Legionella pneumophila and protozoan hosts from the engineered water systems

By

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List of Abbreviations

ANOVA	Analysis of variance
AS	Australian Standard
ATCC	American Type Culture Collection
BCYE-GVPC	Buffered charcoal yeast extract agar supplemented with glycine, vancomycin, polymyxin b and cycloheximide
CAP	Canonical analysis of principal coordinates
CDC	Centres for Disease Control and Prevention
CFU	Colony Forming Unit
Eco-NNA	*Escherichia coli* supplemented non-nutrient agar
ECDC	European Centre for Disease Prevention and Control
ELDSNet	European Legionnaires' disease Surveillance Network
ESGLI	European Study Group for *Legionella* Infections
EU/EEA	European Union/ European Economic Area
EWGLI	European Working Group for *Legionella* Infections
FISH	Fluorescence *in situ* hybridization
GU	Genomic units
h	Hour
HI-FBS- PYG	Heat inactivated-foetal bovine serum supplemented peptone yeast extract glucose broth
HPC	Heterotrophic plate counts
ISO	International Organization for Standardization
NHMRC	National Health and Medical Research Council
L	Litre
LD	Legionnaires' disease
LOD	Limit of detection
$\log_{10}$	Logarithmic base 10
LOQ	Limit of quantification
LSD	Least significant difference
mg	Milligram
min	Minutes
mL	Millilitre
ng	Nanogram
nm	Nanometre
NNDSS	National Notifiable Diseases Surveillance System
NSW Health	New South Wales Ministry of Health
OPPP	Opportunistic premise plumbing pathogen
OTU	Operational taxonomic unit
PBS	Phosphate buffer saline
PCoA	Principal coordinates analyses
PERMANOVA	Permutational multivariate analysis of variance
pg	Picogram
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
q-q plot	Quantile-quantile plot
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
s	Seconds
SA Health	South Australia Ministry of Health
SD	Standard deviation
sg	Serogroup
SIMPER	Similarity percentage analysis
ST	Sequence type

TMV	Thermostatic mixing valve
TO	Thiazole orange
Tukey's HSD	Tukey's honestly significant difference
VBNC	Viable but non-culturable
VFC+qPCR	Viability based flow cytometry-cell sorting and qPCR assay
WHO	World Health Organization
µg	Microgram
µm	Micrometre
µL	Microlitre

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Declaration

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university;

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed:

Date: July 21, 2023

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 appear in the thesis.

Associate Professor Dr Harriet Whiley

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Professor Melissa H. Brown

Associate Professor Richard Bentham

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

Publications

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This thesis is based on the published and unpublished manuscripts, therefore some unavoidable repetition between chapters occurs.

The thesis was written in Australian English; however, words in American English will appear in some chapters that include published and unpublished manuscripts due to the language style used by the journal.

Abstract

Legionella is an opportunistic premise plumbing pathogen and the causative agent of Legionnaires' disease and Pontiac fever. It specifically targets immunocompromised, immunosuppressed, and elderly people. Its prevalence in engineered water systems is an issue of increasing public health significance. Globally, the incidence of *Legionella* infections has been increasing. In the USA, *Legionella* is currently the leading cause of all drinking water related outbreaks. The persistence of *Legionella* in engineered water systems is associated with protozoan hosts, biofilms, failure of disinfection treatments, and water stagnation. Under unfavourable environmental conditions *Legionella* can transform into a viable but nonculturable (VBNC) state. VBNC *Legionella* are potentially pathogenic in nature, and under favourable conditions can transform back into the more pathogenic culturable state.

Globally, routine water testing for *Legionella* is recommended to manage hospital water systems to prevent outbreaks of Legionnaires' disease. The International Organization for Standardization (ISO) recommends two protocols for the detection and quantification of *Legionella* in engineered water systems. ISO11731:2017-05 detects only culturable *Legionella,* whereas ISO/TS12869:2019 is a quantitative PCR (qPCR) based method which detect its genomic DNA. However, both methods are unable to quantify and characterize VBNC *Legionella*. In this study, a culture-independent "viability-based flow cytometry-cell sorting and qPCR (VFC+qPCR)" assay was designed to detect and quantify VBNC *Legionella* from environmental samples. This was the first time that flow cytometry-cell sorting in conjunction with a qPCR assay has been used as a direct and rapid method to quantify VBNC *Legionella* from engineered water systems.

Protozoan hosts, specifically free-living amoebae, are natural hosts and reservoirs of *Legionella*. In this study, a systematic literature review identified that free-living amoebae, most commonly *Acanthamoeba* and *Vermamoeba vermiformis,* are the major hosts of *Legionella* in building water distribution systems. Furthermore, these two free-living amoebae play a significant role in the survival and persistence of *Legionella* in engineered water systems. Based on the findings from the systematic literature review, water and biofilm samples from Australian hospital and domestic water systems were screened for the presence of free-living amoebae and *Legionella*. Both culture-dependant and culture-independent approaches were used for screening and characterization of samples. Direct qPCR assays demonstrated that 41% of samples were positive for *Legionella*, 33% for *L. pneumophila*, 11% for *Acanthamoeba*, and 55% for *V. vermiformis*. Only 7% of samples were positive for culturable *Legionella* (which were *L. pneumophila* serogroup (sg)1, *L. pneumophila* sg2-14, and non-pneumophila

Legionella). In contrast, 41% of samples were positive for culturable free-living amoebae, which were identified as *V. vermiformis*, *Acanthamoeba*, *Stenamoeba*, and *Allovahlkampfia*. These culturable free-living amoebae were highly thermotolerant and osmotolerant and harboured strong broad spectrum bacteriogenic activity. Importantly, all *Legionella*/*L. pneumophila* positive samples were also positive for free-living amoeba, and this co-occurrence was statistically significant (Pearson's chi-squared, $p < 0.05$). Furthermore, using qPCR and fluorescence *in situ* hybridization it was identified that *V. vermiformis* and *Allovahlkampfia* harboured intracellular *L. pneumophila*. Importantly, this is the first time *Allovahlkampfia* and *Stenamoeba* have been demonstrated to be hosts of *L. pneumophila* in engineered potable water systems. In conclusion, the high frequency of free-living amoebae in Australian engineered water systems is a significant public health concern. Therefore, future water management methods should incorporate treatments protocols to control free-living amoebae and reduce the risk to end users.

Stagnation and flow dynamics are important parameters which affect the water quality in engineered water systems. In this study, a systematic literature review demonstrated that both permanent (i.e., dead ends and dead legs) and temporary (through intermittent water usage) stagnation promotes the growth of *Legionella* in engineered water systems. Based on the findings from the systematic literature review, a laboratory scale biofilm model study, and a real world (hospital water system) investigation into the effect of intermittent stagnation was conducted. In both of these studies the new method described above was used to quantify the VBNC *Legionella* present. In the laboratory scale study, a model plumbing system consisting of a water tank and two biofilm reactors was used to investigate the effect of stagnation, and intermitted usage, on *Legionella* and free-living amoebae. Initially, both biofilm reactors were left stagnant for 147 days to allow the formation of biofilm. This was followed by the operational phase during which one biofilm reactor was flushed once a day with 70 L of potable water, and the other biofilm reactor was flushed once a week. It was identified that once-a-day flushing for 28 days significantly (analysis of variance, $p < 0.001$) reduced the amount of biofilm-associated alive (potentially culturable based on VFC+qPCR assay) and culturable *Legionella* and increased the amount of VBNC *Legionella* compared with the once-a-week flushing. Furthermore, it was observed that the concentration of culturable *Legionella* (Spearman's ranking, $p < 0.001$) was positively correlated with heterotrophic plate count (HPC) and the concentration of VBNC *Legionella* (Spearman's ranking, $p < 0.001$) was positively correlated with the concentration of *V. vermiformis*. This laboratory scale study demonstrated that a reduction of water stagnation, and an increase in usage/flushing, significantly decreased the population of total, alive and culturable *Legionella*. The effect of stagnation and flow dynamics was also investigated on water (n = 120) and biofilm (n = 46) samples collected from an

Australian hospital water distribution system. The shower and hand basin water/biofilm samples were collected over 16 months from one hospital and water flushing data was obtained from Enware™ using their Smart Flow® monitoring system. The molecular analysis showed that 22% samples were positive for *Legionella* and 41% for *V. vermiformis* using qPCR. This investigation also suggested that temporary stagnation (< 2 hours water flushing per month) significantly (Kruskal-Wallis test, $p < 0.01$) increased the quantity of VBNC and total (genomic unit) *Legionella.* Moreover, it was also identified that high HPC load was significantly (Kruskal-Wallis test, $p < 0.01$) associated with increased concentrations of *Legionella* and *V. vermiformis* in engineered water system. These three studies demonstrate that stagnation arising through intermitted usage is an important factor influencing the risk of *Legionella* in engineered water systems.

Engineered water systems are a complex environment with a range of variables that can influence the growth and persistence of microbes, especially opportunistic premise plumbing pathogens. In this study, 16S rRNA sequence analysis was used to examine the prokaryotic communities present throughout a hospital distribution system. A total of 46 water samples from showers and hand basins collected during three different sampling periods were examined. The influence of temperature and water flow dynamics (number and total duration of flow events) for one week and six months prior to sample collection was examined. It was found that the hospital water primarily contained six bacterial phyla i.e., Proteobacteria, Actinobacteriota, Bacteroidota, Planctomycetota, Firmicutes, and Cyanobacteria. The diversity of prokaryotic communities present was significantly (Kruskal-Wallis test and PERMANOVA, $p < 0.05$) affected by sampling phase (month) and flow dynamics. Importantly, it was also observed that several biofilm forming (e.g., Pseudomonadales), corrosion responsible (e.g., Desulfobacterales), extremely resistant (e.g., Deinococcales), and potentially pathogenic (e.g., *Pseudomonas*) bacterial taxa were enriched in low flow regimes. This study showed that hospital water system consists of complex prokaryotic communities that is shaped by incoming water quality and the building flow dynamics. It was also identified that in this hospital, the water temperature (most probably, because temperature of hot and cold-water supplies was same) did not influence the composition of prokaryotic communities.

In conclusion, currently guidelines recommend routine monitoring for *Legionella;* however, this study showed that the standard methods fail to detect and quantify VBNC *Legionella* and that VBNC *Legionella* does not follow the same trends as culturable *Legionella*. This could explain long term persistence of *Legionella* contamination in engineered water systems. This study supports the use of HPC as an indicator for water quality, as it followed the same trends as culturable and alive (potentially culturable based on VFC+qPCR assay) *Legionella* but not

VBNC *Legionella*. *Legionella* was always found in the presence of free-living amoeba hosts; therefore, future disinfection and control strategies need to address this and target the free-living amoeba. Stagnation had a greater influence of microbial water quality and *Legionella* concentration compared with water temperature. Importantly, this was not just long-term stagnation, but short-term stagnation arising through intermitted usage. This supports current guidelines recommending flushing of outlet to minimize the risk of legionellosis and more emphasis should be placed on this as a control measure. More research is needed to further examine this relationship and determine the optimum balance between the costs and water usage associated with increased flushing and the risk from *Legionella* to determine to optimum frequency of routine flushing.

1 Introduction

This chapter provides background information on *Legionella* spp., *Legionella pneumophila* and free-living amoebae and their importance from a public health perspective. It also describes factors associated with the persistence of *Legionella* in engineered water systems and methods recommended for the detection and characterization of *Legionella* and free-living amoebae from environmental water samples. This chapter concludes by detailing the aims and objectives of this thesis.

1.1 Opportunistic premise plumbing pathogens

Opportunistic premise plumbing pathogens (OPPPs) are waterborne microbes that are native to engineered water systems and associated with infections in immunocompromised, immunosuppressed, and elderly people (Falkinham III et al., 2015a, Falkinham III, 2023). *Acinetobacter*, *Legionella*, Non-tuberculosis *Mycobacterium*, and *Pseudomonas* are clinically important OPPPs, widely distributed in engineered water systems (Hayward et al., 2022, Falkinham III et al., 2015a, Falkinham III et al., 2015b). Despite members of different taxonomic groups, OPPPs share many unique characteristics, such as resistance to chemical and physical disinfection procedures, survival in nutrient and oxygen deficient environments, intracellular survival and proliferation in protozoan hosts, and biofilm formation (Falkinham III, 2022, Falkinham III, 2023). These unique characteristics support the persistence of OPPPs in engineered water systems.

1.2 *Legionella* and legionellosis

Legionella is a Gram-negative pathogenic bacterium associated with legionellosis, which includes Legionnaires' disease (LD), a severe atypical acute pneumonia infection, and Pontiac fever, an acute "flu-like" illness (Fields et al., 2002). Globally, most cases of LD are predominantly reported in immunosuppressed individuals, older people (> 50 years old) and males (Phin et al., 2014, Fields et al., 2002). Currently, 60 species and 80 distinct serogroups of the genus *Legionella* have been identified (Miyashita et al., 2020, Khodr et al., 2016). *L. pneumophila* and *L. longbeachae* are two clinically important species of *Legionella* associated with the majority of legionellosis cases (Fields et al., 2002, Whiley and Bentham, 2011). Globally, *L. pneumophila* is the primary etiological agent of legionellosis. In the USA and Europe, *L. pneumophila* serogroup (sg1) is responsible for more than 70% of reported *Legionella* cases (Mercante and Winchell, 2015). However, in Australia and New Zealand both *L. pneumophila* and *L. longbeachae* are equally responsible for legionellosis (Graham et al., 2012, Australian Government, 2021b).

Legionella is transmitted via aspiration or inhalation of contaminated water or aerosols (Prussin et al., 2017). Importantly, human to human *Legionella* transmission does not occur (Bartram et al., 2007). In natural and manufactured water systems, protozoa are the natural hosts and reservoirs of *Legionella* (Boamah et al., 2017, Sciuto et al., 2021). Humans are not natural hosts of *Legionella*, but they are considered as accidental hosts. Inhalation or aspiration of *Legionella* contaminated aerosols or water results in bacterial colonization inside the lungs of infection-prone individuals (Newton et al., 2010). The alveolar macrophages engulf the bacterium and develop specialized vacuoles known as "*Legionella* containing vacuole". Inside these vacuoles the *Legionella* utilizes different pathogenicity related proteins and the Dot/Icm bacterial secretion pathway to hijack the host cell endocytic pathway and escape from lysosome mediated death (Allombert et al., 2014). These cells behave like a protozoan host and facilitate its propagation, which results in LD (Oliva et al., 2018).

Legionella is frequently associated with community-acquired and nosocomial legionellosis. According to the European Centre for Disease Prevention and Control (ECDC), in 2021 the majority of LD cases were community-acquired (77.2%), travel-mediated (10.4%), or nosocomial (5.4%) infections (The European Legionnaires' disease Surveillance Network, 2022). Clinically, it is very difficult to distinguish between patients with LD and other types of pneumonia (Fields et al., 2002). Similarly, in the majority of cases self-limiting Pontiac fever remains unnoticed. Therefore, actual incidence of *Legionella* infections is difficult to quantify (Cassell et al., 2019). Globally, the incidence of legionellosis has been increasing. The case fatality rate of LD is 2.2 to 10.3%, lowest in Singapore and highest in European countries (Phin et al., 2014). However, in *L. pneumophila* associated LD nosocomial outbreaks the case fatality rate can reach up to 48 % (O'Mahony et al., 1990, Soda, 2017, Mercante and Winchell, 2015). Over the last 15 years, in the USA and Europe the number of reported LD cases has been significantly increasing (**Figure 1.1**) (The European Legionnaires' disease Surveillance Network, 2022, Centers for Disease Control and Prevention, 2022). In Europe, the number of notified LD cases increased from 4921 in 2011 to 10,723 in 2021, a 200% increase (The European Legionnaires' disease Surveillance Network, 2022). In 2021, the Centres for Disease Control and Prevention (CDC) documented 8260 confirmed cases of LD in USA (Centers for Disease Control and Prevention, 2022). In 2020, 524 confirmed cases of legionellosis were reported in Australia (Australian Government, 2021b). **Figures 1.1** and **1.2** illustrate the number of confirmed cases of legionellosis documented in USA/European countries and Australia.

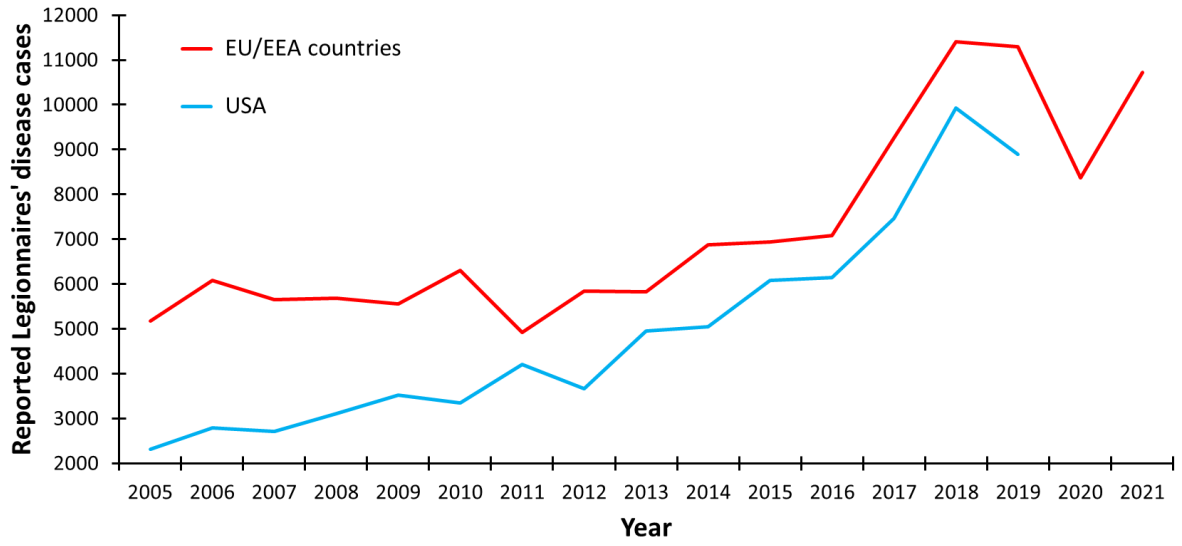

Figure 1.1: The number of confirmed Legionnaires' disease cases recorded from 2005 to 2019/2021 from the European Union/ European Economic Area (red line) (The European Legionnaires' disease Surveillance Network, 2022) and the USA (blue line) (Centers for Disease Control and Prevention, 2022).

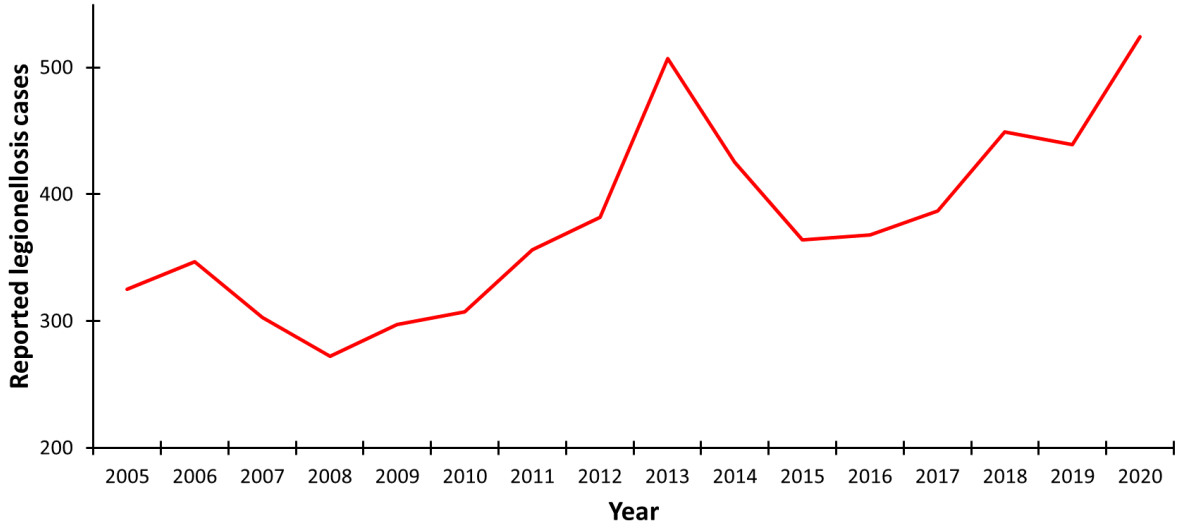

Figure 1.2: The number of confirmed legionellosis cases recorded from 2005 to 2020 in Australia (Australian Government, 2021a).

