of cattle acquiring Johne's disease from environmental sources and are not intended to be representative of human consumption. It is also difficult to make a comparison between dry/wet weights of MAC culture and the actual number of organisms. The lowest dose of MAC bacteria to cause infection was $10^8$ cells ingested by rabbits (Mokresh and Butler 1990). The highest concentration measured in the potable and reuse distribution systems in this study was $10^6$ copies/mL. This suggests that if these were all viable then it would only require ingestion of a few mL to achieve a similar dose.

MAP is also considered a possible causative agent of Crohn's disease, an inflammatory bowel disease, in humans (Mishina 1996; Hermon-Taylor 2000; Chamberlin *et al.* 2001; Naser *et al.* 2004; Sartor 2005; Pierce 2009). However, since the relationship between MAP and Crohn's disease is still widely debated and the variation between animal and human disease manifestation and potential routes of exposure makes it is difficult to infer human infectious dose response data for MAC from these animal models.

**Table 21: Summary of clinical presentations of MAC infections and the susceptible populations**

| Mac caused illness | Susceptible population | General comments | Reference |
|---|---|---|---|
| Cutaneous infection | Immune compromised ( including transplantation patients, chronic corticosteroids users, cancer patients, and A*cquired Immune Deficiency Syndrome (*AIDS) patients)<br>Post-surgery patients<br>Healthy non immune compromised | MAC infection occurs through contact with a wound site. | (Sugita 2000; Karakousis *et al.* 2004) |
| Disseminated infection | Immune compromised patient ( including transplantation patients, chronic corticosteroids users, cancer patients, and AIDS patients) | MAC infection has the potential to spread throughout the body. Disseminated infection has very high mortality rates. | (Wolinsky 1992; von Reyn *et al.* 2002) |
| Pulmonary infection | Elderly patients<br>Children with cystic fibrosis<br>Children and adults with AIDS | Can form pulmonary nodules which get mistaken for cancer. | (Lindegren *et al.* 1996; Olivier *et al.* 2003; Teirstein 2008; Lakhanpal *et al.* 2011) |
| MAC fibrocavity disease | Older men with pre-existing lung conditions and/or a history of smoking and/or alcohol abuse. | | (Field 2004) |
| MAC fibronodular bronchiectasis<br>(Lady Windermere's Syndrome) | Elderly women, without history of lung disease or immunosuppression | | (Huang *et al.* 1999; Field 2004) |
| MAC hypersensitivity pneumonitis | | Inflammation of the alveoli within the lung caused by the body's immune response to MAC contaminated aerosols | (Kenmotsu *et al.* 2005) |
| MAC cervical | Healthy children | Infection of lymph nodes. Treatment is | (Thegerström *et al.* 2008) |

| Mac caused illness | Susceptible population | General comments | Reference |
|---|---|---|---|
| lymphadenitis | | difficult either surgical removal or antibiotic therapy. | |
| Crohn's disease | Crohn's disease can affect anyone, but it is more frequently diagnosed in people aged 15-35, smokers and patients with a close relative who also has the disease. | Evidences suggests that *M. avium* subspecies *paratuberculosis* is the causative agent of Crohn's disease but this still debated. | (Naser *et al.* 2004) |

**Table 22: Studies investigating the dose of *M. avium* subspecies *paratuberculosis* to cause infection in animals (Craven and Morgan, 2000).**

| *M.para-tuberculosis* source | Recipient species | Age at infection | Time of sampling p.i. | Method of infection | Dose | Clinical signs | Gross lesions | Histo-logical lesions | Culture result | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| bovine | sheep | 3 w.o. | 16 mth | fed in milk | $2x10^{10}$ bacteria | + | + | ++ | n.d. | Kluge *et al.* (1968) |
| bovine | sheep | 1 m.o. | 700 days | oral | $2.3x10^{12}$ bacteria | not stated | not stated | ++ | n.d. | Klausen *et al.* (1997) |
| bovine | goats | | | oral | | - | + | ++ | + faeces | Levi (1948) |
| bovine | pigs | 10 d.o. | 3-8 mth | intragastric | 200 mg | - | not stated | ++ | + faeces + tissues | Larsen *et al.* (1971) |
| bovine | pigs | 10 d.o. | 3-8 mth | oral | not stated | - | not stated | ++ | + faeces + tissues | Larsen *et al.* (1971) |
| bovine | horse | 10 d.o. | 120 days | intragastric | 180mg dry wt. | - | - | ++ | + faeces + tissues | Larsen *et al.* (1972) |
| bovine | chickens | 2 w.o. | 30 days | oral | $9x10^{9}$ bacteria | - | - | - | + tissues | Larsen and Moon (1972) |
| bovine | lemming | 6 w.o. | 50-260 d | oral | 5mg dry wt. | - | - | ++ | + tissues | Larsen and Miller (1979) |
| bovine | rabbit | 1 d.o. | 12 mths | oral | $7.8x10^{8}$ cfu | + | - | ++ | + faeces + tissues | Mokresh *et al.* (1989) |
| bovine | rabbit | 1 d.o. | 24 wks | oral | $3.6x10^{8}$ cfu | + | + | ++ | + tissues | Mokresh and Butler (1990) |
| goat | cattle | 3-4 w.o. | 18 mths | oral | 100mg wet wt. | - | - | - | + tissues | Saxegaard (1990) |
| human | chickens | 2 w.o. | 2-9 wks | oral | $1x10^{7}$ bacteria | - | - | ++ | n.d. | van Kruiningen et al. (1991) |
| bighorn sheep | domestic sheep | 4-5 m.o. | 12 mths | oral | 50mg wet wt. | - | + | not stated | + tissues | Williams *et al.* (1983b) |
| bighorn sheep | elk | 4-5 m.o. | 12 mths | oral | 50 mg wet wt. | - | + | ++ | + tissues | Williams *et al.* (1983b) |
| bighorn sheep | mule deer | 4-5 m.o. | 12 mths | oral | 50 mg wet wt. | + | + | ++ | + faeces + tissues | Williams *et al.* (1983b) |

*dry/wet wt. refers to pure *M. avium* subspecies *paratuberculosis* cultures growth on agar plate or broth, dried and then weighed

### 5.2.3 Exposure assessment

Each clinical presentation of MAC has different risk factors and susceptible populations which are covered in Chapter 1 and summarised in Table 21. There have been a number of studies which have identified potential environmental and patient risk factors for MAC infection. A collation of studies investigating patient risk factors is shown in Table 23. There is no consensus with the risk factors identified in these studies and the risk factors are dependent on the population investigated. For example exposure to food is identified as not a risk factor by Reed *et al.* (2006), but exposure to cheese or raw seafood is identified as a risk factor by Horsburgh *et al.* (1994) and Von Reyn *et al.*(2002), respectively.

MAC transmission occurs through inhalation or ingestion of contaminated water, particulate matters or aerosols. Alternatively transmission can occur through contamination of a trauma site (Falkinham III 2003). The role of potable water as a source of MAC infection has been explored in Chapter 1. Briefly MAC infections have been linked to hot water systems (Aronson 1999; Tobin-D'Angelo *et al.* 2004), showers (Falkinham *et al.* 2008; Nishiuchi 2009), spas (Sugita 2000; Lumb *et al.* 2004) and swimming pools (Lynch *et al.* 1992; Rose *et al.* 1998; Koschel *et al.* 2006). Other environmental sources include food (Yajko *et al.* 1995a; Collins 1997; Ellingson *et al.* 2005), soil (Ichiyama *et al.* 1988; Yajko *et al.* 1995a; Ito *et al.* 2012) and environmental waters (von Reyn *et al.* 1993; Pickup *et al.* 2006). However, since the source of a MAC infection is rarely identified it can be difficult to confirm exposure pathways.

There have been studies which have identified showers (Nishiuchi *et al.* 2007; Falkinham *et al.* 2008) and spas (Embil *et al.* 1997) as sources of MAC infection,

linking clinical isolates to the environmental isolates identified from these sources (this is explored in Chapter 1). Direct links between food and MAC infections have also been made. Yoder *et al.*(1999) used a PCR typing method to amplify DNA sequences between the insertion sequences IS1245 and IS1311 and identified a *M. avium* isolate in a patient's food that was identical to the clinical isolate.

**Table 23: Uncertainty associated with current literature regarding patient and environmental risk factors for MAC infection.**

| Country | Risk factor | Not a risk factor | Study cohort | Reference |
|---|---|---|---|---|
| USA | Black race (odds ratio = 3.8, 95% confidence interval: 2.2, 6.6).<br>Birth outside the United States (odds ratio = 2.1, 95% confidence interval: 1.1, 3.9)<br>More than 6 years' cumulative occupational exposure to soil (odds ratio = 2.7, 95% confidence interval: 1.3, 6.0). | Exposure to water, food, or pets was not associated with infection. | General population positive for MAC infection as determined by *M. avium* sensitin skin test. | (Reed *et al.* 2006) |
| Japan | high soil exposure (≥ 2 per week) than control patients (23.6% vs 9.4%, *P* = .032); | Patient characteristics and underlying diseases. | Patients with MAC pulmonary infection. | (Maekawa *et al.* 2011) |
| USA | History of chronic obstructive pulmonary disease (odds ratio, 10; 95% confidence interval 1.2–80).<br>History of pneumonia hospitalization (odds ratio, 3.4; 95% confidence interval, 1.1–11).<br>History of steroid use (odds ratio, 8; 95% confidence interval 1.6–41). | Aerosol-generating activities in the home and garden. | Patients with MAC pulmonary infection. | (Dirac *et al.* 2012) |
| USA | Consuming hard cheese (odds ratio 5.44; 95% confidence interval 1.61-18.4). | Daily showers was protective (odds Ratio 0.55; 95% Confidence interval 0.33-0.94). | Human immunodeficiency virus (HIV) positive with <50 CD4+ cells/mm and without a history of MAC tested for MAC antibodies. | (Horsburgh *et al.* 1994) |
| USA and Finland | History of *Pneumocystis carinii* pneumonia (hazard ratio 1.821).<br>Consumption of spring water (hazard ratio 4.909). | There was no association between *M. avium* colonization of home water and risk of MAC. | HIV positive subjects with CD4 counts <100/mm tested for MAC antibodies. | (von Reyn *et al.* 2002) |

| Country | Risk factor | Not a risk factor | Study cohort | Reference |
|---|---|---|---|---|
| | Consumption of raw seafood (hazard ratio 34.3).<br>Gastrointestinal endoscopy (hazard ratio 2.894)<br>Showering outside the home (hazard ratio 0.388) | | | |

### 5.2.4 Risk characterisation

This is the first study to detect MAC in South Australian potable and reuse water distribution systems. Currently there is little information available regarding human infectious dose of MAC and since qPCR was used in this study the actual number of viable cells is unknown. However, since transmission of MAC occurs through inhalation, ingestion and contact of trauma sites these potable and reuse water source could potentially pose a risk for MAC infection (Table 24 presents common uses for potable and reuse water and the potential exposure routes associated with each).

There have been numerous studies which have indicate MAC is ubiquitous in the environment (Nightingale *et al.* 1992; Norton *et al.* 2004; Turenne *et al.* 2006; Coelho *et al.* 2013). This study indicates that South Australian potable and reuse water distribution systems may also harbour potentially viable MAC. However, the ubiquitous nature of the organism is not reflected by high incidences of infection. One possible explanation is that patient risks factors play a crucial role in acquiring MAC infection. Greater understanding of these patient risk factors is required to ensure risk management practises protect the appropriate susceptible populations.

**Table 24: Common uses for potable and reuse water and the potential exposure routes to MAC contaminated particles**

| Point of use | Potable of reuse water | Potential exposure route to MAC contaminated particles |
|---|---|---|
| Faucets | potable | Inhalation of aerosols<br>Ingestion of aerosols<br>Contact with wound sites |
| Showers | potable | Inhalation of aerosols<br>Ingestion of aerosols<br>Contact with wound sites |
| Dental lines | potable | Inhalation of aerosols<br>Ingestion of aerosols<br>Contact with wound sites |
| Decorative fountains | potable | Inhalation of aerosols |
| Spas | potable | Inhalation of aerosols<br>Ingestion of aerosols<br>Contact with wound sites |
| Swimming pools | potable | Inhalation of aerosols<br>Ingestion of aerosols<br>Contact with wound sites |
| Toilet flushing | Potable and reuse | Inhalation of aerosols |
| Cooling towers | Potable and reuse | Inhalation of aerosols |
| Irrigation systems | Potable and reuse* | Inhalation of aerosols<br>Ingestion of aerosols |

*irrigation using reuse water in public areas occurs at night to reduce the potential for inhalation of aerosols

### 5.2.5 Risk management

It is difficult to create water protection protocols for MAC, as contamination of water distribution systems can originate from a variety of environmental sources (LeChevallier 2004). Current management strategies for the control of MAC and others NTMs in water distribution systems are based on maintenance of disinfection residuals and low nutrient concentrations (Vaerewijck *et al.* 2005a).

Historically, control of NTM growth within water distribution systems has received little attention and the public health focus has concentrated on disease management rather than prevention (Ford *et al.* 2004). Vaerewijck *et al.* (2005a) proposed that a risk assessment model for NTMs would be useful tool for managing potential public health risk from potable water, but also acknowledged that presently this is unachievable due to a lack of information regarding infectious dose, concentration in potable water, virulence factors, patient risk factors and variations in consumer consumption and use of potable water. LeChevallier (2004) suggested that future research into the control and prevention of NTM growth within water systems should move to mechanistic and predictive stages. This would include designing treatment systems based on ecology, survival and risk of NTM in water; however, he also acknowledge the need for improved detection and enumeration methods for NTM in environmental samples before this could happen.

## 5.3 Association of *Legionella* and MAC growth

*Legionella* and MAC are opportunistic pathogens which have been isolated from similar environmental sources (Fields *et al.* 2002; Falkinham 2009; Wang *et al.* 2012). The relationship between the presence of *Legionella* and MAC in the potable

and reuse water pipelines from this study was also explored. Values were not normally distributed so a Spearmen correlation was conducted. The resulting $r^2$ value was 0.652 with the 95% confidence interval ranging from 0.5973 to 0.7007 ($P<0.0001$). This means the more 60% of the observed increases in *Legionella* concentration were associated with increases in MAC concentration and visa-versa. This is important from a risk management perspective, as it suggests that if one of the organisms is detected, then the other is likely to also be present.

# Chapter 6: Conclusion

This study investigated potable and reuse water as potential environmental sources of *Legionella* spp., *L. pneumophila* and MAC in South Australia. The role of potable and reuse water and Legionellosis has previously been established (Cordes *et al.* 1981; Tison and Seidler 1983; Fattal *et al.* 1985; Hanrahan *et al.* 1987; Ruf *et al.* 1988; Palmer *et al.* 1995; Nolde 2000). Presently there is limited research on the incidence of MAC infection that considers all clinical presentations and the role of potable and reuse water as a source of infection. A review of current literature and case studies regarding the wide spectrum of disease caused by MAC and the role of potable water in disease transmission was conducted. Potable water was recognised as a putative pathway for MAC infection. Contaminated potable water sources associated with human infection included warm water distribution systems, showers, faucets, household drinking water, swimming pools and hot tub spas.

The optimum methods for detection had to be chosen to investigate the presence of these opportunistic pathogens in potable and reuse water. Currently there are a range of methods for *Legionella* quantification from environmental sources, but the two most widely used and accepted are culture and real-time polymerase chain reaction (qPCR). A comparison of these two methods was conducted by collating studies from the last 10 years which concurrently used culture and qPCR to quantify *Legionella* spp. from environmental sources. This investigation demonstrated that from the 3967 environmental samples analysed by 28 different studies *Legionella* was detected in 72% of samples (2856 samples) using qPCR compared to 34% (1331 samples) using culture. The main limitation of the culture method of detection was

its inability to detect viable non culturable *Legionella;* whereas, the main limitation of qPCR is its inability to differentiate between live and dead cells, potentially resulting in false positives. *Legionella* is an opportunistic pathogen and hence under detection may have serious consequences, which is why qPCR was the chosen method for enumeration. The chosen method for MAC enumeration was also qPCR as culture based methods are time consuming as it can take up to 6 weeks for MAC growth.

This is the first study to use qPCR to enumerate *Legionella* spp., *L. pneumophila* and MAC along South Australian potable and reuse water distribution pipelines. *Legionella spp*., *L. pneumophila* and MAC were detected in the chlorine and the chloramine disinfected potable water distribution systems throughout the year and were all detected at a maximum concentration of $10^3$ copies/mL in the chlorine disinfected system and $10^6$, $10^3$ and $10^4$ copies/mL respectively in the chloramine disinfected system. The concentrations of these opportunistic pathogens were primarily controlled throughout the distribution network through the maintenance of disinfection residuals. At a pipework dead-end and when the disinfection residual was not maintained, significant ($P<0.05$) increases in concentration were observed when compared to the concentration measured closest to the processing plant in the same pipeline and sampling period. In the two reuse water distribution systems sampled *Legionella spp*., *L. pneumophila* and MAC were detected at maximum concentrations of $10^5$, $10^3$ and $10^6$ copies/mL respectively. During the summer period of sampling the concentration of all three organisms significantly ($P<0.05$) increased along the pipeline, implying multiplication and hence viability. No seasonality in the concentration of in chlorine residual along the pipelines was observed. This suggests that the combined effect of increased temperature and

decrease chlorine residual may be responsible for the observed increase in these opportunistic pathogens.

Creating a risk assessment from the results of the qPCR enumeration of *Legionella* spp., *L. pneumophila* and MAC from the potable and reuse water distribution pipelines was impeded by the uncertainites. Current *Legionella* and MAC risk assessments may be compromised by uncertainties in *Legionella* detection methods, strain infectivity and infectious dose. A review of the literature regarding the uncertainties associated with *Legionella* risk assessment following the EnHealth Risk Assessment Framework (Priestly *et al.* 2012) was completed. The uncertainties highlighted in this thesis affected each component of the risk assessment framework and must be considered when evaluating data used for *Legionella* risk assessment. The uncertainties associated with MAC risk assessment are much greater as there has been limited research into environmental or patient risk factors for MAC infection that consider all the clinical presentations of MAC related illnesses. The limited studies that are available regarding MAC risk factors are conflicting, depending on the population and the presentation of MAC disease that is investigated.

Current risk management strategies for controlling *Legionella* and MAC within artificial aquatic environments are based on maintenance of disinfection residuals and low nutrient concentrations. Typically legislation regarding the management of *Legionella* is better documented compared to that for the control of MAC. The results from this study demonstrated that an observed increase in *Legionella* copies/mL had a relationship with an increase in MAC copies/mL. From a risk manager's perspective this means that if a system has *Legionella* growth then it is

likely to also have MAC growth and management strategies should be targeted at both of these opportunistic pathogens.

This study demonstrated that South Australian potable and reuse water distribution systems contain potentially viable *Legionella spp*., *L. pneumophila* and MAC. This suggests that *Legionella* and MAC may be more ubiquitous than previously considered and that exposure to these organisms may be commonplace. This conclusion however is not supported by high incidences of *Legionella* or MAC infection. This highlights the need for future research aimed at understanding why an outbreak occur and factors which may determine infection. This includes greater research exploring susceptible populations, infectious dose and strain infectivity. This will better inform management strategies for the control of the potential public health risk posed by these organisms.

# Chapter 7: References


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# Chapter 8: Appendix

**Table 25: Monthly average (±SD) water quality parameters of water leaving the potable water DS1 treatment plant throughout the year 2012.**

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100 mL) | E. coli (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| January | Average | 2.18 | 2.34 | 0.35 | 0.00 | 0.00 |
| | n | 12 | 12 | 4 | 4 | 4 |
| | ±SD | 0.15 | 0.15 | 0.18 | 0.00 | 0.00 |
| February | Average | 2.02 | 2.20 | 0.24 | 0.00 | 0.00 |
| | n | 12 | 12 | 4 | 4 | 4 |
| | ±SD | 0.99 | 0.95 | 0.76 | 0.00 | 0.00 |
| March | Average | 2.04 | 2.24 | 0.14 | 0.00 | 0.00 |
| | n | 12 | 12 | 2 | 2 | 2 |
| | ±SD | 0.31 | 0.30 | 0.00 | 0.00 | 0.00 |
| April | Average | 1.99 | 2.17 | 0.20 | 0.00 | 0.00 |
| | n | 13 | 13 | 3 | 3 | 3 |
| | ±SD | 0.19 | 0.21 | 0.09 | 0.00 | 0.00 |
| May | Average | 1.87 | 2.08 | 0.17 | 0.00 | 0.00 |
| | n | 13 | 13 | 13 | 4 | 4 |
| | ±SD | 0.24 | 0.23 | 0.04 | 0.00 | 0.00 |
| June | Average | 1.77 | 1.94 | 0.18 | 0.00 | 0.00 |
| | n | 13 | 13 | 4 | 4 | 4 |
| | ±SD | 0.28 | 0.23 | 0.06 | 0.00 | 0.00 |
| July | Average | 1.50 | 1.61 | 1.38 | 0.00 | 0.00 |
| | n | 13 | 13 | 3 | 3 | 3 |
| | ±SD | 0.18 | 0.15 | 0.02 | 0.00 | 0.00 |
| August | Average | 1.47 | 1.32 | 0.16 | 0.00 | 0.00 |
| | n | 12 | 12 | 3 | 3 | 3 |

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100 mL) | E. coli (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| | ±SD | 0.24 | 0.20 | 0.04 | 0.00 | 0.00 |
| September | Average | 1.48 | 1.57 | 0.14 | 0.00 | 0.00 |
| | n | 11 | 11 | 4 | 4 | 4 |
| | ±SD | 0.29 | 0.25 | 0.02 | 0.00 | 0.00 |
| October | Average | 1.43 | 1.51 | 0.14 | 0.00 | 0.00 |
| | n | 12 | 12 | 3 | 3 | 3 |
| | ±SD | 0.27 | 0.23 | 0.03 | 0.00 | 0.00 |
| November | Average | 1.12 | 1.47 | 0.19 | 0.00 | 0.00 |
| | n | 11 | 11 | 11 | 4 | 4 |
| | ±SD | 0.27 | 0.59 | 0.11 | 0.00 | 0.00 |
| December | Average | 1.35 | 1.49 | 0.14 | 0.00 | 0.00 |
| | n | 13 | 13 | 5 | 5 | 5 |
| | ±SD | 0.16 | 0.14 | 0.02 | 0.00 | 0.00 |