1.3 Environmental habitats of *Legionella*

Legionella is widely distributed in natural and engineered water systems (Schwake et al., 2021). It was estimated that the USA spends US$3.8 billion per year to treat 13 major waterborne diseases (Adam et al., 2017), this includes US$0.43 billion for the treatment of legionellosis (Collier et al., 2012). Historically, cooling towers have been considered the primary source of *Legionella*, but subsequent investigations have suggested that municipal water supplies, engineered water systems, building plumbing systems, humidifiers,

recreational water, public bathhouse, spas, ice machines and dental unit waterlines are also sources of *Legionella* infection (Kanarek et al., 2022, Bartram et al., 2007, Whiley et al., 2014, Sasaki et al., 2008, Benkel et al., 2000, Schuetz et al., 2009, Szymanska, 2004).

1.4 Persistence of *Legionella* in engineered water systems

The availability of clean and safe potable water free from pathogenic microbes and toxic compounds is a main goal of the World Health Organization (WHO) and the European Commission (World Health Organisation, 2002, European Commission, 1998). Potable water supplied for human consumption is chemically or physical disinfected (National Health and Medical Research Council and National Resource Management Ministerial Council, 2022). However, it is necessary to recognize that despite these disinfection procedures, engineered water systems are colonized by diverse prokaryotic and eukaryotic microbial communities which often include *Legionella* (Falkinham III et al., 2015b, Falkinham III, 2022). *Legionella* has been detected in engineered potable water systems from a range of countries (Colbourne and Trew, 1986, Hanrahan et al., 1987, Sabrià et al., 2001, Darelid et al., 2004, O'neill and Humphreys, 2005, Lasheras et al., 2006, Ozerol et al., 2006, Armstrong and Haas, 2007, Eslami et al., 2012, Donohue et al., 2014, Qin et al., 2014, Beer et al., 2015, Rodriguez-Martinez et al., 2015, Totaro et al., 2015, Lesnik et al., 2016, Rakic and Stambuk-Giljanovic, 2016, Hayes-Phillips et al., 2019).

Building plumbing systems are oligotrophic environments, always deficient in nutrients and exposed to multiple environmental stresses, i.e., disinfection treatments (Boe-Hansen et al., 2003). Microbes including *Legionella* persisting in engineered water systems are often either resistant or tolerant to both chemical and physical disinfection treatments (Decker and Palmore, 2014, Falkinham III et al., 2015b, Falkinham III, 2022). Furthermore, there are many biotic and abiotic factors which support growth and persistence of *Legionella* in such harsh environments (Bartram et al., 2007, Boamah et al., 2017).

1.4.1 Biotic factors

In engineered water systems, intrinsic resistance, viable but non culturable state, biofilms and host protozoa play a key role in survival and persistence of *Legionella* (Ashbolt, 2015, Kirschner, 2016).

1.4.1.1 Intrinsically resistant *Legionella* and viable but non culturable state

In *Legionella*, intrinsic resistance is a natural resistance and tolerance against various chemical and physical disinfection treatments. *L. pneumophila* sg1 are intrinsically thermotolerant and survive at ≥ 65°C pasteurization temperature (Whiley et al., 2017, Steinert et al., 1998, Farhat

et al., 2010). Similarly, many different serogroups of *L. pneumophila* tolerate and survive in the presence of chlorine based chemical disinfectant (Totaro et al., 2018, Scaturro et al., 2007). Importantly, both disinfection treatments and nutrient deficiency promote transformation of culturable *Legionella* into a viable but non culturable (VBNC) state (Al-Bana et al., 2014). These cells are potentially pathogenic and infectious in nature but unable to grow on microbiological growth medium (Kirschner, 2016). Standard protocols recommended by International Organization for Standardization (ISO) are unable to either detect or quantify VBNC *Legionella* (International Organization for Standardization, 2017, International Organization for Standardization, 2019). VBNC *Legionella* can survive under these harsh environmental conditions and then subsequently be resuscitated in the presence of suitable protozoa host and favourable environmental conditions (Dietersdorfer et al., 2018). More importantly, VBNC *Legionella* are highly infectious in nature and resuscitate in human macrophage-like and alveolar epithelial cells (Epalle et al., 2015).

1.4.1.2 Biofilms

Biofilms are aggregates of prokaryotic and eukaryotic microbes in which cells are adhered with each other and to a substrate (surface) by extracellular polymeric substances (López et al., 2010). Within engineered waters systems *Legionella* are associated with multispecies biofilms comprised of both prokaryotic and eukaryotic microflora. Engineered water systems are oligotrophic environments that are constantly exposed to different environmental stresses (Abu Khweek and Amer, 2018). Metallic ions and carbon released from the structures/parts of building plumbing systems provide essential nutrients for the colonization of biofilms (Bartram et al., 2007, Proctor and Hammes, 2015). In multispecies biofilms, bacteria compete for the nutrients, therefore these complex microbial communities are comprised of viable and dead cells. *Legionella* is an exceptionally fastidious bacterium in nature; therefore, dead microbes of biofilms provide essential nutrients for its growth and proliferation (Abu Khweek and Amer, 2018). The second mechanism to obtain nutrients and survive in this oligotrophic environment is through the protozoan hosts. These hosts support intracellular growth and proliferation of *Legionella* (Boamah et al., 2017). Bacteria growing in biofilms communicate with each via interspecies and intraspecies quorum sensing and function in a highly coordinated manner (Solano et al., 2014). In these multispecies biofilms, certain bacteria, such as *Flavobacterium*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, and *Pseudomonas putida,* promote growth and persistence of *Legionella* (Stewart et al., 2012, Mampel et al., 2006). Furthermore, these multilayered bacteria communities and extracellular polymeric substances neutralize/hamper the efficacy of chemical disinfectants and protects *Legionella* (Bridier et al., 2011). More importantly, protozoa present in the biofilms, protects *Legionella* from disinfections treatments via intracytoplasmic growth (Abdel-Nour et al., 2013).

1.4.1.3 Protozoan hosts

Humans are accidental hosts of *Legionella* (Marrie, 2012). In nature, protozoa are the real hosts and reservoirs of *Legionella*. Members of three important phyla i.e., Amoebozoa (amoebae), Ciliophora (ciliated protozoa) and Percolozoa (excavates) support intracytoplasmic replication and survival of *Legionella* (Boamah et al., 2017). The protozoa such as *Balamuthia*, *Dictyostelium*, *Echinamoeba*, *Naegleria*, *Paramecium*, and *Vermamoeba* support intracellular replication of *L. pneumophila* (Fields et al., 1989, Newsome et al., 1985, Watanabe et al., 2016, Nahapetian et al., 1991, Shadrach et al., 2005, Solomon et al., 2000), whereas *Acanthamoeba* and *Tetrahymena* allow intracellular replication and packaging of viable bacterium into export vesicles (Berk et al., 2008, Berk et al., 1998). Commonly, in engineered water systems, members of the Gymnamoebae group, also known as the naked or free-living amoebae, are its hosts. These free-living amoebae exists in two different physiological states, i.e., trophozoites (metabolically active state) and cysts (dormant state) (Smirnov et al., 2011, Smirnov and Goodkov, 1999). Their trophozoites provide it essential nutrients (especially amino acids) and support intracytoplasmic proliferation of *Legionella*, whereas the cysts protect *Legionella* from prolonged periods of disinfection treatments or other unfavourable environmental conditions (Dupuy et al., 2014, Boamah et al., 2017). More importantly, under favourable conditions the amoebae trophozoite transforms VBNC *Legionella* into a culturable state (Garcia et al., 2007). The *Legionella* resuscitated by host free-living amoebae are more pathogenic and virulent in nature (Boamah et al., 2017, Fields et al., 2002). *Acanthamoeba* and *Vermamoeba vermiformis* (formerly known as *Hartmannella vermiformis*) are the most common free-living amoebae found in engineered water systems (**Figure 1.3**) (Delafont et al., 2018, Stockman et al., 2011, Wang et al., 2012a). The literature on this topic is investigated in more detail in **Section 5**.

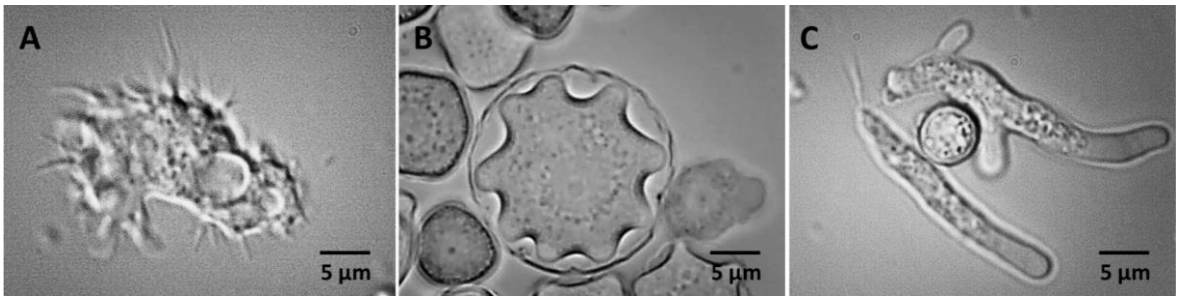

Figure 1.3: Light microscopy images of *Acanthamoeba* and *Vermamoeba vermiformis*. *Acanthamoeba* trophozoite (A) develops acanthopodia and cysts (B) which are double layered. Cysts of *Vermamoeba vermiformis* are also double layered whereas trophozoites (C) predominantly develop prominent monopodial pseudopodia. Scale bar = 5 µm.

(The image was captured by Muhammad Atif Nisar, the author of this thesis)

1.4.2 Abiotic factors

Engineered water systems consist of water tanks, plumbing pipes, plumbing fixtures, valves, water outlet devices (showers, basins, etc.) and other related structures. In this environment many abiotic factors such as plumbing material, age of plumbing system, water temperature, disinfection treatment, and flow dynamics play key roles in survival and persistence of microbial biofilms and *Legionella* (Chan et al., 2019, De Sotto et al., 2020, Lee et al., 2021).

1.4.2.1 Failure of disinfection procedures

There are a range of chemical and physical disinfection processes used in engineered water systems to kill pathogenic microbes including *Legionella* (Bartram et al., 2007). However, these procedures are unable to provide long term disinfection and completely eliminate *Legionella* from engineered water systems (National Academies of Sciences Engineering and Medicine, 2019).

1.4.2.1.1 Chemical disinfection

In many countries, potable water is treated with chemical disinfectants such as chlorine and monochloramine; however, the concentration of these disinfectants decrease along the water distribution system to the point of use (Li et al., 2019, National Health and Medical Research Council and National Resource Management Ministerial Council, 2022, Onyango et al., 2015). World Health Organization (WHO) recommends 0.5 mg/L free chlorine for inactivation of microbial contaminants in potable water (World Health Organisation, 2017, World Health Organization, 2004). Failure of chemical disinfection procedures is associated with plumbing material, stagnation, microbial biomass, and intrinsic resistance of *Legionella* against chemical compounds (Colbourne and Ashworth, 1986, Cullom et al., 2020, Montagnino et al., 2022, Bartram et al., 2007, Dupuy et al., 2011). Generally, the activity and efficacy of chemical disinfectants varies from nature of plumbing materials. Both metallic and synthetic organic plumbing materials inactivates chemical disinfectants (Colbourne and Ashworth, 1986, Cullom et al., 2020). Water aging and stagnation accumulates microbial biomass in building plumbing systems and accelerates the rate of decay of chemical disinfectants (Vieira et al., 2004, Patrick et al., 2012, Ling et al., 2018). Most importantly, certain serogroups of *L. pneumophila* are intrinsically resistants against various oxidizing chemical disinfectants (Totaro et al., 2018, Mustapha et al., 2015).

1.4.2.1.2 Thermal disinfection

Thermal disinfection is considered an effective disinfection method for potable water (Farhat et al., 2010, Bartram et al., 2007). In heat shock treatment, the temperature of hot water is

raised to 71°C to 77°C so that the temperature of the outlet water reaches at least 65°C. This is followed by water flushing through these water outlets for 10 to 30 minutes (Whiley et al., 2017). In healthcare buildings, it is recommended that the temperature of hot water systems should be maintained > 55°C (enHealth, 2015, United States Environmental Protection Agency, 2016b). To avoid scalding, it is recommended that water heaters should be 48.9°C (120°F) (United States Consumer Product Safety Commission, 2022). In building plumbing systems to avoid scalding, Thermostatic Mixing Valves (TMV) are installed for the mixing of hot and cold water, which lowers the water temperature (Watts Industries, 2017).

It has been shown that hot water systems set < 60°C are more likely to be contaminated with *Legionella* (Falkinham III et al., 2015a). Water stagnation and intrinsically thermotolerant *Legionella* are responsible for the failure of heat shock treatment (Falkinham III et al., 2015b). Due to water stagnation, it is not possible to maintain water temperatures of 71°C to 77°C (Falkinham III et al., 2015b, Whiley et al., 2017). Members of *L. pneumophila* sg1 and sg2 are intrinsically thermotolerant and survive at high temperatures (Steinert et al., 1998, Allegra et al., 2008, Allegra et al., 2011). The literature on this topic is explored in more detail in **Section 7**.

1.4.2.2 Plumbing materials

The structure, chemical nature, and age of plumbing materials also play key roles in the survival and persistence of *Legionella* (Bartram et al., 2007, Colbourne and Ashworth, 1986, Cullom et al., 2020, Hayes-Phillips et al., 2019, Wang et al., 2012b). Complex plumbing networks of old building structures are associated with water stagnation, decay of chemical disinfectants, and failure to maintain high temperatures for thermal disinfection (Hayes-Phillips et al., 2019, Bartram et al., 2007). Building plumbing systems are composed of metallic (i.e., copper) and synthetic organic compounds (i.e., polyvinyl chloride). The metallic substances directly inactivate chemical disinfectants, whereas synthetic organic materials release organic carbon which potentially support microbes and indirectly inactivates chemical disinfectants (Colbourne and Ashworth, 1986, Cullom et al., 2020). These organic and inorganic compounds released by plumbing materials can support the growth and proliferation of *Legionella* (Sciuto et al., 2021). It has been identified that the efficacy and stability of chemical disinfectants are impacted by plumbing materials. For example, monochloramine, a chlorine-based disinfectant, is more effective against *L. pneumophila* biofilms growing on metallic (copper) substrates, whereas free-chlorine is more effective against *L. pneumophila* biofilms growing on organic (polyvinyl chloride) substrates (Buse et al., 2019).

1.4.2.3 Flow dynamics and water stagnation

In building plumbing systems, water stagnates temporarily or permanently (Prévost et al., 1997). Temporary stagnation can arise through intermitted usage of water outlets and use of water storage tanks (Peter and Routledge, 2018, Manuel et al., 2009, Rhoads and Hammes, 2021). Permanent stagnation occurs at dead ends and dead legs (Tercelj-Zorman et al., 2004). Flow dynamics within a building can also influence water residence time within a building and water stagnation. For example, in green buildings, the plumbing systems are deliberately designed to reduce the water flow (Rhoads et al., 2015, Rhoads et al., 2016). Both temporary and permanent stagnation accelerates the decay of chemical disinfectants, promote the accumulation of biomass, and deteriorates water quality (Bedard et al., 2018). Restricted water flow and stagnation play an important role in the survival and persistence of *Legionella* in engineered water systems (Ciesielski et al., 1984). The literature on this topic is explored in more detail in **Section 7**.

2 Aims and Objectives

2.1 Aims

The aims of this study were to:

1. Enumerate viable but nonculturable (VBNC) *Legionella* populations and investigate the relationships with host protozoa to understand how this may affect interpretation of standard testing for *Legionella* in water systems.
2. Investigate prevalence and correlations of *Legionella* and *L. pneumophila* with host protozoa in engineered water systems.
3. Investigate the effect of flow dynamics and stagnation on survival of *Legionella* (in both culturable and VBNC forms) host protozoa and bacterial communities.

2.2 Objectives

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

1. Develop and validate a method, using viability-based flow cytometry-cell sorting followed by qPCR enumeration, to the enumerable VBNC *Legionella* spp. and *L. pneumophila* from environmental water samples.
2. Conduct a literature review to examine the potential protozoan hosts of *L. pneumophila* in hospital and domestic building water system.
3. Investigate the prevalence and distribution of the *Legionella* spp., *L. pneumophila*, *Acanthamoeba* and *Vermamoeba vermiformis* in Australian hospital and domestic water samples using qPCR assays.
4. Use a culture based detection method to investigate the presence and diversity of free-living amoebae present in Australian potable water samples.
5. Characterise the pathogenicity of free-living amoeba isolates and potential to be a host for *Legionella* using co-culture assay and cellular imaging.
6. Conduct a literature review to investigate the role of flow dynamics and stagnation on the survival and persistence of *Legionella* and *L. pneumophila* in engineered water systems.
7. Examine the relationships between water flushing arising through usage, water stagnation, *Acanthamoeba*, *Vermamoeba vermiformis*, heterotrophic bacteria, and *Legionella* spp. / *L. pneumophila* (total, culturable, and VBNC) using a laboratory scale model plumbing system.

8. Examine the relationship between *Legionella* spp. and *L. pneumophila* (total DNA, culturable, and VBNC), flow dynamics, water stagnation, water temperature, *Acanthamoeba*, *Vermamoeba vermiformis*, and heterotrophic bacteria within an Australian hospital water distribution system.
9. Use 16S rRNA sequencing analysis to examine the potential role of water temperature, seasons, flow dynamics and water stagnation on the planktonic microbiome of a hospital water system.

3 Materials and methods

This chapter describes the general experimental procedures used throughout the research described in this thesis. It also includes one published article that describes a novel method developed to enumerate viable but nonculturable (VBNC) *Legionella* from environmental samples. This method was validated by quantifying VBNC *Legionella* from environmental water samples and was also used to assess the decontamination assays used in the standard culture method (ISO11731:2017-05). This article addresses objective 1.

3.1 Ethical clearance

This study was approved by the Flinders University Social and Behavioural Research Ethics Committee (SBREC Project Number 7291) as per the recommendations of the National Statement on Ethical Conduct in Human Research, Australia. The study was also approved by Institutional Biosafety Committee, Flinders University (IBC Number: 2017-08, 2017-08.2, 2017-14.1, 2017-15, and 2017-15.2).

3.2 Sample collection and processing

Water and biofilm samples were collected from domestic and hospital water systems and transported according to the recommendations of Centres for Disease Control (Centers for Disease Control and Prevention, 2019) and ISO 5667-3:2018 (International Organization for Standardization, 2018).

3.2.1 Water sampling

Water samples were collected from showers and hand basins (taps). Two samples were collected from each collection point; the first flush and 5 minutes post flushing water. One litre of water was collected in sterile screw capped wide-mouth plastic bottles (Nalgene™). To neutralize residual disinfectants 500 µL of 0.1 N sodium thiosulfate ($Na_2S_2O_3$: 124270010, ACROS Organics™) was added in collected samples.

3.2.2 Biofilm samples

Biofilm samples were collected from tap faucet aerators and showerheads. Briefly, hand basin taps or showers were turned on for couple of seconds to moisten and then turned off (Centers for Disease Control and Prevention, 2019). Using sterile polyurethane-tipped swabs (CleanFoam® TX751B, Texwipe®) visible biofilms were collected. Each swab was placed in a 15 mL sterile screw capped tube and 5 to 10 mL water from the sample hand basin/shower

was added to moisten the sample. To neutralize residual disinfectants 100 µL 0.1 N $Na_2S_2O_3$ was added to collected samples.