N/A = not available

**Table 26: Monthly average (±SD) water quality parameters measured at sampling point A of potable water DS1 throughout the year 2012.**

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Coliforms (MPN/100mL) | *E. coli* (MPN/100mL) |
|---|---|---|---|---|---|
| January | Average | 1.1 | 1.3 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| February | Average | 1.2 | 1.4 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| March | Average | 1 | 1.2 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| April | Average | 1.2 | 1.3 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| May | Average | N/A | N/A | N/A | N/A |
| | n | N/A | N/A | N/A | N/A |
| | ±SD | N/A | N/A | N/A | N/A |
| June | Average | N/A | N/A | N/A | N/A |
| | n | N/A | N/A | N/A | N/A |
| | ±SD | N/A | N/A | N/A | N/A |
| July | Average | 0.9 | 1.1 | 0 | 0 |
| | n | 4 | 4 | 1 | 1 |
| | ±SD | 0.13 | 0 | 0 | 0 |
| August | Average | 1.5 | 1.5 | 0 | 0 |
| | n | 4 | 4 | 1 | 1 |
| | ±SD | 0.17 | 0.16 | 0 | 0 |
| September | Average | 0.7 | 0.8 | 0 | 0 |

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Coliforms (MPN/100mL) | *E. coli* (MPN/100mL) |
|---|---|---|---|---|---|
| | n | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| October | Average | 1.3 | 1.4 | 0 | 0 |
| | n | 3 | 3 | 1 | 1 |
| | ±SD | 0.26 | 0.25 | 0 | 0 |
| November | Average | 1.1 | 1.2 | 0 | 0 |
| | n | 4 | 4 | 1 | 1 |
| | ±SD | 0.28 | 0.28 | 0 | 0 |
| December | Average | 0.6 | 0.7 | 0 | 0 |
| | n | 5 | 5 | 1 | 1 |
| | ±SD | 0.24 | 0.27 | 0 | 0 |

N/A = not available

**Table 27: Monthly average (±SD) water quality parameters measured at sampling point B of potable water DS1 throughout the year 2012.**

| Month | | Free chlorine (mg/L) | total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| January | Average | 0.8 | 0.9 | N/A | 0 | 0 |
| | n | 2 | 2 | N/A | 2 | 2 |
| | ±SD | 0.14 | 0.07 | N/A | 0.00 | 0.00 |
| February | Average | 0.6 | 0.7 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| March | Average | 1.1 | 1.3 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| April | Average | 0.8 | 1 | <0.10 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| May | Average | 1.3 | 1.4 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| June | Average | 1.4 | 1.5 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| July | Average | 0.9 | 1.1 | 0.1 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| August | Average | 1 | 1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| September | Average | 0.9 | 1 | N/A | 0 | 0 |

| Month | | Free chlorine (mg/L) | total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| October | Average | 0.8 | 0.9 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| November | Average | 0.19 | 0.9 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| December | Average | 0.5 | 0.6 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |

N/A = not available

**Table 28: Monthly average (±SD) water quality parameters measured at sampling point C of potable water DS1 throughout the year 2012.**

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| January | Average | 1 | 1.1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| February | Average | 1 | 1.1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| March | Average | 0.8 | 1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| April | Average | 1.2 | 1.3 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| May | Average | 1.2 | 1.4 | <0.10 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| June | Average | 0.9 | 1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| July | Average | 0.6 | 0.8 | 0.12 | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| August | Average | 1.2 | 1.3 | N/A | 0 | 0 |
| | n | 2 | 2 | N/A | 1 | 1 |
| | ±SD | 0.14 | 0.21 | N/A | 0 | 0 |
| September | Average | 1.4 | 1.4 | 0.12 | 0 | 0 |

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| October | Average | 0.5 | 0.6 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| November | Average | 0.5 | 0.6 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| December | Average | 0.2 | 0.3 | 0.1 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |

N/A = not available

**Table 29: Monthly average (±SD) water quality parameters measured at sampling point D of potable water DS1 throughout the year 2012**

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| January | Average | 0.1 | 0.3 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| February | Average | 0.2 | 0.4 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| March | Average | 0.4 | 0.5 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| April | Average | 0.2 | 0.4 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| May | Average | 0.6 | 0.8 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| June | Average | 0.6 | 0.8 | 0.15 | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| July | Average | 0.5 | 0.6 | 0.16 | 0 | 0 |
| | n | 2 | 2 | 1 | 1 | 1 |
| | ±SD | 0.07 | 0 | 0 | 0 | 0 |
| August | Average | 0.4 | 0.5 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| September | Average | 0.8 | 0.9 | N/A | 0 | 0 |

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| October | Average | 0.6 | 0.8 | 0.14 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| November | Average | 0.1 | 0.3 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| December | Average | 0.1 | 0.1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |

N/A = not available

**Table 30: Monthly average (±SD) water quality parameters measured at sampling point E of potable water DS1 throughout the year 2012.**

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| January | Average | 0.1 | 0.2 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| February | Average | 0.2 | 0.4 | 0.24 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| March | Average | 0.1 | 0.2 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| April | Average | 0.4 | 0.5 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| May | Average | 0.1 | 0.2 | <0.10 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| June | Average | 1 | 1.2 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| July | Average | 0.6 | 0.7 | N/A | 0 | 0 |
| | n | 2 | 2 | N/A | 2 | 2 |
| | ±SD | 0.07 | 0 | N/A | 0 | 0 |
| August | Average | 0.6 | 0.7 | <0.10 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| September | Average | 0.4 | 0.5 | N/A | 0 | 0 |

| Month | | Free chlorine (mg/L) | Total chlorine (mg/L) | Turbidity (NTU) | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) |
|---|---|---|---|---|---|---|
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| October | Average | 0.5 | 0.7 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |
| November | Average | 0.8 | 0.9 | <0.10 | 0 | 0 |
| | n | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 |
| December | Average | 0.1 | 0.1 | N/A | 0 | 0 |
| | n | 1 | 1 | N/A | 1 | 1 |
| | ±SD | 0 | 0 | N/A | 0 | 0 |

N/A = not available

**Table 31: Monthly average (±SD) water quality parameters measured in raw water entering potable water DS2 treatment plant throughout the year 2012.**

| Month | | Coliforms (MPN/100mL) | Dissolved Organic Carbon (mg/L) | Turbidity (NTU) | *E. coli* (MPN/100mL) |
|---|---|---|---|---|---|
| January | Average | 8400 | 5 | 84.5 | 57 |
| | n | 1 | 3 | 4 | 1 |
| | ±SD | 0 | 0.8 | 29.5 | 0 |
| February | Average | 4100 | 6.6 | 110.3 | 120 |
| | n | 1 | 4 | 4 | 1 |
| | ±SD | 0 | 0.5 | 91.1 | 0 |
| March | Average | 13000 | 8.0 | 130.8 | 160 |
| | n | 1 | 5 | 6 | 1 |
| | ±SD | 0 | 0.7 | 58.1 | 0 |
| April | Average | 26000 | 9 | 80.5 | 56 |
| | n | 1 | 4 | 4 | 1 |
| | ±SD | 0 | 1.5 | 17.8 | 0 |
| May | Average | 1600 | 12.7 | 55 | 84 |
| | n | 2 | 4 | 4 | 1 |
| | ±SD | 551.5 | 1.3 | 9.6 | 0 |
| June | Average | 310 | 9.6 | 57.3 | 110 |
| | n | 1 | 4 | 5 | 1 |
| | ±SD | 0 | 0.9 | 9.4 | 0 |
| July | Average | 520 | 7.6 | 36 | 180 |
| | n | 1 | 4 | 4 | 1 |
| | ±SD | 0 | 1.3 | 4.7 | 0 |
| August | Average | 520 | 5.3 | 34.5 | 32 |
| | n | 1 | 4 | 4 | 1 |
| | ±SD | 0 | 0.5 | 9.1 | 0 |

| **Month** | | **Coliforms (MPN/100mL)** | **Dissolved Organic Carbon (mg/L)** | **Turbidity (NTU)** | ***E. coli* (MPN/100mL)** |
|---|---|---|---|---|---|
| September | Average | 330 | 6.2 | 57 | 34 |
| | n | 1 | 3 | 5 | 1 |
| | ±SD | 0 | 0.6 | 5.7 | 0 |
| October | Average | 1100 | 5.9 | 61.5 | 28 |
| | n | 1 | 4 | 4 | 1 |
| | ±SD | 0 | 0 | 2.6 | 0 |
| November | Average | 5100 | 6.3 | 63.8 | 1400 |
| | n | 1 | 5 | 5 | 1 |
| | ±SD | 0 | 0.5 | 12.5 | 0 |
| December | Average | 2000 | 5.7 | 55 | 110 |
| | n | 1 | 4 | 4 | 1 |
| | ±SD | 0 | 1.0 | 7.1 | 0 |

**Table 32: Monthly average (±SD) water quality parameters measured at sampling point A of potable water DS2 throughout the year 2012.**

| Month | | coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Dissolved Organic Carbon (mg/L) | Mono-chloramine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|---|
| January | Average | 0 | 0 | 2.5 | 4.4 | 8.8 |
| | n | 4 | 4 | 3 | 4 | 4 |
| | ±SD | 0 | 0 | 0.3 | 0.4 | 0.1 |
| February | Average | 0 | 0 | 3.4 | 4.3 | 9 |
| | n | 4 | 4 | 3 | 4 | 4 |
| | ±SD | 0 | 0 | 0.4 | 0.3 | 0.1 |
| March | Average | 0 | 0 | 4.2 | 3.9 | 8.9 |
| | n | 5 | 5 | 2 | 5 | 5 |
| | ±SD | 0 | 0 | 0.6 | 0.4 | 0.2 |
| April | Average | 0 | 0 | 4.1 | 3.7 | 8.6 |
| | n | 4 | 4 | 2 | 4 | 4 |
| | ±SD | 0 | 0 | 0.2 | 0.5 | 0.1 |
| May | Average | 0 | 0 | 5.0 | 3.8 | 8.9 |
| | n | 4 | 4 | 4 | 4 | 4 |
| | ±SD | 0 | 0 | 0.4 | 0.3 | 0.3 |
| June | Average | 0 | 0 | 5.1 | 3.7 | 8.8 |
| | n | 5 | 5 | 2 | 5 | 5 |
| | ±SD | 0 | 0 | 0.5 | 0.5 | 0.1 |
| July | Average | 0 | 0 | 4.1 | 3.7 | 8.7 |
| | n | 4 | 4 | 2 | 4 | 4 |
| | ±SD | 0 | 0 | 0.2 | 0.2 | 0.2 |
| August | Average | 0 | 0 | 3.1 | 3.9 | 8.3 |
| | n | 4 | 4 | 3 | 4 | 4 |
| | ±SD | 0 | 0 | 0.2 | 0.4 | 0.3 |

| **Month** | | **coliforms (MPN/ 100mL)** | ***E. coli* (MPN/ 100mL)** | **Dissolved Organic Carbon (mg/L)** | **Mono-chloramine (mg/L)** | **pH (pH unit)** |
|---|---|---|---|---|---|---|
| September | Average | 0 | 0 | 3.2 | 3.7 | 8.5 |
| | n | 4 | 4 | 2 | 4 | 4 |
| | ±SD | 0 | 0 | 0.4 | 0.5 | 0.2 |
| October | Average | 0 | 0 | 3.2 | 3.5 | 8.9 |
| | n | 4 | 4 | 2 | 4 | 4 |
| | ±SD | 0 | 0 | 0.1 | 0.3 | 0.4 |
| November | Average | 0 | 0 | 3.5 | 3.6 | 8.8 |
| | n | 5 | 5 | 3 | 5 | 5 |
| | ±SD | 0 | 0 | 0.4 | 0.3 | 0.2 |
| December | Average | 0 | 0 | 3.4 | 3.7 | 9.0 |
| | n | 4 | 4 | 3 | 4 | 4 |
| | ±SD | 0 | 0 | 0.6 | 0.2 | 0.1 |

**Table 33: Monthly average (±SD) water quality parameters measured at sampling point B of potable water DS2 throughout the year 2012.**

| Month | | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Monochloramine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|
| January | Average | 0 | 0 | 3.6 | 9.1 |
| | n | 2 | 2 | 4 | 2 |
| | ±SD | 0 | 0 | 0.4 | 0.2 |
| February | Average | 0 | 0 | 3.3 | 9.1 |
| | n | 2 | 2 | 3 | 2 |
| | ±SD | 0 | 0 | 0.2 | 0.3 |
| March | Average | 0 | 0 | 3.2 | 9.1 |
| | n | 2 | 2 | 4 | 2 |
| | ±SD | 0 | 0 | 0.5 | 0.1 |
| April | Average | 0 | 0 | 3.1 | 9.0 |
| | n | 2 | 2 | 4 | 2 |
| | ±SD | 0 | 0 | 0.5 | 0.1 |
| May | Average | 0 | 0 | 2.5 | 8.9 |
| | n | 2 | 2 | 5 | 2 |
| | ±SD | 0 | 0 | 0.2 | 0.1 |
| June | Average | 0 | 0 | 2.9 | 9 |
| | n | 2 | 2 | 4 | 2 |
| | ±SD | 0 | 0 | 0.2 | 0 |
| July | Average | 0 | 0 | 3.1 | 8.9 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| August | Average | 0 | 0 | 3.8 | 8.7 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| September | Average | 0 | 0 | 2.3 | 9 |

| Month | | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Monochloramine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| October | Average | 0 | 0 | 2.7 | 9 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| November | Average | 0 | 0 | 2.8 | 9 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| December | Average | 0 | 0 | 2.9 | 8.7 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |

**Table 34: Monthly average (±SD) water quality parameters measured at sampling point C of potable water DS2 throughout the year 2012.**

| Month | | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Mono-chloramine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|
| January | Average | 0 | 0 | 4.0 | N/A |
| | n | 2 | 2 | 2 | N/A |
| | ±SD | 0 | 0 | 0.6 | N/A |
| February | Average | 0 | 0 | 3.2 | N/A |
| | n | 2 | 2 | 2 | N/A |
| | ±SD | 0 | 0 | 0.1 | N/A |
| March | Average | 0 | 0 | 3.0 | N/A |
| | n | 2 | 2 | 2 | N/A |
| | ±SD | 0 | 0 | 0.8 | N/A |
| April | Average | 0 | 0 | 3.4 | N/A |
| | n | 2 | 2 | 2 | N/A |
| | ±SD | 0 | 0 | 0.3 | N/A |
| May | Average | 0 | 0 | 2.4 | N/A |
| | n | 2 | 2 | 2 | N/A |
| | ±SD | 0 | 0 | 0.1 | N/A |
| June | Average | 0 | 0 | 2.4 | 8.7 |
| | n | 2 | 2 | 2 | 1 |
| | ±SD | 0 | 0 | 0.7 | 0 |
| July | Average | 0 | 0 | 2.2 | 8.5 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| August | Average | 0 | 0 | 3.2 | 8.4 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| September | Average | 0 | 0 | 3.5 | 8.5 |

| Month | | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Mono-chloramine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| October | Average | 0 | 0 | 2.8 | 8.9 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| November | Average | 0 | 0 | 3.9 | 8.7 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| December | Average | 0 | 0 | 3.5 | 8.6 |
| | n | 2 | 2 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |

NA = data unavailable

**Table 35: Monthly average water quality parameters measured at sampling point D of potable water DS2 throughout the year 2012.**

| | | Alkalinity as Calcium Carbonate (mg/L) | Coilforms /100mL | True colour (456nm) (HU) | Cond-uctivity (µScm) | Dissolved Organic Carbon (mg/L) | E.coli /100mL | Mono-chloramine (mg/L) | pH (pH units) | Total Dissolved Solids (mg/L) | Total Hardness as CaCO3 (mg/L) | Turbidity (NTU) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| January | Average | 54 | 0 | 2 | 464 | 3.6 | 0 | 2.5 | 9.3 | 250 | 74 | 0.61 |
| | n | 1 | 4 | 1 | 1 | 1 | 4 | 4 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0.6 | 0 | 0 | 0 | 0 |
| February | Average | N/A | 0 | 1 | N/A | N/A | 0 | 2.7 | 9.5 | N/A | N/A | 0.15 |
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 5 | 1 | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.6 | 0 | N/A | N/A | 0 |
| March | Average | 77 | 0 | 2 | 400 | 4.5 | 0 | 1.9 | 9 | 220 | 74 | <0.1 |
| | n | 1 | 5 | 1 | 1 | 1 | 5 | 4 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 |
| April | Average | N/A | 0 | 3 | N/A | N/A | 0 | 2.6 | 8.9 | N/A | N/A | <0.1 |
| | n | N/A | 5 | 1 | N/A | N/A | 5 | 4 | 1 | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.7 | 0 | N/A | N/A | 0 |
| May | Average | N/A | 0 | 2 | N/A | N/A | 0 | 1.7 | N/A | N/A | N/A | 0.21 |

| | | Alkalinity as Calcium Carbonate (mg/L) | Coilforms /100mL | True colour (456nm) (HU) | Cond-uctivity (µScm) | Dissolved Organic Carbon (mg/L) | E.coli /100mL | Mono-chloramine (mg/L) | pH (pH units) | Total Dissolved Solids (mg/L) | Total Hardness as CaCO3 (mg/L) | Turbidity (NTU) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 4 | N/A | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.3 | N/A | N/A | N/A | 0 |
| June | Average | N/A | 0 | 3 | N/A | N/A | 0 | 1.3 | N/A | N/A | N/A | 0.38 |
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 4 | N/A | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.2 | N/A | N/A | N/A | 0 |
| July | Average | 77 | 0 | 2 | 397 | 5 | 0 | 1.5 | 8.9 | 220 | 80 | <0.1 |
| | n | 1 | 4 | 1 | 1 | 1 | 4 | 5 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 |
| August | Average | N/A | 0 | 1 | N/A | N/A | 0 | 0.8 | 8.6 | N/A | N/A | 0.1 |
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 4 | 1 | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.4 | 0 | N/A | N/A | 0 |
| September | Average | N/A | 0 | 1 | N/A | N/A | 0 | 1.5 | 8.6 | N/A | N/A | <0.1 |
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 4 | 1 | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.3 | 0 | N/A | N/A | 0 |

| | | Alkalinity as Calcium Carbonate (mg/L) | Coilforms /100mL | True colour (456nm) (HU) | Cond-uctivity (µScm) | Dissolved Organic Carbon (mg/L) | E.coli /100mL | Mono-chloramine (mg/L) | pH (pH units) | Total Dissolved Solids (mg/L) | Total Hardness as CaCO3 (mg/L) | Turbidity (NTU) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| October | Average | 49 | 0 | 2 | 277 | 3 | 0 | 2.0 | 9.2 | 150 | 51 | 0.12 |
| | n | 1 | 4 | 1 | 1 | 1 | 4 | 4 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0 | 0 |
| November | Average | N/A | 0 | 3 | N/A | N/A | 0 | 2.3 | 8.9 | N/A | N/A | 0.11 |
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 4 | 1 | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.2 | 0 | N/A | N/A | 0 |
| December | Average | N/A | 0 | 2 | N/A | N/A | 0 | 2.5 | 9.2 | N/A | N/A | 0.13 |
| | n | N/A | 4 | 1 | N/A | N/A | 4 | 4 | 1 | N/A | N/A | 1 |
| | ±SD | N/A | 0 | 0 | N/A | N/A | 0 | 0.2 | 0 | N/A | N/A | 0 |

N/A = data unavailable

**Table 36: Monthly average water quality parameters measured at sampling point E of potable water DS2 throughout the year 2012.**

| Month | | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Mono-chlormaine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|
| January | Average | 0 | 0 | 0.05 | 8 |
| | n | 4 | 4 | 1 | 2 |
| | ±SD | 0 | 0 | 0 | 0.1 |
| February | Average | 0 | 0 | 0.05 | 8.2 |
| | n | 4 | 4 | 1 | 2 |
| | ±SD | 0 | 0 | 0 | 0.1 |
| March | Average | 0 | 0 | 0.05 | 8.2 |
| | n | 4 | 4 | 1 | 2 |
| | ±SD | 0 | 0 | 0 | 0.1 |
| April | Average | 0 | 0 | 0.1 | 8.5 |
| | n | 4 | 4 | 1 | 2 |
| | ±SD | 0 | 0 | 0 | 0.1 |
| May | Average | 0 | 0 | 0.05 | 7.7 |
| | n | 4 | 4 | 1 | 2 |
| | ±SD | 0 | 0 | 0 | 0.3 |
| June | Average | 0 | 0 | 0.05 | 7.7 |
| | n | 4 | 4 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 |
| July | Average | 0 | 0 | N/A | N/A |
| | n | 3 | 3 | N/A | N/A |
| | ±SD | 0 | 0 | N/A | N/A |
| August | Average | 0 | 0 | N/A | N/A |
| | n | 4 | 4 | N/A | N/A |
| | ±SD | 0 | 0 | N/A | N/A |
| September | Average | 0 | 0 | N/A | N/A |

| Month | | Coliforms (MPN/ 100mL) | *E. coli* (MPN/ 100mL) | Mono-chlormaine (mg/L) | pH (pH unit) |
|---|---|---|---|---|---|
| | n | 4 | 4 | N/A | N/A |
| | ±SD | 0 | 0 | N/A | N/A |
| October | Average | 0 | 0 | N/A | N/A |
| | n | 4 | 4 | N/A | N/A |
| | ±SD | 0 | 0 | N/A | N/A |
| November | Average | 0 | 0 | N/A | N/A |
| | n | 4 | 4 | N/A | N/A |
| | ±SD | 0 | 0 | N/A | N/A |
| December | Average | 0 | 0 | N/A | N/A |
| | n | 4 | 4 | N/A | N/A |
| | ±SD | 0 | 0 | N/A | N/A |

N/A = data unavailable

**Table 37: Monthly average water quality parameters of sewage entering the treatment plant of reuse DS1 throughout the year 2012.**