3.2.3 Sample transport and storage

Water and biofilm samples were transported from the collection site to the laboratory at room temperature and processed within 24 h or stored at 5°C and analysed within 72 h.

3.2.4 Sample processing

One litre of water and 10 mL biofilm samples were vacuum filtered onto 47 mm diameter 0.2 µm polycarbonate membranes (GTTP04700, Isopore™). Using sterile forceps, the membrane was transferred to a sterile tube containing 5 mL of sterile water and vortexed vigorously. This suspension was used for further microbiological and molecular testing (**Figure 3.1**).

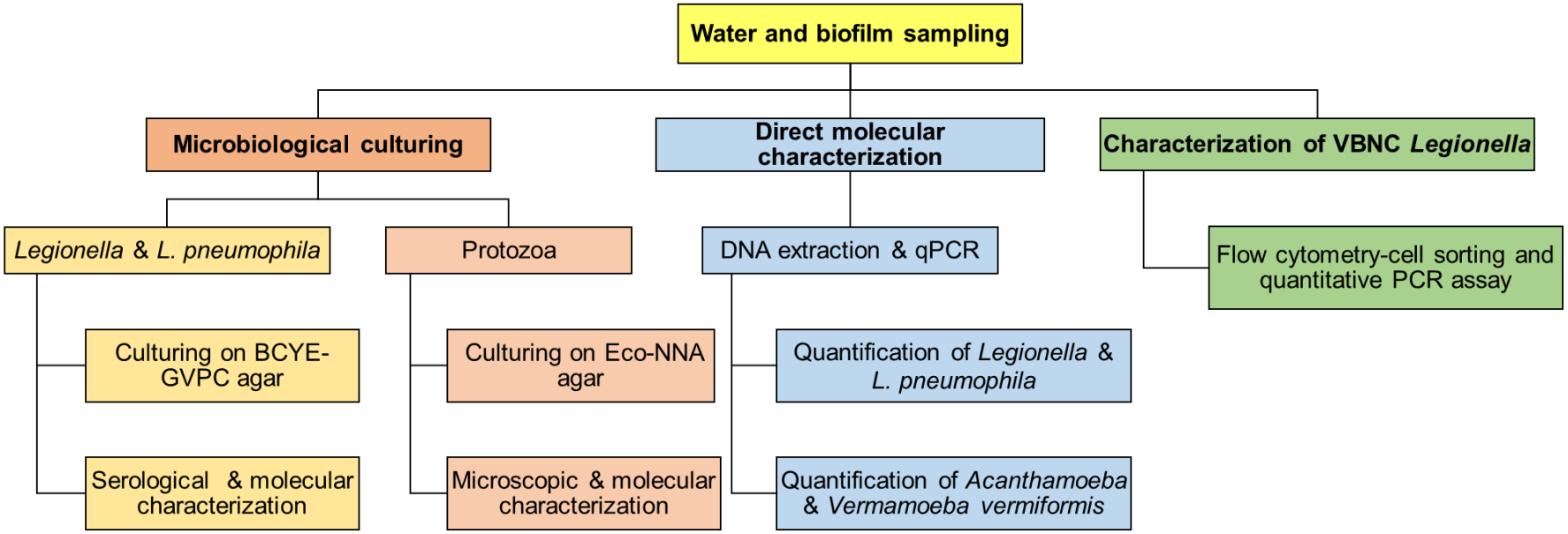

Figure 3.1: Flow chart presenting methodology used for characterization of both water and biofilm samples ant microbiological and molecular levels. qPCR: quantitative polymerase chain reaction; VBNC *Legionella*: viable but nonculturable *Legionella*; BCYE-GVPC: buffered charcoal yeast extract agar supplemented with glycine, vancomycin, polymyxin B and cycloheximide; Eco-NNA: *Escherichia coli* supplemented non-nutrient agar.

3.3 Microbiological isolates

3.3.1 *Legionella* and *L. pneumophila*

3.3.1.1 Microbiological culturing

Standard protocols i.e., AS5132:2017 (Standards Australia, 2017) and ISO11731:2017-05 (International Organization for Standardization, 2017) were followed to isolate and cultivate *Legionella*. According to the standard protocols, *Legionella* was cultured on buffered charcoal yeast extract agar (BCYE) supplemented with glycine, vancomycin, polymyxin B and cycloheximide (GVPC) and growth (buffer/potassium hydroxide, ferric pyrophosphate, L-cysteine and α-ketoglutarate) supplements. The medium was prepared as per guidelines provided by the manufacturer. Briefly, 12.5 g CYE agar (CM0655, Oxoid Ltd.) was resuspended in 450 mL distilled water and steam sterilized by autoclaving at 121°C for 20 minutes. Sterile CYE agar was cooled in a water bath to 50°C. Vials of GVPC (SR0152, Oxoid Ltd.) and *Legionella* growth supplement (SR0110C, Oxoid Ltd.) were dissolved in 20 mL and 30 mL sterile water, respectively. Reconstituted supplements were aseptically added to the molten CYE agar and mixed gently. The final pH was adjusted to 6.9 ± 0.1 with 0.2 M potassium hydroxide or 0.5 M hydrochloric acid and under aseptic conditions agar was poured in the sterile disposable Petri plates and stored at 4°C for 1 month.

L. *pneumophila* sg1 and sg2-14 clinical isolates obtained from SA Pathology (Adelaide, Australia) and *L*. *pneumophila* subsp. *pneumophila* Philadelphia sg1 ATCC® 33152™ were used as control strains (**Figure 3.2 A**). Isolates were streaked onto BCYE-GVPC agar and incubated under aerobic conditions at 37 ± 1°C for 3 days. Isolates developed into circular grey/white colonies with a textured cut glass appearance. The plates were stored at 4°C for 1 month.

Filtered environmental samples were either heat or acid treated (International Organization for Standardization, 2017). For heat treatment, the sample was incubated at 50 ± 1°C for 30 ± 2 minutes and labelled as treated sample. In acid treatment, 1 mL sample was added in sterile tube containing 9 mL of acid buffer (3.5 mL 0.2 M HCl and 25 mL 0.2 M KOH, final pH 2.2) and incubated at room temperature for 5 ± 0.5 minutes and labelled as treated sample. One hundred microlitres of treated sample was spread on BCYE-GVPC agar and incubated under aerobic conditions at 37 ± 1°C for 3 to 5 days. Presumptive *Legionella* colonies were subcultured on fresh agar plates.

3.3.1.2 Serotyping and molecular identification

To identify *Legionella*, *L. pneumophila sg*1 and *L. pneumophila sg*2-14. *Legionella* a latex agglutination test was performed (DR0800, Oxoid Ltd.). Using a plastic disposable loop, a

single colony was placed on the test slide and resuspended in one drop of suspension buffer. One drop of test reagent was added onto this bacterial suspension and the test slide was gently rocked for 30 s. The mixture was observed for the agglutination reaction (**Figure 3.2 B**).

For molecular identification, the isolated *Legionella* colonies were dispatched to the Australian Genome Research Facility (AGRF Ltd.) for microbial identification. Briefly, a 0.7 kb region of the bacterial 16S rRNA gene was amplified and sequenced. Retrieved sequences were trimmed and analysed on basic local alignment search tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on BLAST results, bacteria were identified to genus and species level.

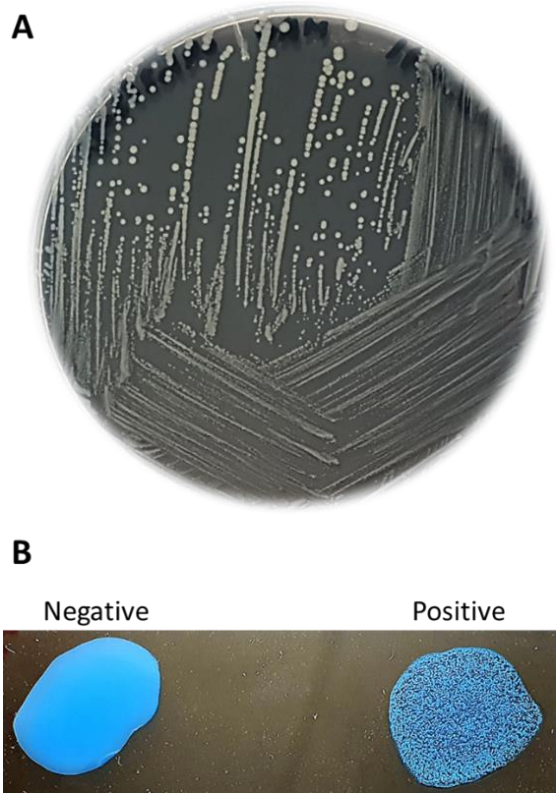

Figure 3.2: Growth of *Legionella pneumophila* subsp. *pneumophila* Philadelphia sg1 ATCC® 33152™ on BCYE-GVPC agar (A) and serological identification using Legionella latex test kit (B). In B, the absence of agglutination reaction demonstrates negative reaction for *L. pneumophila* sg1, whereas agglutination reaction within 30 s demonstrates positive reaction for *L. pneumophila* sg1.

(The image was captured by Muhammad Atif Nisar, the author of this thesis)

3.3.1.3 Preservation and storage

For long term storage, *Legionella* isolates were preserved in cell storage medium. Ninety millilitres of sterilised glycerol: water solution in 1:1 ratio was prepared. The solution was allowed to cool at room temperature. A vial of *Legionella* growth supplement (SR0110A, Oxoid Ltd.) was reconstituted in 10 mL sterile water and added in the water-glycerol solution. 1 mL

storage medium was aliquoted into sterile 1.5 mL cryovial. Individual bacterial colonies were resuspended in the cryovials and stored at –80°C.

3.3.2 Selected Gram-negative and Gram-positive bacteria

Acinetobacter baumannii Bouvet and Grimont ATCC 17978™, *Acinetobacter calcoaceticus* (environmental isolate), *Escherichia coli* HS(pF*amp*)R ATCC® 700891™, *Klebsiella pneumoniae* Flinders Culture Collection 575, and *Pseudomonas aeruginosa* (environmental isolate) were used as representatives of Gram-negative bacteria. *Enterococcus faecium* (Orla-Jensen) Schleifer and Kilpper-Balz 19434™ and *Staphylococcus aureus* subsp*. aureus* Rosenbach 6538P™ were used as representatives of Gram-positive bacteria.

3.3.2.1 Microbiological culturing

Cetrimide agar (CM059, Oxoid Ltd.) was used for *P. aeruginosa*; MacConkey agar (CM0007, Oxoid Ltd.) for *A. baumannii* and *A. calcoaceticus*; nutrient agar (CM0003, Oxoid Ltd.) for *E. coli*; and tryptone soya agar (CM0131B, Oxoid Ltd.) for *E. faecium*, *S. aureus*, and *K. pneumoniae* culturing. Culture media were prepared according to the manufacturer's instruction and steam sterilized by autoclaving at 121°C for 20 minutes. Sterile agar was allowed to cool to 50°C and poured in sterile disposable Petri plates and stored at 4°C for 1 month. Each isolate was streaked onto the respective agar plates and incubated under aerobic conditions at 37 ± 1°C for 16 ± 2 hours.

3.3.2.2 Preservation and storage

For long term storage, bacteria were preserved in cell storage medium. One hundred millilitres of nutrient broth (CM0001, Oxoid Ltd.) and glycerol in 1:1 ratio was prepared and autoclaved. One millilitre storage medium was added into a 1.5 mL cryovial, and a single colony was inoculated and incubated under aerobic conditions at 37 ± 1°C for 16 ± 2 h. Then cryovials were stored at –80°C.

3.3.3 Free-living amoebae

Protocols published by Health Protection Agency, UK (2004) and Page (Page, 1988) were used for isolation and cultivation of amoebae. Amoebae were cultured on heat inactivated *E. coli* supplemented non-nutrient agar (Eco-NNA) plates and heat inactivated-foetal bovine serum (HI-FBS) supplemented peptone yeast extract glucose (PYG) broth.

3.3.3.1 Amoeba monoxenic cultures

Eco-NNA plates were used for amoebae monoxenic cultures. One and a half grams of agar no. 1 (LP0011, Oxoid Ltd.) was resuspended in 100 mL 1X Page's saline (0.12 g NaCl, 0.004 g $MgSO_4.5H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.142 g Na_2HPO_4 and 0.136 g KH_2PO_4 per litre distilled

water, pH 6.8 ± 0.2) and autoclaved. Sterile agar was cooled to 50°C and poured in sterile disposable Petri dishes and stored at 4°C for 1 month. Overnight grown cultures of *E. coli* (**Section 3.3.2.1**) were harvested in 2 mL sterile 1X Page's saline and thermally inactivated at 50°C for 30 ± 2 minutes. Heat inactivated cell suspension (200 µL) was spread on NNA plates and placed at 25 ± 1°C for 30 minutes. Eco-NNA plates were stored at 4°C for 3 days.

Amoebae suspension (50 µL) or filtered environmental sample (100 µL, **Section 3.2.4**) was spread on Eco-NNA plates and incubated under aerobic conditions at 25 ± 1°C for 5 to 14 days. Cultured plates were regularly monitored under upright (BX43, Olympus) and inverted light microscopes (EVOS™ FL, Thermo Fisher Scientific) after 48 h. Appearance of clear zones indicated amoebae growth. To subculture the amoebae isolates, two streaks of freshly grown *E. coli* (diameter 7 to 15 mm) were made on NNA plates. The clear zone developed on the Eco-NNA plate was removed using a sterile scalpel. Excised agar block (< 7 mm) was placed on the *E. coli* streaks and plates were incubated under aerobic conditions at 25 ± 1°C for 3 to 7 days. Development of clearance zones indicated amoebae migration (**Figure 3.3**).

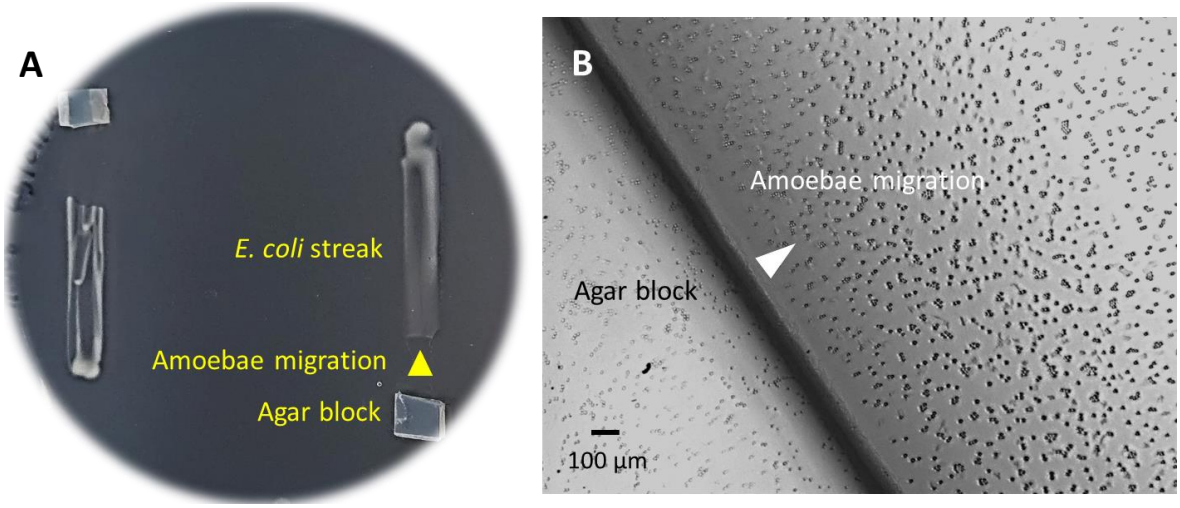

Figure 3.3: Amoebae growing on *Escherichia coli* streaked on non-nutrient agar plate (A). Light microscopic examination clearly demonstrated migration of amoebae from agar block to *E. coli* streak (B). The arrow indicates direction of amoebae migration. Scale bar = 100 µm.

(The image was captured by Muhammad Atif Nisar, the author of this thesis)

3.3.3.2 *Acanthamoeba polyphaga* axenic cultures

HI-FBS supplemented PYG broth was used for amoebae axenic cultures. Two grams of peptone (LP0037B, Oxoid Ltd.) and 0.2 g yeast extract (LP0021, Oxoid Ltd.) were dissolved in 90 mL distilled water and autoclaved. Sterile broth was allowed to cool at room temperature. 1.8 g $_{D}$-glucose was dissolved in 10 mL distilled water and filter sterilized using 0.2 µm syringe filter. Filtered glucose solution was added in sterile broth. To prepare FBS-PYG broth, 5 mL

HI-FBS (10100139, Gibco™ Thermo Fisher Scientific) was mixed in 45 mL PYG broth and stored at 4°C for 1 week.

Acanthamoeba polyphaga (Puschkarew) Page ATCC® 30461™ was cultured in FBS-PYG broth. 500 µL *A. polyphaga* suspension and 4 mL FBS-PYG broth were inoculated in a T25 (156367, Nunc™ EasYFlask™, Thermo Fisher Scientific) culture flask and incubated under aerobic conditions at 25 ± 1°C for 5 to 7 days. Cells were harvested using a cell scraper and centrifuged at 1500 *g* for 10 minutes. The cell pellet was resuspended in 2 mL sterile 1X PAGE saline and examined under light and phase contrast microscopes (**Figure 3.4**).

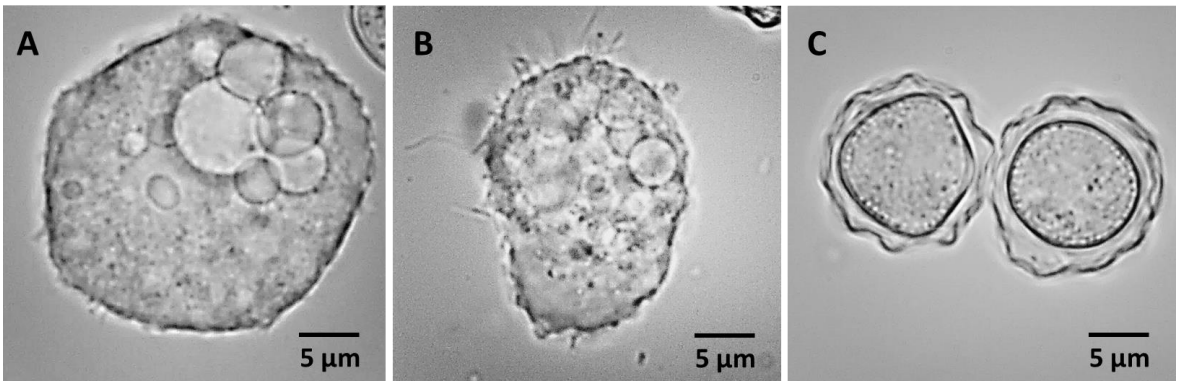

Figure 3.4: Light microscopic images of *Acanthamoeba polyphaga* (Puschkarew) Page ATCC® 30461™ trophozoite (A and B) and cysts (C) growing in heat inactivated-foetal bovine serum supplemented peptone yeast extract glucose broth. Scale bar = 5 µm.

(The image was captured by Muhammad Atif Nisar, the author of this thesis)

3.3.3.3 Morphological and molecular identification

Amoebae isolates were identified on the basis of trophozoite and cyst morphology as suggested by Page (Page, 1988). Briefly, amoebae grown on Eco-NNA plates were harvested and transferred to a screw capped tube containing 2 mL Page's saline. The tube was incubated under aerobic conditions at 25 ± 1°C for 2 hours. Using a micropipette, 20 µL sample was taken from the bottom of tube and placed onto a glass slide. A coverslip was placed onto the sample spot and sealed with sealant nail polish. Slides were examined using upright light microscope (BX43, Olympus) at 10X, 20X, 40X and 100X objective lens and images were captured.