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Biochemical Oxygen Demand (mg/L) | Average | 216.4 | 200.0 | 221.6 | 166.6 | 184.2 | 172.0 | 197.5 | 211.2 | 163.4 | 201.2 | 174.4 | 239.3 |
| | n | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 6 | 5 | 4 |
| | ±SD | 25.6 | 40.8 | 43.7 | 26.5 | 33.5 | 32.1 | 18.5 | 24.2 | 32.9 | 55.0 | 32.7 | 37.6 |
| Suspended Solids (mg/L) | Average | 281.8 | 224 | 311.4 | 264.8 | 306 | 304.8 | 265.5 | 307.6 | 272.2 | 295.3 | 290.6 | 339.8 |
| | n | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 6 | 5 | 4 |
| | ±SD | 52.8 | 72.8 | 79.2 | 27.3 | 60.4 | 46.5 | 34.5 | 57.1 | 24.0 | 53.6 | 30.4 | 26.9 |
| Chemical Oxygen Demand (mg/L) | Average | 562 | 522 | 615 | 546 | 581 | 575 | 531 | 568 | 561 | 589 | 616 | 689 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| pH (pH units) | Average | 7.2 | 7.3 | 7.3 | 7.3 | 7.3 | 7.4 | 7.4 | 7.5 | 7.4 | 7.5 | 7.3 | 7.3 |
| | n | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 6 | 5 | 4 |
| | ±SD | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.2 | 0.2 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ammonia as N (mg/L) | Average | 41.2 | 39.8 | 42.5 | 45.0 | 41.2 | 35.9 | 33.0 | 33.0 | 34.4 | 38.0 | 36.9 | 40.7 |
| | n | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 5 | 4 |
| | ±SD | 2.4 | 2.5 | 3.0 | 3.4 | 2.1 | 10.5 | 2.7 | 4.7 | 2.1 | 1.9 | 4.4 | 5.3 |
| Total Dissolved Solids (by EC) (mg/L) | Average | 1080 | 1080 | 996 | 1008 | 1008 | 1038 | 1225 | 1200 | 1200 | 1133.333 | 1060 | 1100 |
| | n | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 6 | 5 | 4 |
| | ±SD | 44.7 | 83.7 | 8.9 | 55.4 | 54.0 | 172.4 | 50.0 | 70.7 | 0.0 | 51.6 | 54.8 | 0.0 |
| TKN as Nitrogen (mg/L) | Average | 63.7 | 59.3 | 63.6 | 64.6 | 56.0 | 56.0 | 51.9 | 57.1 | 53.4 | 56.7 | 53.4 | 60.6 |
| | n | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 5 | 4 |
| | ±SD | 5.5 | 1.4 | 7.9 | 5.4 | 6.1 | 7.7 | 3.3 | 10.9 | 7.9 | 6.9 | 1.4 | 7.7 |
| Phosphorus - Total (mg/L) | Average | 10.1 | 9.8 | 9.5 | 11.5 | 10.4 | 10.8 | 8.5 | 7.9 | 9.9 | 8.4 | 9.3 | 10.5 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Aluminium - Total | Average | 0.364 | 0.511 | 0.374 | 0.457 | 0.407 | 0.494 | 0.396 | 0.544 | 0.439 | 0.406 | 0.42 | 0.462 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimony - Total (mg/L) | Average | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arsenic - Total (mg/L) | Average | 0.001 | 0.0012 | 0.001 | 0.0012 | 0.0011 | 0.001 | 0.0009 | 0.001 | 0.001 | 0.0008 | 0.0007 | 0.001 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Beryllium - Total (mg/L) | Average | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Boron - Soluble (mg/L) | Average | 0.344 | 0.314 | 0.3 | 0.256 | 0.279 | 0.424 | 0.378 | 0.542 | 0.455 | 0.537 | 0.396 | 0.3935 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cadmium - Total (mg/L) | Average | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0001 | 0.0001 | 0.0003 | 0.0002 | 0.0002 | 0.0002 | 0.00025 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chromium - Total (mg/L) | Average | 0.0115 | 0.0168 | 0.0139 | 0.013 | 0.0181 | 0.0246 | 0.0222 | 0.0244 | 0.0204 | 0.0154 | 0.0163 | 0.01545 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cobalt - Total (mg/L) | Average | 0.0007 | 0.0006 | 0.0005 | 0.0004 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0004 | 0.0005 | 0.0005 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Copper - Total (mg/L) | Average | 0.1008 | 0.1313 | 0.0966 | 0.1311 | 0.124 | 0.1208 | 0.0788 | 0.1016 | 0.099 | 0.0885 | 0.0747 | 0.09215 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Iron - Total (mg/L) | Average | 0.3421 | 0.4526 | 0.3458 | 0.3898 | 0.4099 | 0.4342 | 0.292 | 0.4295 | 0.38 | 0.3589 | 0.3294 | 0.37715 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lead - Total (mg/L) | Average | 0.0048 | 0.0103 | 0.0067 | 0.0057 | 0.0057 | 0.0072 | 0.0056 | 0.0063 | 0.0058 | 0.0045 | 0.0069 | 0.0079 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lithium - Total (mg/L) | Average | 0.0082 | 0.007 | 0.0072 | 0.0067 | 0.0068 | 0.0087 | 0.0086 | 0.0096 | 0.0087 | 0.0071 | 0.0075 | 0.00755 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Manganese - Total (mg/L) | Average | 0.0322 | 0.0363 | 0.0364 | 0.0353 | 0.0353 | 0.0373 | 0.0362 | 0.0402 | 0.0379 | 0.0325 | 0.0358 | 0.0355 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mercury - Total | Average | 0.00008 | 0.00068 | 0.00005 | 0.00011 | 0.00076 | 0.00013 | 0.00007 | 0.00062 | 0.00007 | 0.00013 | 0.00014 | 0.00013 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Molybdenum - Total (mg/L) | Average | 0.0026 | 0.0034 | 0.0028 | 0.003 | 0.003 | 0.0032 | 0.0032 | 0.0038 | 0.0026 | 0.0016 | 0.0012 | 0.00155 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nickel - Total (mg/L) | Average | 0.007 | 0.0082 | 0.0094 | 0.007 | 0.0095 | 0.0106 | 0.0139 | 0.0132 | 0.0124 | 0.0117 | 0.0158 | 0.01115 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Selenium - Total (mg/L) | Average | 0.0019 | 0.0016 | 0.0004 | 0.0006 | 0.0025 | 0.0015 | 0.0016 | 0.0003 | 0.0019 | 0.0024 | 0.0024 | 0.0039 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Silver - Total (mg/L) | Average | 0.0009 | 0.00096 | 0.00095 | 0.00093 | 0.00102 | 0.00111 | 0.00078 | 0.00125 | 0.00089 | 0.00084 | 0.00153 | 0.001695 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thallium - Total (mg/L) | Average | 0.0002 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tin - Total (mg/L) | Average | 0.0033 | 0.0044 | 0.0034 | 0.0043 | 0.0044 | 0.0044 | 0.0031 | 0.0043 | 0.0036 | 0.0038 | 0.0035 | 0.0044 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Vanadium - Total (mg/L) | Average | 0.0001 | 0.0001 | 0.0004 | 0.0001 | 0.001 | 0.0001 | 0.0016 | 0.0011 | 0.0011 | 0.0006 | 0.0001 | 0.0016 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Zinc - Total (mg/L) | Average | 0.0981 | 0.1165 | 0.1007 | 0.1055 | 0.1143 | 0.0995 | 0.071 | 0.0988 | 0.0961 | 0.09 | 0.0882 | 0.0957 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Methylene Blue Active Substance(mg/L) | Average | 3.87 | 3.6 | 4.55 | 5.16 | 1.86 | 3.51 | 4.23 | 3.69 | 2.58 | 3.39 | 3.4 | 2.1 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Phenols (mg/L) | Average | 0.01 | | 0.084 | 0.01 | 0.01 | 0.196 | 0.01 | 0.01 | 0.018 | 0.093 | 0.012 | 0.127 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Copper - Total (mg/L) | Average | 0.1008 | 0.1313 | 0.0966 | 0.1311 | 0.124 | 0.1208 | 0.0788 | 0.1016 | 0.099 | 0.0885 | 0.0747 | 0.09215 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Grease (mg/L) | Average | 50 | 24 | 58 | 60 | 58 | 70 | 42 | 78 | 51 | 26 | 39 | 55 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cyanide as CN - | Average | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |

| | | **January** | **February** | **March** | **April** | **May** | **June** | **July** | **August** | **September** | **October** | **November** | **December** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total (mg/L) | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 38: Monthly average water quality parameters measured at sampling point A of reuse water DS1 throughout the year sampled**

| Month | | Temperature (°C) | *E. coli* (MPN/100mL) | Coliforms | pH (pH units) | Biochemical Oxygen Demand (mg/L) | Sodium Adsorption Ratio - Calculation | Nitrogen - Total (mg/L) | Phosphorus - Total (mg/L) | Ammonia as N (mg/L) | TKN as Nitrogen (mg/L) | Conductivity (µscm) | Total Dissolved Solids (by EC) | Nitrate + Nitrite as N (mg/L) | Potassium (mg/L) | Sodium (mg/L) | Magnesium(mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| January | Average | N/A | 0.0 | N/A | 7.1 | N/A | 6.9 | 7.7 | 6.0 | 0.0 | 1.5 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | N/A | 4 | N/A | 1 | N/A | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | 0.0 | N/A | 0.0 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| February | Average | N/A | 0.0 | N/A | 7.1 | N/A | 6.7 | 8.1 | 5.7 | 0.0 | 1.3 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | N/A | 5 | N/A | 2 | N/A | 2 | 2 | 2 | 2 | 2 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | 0.0 | N/A | 0.1 | N/A | 0.021 213 | 0.735 391 | 0.063 64 | 0.000 707 | 0.261 63 | N/A | N/A | N/A | N/A | N/A | N/A |
| March | Average | N/A | 0.0 | N/A | 7.1 | 2.0 | 5.6 | 5.3 | 4.8 | 0.2 | 1.5 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | N/A | 4 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | 0.0 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| April | Average | N/A | 0.0 | N/A | 7.0 | 2.0 | 6.5 | 10.8 | 5.8 | 0.0 | 1.5 | N/A | N/A | N/A | N/A | N/A | N/A |

| Month | | Temperature (°C) | *E. coli* (MPN/100mL) | Coliforms | pH (pH units) | Biochemical Oxygen Demand (mg/L) | Sodium Adsorption Ratio - Calculation | Nitrogen - Total (mg/L) | Phosphorus - Total (mg/L) | Ammonia as N (mg/L) | TKN as Nitrogen (mg/L) | Conductivity (µscm) | Total Dissolved Solids (by EC) | Nitrate + Nitrite as N (mg/L) | Potassium (mg/L) | Sodium (mg/L) | Magnesium(mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | N/A | 4 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | 0.0 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| May | Average | N/A | 0.0 | N/A | 6.9 | 2.0 | 6.2 | 11.2 | 8.3 | 0.0 | 1.7 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | N/A | 5 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | 0.0 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| June | Average | N/A | 0 | N/A | 7 | 3 | 7 | 9 | 5 | 0 | 2 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | N/A | 4 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | 0 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| July | Average | N/A | 0 | 475 | N/A | 3 | 7.77 | 5.96 | 8.38 | 0.02 | 2.43 | 2110 | 1200 | 3.53 | 25.1 | 317 | 48.3 |
| | n | N/A | 4 | 4 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | N/A | 0 | 200.4 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 28.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

| Month | | Temperature (°C) | *E. coli* (MPN/100mL) | Coliforms | pH (pH units) | Biochemical Oxygen Demand (mg/L) | Sodium Adsorption Ratio - Calculation | Nitrogen - Total (mg/L) | Phosphorus - Total (mg/L) | Ammonia as N (mg/L) | TKN as Nitrogen (mg/L) | Conductivity (µscm) | Total Dissolved Solids (by EC) | Nitrate + Nitrite as N (mg/L) | Potassium (mg/L) | Sodium (mg/L) | Magnesium(mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| August | Average | N/A | 0 | 1070 | N/A | 2 | 7.36 | 7.39 | 4.98 | 0.006 | 1.24 | 2060 | 1100 | 6.15 | 23.8 | 268 | 37.7 |
| | n | N/A | 5 | 5 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 5 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | N/A | 0 | 896.4 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 211.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| September | Average | N/A | 0 | 782.5 | N/A | N/A | 7.6 | 6.34 | 4.4 | 0.01 | 0.95 | 2240 | 1200 | 5.39 | 23.6 | 310 | 46.6 |
| | n | N/A | 4 | 4 | N/A | N/A | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | N/A | 0 | 470.3 | N/A | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 56.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| October | Average | N/A | 0 | 2300 | N/A | N/A | 7.15 | 7.8 | 5.3 | 0.014 | 0.94 | 1880 | 1000 | 6.86 | 23.4 | 264 | 36.7 |
| | n | N/A | 4 | 4 | N/A | N/A | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | N/A | 0 | 2684.2 | N/A | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 101.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| November | Average | N/A | 0 | 134 | N/A | 2 | 6.81 | 8.04 | 5.22 | 0.014 | 1.01 | 1710 | 940 | 7.03 | 21.7 | 231 | 29.9 |
| | n | N/A | 4 | 4 | N/A | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 |

| Month | | Temperature (°C) | *E. coli* (MPN/100mL) | Coliforms | pH (pH units) | Biochemical Oxygen Demand (mg/L) | Sodium Adsorption Ratio - Calculation | Nitrogen - Total (mg/L) | Phosphorus - Total (mg/L) | Ammonia as N (mg/L) | TKN as Nitrogen (mg/L) | Conductivity (µscm) | Total Dissolved Solids (by EC) | Nitrate + Nitrite as N (mg/L) | Potassium (mg/L) | Sodium (mg/L) | Magnesium(mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ±SD | N/A | 0 | 87.8 | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 35.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| December | Average | N/A | 0 | 221.8 | N/A | N/A | 6.64 | | 5.82 | | 1.35 | 1850 | 1000 | | 26.1 | 244 | 35.2 |
| | n | N/A | 5 | 5 | N/A | N/A | 1 | 1 | 1 | 1 | 1 | 5 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | N/A | 0 | 210.0 | N/A | N/A | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 57.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

*N/A = data unavailable

**Table 39: Monthly average water quality parameters measured at sampling point B of reuse water DS1 throughout the year sampled**

| Month | | Temperature ($^{o}$C) | Chlorine - Free (mg/L) | Chlorine - Total (mg/L) | *E. coli* (MPN/ 100mL) | Coliforms (MPN/ 100mL) | Conductivity (µscm) | Total Dissolved Solids (by EC) (mg/L) | Colour - True (456nm) | Ammonia as N (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| January | Average | N/A | 0.1 | 0.1 | 0 | 2024 | 1846.0 | 998.00 | 11.00 | 0.05 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.0 | 0 | 1656 | 45.60702 | 4.47 | 0.71 | 0.01 |
| February | Average | N/A | 0.1 | 0.1 | 0 | 304 | 1747.5 | 965.00 | 11.00 | 0.06 |
| | n | N/A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | 0.0 | 0.1 | 0 | 324 | 41.12988 | 20.82 | 1.83 | 0.02 |
| March | Average | N/A | 0.1 | 0.1 | 0 | 2828 | 1707.5 | 942.50 | 10.50 | 0.11 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.1 | 0 | 2736 | 15 | 9.57 | 1.91 | 0.03 |
| April | Average | N/A | 0.1 | 0.1 | 0 | 630 | 1740.0 | 960.00 | 11.75 | 0.07 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.0 | 0 | 1180 | 40.82483 | 23.09 | 2.63 | 0.02 |
| May | Average | N/A | 0.1 | 0.1 | 0 | 118 | 1760.0 | 970.00 | 7.60 | 0.07 |

| Month | | Temperature (°C) | Chlorine - Free (mg/L) | Chlorine - Total (mg/L) | *E. coli* (MPN/ 100mL) | Coliforms (MPN/ 100mL) | Conductivity (µscm) | Total Dissolved Solids (by EC) (mg/L) | Colour - True (456nm) | Ammonia as N (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.0 | 0 | 163 | 42.42641 | 21.21 | 4.04 | 0.01 |
| June | Average | N/A | 0 | 0 | 0 | 159 | 1710 | 942.50 | 4.67 | 0.08 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0 | 0 | 0 | 82 | 48.30459 | 29.86 | 0.58 | 0.00 |
| July | Average | N/A | 0.1 | 0.1 | 0 | 475 | 1667.5 | 920.00 | 4.50 | 0.09 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0 | 0 | 0 | 200 | 28.72281 | 14.14 | 0.58 | 0.01 |
| August | Average | N/A | 0.1 | 0.1 | 0 | 1070 | 1936 | 1080.00 | 5.60 | 0.11 |
| | n | N/A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | 0 | 0 | 0 | 896 | 211.4947 | 125.50 | 1.34 | 0.00 |
| September | Average | N/A | N/A | 0.1 | 0 | 783 | 2117.5 | 1150.00 | 3.75 | 0.07 |
| | n | N/A | N/A | 1 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | N/A | #DIV/0! | 0 | 470 | 56.78908 | 57.74 | 0.50 | 0.03 |

| Month | | Temperature (°C) | Chlorine - Free (mg/L) | Chlorine - Total (mg/L) | *E. coli* (MPN/ 100mL) | Coliforms (MPN/ 100mL) | Conductivity (µscm) | Total Dissolved Solids (by EC) (mg/L) | Colour - True (456nm) | Ammonia as N (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| October | Average | N/A | N/A | 0.125 | 0 | 2300 | 2040 | 1125.00 | 3.50 | 0.03 |
| | n | N/A | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | N/A | 0.05 | 0 | 2684 | 100.995 | 50.00 | 0.58 | 0.02 |
| November | Average | N/A | 0.1 | 0.2 | 0 | 134 | 1812.5 | 992.50 | 4.00 | 0.02 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0 | 0.08165 | 0 | 88 | 35.93976 | 9.57 | 0.82 | 0.01 |
| December | Average | N/A | N/A | 0.2 | 0 | 222 | 1818 | 990.00 | 5.80 | 0.03 |
| | n | N/A | N/A | 4 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | N/A | 0.08165 | 0 | 210 | 57.61944 | 14.14 | 0.84 | 0.01 |

*N/A = data unavailable

**Table 40: Monthly average water quality parameters measured at sampling point C of reuse water DS1 throughout the year sampled**

| Month | | Temperature ($^{o}$C) | Chlorine - Free (mg/L) | Chlorine - Total (mg/L) | *E. coli* (MPN/ 100mL) | Coliforms (MPN/ 100mL) | Conductivity (µscm) | Total Dissolved Solids (by EC) (mg/L) | Colour - True (456nm) | Ammonia as N (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| January | Average | N/A | 0.1 | 0.1 | 0.0 | 429.4 | 1792.0 | 978.0 | 10.2 | 0.0 |
| | n | N/A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | 0.0 | 0.0 | 0.0 | 305.9 | 71.9 | 29.5 | 0.8 | 0.0 |
| February | Average | N/A | 0.1 | 0.1 | 0.0 | 230.8 | 1752.5 | 967.5 | 10.5 | 0.0 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.1 | 0.0 | 285.8 | 9.6 | 5.0 | 2.1 | 0.0 |
| March | Average | N/A | 0.1 | 0.2 | 0.0 | 2492.5 | 1705.0 | 942.5 | 9.3 | 0.1 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.1 | 0.0 | 3357.4 | 19.1 | 9.6 | 2.9 | 0.0 |
| April | Average | N/A | 0.1 | 0.1 | 0.0 | 2233.3 | 1752.5 | 965.0 | 10.8 | 0.1 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.0 | 0.0 | 3990.3 | 44.3 | 23.8 | 2.2 | 0.1 |
| May | Average | N/A | 0.1 | 0.1 | 0.0 | 1692.0 | 1748.0 | 966.0 | 6.2 | 0.1 |

| Month | | Temperature (°C) | Chlorine - Free (mg/L) | Chlorine - Total (mg/L) | *E. coli* (MPN/ 100mL) | Coliforms (MPN/ 100mL) | Conductivity (µscm) | Total Dissolved Solids (by EC) (mg/L) | Colour - True (456nm) | Ammonia as N (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | N/A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | 0.0 | 0.0 | 0.0 | 2863.7 | 32.7 | 18.2 | 3.0 | 0.0 |
| June | Average | N/A | 0 | 0 | 0 | 1463 | 1683 | 930 | 3 | 0 |
| | n | N/A | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | ±SD | N/A | 0.0 | 0.0 | 0.0 | 866.6 | 32.1 | 17.3 | 0.0 | 0.0 |
| July | Average | N/A | 0.1 | 0.1 | 0 | 1317.5 | 1747.5 | 975 | 4.25 | 0.12425 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.0 | 0.0 | 734.8 | 115.3 | 83.5 | 0.5 | 0.0 |
| August | Average | N/A | 0.1 | 0.1 | 0 | 944 | 2148 | 1200 | 3.8 | 0.1314 |
| | n | N/A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | 0.0 | 0.0 | 0.0 | 491.7 | 37.0 | 0.0 | 1.6 | 0.0 |
| September | Average | N/A | N/A | 0.1 | 0 | 454 | 2127.5 | 1150 | 3.25 | 0.0815 |
| | n | N/A | N/A | 2 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | N/A | 0.0 | 0.0 | 472.6 | 58.5 | 57.7 | 0.5 | 0.0 |

| Month | | Temperature ($^{o}$C) | Chlorine - Free (mg/L) | Chlorine - Total (mg/L) | *E. coli* (MPN/ 100mL) | Coliforms (MPN/ 100mL) | Conductivity (µscm) | Total Dissolved Solids (by EC) (mg/L) | Colour - True (456nm) | Ammonia as N (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| October | Average | N/A | 0.1 | 0.225 | 0 | 44.75 | 1992.5 | 1100 | 3 | 0.023 |
| | n | N/A | 1 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.0 | 0.1 | 0.0 | 77.1 | 81.8 | 81.6 | 0.0 | 0.0 |
| November | Average | N/A | 0.15 | 0.25 | 0 | 25.5 | 1837.5 | 1017.5 | 3.75 | 0.01475 |
| | n | N/A | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | ±SD | N/A | 0.1 | 0.1 | 0.0 | 45.0 | 75.0 | 56.8 | 1.0 | 0.0 |
| December | Average | N/A | N/A | 0.24 | 0 | 3 | 1816 | 992 | 5.6 | 0.016 |
| | n | N/A | N/A | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | ±SD | N/A | N/A | 0.2 | 0.0 | 5.7 | 45.6 | 11.0 | 0.9 | 0.0 |

*N/A = data unavailable

**Table 41: Monthly average water quality parameters of sewage entering water treatment plant of reuse water DS2 throughout the year sampled**