Genomic DNA of each isolated amoebae was extracted using Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.) (**Section 3.4.1**) and dispatched to AGRF Ltd. for amplification and sequencing of the 18S rDNA gene (Moreno et al., 2018). Retrieved sequences were trimmed and analysed on basic local alignment search tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on BLAST results, amoebae isolates were identified to either genus or species level.

3.3.3.4 Amoeba storage and preservation

Amoebae were preserved on Eco-NNA slants for 6 months and for long term storage 20% dimethyl sulfoxide (DMSO) stocks were prepared. To prepare Eco-NNA slants, sterile agar was poured in 50 mL tubes and allowed to solidify at a slanted angle. A loop of overnight grown *E. coli* culture was streaked onto the slant. An agar block of amoebae was placed on the *E. coli* steak and tubes were incubated under aerobic conditions at 25 ± 1°C for 5 days. The tubes were sealed with parafilm tape and stored at 4°C for 6 months. For long term storage, amoebae preservation medium was prepared. Ten millilitres of DMSO were added in 40 mL FBS-PYG broth. One millilitre of preservation medium was inoculated into 1.5 mL cryovials, and 10^5 to 10^7 cells were resuspended and stored at –80°C.

3.4 DNA extraction and amplification

3.4.1 Genomic DNA extraction and storage

Total genomic DNA was extracted using an Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.) following the manufacturer's guidelines. The filter membrane and 1 mL of processed sample (**Section 3.2.4**) were placed into 15 mL tubes containing 2 mL R1 buffer. Each tube was vigorously vortexed for 20 seconds and incubated in a 90 ± 5°C water bath for 15 minutes. The tube was vortexed again for 20 seconds and the membrane filter was removed from the tube and discarded. The tube was placed at room temperature for 15 min then centrifuged at 900 *g* for 3 min. Five hundred microlitres of clear supernatant was added into the purification column and centrifuged at 6000 *g* for 10 min and the eluent discarded. For the remaining supernatant, this step was repeated. One hundred microlitres of R2 buffer was transferred into the column, and this placed into the collection tube in an upside-down orientation and centrifuged at 1000 *g* for 3 minutes. The column was discarded, and purified DNA was stored at –20°C.

3.4.2 Quantitative polymerase chain reaction (qPCR)

Extracted DNA was subjected to qPCR for the molecular detection and quantification of *Legionella*, *L. pneumophila*, *Acanthamoeba* and *Vermamoeba* genomic unit (GU). All reactions were conducted on a Rotor-Gene Q thermal cycler (QIAGEN Ltd.). Each qPCR reaction mix consisted of 10 µL 2X SsoAdvanced™ universal probes supermix (172-5281, BIO-RAD Laboratories Ltd.), 1 µL primers and fluorogenic probe mix (see **Sections 3.4.3.1** to **3.4.3.4** for the primer and probe sequences used), 4 µL double autoclaved Milli-Q® water and 5 µL DNA template. gBlocks gene fragments (IDT™) were used as positive controls and for plotting standard curves. To detect the presence of environmental inhibitors in the purified DNA, the

sample was diluted 1:10 using double autoclaved Milli-Q® water and amplified under the same qPCR conditions (Hayes-Phillips et al., 2019).

3.4.3 DNA standard curve

The gBlocks gene fragments (IDT™) were used as standard DNA to plot the standard curve. The DNA was dissolved in double autoclaved Milli-Q® water to make 10 ng/mL stock solution. The stock was serially diluted to obtain 100 pg/µL, 10 pg/µL, 1 pg/µL, 0.1 pg/µL, 0.01 pg/µL, 0.001 pg/µL, 0.0001 pg/µL and 0.00001 pg/µL concentrations. These dilutions were used as DNA templates to generate a qPCR standard curve.

3.4.3.1 *Legionella* 16S rDNA gene standard curve

The primers, probe and protocol recommended by ISO/TS12869:2019 (International Organization for Standardization, 2019) were used for the amplification of the *Legionella* 16S rDNA gene. The primers and fluorogenic probe used in the study were:

Forward primer	5'-GGAGGGTTGATAGGTTAAGAGCT-3'
Reverse primer	5'-CCAACAGCTAGTTGACATCGTTT-3'
Fluorogenic probe	5'-FAM-AGTGGCGAAGGCGGCTACCT-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

DNA fragment used for synthesis of gBlock was as follows:

Accession Number CP021281 (underlined sequences show primers and probe binding regions)

TAAAGCACTTTCAGTGGGGAGGAGGGTTGATAGGTTAAGAGCTGATTAACTGGACGTTA
CCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CGAGCGTTAATCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTGATTAAGTTATCT
GTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTTGACTCGAGTATGGG
AGAGGGTAGTGGAATTTCCGGTGTAGCGGTGAAATGCGTAGAGATCGGAAGGAACACC
AGTGGCGAAGGCGGCTACCTGGCCTAATACTGACACTGAGGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCTGTTGG
TTATATGAAAATAATTAGTG

The qPCR assay conditions used for the amplification were: 95°C for 3 min, followed by 43 cycles of 95°C for 20 s and 60°C for 60 s. The standard curves demonstrated 89% efficiency of the qPCR assay with a 35 GU/reaction limit of detection (**Figure 3.5**).

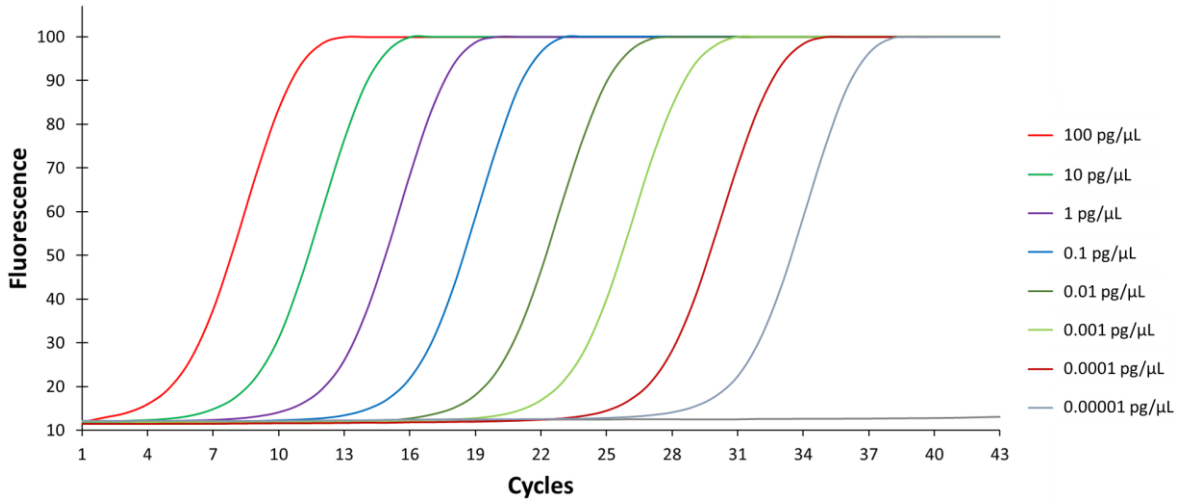

Figure 3.5: qPCR assay standard curve of *Legionella* 16S rDNA gene gBlocks gene fragments. The standard DNA was serially diluted from 100 pg/µL to 0.00001 pg/µL. X-axis: numbers of qPCR cycles, and Y-axis: fluorescence (arbitrary units).

3.4.3.2 *Legionella pneumophila mip* gene standard curve

The oligos and protocol suggested by ISO/TS12869:2019 (International Organization for Standardization, 2019) were used to PCR the *L. pneumophila mip* gene. Sequences of primers and probe were:

Forward primer	5'-CCGATGCCACATCATTAGC-3'
Reverse primer	5'-CCAATTGAGCGCCACTCATAG-3'
Fluorogenic probe	5'-FAM-TGCCTTTAGCCATTGCTTCCG-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Sequence of DNA fragment used for designing gBlock was:

Accession Number KR902705 (underlined sequences show primers and probe binding regions)

GTCAACAGCAATGGCTGCAACCGATGCCACATCATTAGCTACAGACAAGGATAAGTTGT
CTTATAGCATTGGTGCCGATTTGGGGAAGAATTTTAAAAATCAAGGCATAGATGTTAATC
CGGAAGCAATGGCTAAAGGCATGCAAGACGCTATGAGTGGCGCTCAATTGGCTTTAAC
CGAACAGCAAATG

The qPCR assay conditions used were: 95°C for 3 min, followed by 43 cycles of 95°C for 20 s and 60°C for 60 s. The standard curves demonstrated 99% efficiency of the qPCR assay with a 35 GU/reaction limit of detection (**Figure 3.6**).

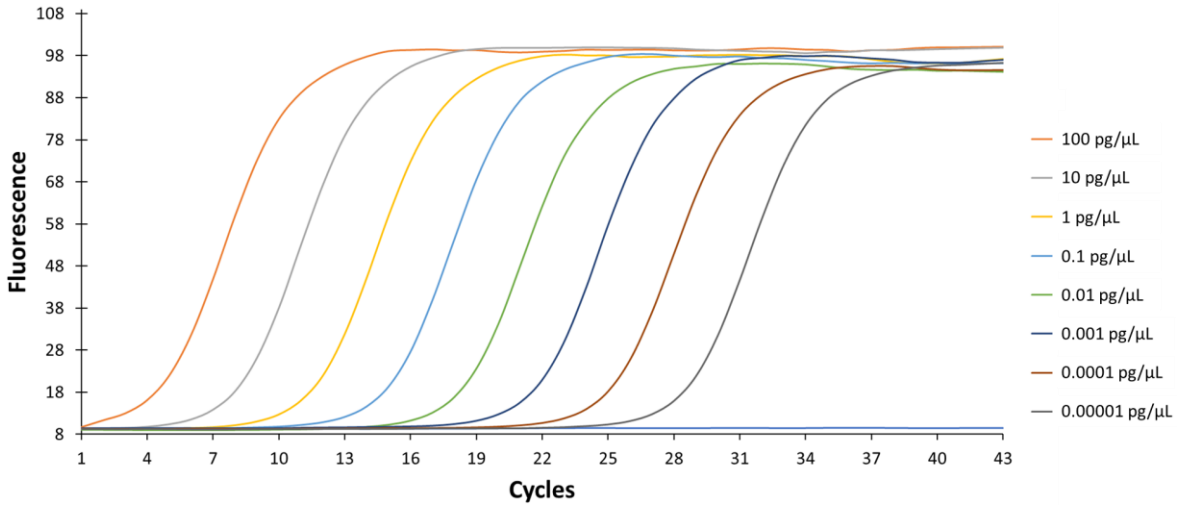

Figure 3.6: qPCR assay standard curve of *Legionella pneumophila mip* gene gBlocks gene fragments. The standard DNA was serially diluted from 100 pg/µL to 0.00001 pg/µL. X-axis: numbers of qPCR cycles, and Y-axis: fluorescence (arbitrary units).

3.4.3.3 *Acanthamoeba* 18S rDNA gene standard curve

The primers, probe and protocol used by Qvarnstrom et al., (2006) were selected for *Acanthamoeba* 18S rDNA gene real time PCR. Sequences of fluorogenic probe and primers were as follows:

Forward primer	5'-CCCAGATCGTTTACCGTGAA-3'
Reverse primer	5'-TAAATATTAATGCCCCCAACTATCC-3'
Fluorogenic probe	5'-FAM-CTGCCACCGAATACATTAGCATGG-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

DNA fragment used for synthesis of gBlock was as follows:

Accession Number U07413 (underlined sequences show primers and probe binding regions)

GCGGCGGTGGGTCCCTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAA
GCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACC
CTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGG
GATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCT

The qPCR assay conditions were: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 63°C for 60 s. The standard curves demonstrated 81% efficiency of the qPCR assay with a 40 GU/reaction limit of detection (**Figure 3.7**).

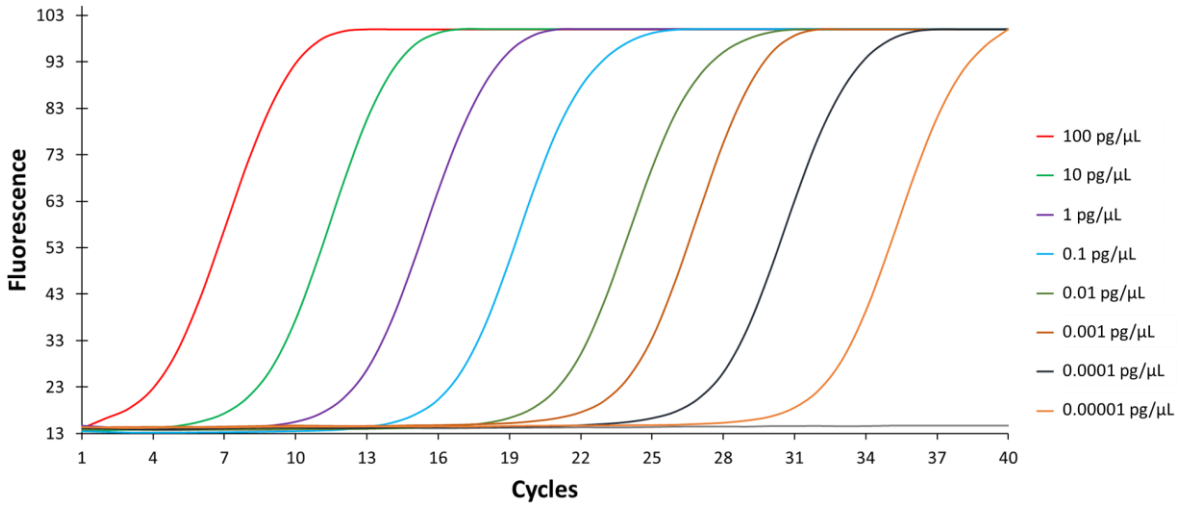

Figure 3.7: qPCR assay standard curve of *Acanthamoeba* 18S rDNA gene gBlocks gene fragments. The standard DNA was serially diluted from 100 pg/µL to 0.00001 pg/µL. X-axis: numbers of qPCR cycles, and Y-axis: fluorescence (arbitrary units).

3.4.3.4 *Vermamoeba vermiformis* 18S rDNA gene standard curve

The oligos and protocol used by Scheikl et al., (2016) were selected for the amplification of *Vermamoeba vermiformis* 18S rDNA gene. Sequences of fluorogenic probe and primers were as follows:

Forward primer	5'-TAACGATTGGAGGGCAAGTC-3'
Reverse primer	5'-ACGCCTGCTTTGAACACTCT-3'
Fluorogenic probe	5'-FAM-TGGGGAATCAACCGCTAGGA-Q-3'

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm

Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

Sequence of DNA fragment used for designing gBlock was as follows:

Accession Number KT185625 (underlined sequences show primers and probe binding regions)

AATTTAAATCCCTTAACGAGTAACGATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGT
AATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATT
TCGGAAGGTCTTTAGCAGTCCGCCCCTTCGGGGAGCGGGTTGCTGGCCTCCTATGTTC
CTAACGGTCCTCATCCGCGAGGGTGGGGAATCAACCGCTAGGATCGTTTACTTTGAGG
AAATTAGAGTGTTCAAAGCAGGCGTAACTCGCCTCCGAATACGTT

The qPCR assay conditions used for the amplification were: 95°C for 5 min, followed by 45 cycles of 95°C for 20 s and 60°C for 60 s. The standard curves demonstrated 75% efficiency of the qPCR assay with a 44 GU/reaction limit of detection (**Figure 3.8**).

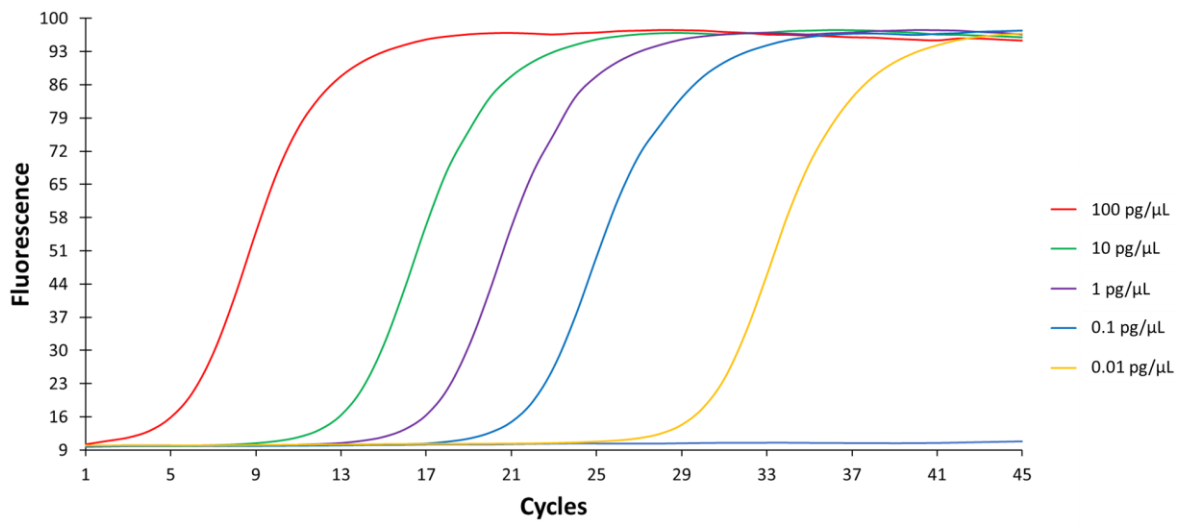

Figure 3.8: qPCR assay standard curve of *Vermamoeba vermiformis* 18S rDNA gene gBlocks gene fragments. The standard DNA was serially diluted from 100 pg/µL to 0.01 pg/µL. X-axis: numbers of qPCR cycles, and Y-axis: fluorescence (arbitrary units).

3.5 *Legionella*-amoebae co-culture assay

Legionella-amoebae co-culture assays were performed to study infectivity of culturable and VBNC *L. pneumophila*. The co-culture assay designed by Moffat and Tompkins (1992) was used with some modifications. Briefly, *L. pneumophila* were harvested and washed with 1X Page's saline by centrifugation at 3200 *g* for 15 min. The washed cell pellet was resuspended in 1X Page's saline and 10^8 to 10^9 CFU/mL ($\lambda_{(600nm)}$ = 0.2 to 0.25) final concentration was

adjusted. *A. polyphaga* growing in FBS-PGY broth was harvested and washed with 1X Page's saline by centrifugation at 1500 *g* for 10 min. Washed cells were resuspended in 1X Page's saline and the final concentration was adjusted to 10^5 cells/mL. Both bacterial and amoebae suspensions were mixed in a 1:1 ratio and incubated at 37°C for 1 to 2 h. The mixture was centrifuged at 1500 *g* for 10 minutes and supernatant was discarded. The cell pellet was washed with 1X Page's saline by centrifugation at 1500 *g* for 10 min. To kill extracellular *L. pneumophila*, the washed cell pellet was resuspended in 1X Page's saline supplemented with 100 µg gentamicin and incubated at 37°C for 2 to 3 hours. The mix was centrifuged at 1500 *g* for 10 minutes and the cell pellet was washed with 1X Page's saline. The washed cell pellet was resuspended in 4 mL treatment broth (FBS-PGY broth and 1X Page's saline, 1:10 ratio) and plated in a T25 culture flask (156367, Nunc™ EasYFlask™, Thermo Fisher Scientific). The flask was incubated under aerobic conditions at 25 ± 1°C for 3 to 5 days. Internalization of culturable *Legionella* (infectivity) and resuscitation of VBNC *Legionella* was confirmed using fluorescence *in situ* hybridization and fluorescence microscopy (see **Section 3.6**).