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Biochemical Oxygen Demand (mg/L) | Average | 375 | 332.8 | 309.2 | 373 | 340 | 254.4 | 308.4 | 413.3 | 379.3 | 364.2 | 286 | 359.5 |
| | n | 4 | 5 | 5 | 5 | 3 | 13 | 5 | 3 | 3 | 5 | 5 | 4 |
| | ±SD | 37.9 | 54.3 | 96.9 | 215.2 | 20.0 | 126.6 | 100.5 | 25.2 | 56.8 | 82.3 | 58.3 | 27.9 |
| Suspended Solids (mg/L) | Average | 434.8 | 382.6 | 443.4 | 453.2 | 521.3 | 266.9 | 424.7 | 476.3 | 466.7 | 466.0 | 390.2 | 430.5 |
| | n | 4 | 5 | 5 | 5 | 3 | 13 | 6 | 3 | 3 | 5 | 5 | 4 |
| | ±SD | 88.4 | 120.5 | 63.4 | 168.5 | 45.7 | 233.1 | 116.2 | 39.2 | 10.1 | 87.5 | 152.5 | 49.4 |
| Chemical Oxygen Demand (mg/L) | Average | 788.0 | 940.0 | 552.0 | 927.0 | 1070.0 | 600.6 | 719.5 | | 908.0 | 944.0 | 620.0 | 992.0 |
| | n | 1 | 1 | 1 | 1 | 1 | 12 | 2 | 0 | 1 | 2 | 2 | 2 |
| | ±SD | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 302.8 | 200.1 | 0.0 | 0.0 | 93.3 | 186.7 | 39.6 |
| pH (pH units) | Average | 7.0 | 7.2 | 7.3 | 7.2 | 7.2 | 7.2 | 7.2 | 7.2 | 7.2 | 7.3 | 7.3 | 7.1 |
| | n | 4 | 5 | 5 | 5 | 3 | 13 | 6 | 3 | 3 | 5 | 5 | 4 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ±SD | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 |
| Ammonia as N (mg/L) | Average | 44.9 | 42.8 | 47.6 | 48.4 | 50.1 | 44.9 | 45.6 | 50.0 | 50.1 | 50.0 | 49.9 | 47.2 |
| | n | 4 | 5 | 5 | 5 | 3 | 13 | 6 | 3 | 3 | 5 | 5 | 4 |
| | ±SD | 4.2 | 3.8 | 2.7 | 1.2 | 1.1 | 6.7 | 3.9 | 2.7 | 2.1 | 2.1 | 4.4 | 3.8 |
| Total Dissolved Solids (by EC) (mg/L) | Average | 1075.0 | 982.5 | 996.0 | 972.0 | 1033.3 | 1042.0 | 1160.0 | 1133.3 | 1233.3 | 1140.0 | 1140.0 | 1100.0 |
| | n | 4 | 4 | 5 | 5 | 3 | 5 | 5 | 3 | 3 | 5 | 5 | 4 |
| | ±SD | 95.7 | 28.7 | 5.5 | 34.2 | 57.7 | 135.4 | 89.4 | 57.7 | 57.7 | 54.8 | 89.4 | 0.0 |
| TKN as Nitrogen (mg/L) | Average | 70.6 | 64.9 | 75.2 | 75.0 | 76.2 | 61.7 | 72.1 | 76.9 | 79.7 | 73.4 | 69.1 | 70.6 |
| | n | 4 | 5 | 5 | 5 | 3 | 13 | 5 | 3 | 3 | 5 | 5 | 4 |
| | ±SD | 5.6 | 5.8 | 13.3 | 9.1 | 7.0 | 12.1 | 11.4 | 5.5 | 8.8 | 5.5 | 11.9 | 4.9 |
| Phosphorus - Total (mg/L) | Average | 10.6 | 10.6 | 9.4 | 16.1 | 15.1 | 8.5 | 9.0 | N/A | 10.9 | 13.8 | 6.5 | 9.9 |
| | n | 1 | 1 | 1 | 1 | 1 | 12 | 1 | N/A | 1 | 1 | 1 | 1 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ±SD | 0 | 0 | 0 | 0 | 0 | 5.1 | 0.0 | N/A | 0.0 | 0.0 | 0.0 | 0.0 |
| Grease (mg/L) | Average | 81 | 90 | 56 | 133 | 52 | 96 | 54 | N/A | 73 | 96 | 32 | 57 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | N/A | 0 | 0 | 0 | 0 |
| Aluminium - Total (mg/L) | Average | 7.7 | | 9.6 | 7.5 | 4.9 | 3.4 | 3.5 | 6.4 | 8.0 | 4.8 | 8.2 | 5.7 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Antimony - Total (mg/L) | Average | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0005 | 0.0009 | 0.0009 | 0.0005 | |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arsenic - Total (mg/L) | Average | 0.0017 | 0.0019 | 0.0029 | 0.0027 | 0.0022 | 0.0016 | 0.0015 | 0.0022 | 0.0024 | 0.0019 | 0.0008 | 0.0017 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Beryllium - Total (mg/L) | Average | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | 0.0003 | N/A | N/A | 0.0003 | |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | 0 | 0 |
| Boron - Soluble (mg/L) | Average | 0.336 | 0.243 | 0.241 | 0.243 | 0.248 | 0.353 | 0.385 | 0.44 | 0.375 | 0.383 | 0.416 | 0.288 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cadmium - Total (mg/L) | Average | 0.0002 | 0.0001 | 0.0002 | 0.0003 | 0.0002 | 0.0002 | 0.0004 | 0.0004 | 0.0003 | 0.0003 | 0.0004 | 0.0003 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chromium - Total (mg/L) | Average | 0.0039 | 0.0046 | 0.0063 | 0.0081 | 0.0059 | 0.0057 | 0.0044 | 0.0114 | 0.0069 | 0.0075 | 0.005 | 0.0043 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cobalt - Total | Average | 0.0008 | 0.0008 | 0.0012 | 0.0013 | 0.0011 | 0.001 | 0.0008 | 0.0014 | 0.0014 | 0.0012 | 0.0013 | 0.0008 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Copper - Total (mg/L) | Average | 0.0947 | 0.119 | 0.1341 | 0.1399 | 0.129 | 0.1314 | 0.0823 | 0.1494 | 0.1521 | 0.1468 | 0.1464 | 0.0907 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Iron - Total (mg/L) | Average | 0.9396 | 1 | 1.739 | 2.294 | 1.53 | 0.978 | 0.8103 | 2.358 | 1.828 | 1.728 | 1.496 | 1.14 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lead - Total (mg/L) | Average | 0.0169 | 0.0064 | 0.0172 | 0.0136 | 0.01 | 0.0135 | 0.0121 | 0.0291 | 0.0143 | 0.016 | 0.0212 | 0.0091 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lithium - Total (mg/L) | Average | 0.0096 | 0.0075 | 0.0077 | 0.0078 | 0.0074 | 0.0091 | 0.0092 | 0.0112 | 0.0099 | 0.0087 | 0.0076 | 0.0069 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Manganese - Total (mg/L) | Average | 0.1103 | 0.1071 | 0.1233 | 0.1139 | 0.0942 | 0.0992 | 0.1011 | 0.1135 | 0.1357 | 0.0887 | 0.0968 | 0.1031 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mercury - Total (mg/L) | Average | 0.00004 | 0.00045 | 0.00014 | 0.00027 | 0.00016 | 0.0001 | 0.00015 | 0.00025 | 0.00027 | 0.00041 | 0.00018 | 0.00016 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Molybdenum - Total (mg/L) | Average | 0.0053 | 0.0072 | 0.0075 | 0.0059 | 0.0071 | 0.0057 | 0.0051 | 0.0083 | 0.0077 | 0.0072 | 0.0043 | 0.0039 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nickel - Total (mg/L) | Average | 0.0069 | 0.0062 | 0.0095 | 0.0078 | 0.0088 | 0.0077 | 0.0079 | 0.0156 | 0.0091 | 0.0097 | 0.0107 | 0.0081 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Selenium - Total (mg/L) | Average | 0.0043 | 0.0032 | 0.0011 | 0.005 | 0.0046 | 0.0041 | 0.0015 | 0.0037 | 0.0019 | 0.0072 | 0.0001 | 0.0042 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Silver - Total (mg/L) | Average | 0.00068 | 0.00069 | 0.00147 | 0.00095 | 0.00069 | 0.00058 | 0.00051 | 0.00128 | 0.00105 | 0.00122 | 0.00156 | 0.00084 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thallium - Total (mg/L) | Average | 0.0003 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | N/A | N/A | 0.0001 | |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | 0 | 0 |
| Tin - Total (mg/L) | Average | 0.0051 | 0.004 | 0.0059 | 0.0051 | 0.0054 | 0.0063 | 0.0059 | 0.0084 | 0.0065 | 0.0083 | 0.0046 | 0.0061 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Vanadium - Total | Average | 0.0015 | 0.0001 | 0.0091 | 0.0053 | 0.0047 | 0.0032 | 0.0042 | 0.0068 | 0.0124 | 0.0056 | 0.0045 | 0.0073 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Zinc - Total (mg/L) | Average | 0.1131 | 0.1039 | 0.1619 | 0.1607 | 0.1458 | 0.1557 | 0.1267 | 0.1941 | 0.1973 | 0.1742 | 0.1747 | 0.0962 |
| | n | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*N/A = data unavailable

**Table 42: Monthly average water quality parameters measured at sampling point A of reuse water DS2 throughout the year sampled.**

| | | **January** | **February** | **March** | **April** | **May** | **June** | **July** | **August** | **September** | **October** | **November** | **December** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature ($^{o}$C) | Average | 23.6 | 23.8 | 23.0 | 20.9 | 17.6 | 14.9 | 14.0 | 14.8 | 15.6 | 18.3 | 19.0 | 17.8 |
| | n | 4 | 4 | 4 | 4 | 5 | 4 | 4 | 5 | 4 | 4 | 4 | 4 |
| | ±SD | 2.1 | 1.3 | 0.8 | 1.3 | 1.1 | 1.0 | 0.0 | 0.5 | 0.5 | 0.8 | 1.4 | 2.8 |
| *E. coli* (MPN/ 100mL) | Average | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Free Chlorine (mg/L) | Average | 0.5 | 0.1 | 0.2 | 0.2 | 0.2 | 0.6 | 1.1 | 1.3 | 1.2 | 1.4 | 1.5 | 0.9 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.3 | 0.0 | 0.1 | 0.2 | 0.3 | 0.3 | 0.4 | 0.4 | 0.4 | 0.2 | 0.5 | 0.8 |
| Total Chlorine - (mg/L) | Average | 0.8 | 0.4 | 0.3 | 0.3 | 0.3 | 0.9 | 1.4 | 1.6 | 1.5 | 1.6 | 1.7 | 1.0 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.5 | 0.1 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 | 0.5 | 0.5 | 0.3 | 0.5 | 0.7 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Conductivity (mg/L) | Average | 1322.0 | 1315.0 | 903.0 | 702.8 | 720.8 | 1195.8 | 1406.0 | 1385.0 | 1430.0 | 1295.8 | 454.0 | 962.5 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 169.9 | 48.0 | 272.8 | 17.9 | 111.8 | 311.5 | 27.0 | 82.3 | 48.3 | 401.4 | 39.5 | 573.8 |
| Total Dissolved Solids (by EC) (mg/L) | Average | 728.0 | 722.5 | 497.5 | 387.5 | 394.0 | 657.5 | 776.0 | 762.5 | 787.5 | 714.0 | 250.0 | 532.5 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 94.7 | 27.5 | 153.5 | 9.6 | 61.5 | 173.5 | 13.4 | 41.1 | 26.3 | 220.7 | 21.6 | 317.7 |
| Colour - True (456nm) (HU) | Average | 4.2 | 3.3 | 2.5 | 1.8 | 2.8 | 1.0 | 1.0 | 1.5 | 1.5 | 2.0 | 1.5 | 3.0 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 4.7 | 1.0 | 0.6 | 0.5 | 0.8 | 0.0 | 0.0 | 0.6 | 0.6 | 1.0 | 1.0 | 1.2 |
| Manganese - Soluble (mg/L) | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 2 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0.0220 | 0.0024 | 0.0006 | 0.0002 | 0.0039 | 0.0003 | 0.0004 | 0.0000 | 0 | 0 | 0 | 0 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Manganese - Total (mg/L) | Average | 0.0192 | 0.0046 | 0.0017 | 0.0006 | 0.0108 | 0.0016 | 0.0096 | 0.0166 | 0.0258 | 0.0307 | 0.0254 | 0.0567 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 2 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0.0237 | 0.0027 | 0.0011 | 0.0002 | 0.0125 | 0.0008 | 0.0100 | 0 | 0 | 0 | 0 | 0 |
| Iron - Soluble (mg/L) | Average | 0.0665 | 0.0035 | 0.0014 | 0.0017 | 0.0124 | 0.0017 | 0.0060 | 0.0088 | 0.0224 | 0.0161 | 0.0094 | 0.0034 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 2 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 0.0947 | 0.0048 | 0.0008 | 0.0014 | 0.0133 | 0.0012 | 0.0016 | 0 | 0 | 0 | 0 | 0 |
| Iron - Total (mg/L) | Average | 0.1592 | 0.0202 | 0.0062 | 0.0054 | 0.1034 | 0.0153 | 0.1225 | 0.1919 | 0.3552 | 0.1843 | 0.3316 | 0.1333 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0.2055 | 0.0313 | 0.0032 | 0.0029 | 0.1057 | 0.0126 | N/A | N/A | N/A | N/A | N/A | N/A |