3.6 Fluorescence *in situ* hybridization and fluorescence microscopy

A fluorescence *in situ* hybridization (FISH) protocol used by Whiley et al. (2011) was used to confirm internalization of *Legionella* in *Acanthamoeba* host and to visualise *Legionella* and amoeba within biofilms samples. The following fluorogenic probes (Invitrogen™) were used:

LEG705: *Legionella* 16S rDNA probe, target site 705–722 (Manz et al., 1995)

5′-CTGGTGTTCCTTCCGATC-3′ labelled with Alexa Fluor 488

EUB338: Eubacterial 16S rDNA probe, target 338–355 (Amann et al., 1990)

5′-GCTGCCTCCCGTAGGAGT-3′ labelled with Alexa Fluor 546

EUK1209: Eukaryota 18S rDNA probe, target site 1431–1446 (Lim et al., 1993)

5′-GGGCATCACAGACCTG-3′ labelled with Alexa Fluor 647

L. pneumophila infected *A. polyphaga* cells or intact biofilms were used as samples. In the case of *L. pneumophila* infected *A. polyphaga* cells, the cell suspension was harvested and washed with 1X phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 per litre distilled water, pH 7.6 ± 0.2) by centrifugation at 1500 *g* for 10 min. The washed cell pellet was resuspended in 1X PBS. Either 1 mL cell suspension or intact biofilm was placed in a screw capped tube containing 1 mL chilled 4% paraformaldehyde (4 g/100 mL 1X PBS) and incubated overnight at 4°C. The cell pellet was harvested and washed

with 1X PBS by centrifugation at 1500 *g* for 10 min. The washed pellet was resuspended in 500 µL 1X PBS. Using a micropipette, 20 µL sample (either washed cell pellet or fixed intact biofilm) was inoculated into each well of a 3-well PTFE printed glass slide (G35-0308, ProSciTech) and air dried at 35 ± 2°C for 15 minutes. After fixation, the slide was washed with 1X PBS and dehydrated in an ethanol series: 50% ethanol/2 minutes, 80% ethanol/2 minutes and 96% ethanol/2 minutes. The sample was covered with 200 µL hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl with pH = 7.6) containing 100 ng of each probe and covered with RNase free hybridization cover slips (H582, ProSciTech). The slide was placed in a dark humidified chamber and incubated at 55°C for 100 min. To remove hybridization cover slips and unbound probes, the slide was dipped in warm wash buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl with pH = 7.6) and incubated in the dark at 55°C for 15 min. The slide was washed with Milli-Q® water and air dried at 35 ± 2°C for 15 min. Twenty microlitres of CitiFluor™ AF1 (17970-25, Electron Microscopy Sciences) was added to the sample and covered with a clean coverslip and sealed with sealant nail polish.

The slide was examined using an upright fluorescence microscope (AX70, Olympus) at 10X, 20X, 40X and 100X objective lens, with 470 nm green and 635 nm red filters. The slides were also examined by confocal microscopy (LSM 880 fast airyscan confocal, Zeiss) using an oil immersion objective (C Plan-Apochromat 63x/1.4 oil DIC M27, Zeiss). Finally, captured images were processed using Fiji software (https://imagej.net/software/fiji/). Pure cultures of *L. pneumophila* subsp. *pneumophila* Philadelphia sg1 ATCC® 33152™ and *A. polyphaga* (Puschkarew) Page ATCC® 30461™ were used as controls (**Figure 3.9**).

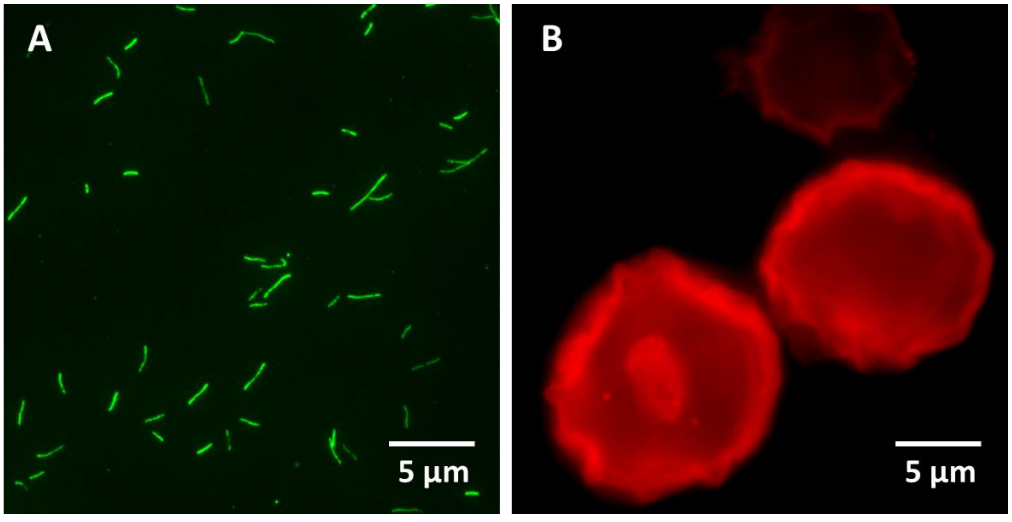

Figure 3.9: Fluorescence *in situ* hybridization of *Legionella pneumophila* subsp. *pneumophila* Philadelphia sg1 ATCC® 33152™ (A) and *Acanthamoeba polyphaga* (Puschkarew) Page ATCC® 30461™ (B) stained with Alexa Fluor 488 labelled LEG705 (green) and Alexa Fluor 647 labelled EUK1209 (red) probes, respectively. Scale bar = 5 µm.

(The image was captured by Muhammad Atif Nisar, the author of this thesis)

3.7 Detection and quantification of VBNC *Legionella*

The ISO recommends microbiological culturing (ISO11731:2017-05) and qPCR (ISO/TS12869:2019) assays for detection and quantification of *Legionella* and *L. pneumophila* from engineered water systems (International Organization for Standardization, 2017, International Organization for Standardization, 2019). However, in the environment, *Legionella* also exists in a nonculturable state, which neither method is able to detect and quantify (Kirschner, 2016). Generally, during the environmental stresses *Legionella* enters into this VBNC state. After the end of the stress period and in presence of suitable protozoan hosts these VBNC cells transforms back into culturable state (Dietersdorfer et al., 2018). By definition, it is a nonculturable state and classical culturing methods are unable to identify its viability (Kirschner, 2016). The *in vitro Legionella*-amoebae coculture assays is gold standard method to detect VBNC cells (Garcia et al., 2007). *Acanthamoeba* is the preferred host for *Legionella*-amoebae coculture assays (Conza et al., 2013, Steinert et al., 1997). Alternatively, fluorescence *in situ* hybridization (FISH), viability quantitative polymerase chain reaction (qPCR), and differential live/dead stain flow cytometry assays are good approaches to detect or estimate cellular viability (Delgado-Viscogliosi et al., 2005, Ditommaso et al., 2014, Mustapha et al., 2015, Wang et al., 2010, Allegra et al., 2008, Allegra et al., 2011). However, due to either non-specificity or time consumed to perform or competing environment microflora, these assays have limited application (Kirschner, 2016). Therefore, in this study viability-based flow cytometry-cell sorting and quantitative PCR (VFC+qPCR) assay was designed for detection and quantification of VBNC *Legionella* spp. and *L. pneumophila*.

4 Detection and quantification of viable but non-culturable (VBNC) *Legionella pneumophila* from water samples using flow cytometry-cell sorting and quantitative PCR

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4.1 Abstract

Legionella pneumophila is a waterborne pathogen and, as the causative agent of Legionnaires' disease, a significant public health concern. Exposure to environmental stresses, and disinfection treatments, promotes the formation of resistant and potentially infectious viable but non-culturable (VBNC) *Legionella.* The management of engineered water systems to prevent Legionnaires' disease is hindered by the presence of VBNC *Legionella* that cannot be detected using the standard culture (ISO11731:2017-05) and quantitative polymerase reaction (ISO/TS12869:2019) methods. This study describes a novel method to quantify VBNC *Legionella* from environmental water samples using a “viability-based flow cytometry-cell sorting and qPCR” (VFC+qPCR) assay. This protocol was then validated by quantifying the VBNC *Legionella* genomic load from hospital water samples. The VBNC cells were unable to be cultured on Buffered Charcoal Yeast Extract (BCYE) agar; however, their viability was confirmed through their ATP activity and ability to infect amoeba hosts. Subsequently, an assessment of the ISO11731:2017-05 pre-treatment procedure demonstrated that acid or heat treatment cause underestimation of alive *Legionella* population. Our results showed that these pre-treatment procedures induce culturable cells to enter a VBNC state. This may explain the observed insensitivity and lack of reproducibility often observed with the *Legionella* culture method. This study represents the first time that flow cytometry-cell sorting in conjunction with a qPCR assay has been used as a rapid and direct method to quantify VBNC *Legionella* from environmental sources. This will significantly improve future research evaluating *Legionella* risk management approaches for the control of Legionnaires' disease.

4.2 Introduction

Legionella is an opportunistic premise plumbing pathogen associated with Legionnaires' disease (LD) and Pontiac fever. It is ubiquitously present in natural and engineered water systems (Fields et al., 2002). Aerosols generated from cooling towers, showers, humidifiers and other fabricated water distribution systems are major sources of LD (Bartram et al., 2007). Within these engineered water sources, microbial biofilms and host protozoa protect *Legionella* from environmental stresses and disinfection procedures. Furthermore, disinfection protocols (Casini et al., 2018, Allegra et al., 2008) and environmental stresses (Li et al., 2014) cause culturable *Legionella* to enter a viable but non-culturable (VBNC) state. VBNC *Legionella* tolerate environmental stresses and are still able to infect human macrophages (Dietersdorfer et al., 2018) and alveolar epithelial cells (Epalle et al., 2015). Under favourable conditions, VBNC *Legionella* can be resuscitated using protozoan hosts to generate culturable *Legionella* (Garcia et al., 2007, Steinert et al., 1997).

Over the last decade, in the USA and across Europe the number of reported LD cases has been significantly increasing (National Notifiable Diseases Surveillance System and Centers for Disease Control and Prevention, 2018, Smith et al., 2007, The European Centre for Disease Prevention and Control). In 2022, the European Centre for Disease Prevention and Control reported 8372 confirmed cases of LD, of which 66.9% were community associated and 5.1% were nosocomial infections(The European Centre for Disease Prevention and Control). During COVID-19 pandemic, lockdown resulted in water stagnation in some buildings, which ultimately increased *Legionella pneumophila* contamination (Liang et al., 2021, Chao and Lai, 2022). Furthermore, climate change, urbanization and new energy conservation approaches are also increasing the risk of legionellosis (both LD and Pontiac fever) (Connolly et al., 2021, Gattuso et al., 2022). As such there is a need for improved risk management protocols to reduce the risk of legionellosis.

One of the challenges preventing the improved control of *Legionella* in engineered water systems is the uncertainty associated with standard detection methods (Whiley, 2016). The International Organization for Standardization (ISO) provides two protocols for the detection and quantification of *Legionella* contamination in potable water. ISO11731:2017-05 is a culture based method that detects only culturable *Legionella* (International Organization for Standardization, 2017); whereas, ISO/TS12869:2019 is a quantitative PCR (qPCR) based assay which estimates the bacterial genomic load (International Organization for Standardization, 2019). The culture method is considered the gold standard for *Legionella* detection and characterization. However, it is time consuming, taking 10 to 14 days*,* and does not detect VBNC *Legionella* resulting in underestimation of *Legionella* numbers and false negatives (International Organization for Standardization, 2017, Whiley and Taylor, 2016). In contrast, the qPCR method is faster (International Organization for Standardization, 2019); however, it quantifies both the live, VBNC and dead *Legionella,* resulting in overestimations and false positive results (Whiley and Taylor, 2016).

The concentration of culturable *Legionella* is typically low in potable water, however, previous studies have found high concentration of *Legionella* DNA using qPCR quantification (e.g., 10^3 to 10^7 GU/L) in culture negative potable water samples (Casini et al., 2018, Dai et al., 2019, Hayes-Phillips et al., 2019). Several methods have been used to try to overcome this discrepancy observed between the two standard methods (culture and qPCR). Ethidium monoazide (EMA) or propidium monoazide (PMA) based viability qPCR (Taylor et al., 2014, Delgado-Viscogliosi et al., 2009, Zacharias et al., 2015) and fluorescence *in situ* hybridization (FISH) (Whiley et al., 2011) assays are alternative approaches designed to differentiate between viable and non-viable *Legionella* from environmental samples. However, background

microbial populations compromise the validity of both techniques (Zacharias et al., 2015) and PMA and EMA have a concentration depended cytotoxic effect that can make it challenging to use in environmental samples of with unknown cell concentrations (Fittipaldi et al., 2012). Also, VBNC bacterial cells show low metabolic activity and rRNA contents, as such, FISH is not an effective technique to detect and characterise VBNC *Legionella* (Satoh et al., 2002). Catalysed reporter deposition FISH (CARD-FISH) technique was designed to overcome lower contents of rRNA. It was used for direct enumeration of *L. pneumophila* from hospital water system (Kirschner et al., 2012). However, CARD-FISH detect and enumerate non-viable cells as well. Alternatively, combined microcolonies cultivation-FISH-solid phase cytometry assay was designed to detect and quantify viable *Legionella* (Baudart et al., 2015). However, this method cannot detect VBNC *Legionella. Legionella*-amoebae coculture assay is another technique used to detect VBNC *Legionella* from environmental samples (Conza et al., 2013). However, it is very time-consuming and is unable to quantify the population of VBNC *Legionella*.

Flow cytometric analysis of environmental samples has previously been used to characterize total bacterial VBNC populations (Khan et al., 2010, Sachidanandham et al., 2005). However, this method does not enable the discrimination and quantification of specific species from mixed bacterial population (Zacharias et al., 2015). Previous studies have also used flow cytometry to characterize VBNC *Legionella* generated by physical and chemical disinfection procedures, but these studies used pure cultures of *L. pneumophila* (Mustapha et al., 2015, Allegra et al., 2008). Another method used flow cytometry with specific fluorogenic antibodies to detect *L. pneumophila* from environmental samples (2010). In this method, FITC (ab20818, abcam) and Alexa (GTX40943, GeneTex) conjugated *L. pneumophila* sg1 (ATCC® 33152™) specific polyclonal antibodies were applied to detected *L. pneumophila* sg1 (ATCC® 33152™) which had been used to spike a water sample. Antibodies required for a universal assay must target epitope(s) that are well expressed in all serogroups and VBNC *Legionella*. In our study, to overcome this problem, instead of bacteria specific antibodies, dual staining flow cytometry-cell sorting was used, and genomic load estimated by a standard qPCR assay.

The aim of this study was to develop a rapid and robust “viability-based flow cytometry-cell sorting and qPCR” (VFC+qPCR) assay to detect and quantify VBNC *Legionella* from engineered water systems. Following the optimization of the assay using pure *Legionella* cultures, its performance was validated using environmental water samples. It was also used to assess the impact of the pre-treatments described in the ISO11731:2017-05 culture method on *Legionella* recovery.

4.3 Materials and Methods

4.3.1 Microbial strains, culture media and growth conditions

Legionella pneumophila subsp. *pneumophila* Philadelphia serogroup 1 (ATCC® 33152™) was cultured on BCYE (buffered charcoal yeast extract: CM0655, Oxoid Ltd.) agar supplemented with GVPC (glycine, vancomycin, polymyxin B and cycloheximide: SR0152, Oxoid Ltd.) and *Legionella* growth supplement (buffer/potassium hydroxide, ferric pyrophosphate, $_{L}$-cysteine, and α-ketoglutarate: SR0110C, Oxoid Ltd.); and incubated at 37 ± 1°C for 3 to 4 days. *Escherichia coli* HS(pF*amp*)R (ATCC® 700891™) and *Acinetobacter calcoaceticus* (environmental isolate) were cultured on nutrient and MacConkey agar, respectively; and incubated at 37 ± 1°C for 24 hours. *Acanthamoeba polyphaga* (ATCC® 30461™) cells were cultured in a T25 flask (156367, Nunc™ EasYFlask™, Thermo Fisher Scientific) containing 4.5 mL PYG broth (2% peptone, 0.2% yeast extract and 1.8% $_{D}$-glucose) supplemented with 10% heat inactivated foetal bovine serum (FBS: 10100139, Gibco®, Thermo Fisher Scientific) and incubated at 25 ± 1°C for 4 to 5 days. Whenever washing was required 1X phosphate-buffered saline (PBS: 003002, Invitrogen™, Thermo Fisher Scientific) and 1X Page's saline (0.12 g NaCl, 0.004 g $MgSO_4.5H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.142 g Na_2HPO_4 and 0.136 g KH_2PO_4 per litre distilled water, pH 6.8 ± 0.2) were used for bacterial strains and amoebae, respectively.

4.3.2 Determining the impact of sample processing according to ISO11731:2017-05 on *Legionella* culturability

A water sample spiked with 10^6 CFU/L *L. pneumophila* was prepared. The sample was concentrated through filtration. After filtration, the contents present on the membrane were carefully resuspended in 1X PBS. This concentrated sample was processed according to the ISO11731:2017-05 standard culture method, which recommends heat or acid pre-treatment to remove background microbes from water samples. For the heat treated sample, 2 mL of sample was heated at 50 ± 1°C for 30 ± 2 min, whereas for the acid pre-treatment procedure, 1 mL sample was mixed with 9 mL of acid buffer (3.5 mL 0.2 M HCl and 25 mL 0.2 M KOH, final pH 2.2) and incubated at room temperature for 5 ± 0.5 min (International Organization for Standardization, 2017). Then untreated, heat and acid treated samples were cultured on BCYE-GVPC agar and tested using the VFC+qPCR assay described below. The data was analysed using R (version 4.2.2) package agricolae (version 1.3-5) (de Mendiburu, 2021). Firstly, normality was assessed using the Shapiro-Wilk test, then one way ANOVA (analysis of variance) was performed followed by Tukey's HSD (honestly significant difference) test. Finally, graph was designed using ggplot2 (version 3.3.6) in the R environment (Wickham, 2016).

4.3.3 Viability based flow cytometry-cell sorting and qPCR assay (VFC+qPCR) development

4.3.3.1 Preparation of *Legionella* suspension

To prepare *Legionella* suspension, 5 mL of 1X PBS (temperature preadjusted to 37 ± 1°C) was pipetted onto an agar plate containing *Legionella* colonies. These colonies were harvested by gently scrapping with a spreader to resuspended them in the PBS. The suspension containing harvested cells was then pipetted into a sterile 10 mL tube and repeatedly pipette mixed. Finally, 2.5 mL of the cell suspension was transferred to a 10 mL sterile tube containing 2.5 mL of PBS and again pipette mixed thoroughly. This was used as bacterial stock solution and diluted accordingly.

4.3.3.2 Flow cytometry-cell sorting assay

The BD™ cell viability kit (349480, BD™) was used for staining and estimation of alive, dead and injured cell populations (Alsharif and Godfrey, 2002). Briefly, 100 µL of *Legionella* cells were mixed in 400 µL filter sterilised staining buffer (1 mM EDTA and 0.01% tween-20 in 1X PBS, pH 7.4 ± 0.1). In this mixture 420 nM of thiazole orange (TO; $\lambda_{(excitation)}/\lambda_{(emission)}$: 512/533 nm) and 48 µM propidium iodide (PI; $\lambda_{(excitation)}/\lambda_{(emission)}$: 537/618 nm) were added and vortexed. Sample tubes were incubated at 5°C for 15 min, then 50 µL of counting beads were added in each tube. Using a BD™ FACSAria™ Fusion instrument (BD Biosciences), cells were analysed and the TO (FL1) vs PI (FL3) fluorescence plots were used to distinguish alive, dead, and injured cells. Untreated cells and heat killed cell (75°C for 10 min) were used as controls to define gates distinguishing alive and dead cell populations, respectively. The bacterial populations were first gated based on their forward (FSC) and side scatter (SSC) properties (Appendix-1, **Figure 12.2**). During optimization of the protocol, control samples containing predominantly dead or alive *Legionella* cells were used to position the quadrant gate. A blank control of buffer containing dyes was also included to confirm that debris was not being stained with the dyes and was not included in the gated populations (Appendix-1, **Figure 12.1**). All gated cell populations were examined to monitor viability and culturability of *Legionella*. Depending upon samples type and cell number, approximately 10^3 to 10^6 alive, dead, and injured cell fractions were then sorted into different tubes for further characterization.

4.3.3.3 Interference of viability dyes in quantitative PCR

To confirm that TO and PI dyes did not interfere with the qPCR assay, sorted cell fractions (10^3 to 10^5 cells from each fraction) were subjected to DNA extraction and qPCR. The results of TO and PI dyed cell fractions were compared with the unstained *Legionella* and gBlocks gene fragments (IDT™). DNA was extracted using the Aquadien™ DNA extraction and purification kit (3578121, BIO-RAD Laboratories Ltd.). According to the guidelines of ISO/TS12869:2019 (International Organization for Standardization, 2019), the 16S rDNA gene was amplified and

quantified. Briefly, gBlocks gene fragments (IDT™) were used as standard DNA with 10-fold serial dilution with concentrations ranging from 100 pg/µL to 0.00001 pg/µL. Using 2X SsoAdvanced™ universal probes supermix (172-5281, BIO-RAD Laboratories Ltd.) and 16S rDNA specific oligos (BIO-RAD Laboratories Ltd., **Table 4.1**), the reaction mix was prepared and subjected to a Rotor-Gene Q thermal cycler (QIAGEN Ltd.) for qPCR assay. In the assays, a channel with $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm was used for detection of 6-carboxyfluorescein ($\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm) and Iowa Black® FQ quencher labelled fluorogenic probe (BIO-RAD Laboratories Ltd.). Based on 10-fold serial dilution and qPCR assay, the limit of detection and limit of quantification were estimated. A standard curve was used to estimate the *Legionella* GU/L from alive, dead, and injured cell fractions. All qPCR assays were performed in triplicate and mean C_T values were used for all calculations.

Table 4.1: Sequence of oligos and fluorogenic probes.

Name	Sequence/ Fluorogenic signal (5´→3´)	Reference
***Legionella* PCR primers and probe**		
Forward Primer	GGAGGGTTGATAGGTTAAGAGCT	(International Organization for Standardization, 2019)
Reverse Primer	CCAACAGCTAGTTGACATCGTTT	
Probe	FAM-AGTGGCGAAGGCGGCTACCT-Q	
Fluorescence *in situ* hybridization probes		
LEG705	Alexa Fluor 488-CTGGTGTTCCTTCCGATC	(Manz et al., 1995)
EUK1209	Alexa Fluor 647-GGGCATCACAGACCTG	(Lim et al., 1993)

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm
Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm

4.3.4 Assay validation using *Legionella* pure cultures

4.3.4.1 Thermal treatment

To study thermal tolerance, *L. pneumophila* was resuspended in 1X PBS at ≈ 10^8 CFU/mL (λ_{600nm} 0.2) and heat treated at 37°C to 75°C for 10 min, with gentle shaking after regular intervals of three minutes. To investigate effect of exposure time, cells were heated at 55°C in 1 min increments from 0 to 10 min. The time required to attain desired temperature within the bacterial vials was also considered. Following the thermal treatment vials were placed at 5°C for 60 min. All treated cells were analysed by flow cytometry and sorted into alive, dead, and injured (suspected VBNC) cell fractions (as described in **Section 4.3.3.2**).

4.3.4.2 Culturability of VBNC *Legionella*

Culturability of sorted cell fractions was determined using BCYE agar (CM0655, Oxoid Ltd.) containing *Legionella* growth supplement (SR0110C, Oxoid Ltd.) and *Legionella* enrichment broth (M1399-100G, Himedia®) containing *Legionella* growth supplement (FD016A-5VL, Himedia®). For culturing, from 100 to 300 sorted cells from each fraction was inoculated onto each growth medium and incubated at 37 ± 1°C for 5 to 7 days.

4.3.4.3 Viability of VBNC *Legionella*

The viability of non-culturable cells was determined using two methods: an ATP estimation assay and an amoebae coculture assay.

4.3.4.3.1 ATP estimation assay

ATP contents were estimated using a luciferase-based ATP determination kit (A22066, Thermo Fisher scientific) according to the manufacturer's instructions. The quantity of ATP in sorted cell fractions was estimated as a function of luminescence measured at 540 nm using a CLARIOstar® microplate reader (BMG Labtech GmbH).

4.3.4.3.2 Amoebae coculture assay

In this assay, 100 µL (10^7 *Legionella*) of each of the sorted fractions, or a filter concentrated environmental water sample, were inoculated on 10^6 *A. polyphaga* cells growing in infection medium (FBS-PYG broth and Page's saline in 1:10 ratio) and incubated at 25 ± 1°C for 7 days (multiplicity of infection of 10) (Moffat and Tompkins, 1992). Internalization and resuscitation of *Legionella* within amoeba hosts was confirmed through FISH using Alexa Fluor 488 labelled *Legionella* LEG705 and Alexa Fluor 647 labelled eukaryotic universal EUK1209 fluorogenic probes (Invitrogen™, **Table 4.1**) (Whiley et al., 2011, Manz et al., 1995, Amann et al., 1990). Briefly, infected cells resuspended in 1X PBS were fixed with 4% paraformaldehyde. Fixed cells were placed on a slide, air dried and dehydrated in an ethanol series: 50% ethanol/2 min,

80% ethanol/2 min and 96% ethanol/2 min. Fixed, and dehydrated cells were covered with 200 µL hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl pH 7.6) containing 100 ng of each probe and placed in a dark humidified chamber at 55 ± 1°C for 100 min. To remove unbound probes, cells were washed with warm wash buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl pH 7.6). Finally, the slide was washed with Milli-Q® water, air dried, mounted with 20 µL of CitiFluor™ AF1 (17970-25, Electron Microscopy Sciences) and examined under fluorescence microscope (AX70, Olympus). Nonspecific binding of these probes was tested on *E. coli*.

4.3.5 Assay validation using mixed microbial samples

Four water samples spiked with different concentrations of *L. pneumophila*, *E. coli*, *A. calcoaceticus* and *A. polyphaga* were prepared (**Table 4.2**). All water samples were heated at 50°C for 15 min, then vacuum filtered onto a 47 mm diameter 0.2 µm polycarbonate membrane (GTTP04700, Isopore™). The filter membrane was transferred into a sterile tube containing 1000 µL 1X PBS and vortexed. Presence of culturable *Legionella* in all four samples were confirmed using standard culturing method (International Organization for Standardization, 2017). From each resuspended sample, 200 µL was mixed in 300 µL filter sterilised staining buffer and used for flow cytometry-cell sorting. The sorted VBNC fractions were subjected to qPCR to estimate *Legionella* GU/L (as described in **Section 4.3.3.3**).

Table 4.2: Mixed microbial water samples spiked with *Legionella pneumophila*.

Name	Microorganism	Concentration/L
Sample 1	*Legionella pneumophila*	≈ 4.5 x 10^6 CFU
Sample 2	*Legionella pneumophila*	≈ 4.5 x 10^6 CFU
	Acanthamoeba polyphaga	≈ 4.5 x 10^6 Cells
Sample 3	*Legionella pneumophila*	≈ 4.5 x 10^6 CFU
	Acinetobacter calcoaceticus	≈ 9 x 10^7 CFU
	Escherichia coli	≈ 9 x 10^7 CFU
Sample 4	*Legionella pneumophila*	≈ 4.5 x 10^6 CFU
	Acinetobacter calcoaceticus	≈ 9 x 10^7 CFU
	Escherichia coli	≈ 9 x 10^7 CFU
	Acanthamoeba polyphaga	≈ 4.5 x 10^6 Cells

4.3.6 Assay validation using environmental water samples

From an Australian hospital, shower water (1.15 L) samples were collected in sterile screw capped wide-mouth plastic bottle. Culturable *Legionella* was detected using Legiolert kit (IDEXX) and standard culture method (International Organization for Standardization, 2017). For the standard culture method, 1 L water sample was concentrated through filtration. The concentrated residues were resuspended in 5 mL distilled water and subjected to both acid and heat pre-treatment steps. The treated sample was plated on BCYE-GVPC agar and incubated under standard culturing conditions (as described in **Section 4.3.1**). For the

Legiolert kit method, 100 mL sample water was processed according to the manufacturer's protocols. Using the Aquadien™ DNA extraction and purification kit DNA was extracted from the water sample and *Legionella* GU/L was estimated. To detect presence of any VBNC *Legionella*, the PCR positive and culture negative samples were subjected to amoeba coculture assay. Finally, these *Legionella* PCR positive and culture negative shower water samples were processed to detect and quantify VBNC *Legionella* as described in **Section 4.3.3**.

4.4 Results

4.4.1 ISO sample processing protocol and *Legionella* culturability

L. pneumophila spiked water samples were assayed according to ISO11731:2017-05 (International Organization for Standardization, 2017). **Figure 4.1** shows that the highest culturable *Legionella* numbers were obtained by directly inoculating the sample after membrane filtration (1.136 ± 0.087 CFU/L). There were statistically significant differences between the control sample (no pre-treatment) and the two pre-treatments ($p < 0.001$). Both acid and heat decontamination steps transformed culturable *Legionella* to non-culturable cells and decreased the cell numbers to 0.774 ± 0.043 CFU/L and 0.766 ± 0.097 CFU/L, respectively. These results demonstrate that both sample pre-treatment procedures also effect the reliability of the ISO culturing assay (ISO11731:2017-05).

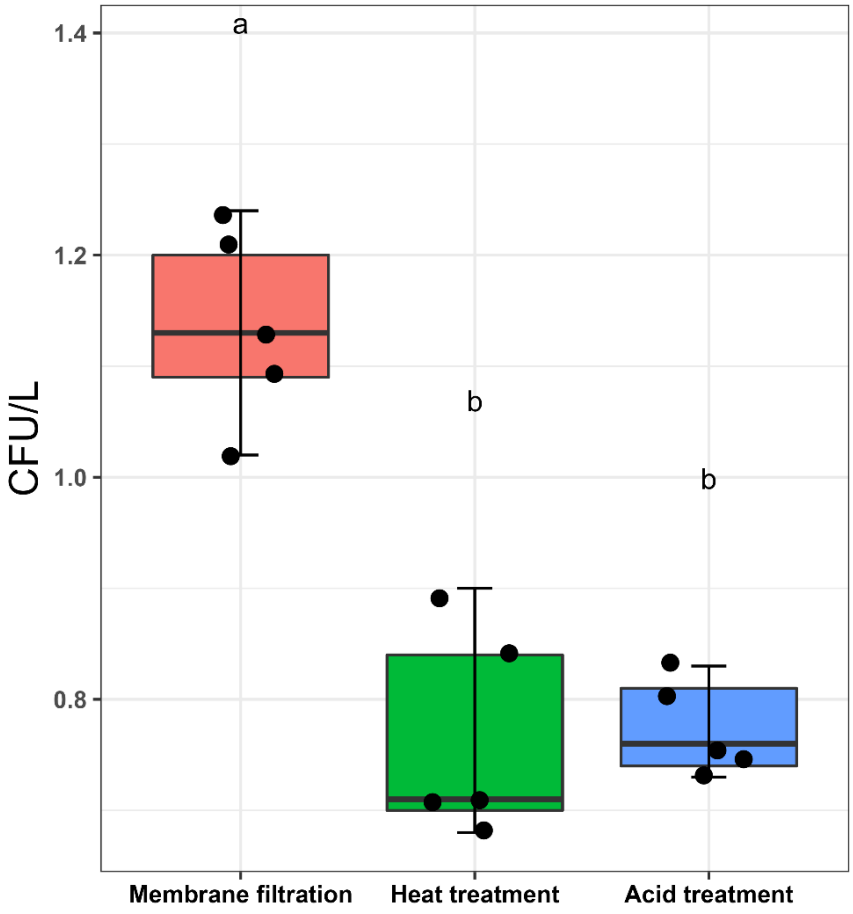

Figure 4.1: Effect of sampling processing methods (ISO11731:2017-05) on *L. pneumophila* culturability. *L. pneumophila* spiked water samples were membrane filtered (no pre-treatment used as control), followed by heat (50°C/30 min) and acid (KCl-HCl with pH 2.2/5 min) pre-treatment method. These pre-treatment methods transformed ≈30% culturable *Legionella* into a non-culturable state. Data are the mean with standard deviation of five replicates, while the same letter within the same treatment is statistically similar according to Tukey's HSD test ($p < 0.001$).

4.4.2 Assay optimization

4.4.2.1 Flow cytometry and differentiation of cells

Three distinct regions can be distinguished on the cytograms: PI-stained dead cells, TO-stained alive cells and TO-stained injured (transition state) cells (**Figure 4.2 A**). Injured or intermediate cells were observed in between the alive and dead cell regions. The principle of the PI/TO assay is to selectively stain dead bacteria (damaged membrane) with the dye PI, whereas TO can enter into all cells (Alsharif and Godfrey, 2002). The PI staining divides cells into two major populations i.e., PI-staining (dead) and PI-unstained (alive and injured) cells. In PI unstained cell population, fluorescence of TO dye become very prominent. Injured cells are in a transitional physiological state and stain positive with TO dye. In these experiments *Legionella* was grown at 37°C on agar for three to four day and all samples consisted of some dead, injured, and alive cells although the proportion of alive cells was the highest. It is possible that the washing, and centrifugation steps could have generated these small number of injured and dead cells. Different handling and processing steps have been shown to cause *Legionella* to lose culturability (Whiley, 2016). **Figure 4.2 A** shows that thermal treatment at 75°C for 10 min completely inactivated the cells and these dead cells are stained with PI. These results clear demonstrated that using flow cytometry-cell sorting, it was possible to isolate and characterise all three cell fractions.

4.4.2.2 Validation of qPCR and detection of VBNC *Legionella*

Mean C_T values obtained from qPCR assays and logarithmic concentration of 10-fold dilutions of *Legionella* 16S rDNA gBlocks were used to plot a standard curve with a 0.9995 regression coefficient and 88.9912% amplification efficiency. In this study, the limit of detection and limit of quantification were 5 GU and 25 GU, respectively. To investigate the effect of the TO dye, 10^3 to 10^5 sorted *Legionella* cells were used for DNA extraction. The extracted DNA was subjected to qPCR assay. **Figure 4.2 B** illustrates that the TO dye did not interfere with DNA extraction and amplification.

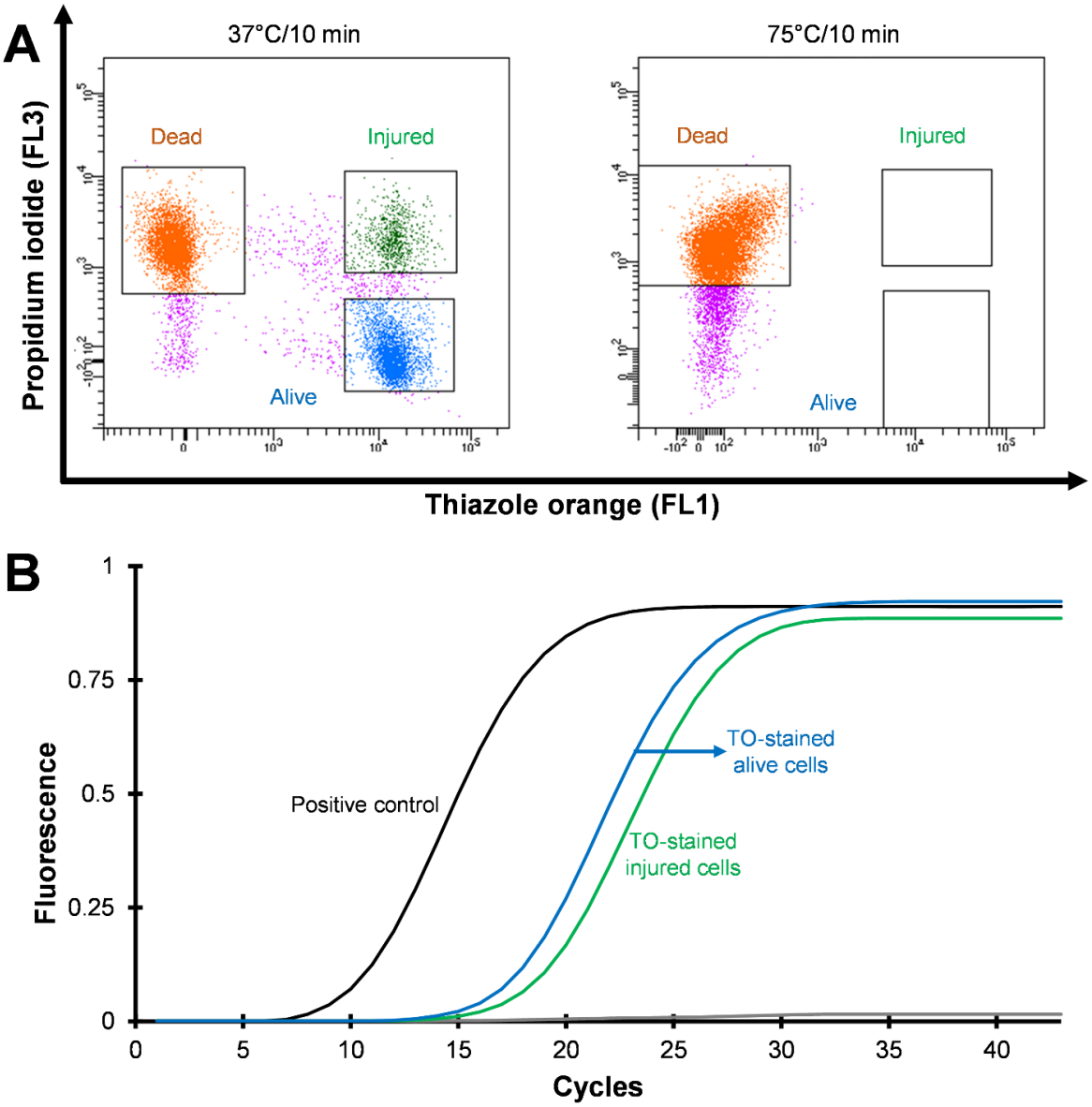

Figure 4.2: Viability based flow cytometry-cell sorting and qPCR assay. *L. pneumophila* grown on BCYE-GVPC agar at 37°C were used as a positive control and cells treated at 75°C for 10 min were used as a negative control A: Cytograms represent three populations: thiazole orange-stained alive (blue), thiazole orange-stained injured (green) and propidium iodide-stained dead (orange) cells. Propidium iodide selectively stained dead cells. Pink coloured events are debris. B: qPCR assay of thiazole orange stained alive and injured cells. The results showed that thiazole orange (TO) does not interfere with DNA extraction and qPCR.

4.4.3 Assay validation using pure cultures of *Legionella*

4.4.3.1 Heat treatment promoted formation of VBNC *Legionella*

In order to study thermal tolerance of *Legionella*, bacteria were heat shocked sequentially in 5°C increments from 40°C to 70°C for 10 min and examined by flow cytometry (**Figure 4.3 A**). From the cytograms, PI-stained cells were non-culturable and non-viable *Legionella* and defined as dead cells. TO-stained alive cells grew on culture medium, whereas TO-stained injured cells obtained from ≤ 45°C treatment was unable to grow on both BCYE agar and *Legionella* enrichment broth (**Figure 4.3 B**). The 50°C treatment triggered the formation of non-culturable injured cells, which were considered VBNC *Legionella*. Heat treatment at 55°C for 0 to 3 min increased the rate of transformation of alive cells into VBNC cells (**Figure 4.3 C**). It is worth noting that a considerable number of *Legionella* converted into VBNC cells after three minutes of treatment. During the 55°C thermal treatment for 0 to 10 min, the number of PI-stained dead cells increased proportionally with the duration of thermal treatment. After 10 min of heat shock treatment at 55°C, all bacteria clustered in the dead cell region on the cytogram and were no longer culturable on BCYE agar or in *Legionella* enrichment broth. Thus, according to the cytograms (**Figure 4.3 A** and **C**), the PI/TO membrane integrity assay is an effective way to study the effect of disinfection treatment on *Legionella*.