*N/A = data unavailable

**Table 43: Monthly average water quality parameters measured at sampling point B of reuse water DS2 throughout the year sampled**

| | | **January** | **February** | **March** | **April** | **May** | **June** | **July** | **August** | **September** | **October** | **November** | **December** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature ($^{o}$C) | Average | 28.6 | 27.3 | 27.0 | 23.5 | 19.6 | 15.3 | 14.4 | 15.0 | 17.9 | 20.6 | 24.1 | 25.3 |
| | n | 4 | 4 | 4 | 4 | 5 | 4 | 4 | 5 | 4 | 4 | 4 | 4 |
| | ±SD | 0.5 | 0.5 | 1.4 | 2.1 | 1.1 | 2.9 | 0.5 | 0.8 | 0.9 | 1.5 | 1.5 | 1.0 |
| *E. coli* (MPN/ 100mL) | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Free Chlorine (mg/L) | Average | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.4 | 0.5 | 0.3 | 0.9 | 1.4 | 0.6 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 0.2 | 0.3 | 0.3 | 0.2 | 0.6 |
| Total Chlorine - (mg/L) | Average | 0.3 | 0.2 | 0.1 | 0.1 | 0.1 | 0.3 | 0.6 | 0.8 | 0.6 | 1.1 | 1.5 | 0.7 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.2 | 0.1 | 0.0 | 0.1 | 0.1 | 0.2 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.6 |
| Conductivity | Average | 1344.0 | 1317.5 | 940.0 | 712.5 | 692.8 | 1150.0 | 1396.0 | 1452.5 | 1422.5 | 1335.4 | 459.3 | 942.3 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 98.9 | 55.6 | 403.9 | 19.7 | 32.1 | 272.4 | 24.1 | 60.8 | 38.6 | 407.9 | 22.1 | 575.3 |
| Total Dissolved Solids (by EC) (mg/L) | Average | 742.0 | 727.5 | 517.5 | 390.0 | 380.0 | 632.5 | 768.0 | 800.0 | 780.0 | 736.0 | 252.5 | 517.5 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 58.1 | 28.7 | 225.4 | 14.1 | 17.3 | 149.3 | 16.4 | 34.6 | 21.6 | 227.3 | 9.6 | 317.5 |
| Colour - True (456nm) (HU) | Average | 5.0 | 3.0 | 7.0 | 2.0 | 2.0 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| Manganese - Soluble (mg/L) | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Manganese - Total (mg/L) | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Iron - Soluble | Average | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Iron - Total (mg/L) | Average | 0.2 | 0.1 | 0.0 | 0.0 | 0.1 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |

*N/A = data unavailable

**Table 44: Monthly average water quality parameters measured at sampling point C of reuse water DS2 throughout the year sampled**

| | | **January** | **February** | **March** | **April** | **May** | **June** | **July** | **August** | **September** | **October** | **November** | **December** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature ($^{o}$C) | Average | 25.0 | 24.0 | 23.5 | 22.0 | 18.3 | 15.5 | 14.4 | 14.8 | 16.3 | 18.8 | 21.0 | 20.3 |
| | n | 4 | 4 | 4 | 4 | 5 | 4 | 4 | 5 | 4 | 4 | 4 | 4 |
| | ±SD | 1.3 | 0.8 | 0.6 | 1.6 | 1.0 | 0.6 | 0.5 | 0.5 | 0.5 | 0.8 | 3.4 | 2.6 |
| *E. coli* (MPN/ 100mL) | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Free Chlorine (mg/L) | Average | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 0 | 0.9 | 1.0 | 0.8 | 1.0 | 1.2 | 0.7 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.2 | 0.0 | 0.0 | 0.1 | 0.2 | 0.4 | 0.3 | 0.5 | 0.3 | 0.2 | 0.7 | 0.7 |
| Total Chlorine - (mg/L) | Average | 0.5 | 0.3 | 0.2 | 0.2 | 0.2 | 1 | 1.2 | 1.2 | 1.2 | 1.3 | 1.3 | 0.8 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.5 | 0.3 | 0.5 | 0.3 | 0.3 | 0.7 | 0.7 |
| Conductivity | Average | 1303.8 | 1305.0 | 929.3 | 706.0 | 675.4 | 1160 | 1384.0 | 1427.5 | 1367.5 | 1302.6 | 619.5 | 941.8 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (mg/L) | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 171.4 | 23.8 | 304.7 | 17.7 | 41.7 | 298.0 | 30.5 | 39.5 | 158.8 | 396.9 | 333.7 | 559.0 |
| Total Dissolved Solids (by EC) (mg/L) | Average | 718.0 | 720.0 | 510.0 | 387.5 | 372.0 | 640 | 762.0 | 782.5 | 752.5 | 720.0 | 340.0 | 517.5 |
| | n | 5 | 4 | 4 | 4 | 6 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 94.7 | 14.1 | 167.5 | 9.6 | 21.7 | 162.7 | 17.9 | 22.2 | 88.5 | 218.2 | 186.7 | 307.7 |
| Colour - True (456nm) (HU) | Average | 6.0 | 2.0 | 4.0 | 3.0 | 2.5 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0.7 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Manganese - Soluble (mg/L) | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Manganese - | Average | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total (mg/L) | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Iron - Soluble (mg/L) | Average | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Iron - Total (mg/L) | Average | 0.2 | 0.0 | 0.0 | 0.0 | 0.1 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 1 | 1 | 1 | 1 | 2 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0 | 0 | 0 | 0 | 0.2 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |

*N/A = data unavailable

**Table 45: Monthly average water quality parameters measured at sampling point D of reuse water DS2 throughout the year sampled**

| | | **January** | **February** | **March** | **April** | **May** | **June** | **July** | **August** | **September** | **October** | **November** | **December** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Temperature ($^{o}$C) | Average | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| *E. coli* (MPN/ 100mL) | Average | 0 | 0 | 0 | 0 | 0 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Free Chlorine (mg/L) | Average | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.8 | 0.4 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.2 | 0.3 | 0.4 |
| Total Chlorine - (mg/L) | Average | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 0.9 | 0.5 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.1 | 0.2 | 0.4 | 0.4 |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Conductivity (mg/L) | Average | 1400.0 | 1315.0 | 996.0 | 708.3 | 709.8 | 1200.3 | 1358.0 | 1440.0 | 1447.5 | 1310.6 | 468.0 | 824.8 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 28.3 | 25.2 | 357.2 | 16.5 | 113.1 | 144.0 | 63.8 | 56.6 | 64.0 | 396.0 | 22.5 | 536.6 |
| Total Dissolved Solids (by EC) (mg/L) | Average | 772.0 | 722.5 | 550.0 | 387.5 | 390.0 | 662.5 | 750.0 | 795.0 | 797.5 | 722.0 | 257.5 | 452.5 |
| | n | 5 | 4 | 4 | 4 | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 4 |
| | ±SD | 17.9 | 12.6 | 196.6 | 9.6 | 65.2 | 78.0 | 35.4 | 33.2 | 35.9 | 219.4 | 12.6 | 295.7 |
| Colour - True (456nm) (HU) | Average | 8.5 | 4.0 | 3.0 | 2.0 | 3.0 | 2.0 | 1.0 | 2.0 | 4.0 | 2.0 | 1.0 | 2.0 |
| | n | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | ±SD | 9.1 | 0 | 0 | 0 | 0 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Manganese - Soluble (mg/L) | Average | 0.0248 | 0.0015 | 0.0004 | 0.0012 | 0.001 | 0.0008 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 2 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0.0321 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |

| | | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Manganese - Total (mg/L) | Average | 0.0284 | 0.0025 | 0.0006 | 0.0013 | 0.0018 | 0.0013 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 2 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0.0361 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Iron - Soluble (mg/L) | Average | 0.0983 | 0.0008 | 0.002 | 0.003 | 0.0254 | 0.0016 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 2 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0.1346 | 0 | 0 | 0 | 0 | 0 | N/A | N/A | N/A | N/A | N/A | N/A |
| Iron - Total (mg/L) | Average | 0.1905 | 0.0032 | 0.0041 | 0.0086 | 0.1029 | 0.0340 | N/A | N/A | N/A | N/A | N/A | N/A |
| | n | 2 | 1 | 1 | 1 | 1 | 1 | N/A | N/A | N/A | N/A | N/A | N/A |
| | ±SD | 0.2596 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N/A | N/A | N/A | N/A | N/A | N/A |

*N/A = data unavailable

Anecdotally, it is acknowledged that within water systems if chlorine residual is not maintained there is a risk for *Legionella* growth. *Legionella* may be much more ubiquitous to aquatic environments, including potable water supplies, than previously considered. However despite this, there are fewer cases of Legionellosis than would be expected. Supporting this, in South Australia, all hospital diagnosed pneumonia cases are tested for *L. pneumophila* and yet the reported incidence is still only 8-10 cases annually. This is less than half the number of *L. longbeachae* cases, but since *L. longbeachae* is transmitted primarily through potting mix there is little chance of an outbreak. Therefore greater emphasis is placed on investigating cases from aquatic routes of exposure because of the outbreak potential. In regards to *Legionella* management within water systems, it is generally accepted that a detection using culture indicates that a failure with system management has occurred.

(Personal communication, risk manager 2014)

**Figure 41: *Legionella* risk assessment from the perspective of risk manager.**