4.4.3.2 Culturability and viability of VBNC *Legionella*

The injured cells isolated following 50°C heat shock was unable to grow on either agar or in broth medium (**Figure 4.4 A** and **B**). The viability of these cells was confirmed by quantification of ATP contents and resuscitation in *A. polyphaga*. According to the luciferase-based ATP detection assay, TO-stained alive culturable cells produced the highest amount of ATP (11030 ± 860 units), whereas the least amount of ATP was detected from PI-stained dead cells (85 ± 10 units). TO-stained injured non-culturable cells were found to be metabolically active and able to produce 4645 ± 345 units ATP.

Axenic *A. polyphaga* cultures were used to study infectivity of suspected VBNC (TO-stained injured) and culturable *Legionella*. This was achieved by labelling *Legionella* infected *A. polyphaga* cells with fluorescent probes and examining them under a fluorescent microscope. In the FISH images (**Figure 4.4 C**), the LEG705 *Legionella* probe appears green and the EUK1209 eukaryotic probe is coloured red. *A. polyphaga* appeared as red coloured spherical bodies (**Figure 4.4 C**), whereas *Legionella* appears as distinct cells localised within the amoebae cytoplasm (**Figure 4.4 C-ii** and **iii**). Unlike the VBNC *Legionella*, culturable *Legionella* were able to proliferate within *A. polyphaga* after 60 h of infection. The results from 7 days post-infection showed internalization and multiplication of suspected VBNC *Legionella* in *A. polyphaga* (**Figure 4.4 C-ii)**. After 10 days of infection, host amoebae lost its cellular

integrity and *Legionella* egressed **(Figure 4.4 C-iii**). This demonstrates that thermally generated non-culturable injured cells exhibit intracellular proliferation within *A. polyphaga*. After 120 h of infection, the alive *Legionella* infected a higher number of *A. polyphaga* ≈ 23%, whereas the VBNC *Legionella* infected only ≈ 7% of *A. polyphaga*. Unlike alive and VBNC *Legionella* cells, the thermally inactivated *Legionella* (75°C for 10 min) were unable to proliferate within *A. polyphaga* cells. Furthermore, the differences with proliferation and infection rates suggest that cellular behaviour and pathogenicity of culturable and VBNC *Legionella* is not identical. Both assays clearly establish that TO-stained injured non-culturable cells are metabolically active and infect *A. polyphaga* and can be regarded as VBNC *Legionella*.

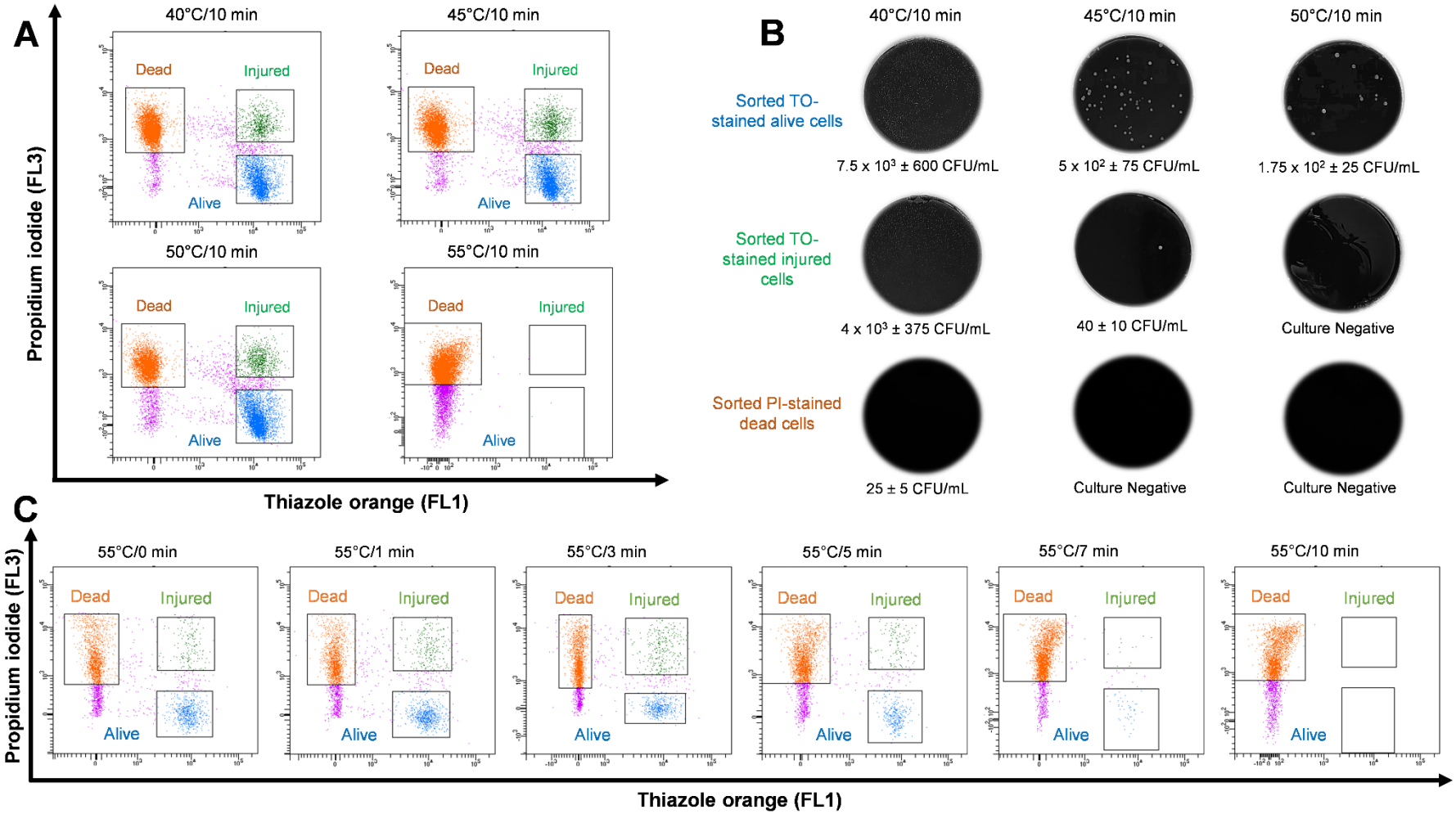

Figure 4.3: *L. pneumophila* thermal profile and culturability assay. A: Bacterial cells were heat shocked at 40°C to 55°C for 10 minutes and analysed using flow cytometry. B: Sorted cell fractions cultured on BCYE agar. Thermal shock at 50°C for 10 min transformed TO-stained injured cells into VBNC *Legionella*, as indicated by the lack of growth on the BCYE agar. C: Pure culture of *L. pneumophila* subjected to 55°C thermal treatment in increments from 0 to 10 min. The population of VBNC *L. pneumophila* increased gradually with time 9% at 1 min, 20% at 3 min and then decreased to 5% at 5 min.

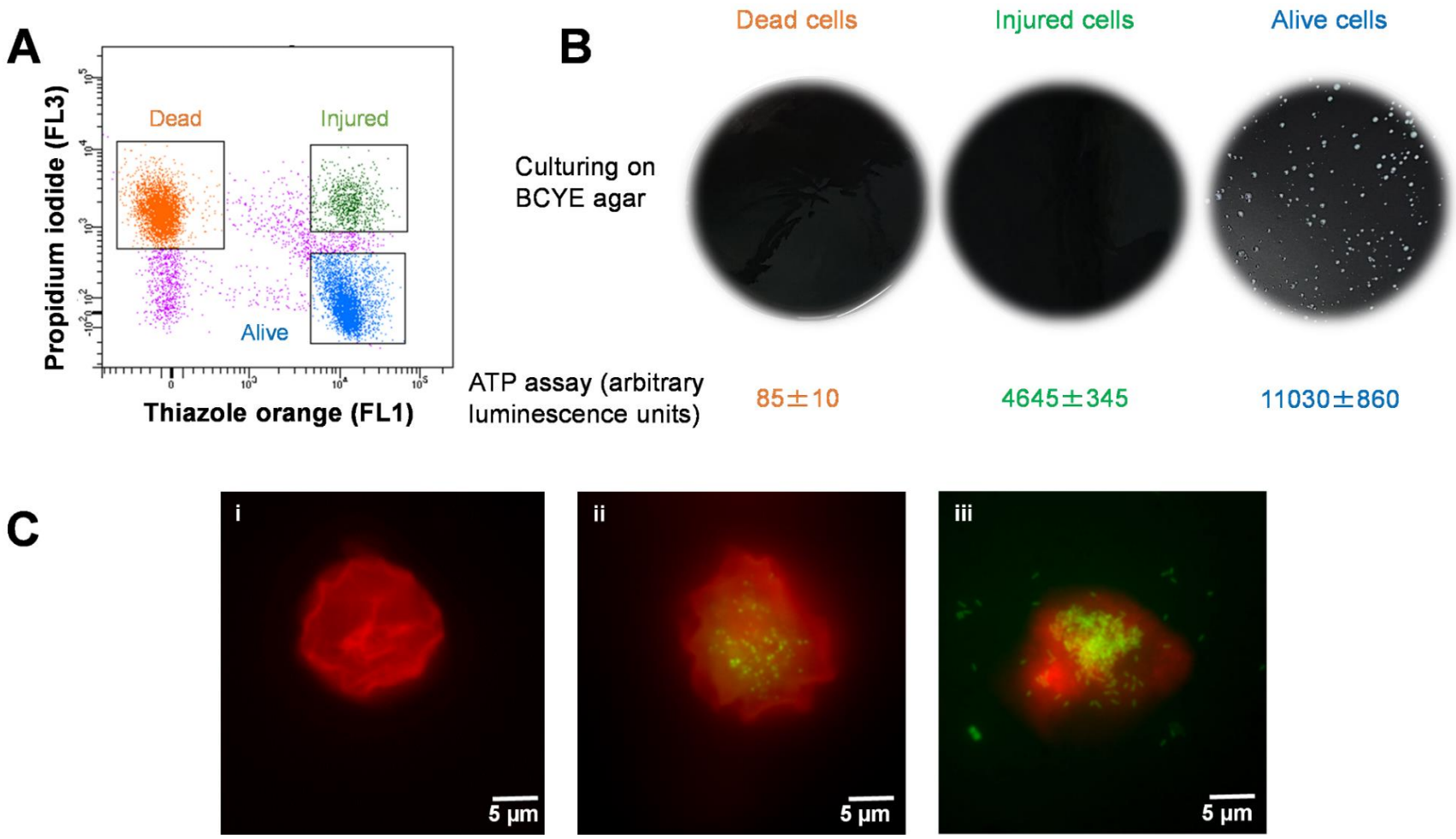

Figure 4.4: Characterization of thermally generated (50°C/10 min) VBNC *L. pneumophila*. A: Cytogram representing thiazole orange-stained alive (blue), thiazole orange-stained injured (green) and propidium iodide-stained dead (orange) cells generated by 50°C heat shock for 10 min. B: These three fractions were cultured on BCYE agar, and their ATP contents measured using a luciferase-based ATP estimation assay. C: FISH of *A. polyphaga* with Alexa Fluor 647 labelled EUK1209 (red) and *Legionella* with Alexa Fluor 488 labelled LEG705 (green) probes. C: i: uninfected *A. polyphaga*. C: ii and iii: These micrographs show intracellular proliferation of VBNC *Legionella* within *A. polyphaga* after seven and ten days of infection, respectively.

4.4.4 Detection and quantification of VBNC from *Legionella* spiked water samples

Four different water samples spiked with *L. pneumophila* were prepared and heat treated at 50°C for 15 mins. Standard culturing method confirmed that all samples were negative for culturable *Legionella*. Using the flow cytometry-cell sorting assay these water samples were examined. It was observed that water samples (sample 3 and 4) consisted of mixed bacterial population generated a large population of TO-stained injured cells and a smaller population of PI-stained dead cells (**Figure 4.5 A**). The qPCR results of injured cell fractions showed that water samples containing mixed bacterial populations harboured the highest amount of TO-stained injured *Legionella* cells (potentially VBNC cells ≈ 2 x 10^6 GU/L, **Figure 4.5 B**). It demonstrated that heat treatment of *Legionella* spiked water samples resulted in generation of VBNC *Legionella* and mixed bacterial cultures significantly increased the numbers of VBNC *Legionella*. This could potentially be due to the additional mixed bacteria cells providing a slight protection to the *Legionella* during the heat treatment or the mixed bacterial population harboured more biomass which increased the efficiency of DNA extraction and artificially increased the counts of *Legionella*. More importantly, in combination the PI/TO staining flow cytometry-cell sorting and qPCR assays can detect and quantify VBNC *Legionella* from potable water.

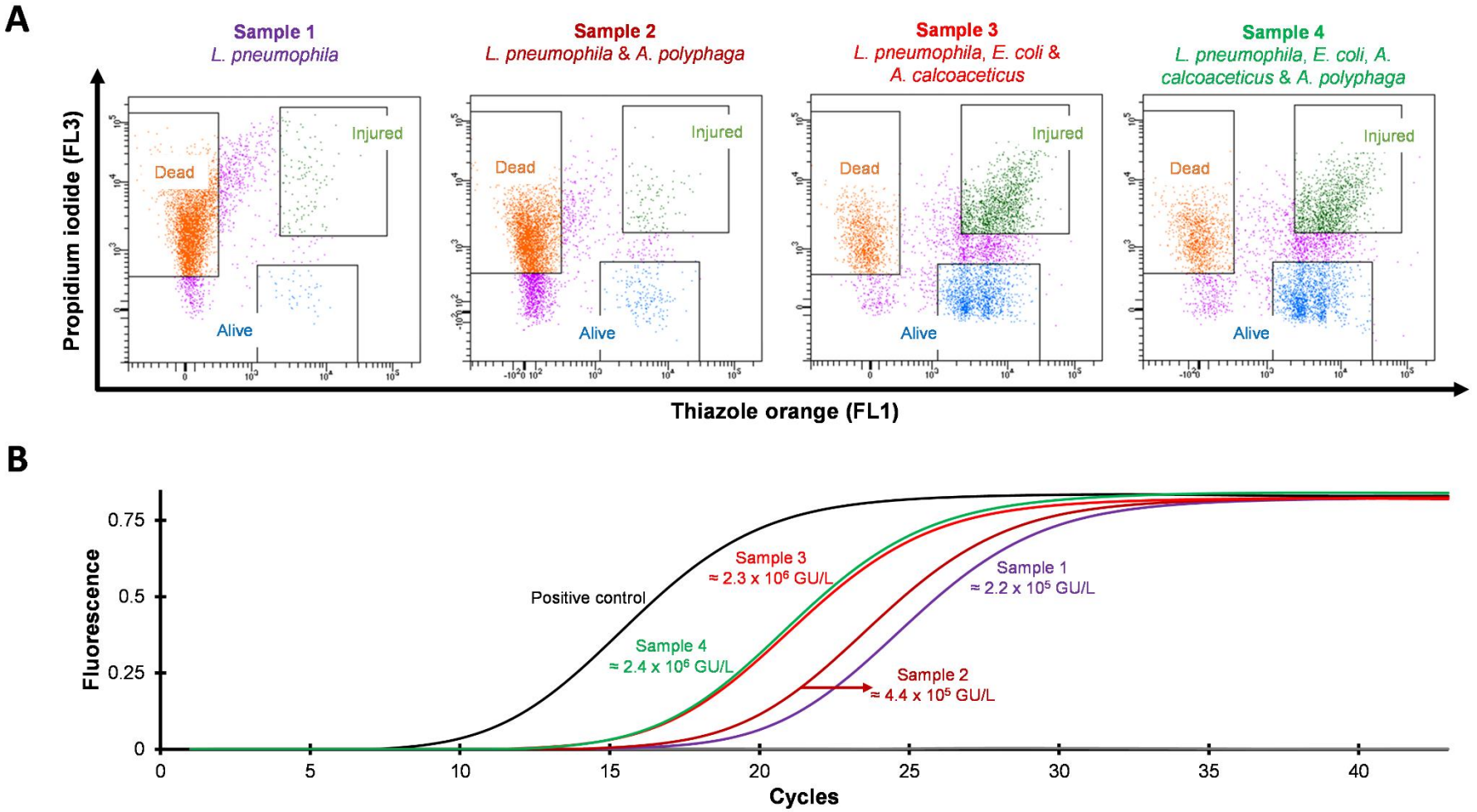

Figure 4.5: Detection and quantification of VBNC *L. pneumophila* from spiked water samples. All four spiked water samples were heated at 50°C for 15 min to generate VBNC *Legionella*. A: Cytograms of four different water samples spiked with 4.5 x 10^6 CFU/L *L. pneumophila*. Samples 3 and 4 generated the highest amount of VBNC cells. B: qPCR of VBNC (collected injured fraction) *Legionella* taken from the four samples.

4.4.5 Detection and quantification of VBNC *Legionella* from environmental samples

Hospital shower water samples were screened for *Legionella* using the Legiolert kit, standard *Legionella* culturing and qPCR assays. The two samples were selected that were negative for culturable *Legionella,* but the qPCR assay demonstrated the presence of *Legionella* DNA (Appendix-1, **Table 12.1** shows the concentrations of the different *Legionella* populations enumerated using each method). Furthermore, the amoebae coculture assay and FISH analysis confirmed that non-culturable *Legionella* present in both samples effectively propagate in *A. polyphaga* after 7 days of infection (≈ 2% infected cells) (**Figure 4.6**). To test efficacy of the VFC+qPCR assay both samples were stained with PI/TO dyes and analysed by flow cytometry and injured cell fractions were sorted out. **Figure 4.7 A** shows that both samples contained a significant amount of TO-stained injured cells, with environmental sample 1 having 1.47 x 10^5 TO-stained injured cells and sample 2 contained 7.4 x 10^4 TO-stained injured cells. DNA was extracted from the whole water sample and TO-stained injured cells and subjected to qPCR assay for the estimation of *Legionella* GU/L. **Figure 4.7 B** clearly shows the difference between *Legionella* GU/L of the whole water sample and TO-stained injured cells. Environmental sample 1 contained 3.9 x 10^4 GU/L *Legionella* whereas in TO-stained injured cells it was 3.2 x 10^3 GU/L. Similarly, in sample 2, the *Legionella* concentration was 3.5 x 10^4 GU/L in the whole sample whereas TO-stained injured cells contained 1 x 10^3 GU/L *Legionella*. The difference (1 to 1.5 Log_{10}) between *Legionella* genomic load of whole potable water sample and TO-stained injured cells indicated presence of dead *Legionella* or its residual DNA. These results challenge the efficacy and reliability of the culturing (ISO11731:2017-05) and standard qPCR assays (ISO/TS12869:2019).

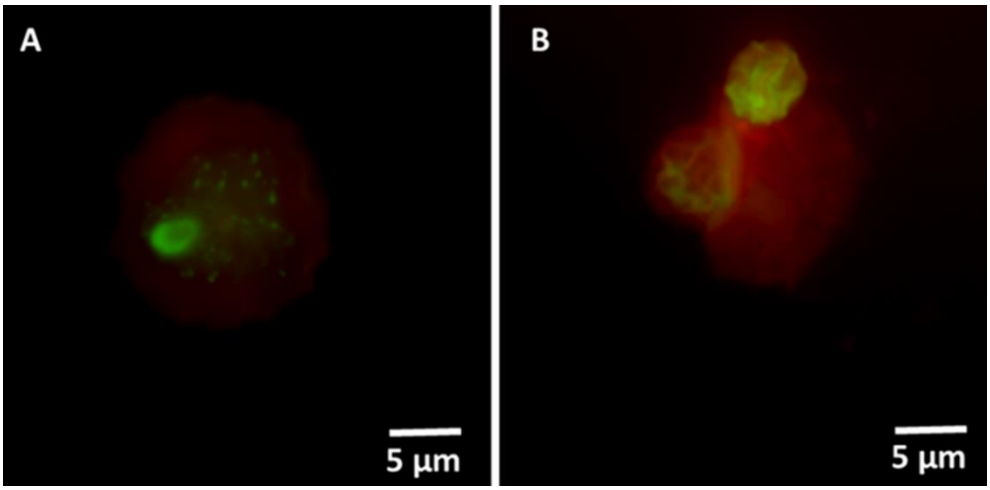

Figure 4.6: FISH of *A*. *polyphaga* stained with Alexa Fluor 647 labelled EUK1209 (red) and *Legionella* stained with Alexa Fluor 488 labelled LEG705 (green) probes. These micrographs show intracellular proliferation of environmental (shower water sample 1: A and sample 2: B) non-culturable *Legionella* within *A*. *polyphaga*.

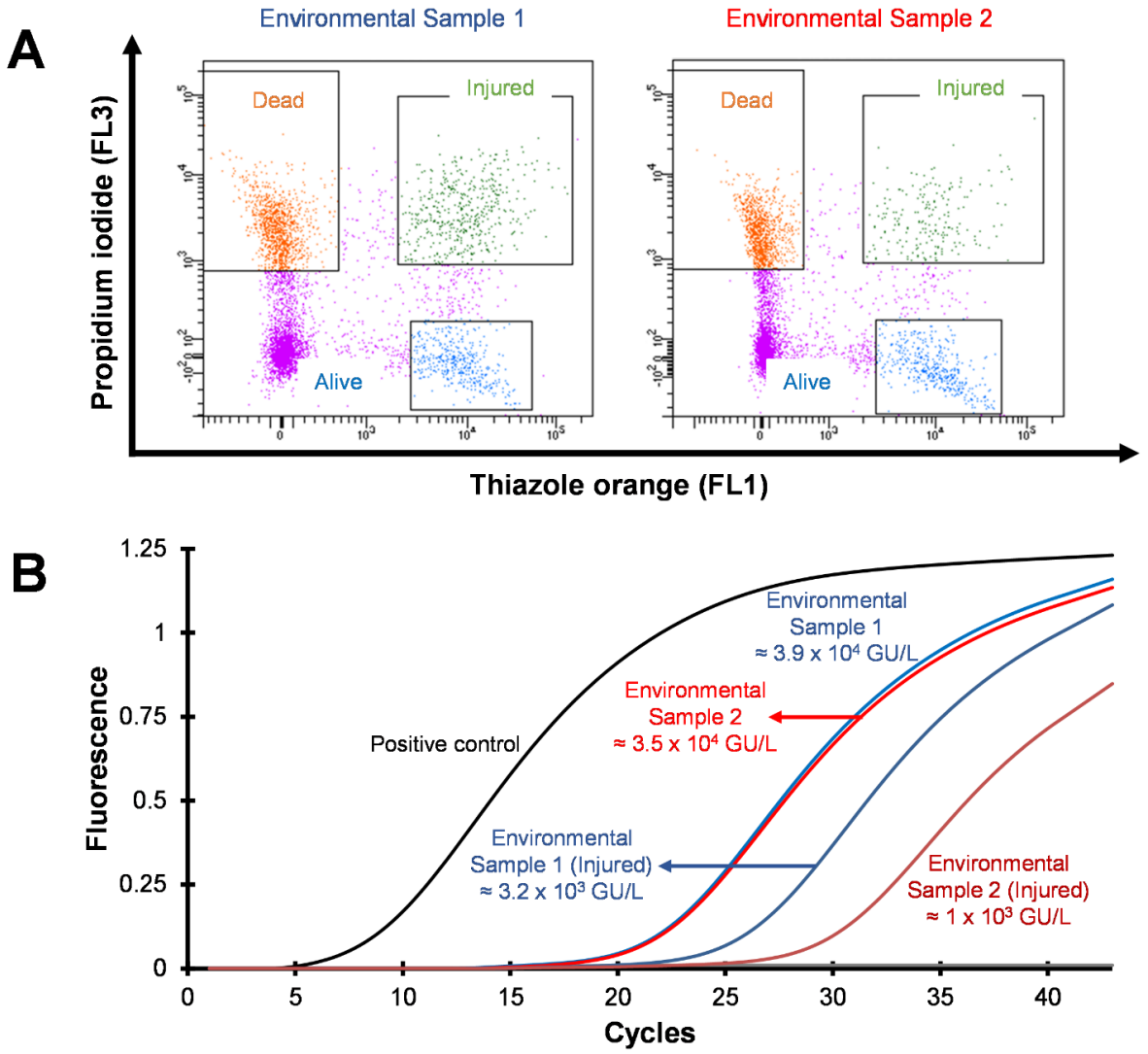

Figure 4.7: Detection and quantification of VBNC *L. pneumophila* from potable water samples. A: Cytograms of culture negative potable water samples. B: qPCR of whole water sample and VBNC *Legionella* and estimation of GU/L after viability cell sorting using flow cytometry shown in A. This image clearly demonstrates the difference in the concentration of *Legionella*, indicating presence of dead *Legionella* of its DNA in the water sample.

4.4.6 Discussion

VBNC state is a physiological condition in which *Legionella* lose its ability to grow on standard microbiological culture media but retain cellular viability and pathogenicity (Epalle et al., 2015). It is unclear whether the VBNC state is an adaptive response supporting survival and persistence of *Legionella* under unfavourable conditions or a form of cellular damage, which results in loss of culturability. This presents an important challenge in *Legionella* research, as it is vital to understand the role of VBNC *Legionella* within manufactured water systems to improve control and risk management strategies to prevent LD. One cause of long-term persistence of *Legionella* contamination in engineered water systems is regular reseeding of *Legionella* and VBNC *Legionella* from the supply/source water (Whiley et al., 2017, Nisar et al., 2020b). Common building water disinfection procedures including pasteurization,

chlorination, and chlorine dioxide treatment, induces and selects VBNC *Legionella* (Whiley et al., 2017, Allegra et al., 2008, Mustapha et al., 2015). Importantly, in hospital buildings thermal disinfection can act as a selection pressure that results in the persistence of heat resistant *Legionella* (Allegra et al., 2011). The method described in this study successfully detected VBNC *Legionella* from environmental water samples (hospital showers). The viability-based flow cytometry assay clearly discriminated *Legionella* into dead and viable fractions (**Figure 4.2 A**). The injured cells sorted in this assay, met the definition of VBNC *Legionella* as they were unculturable on standard BCYE agar (**Figure 4.4 B**); however, were still viable as determined by ATP quantification and the ability to infect amoebae hosts (**Figure 4.4 C**). These are accepted methods to measure viability, with ATP estimation kits widely used to discriminate metabolically active (alive or injured) cells from metabolically inactive (dead) cells (Allegra et al., 2008). Amoebae coculture assay is described as most suitable system to determine pathogenicity of VBNC *Legionella* (La Scola et al., 2001, Cervero-Arago et al., 2019). According to these results, the viability-based flow cytometry assay is an effective parameter to study behaviour and physiology of *Legionella* under different disinfection treatments.

Complete eradication of *Legionella* from manufactured water systems is not practically possible; therefore, environmental monitoring and risk assessment of *Legionella* on a regular basis are crucial to reduce the risk of legionellosis outbreaks. Despite recent advances in molecular biology and biotechnology, the culture based approach is regarded as the standard method to detect *Legionella* contamination in engineered water systems (International Organization for Standardization, 2017, Standards Australia, 2017). Importantly in engineered water systems, the majority of the *Legionella* population exists in the VBNC state (Diederen et al., 2007). Routine detection and quantification methods, culture based or qPCR assay, either over or underestimate *Legionella* contamination and do not provide information about VBNC *Legionella* (Whiley, 2016).

There are previously described alive/dead dye-based flow cytometry protocols used to differentiate between alive and VBNC *Legionella* (Braun et al., 2019, Allegra et al., 2008, Zacharias et al., 2015). However, these previous protocols are designed to study pure cultures of *Legionella* and are not designed for analysing environmental samples in which *Legionella* are present in complex microbial communities. When these previously described methods are applied to environmental samples they can differentiate the dead, VBNC, and alive total bacterial populations, but they cannot determine if *Legionella* is part of these populations (Ramamurthy et al., 2014, Li et al., 2014). One approach that has been previously used to overcome this limitation is the use of specific fluorogenic antibodies; however, this approach is currently strain specific and cannot be used to detect different environmental strains of

Legionella (Füchslin et al., 2010). In contrast, the method described in this paper (VFC+qPCR) can detect and quantify VBNC *Legionella* from environmental samples. It combines alive/dead dye-based flow cytometry with cell sorting of the separate dead, VBNC and alive bacterial populations followed by qPCR enumeration of *Legionella* spp. and *L. pneumophila* present in the VBNC and alive populations. This study used PI and TO dyes to differentiate the alive, dead and VBNC bacterial populations. The PI selectively stains dead cells with damaged cell membranes (Crowley et al., 2016). The TO dye is then used to characterize the cells which do not uptake the PI (Allegra et al., 2008, Alsharif and Godfrey, 2002). In contrast to widely used PMA/EMA-qPCR assay, here PI was used for discriminating dead cells and other cells were stained with only TO dye. Secondly, unlike PMA/EMA-qPCR assay the VBNC cells were selectively sorted out the processed for DNA extraction and qPCR assay (Taylor et al., 2014). This additional step removed unwanted dead cells and any inhibition associated with DNA binding dyes. A limitation of this approach is that PI has been shown to have reduce penetration of biofilm associated bacterial cells, which can lead to and overestimation of bacterial viability (Rosenberg et al., 2019).

This study explored the potential of thermal disinfection to induce the VBNC state. The findings demonstrated that culturability on the standard medium (BCYE and BCYE-GVPC agar) was the least reliable cellular viability indicator (**Figures 4.3 B** and **4.4 B**). In addition, the effect of the acid and heat pre-treated methods (prescribed in the ISO11731:2017-05 culture method) on *Legionella* recovery was examined. These pre-treatments are included in the standard culture method to selectively kill other environmental bacteria and prevent plates from becoming overgrown(International Organization for Standardization, 2017). However, this study (**Figure 4.1**), demonstrated that both selective decontamination methods statistically significantly reduced the recovery of culturable *Legionella* and analysis using this described method showed that this reduction was caused by the induction of the VBNC state. This could potentially explain some of the unreliability and lack of reproducibility often observed with the ISO11731:2017-05 method (Lucas et al., 2011, De Luca et al., 1999). Other factors which compromise the validity of culture-based techniques are sample holding time (McCoy et al., 2012) and residual disinfectant (Wiedenmann et al., 2001) present in collected water sample. In this study we examined the relationship between CFU/L vs GU/L for *L. pneumophila* qPCR (Appendix-1, **Figure 12.3**) and found they were comparable at concentrations less than 10^7 CFU/L or GU/L. The limit of detection for this described method (VFC+qPCR) when applied to the environmental water samples was 10^2 GU/L. This is comparable to the limit of detections for the standard ISO methods (> 10 CFU/plate for ISO11731:2017-05 and > 5GU/PCR reaction for ISO/TS12869:2019, theoretically equals to 10^2 CFU/L or GU/L for environmental samples).

In conclusion, this study describes a direct and rapid (results within 5-6 hours) assay to detect and quantify VBNC *Legionella* from potable water samples. This assay offers following advantages: (a) viable and dead *Legionella* quantification without culturing (b) quantification of viable and dead *Legionella*; and (c) rapid and direct screening of VBNC *Legionella*.

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Author contributions

MAN, HW and RB conceived and designed research. MAN performed the experiments. GB and MAN conducted flow cytometry-cell sorting assay. KER, MHB, HW and RB provided technical assistance. MAN, and HW drafted and edited the manuscript, HW, KER, MHB, GB and RB corrected and contributed to the manuscript. All authors approved the final manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Legionella and free-living amoebae

Naturally, *Legionella* are intracellular parasites of free-living amoebae. This chapter addresses objectives 2 to 5 and explores the relationship between *Legionella* and free-living amoebae in engineered water systems. It consists of two published articles. The first is a systematic literature review article describing relationship between *Legionella* and free-living amoebae in hospital and domestic water systems (objective 2). The second manuscript is a research article in which prevalence of *Legionella* and free-living amoebae in an Australian hospital, and domestic water systems is studied (objective 3 to 5).

5 *Legionella pneumophila* and protozoan hosts: Implications for the control of hospital and potable water systems

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5.1 Abstract

Legionella pneumophila is an opportunistic waterborne pathogen of public health concern. It is the causative agent of Legionnaires' disease (LD) and Pontiac fever and is ubiquitous in manufactured water systems, where protozoan hosts and complex microbial communities provide protection from disinfection procedures. This review collates the literature describing interactions between *L. pneumophila* and protozoan hosts in hospital and municipal potable water distribution systems. The effectiveness of currently available water disinfection protocols to control *L. pneumophila* and its protozoan hosts is explored. The studies identified in this systematic literature review demonstrated the failure of common disinfection procedures to achieve long term elimination of *L. pneumophila* and protozoan hosts from potable water. It has been demonstrated that protozoan hosts facilitate the intracellular replication and packaging of viable *L. pneumophila* in infectious vesicles, whereas cyst-forming protozoans provide protection from prolonged environmental stress. Disinfection procedures and protozoan hosts also facilitate biogenesis of viable but non-culturable (VBNC) *L. pneumophila* which have been shown to be highly resistant to many water disinfection protocols. In conclusion, a better understanding of *L. pneumophila*-protozoan interactions and the structure of complex microbial biofilms is required for the improved management of *L. pneumophila* and the prevention of LD.

5.2 Introduction

Legionella pneumophila is an opportunistic pathogen associated with community acquired and nosocomial infections. It is the causative agent of legionellosis which includes Legionnaires' disease (LD), a severe atypical pneumonia infection, and Pontiac fever, an acute "flu-like" illness (Cunha et al., 2016). Globally, the incidence of LD has been increasing. In Europe, the number of notified cases increased from 4921 in 2011 to 11343 in 2018 (The European Legionnaires' disease Surveillance Network and The European Centre for Disease Prevention and Control, 2019). In the US, the number of notified LD cases has increased from 2301 in 2005 (Smith et al., 2007) to 7104 in 2018 (National Notifiable Diseases Surveillance System and Centers for Disease Control and Prevention, 2018), a 300% increase. Globally, the fatality rate of LD ranges from 2.2 to 10.3%, with the lowest in Singapore and the highest in European countries (Phin et al., 2014). In nosocomial outbreaks the fatality rate can reach up to 48% (O'Mahony et al., 1990, Soda, 2017, Mercante and Winchell, 2015b).

The genus *Legionella* is comprised of 60 species and 80 distinct serogroups (Miyashita et al., 2020). Globally, *L. pneumophila* is the primary aetiological agent of LD. In Europe and the US, *L. pneumophila* serogroup (sg1) is responsible for 70 to 92% reported cases (Mercante and

Winchell, 2015). According to World Health Organization (WHO), 20 to 30% infections are caused by other *L. pneumophila* serogroups and only 5 to 10% are caused by other *Legionella* species (*L. micdadei*, *L. bozemanii*, *L. dumoffii* and *L. longbeachae*) (Bartram et al., 2007). However, unlike rest of the world, in Australia and New Zealand *L. longbeachae* is associated with ≈ 50% reported cases of legionellosis (National Notifiable Diseases Surveillance System Annual Report Working Group, 2019, Graham et al., 2012).

L. pneumophila is ubiquitous in manufactured water systems (Bartram et al., 2007) and in the USA has been identified as the primary cause of all potable water related outbreaks (Benedict et al., 2017). Manufactured water systems, building plumbing systems, recreational water, cooling towers and humidifiers are major sources of *L. pneumophila* (Bartram et al., 2007). Inside these plumbing structures, *Legionella* and protozoan hosts are incorporated within biofilms. Factors like water stagnation, higher levels of organic carbon and moderate temperatures can increase the rate of biofilm formation (Liu et al., 2006, Abdel-Nour et al., 2013). Transmission occurs through inhalation or aspiration of contaminated aerosols or water (Prussin et al., 2017). *L. pneumophila* maintains long term contamination of manufactured water systems through its growth within protozoan hosts, association with biofilms and disinfectant resistance or tolerance (Ashbolt, 2015, Falkinham III et al., 2015b). Freshwater amoebae are the natural eukaryotic hosts of *Legionella*, whereas humans are considered accidental hosts (Boamah et al., 2017). In the human body, *Legionella* contaminated aerosols are inhaled into the lungs and phagocytosed by alveolar macrophages. The alveolar macrophages behave like amoebae hosts and facilitate the intracellular division and propagation of *Legionella*, resulting in LD (Newton et al., 2010, Oliva et al., 2018).

Understanding the interactions between *L. pneumophila* and protozoan hosts is essential to inform water treatment and risk management strategies for the prevention of LD. Protozoan hosts play an important role in the ability of *L. pneumophila* to survive exposure to physiochemical and environmental stresses. Protozoans facilitate the intracellular replication and packaging of live bacterial cells in the stress resistant membrane bound infectious export vesicles (Bouyer et al., 2007, Koubar et al., 2011). The cysts of cyst-forming amoebae provide a protective shelter from prolonged environmental stress (Jjemba et al., 2015). There are numerous reports describing existence of *L. pneumophila* harbouring within protozoans from thermally-, chemically-, and UV radiation-treated potable water supplies and storage reservoirs (Kim et al., 2002). Protozoan hosts and environmental stress may facilitate the genesis of highly resistant and potentially infectious viable but non-culturable (VBNC) *L. pneumophila* (Casini et al., 2018, Buse et al., 2013). Importantly, water storage facilities and distribution networks of many countries have been shown to be highly contaminated with protozoans that

may act as hosts for *L. pneumophila* (> 0 to 4500 cell/L cell density) (Thomas and Ashbolt, 2011).

This systematic literature review collated studies which detected *L. pneumophila* in association/connection with protozoan hosts from hospital or municipal potable water distribution systems and discusses this relationship under diverse environmental conditions. The effectiveness of different physical and chemical water treatment methods to control the *L. pneumophila* and its protozoan hosts is described and implications for the control and management of these water distribution systems is explored.

5.3 Materials and Methods

The databases Scopus and Web of Science were searched for articles written in English containing the keywords (*Legionella pneumophila OR L. pneumophila*) AND (*Acanthamoeba* OR *Vermamoeba* OR *Hartmannella* OR *Dictyostelium* OR *Naegleria* OR *Tetrahymena* OR *Echinamoeba* OR *Paramecium* OR *Balamuthia* OR *Oxytricha* OR *Stylonychia* OR *Diphylleia* OR *Stenamoeba* OR *Singhamoeba* OR *Filamoeba* OR Protozoa OR Protozoan OR Amoeba). The above search terms were modified from the review conducted by Boamah et al. (2017). **Figure 5.1** presents the systematic approach to article inclusion or exclusion. Articles were screened by reading the titles and abstracts and initially excluded if they did not refer to a study that detected *L. pneumophila* and a potential protozoan host from a hospital or potable/drinking water source. Articles were then read in full and excluded if they only described laboratory based simulated or pilot-scale experiments on registered bacterial and protozoan strains.

5.4 Results

One thousand two hundred and seventy abstracts were obtained from the Web of Science and SCOPUS. After applying the described criteria (see **Figure 5.1** and **Section 5.3** Materials and Methods), twenty-nine research manuscripts discussing *L. pneumophila* and its protozoan hosts in hospital and potable water systems were included in the study (**Table 5.1**). Potential protozoan hosts playing crucial role(s) in the *L. pneumophila* life cycle and living in both types of water systems are compiled in **Table 5.2**. These protozoan hosts have the potential to provide an appropriate habitat for replication and survival of *L. pneumophila*.

The articles from hospital settings showed that *L. pneumophila* serogroup 1 (hereafter sg1) is the most common serogroup causing infection in USA and European countries. Globally, *L. pneumophila* sg1 is also associated with community acquired legionellosis (Yu et al., 2002, Beaute et al., 2013). However, a limitation was that most municipal potable water supply studies did not characterize the *L. pneumophila* serogroups. To investigate the different *L.*

pneumophila-protozoan interactions, some studies used co-isolation and coculturing techniques or PCR. Other approaches included techniques like scanning electron microscopy or DVC-FISH to demonstrate the fate of internalized bacteria. The electron microscope studies conducted in hospital settings found that *L. pneumophila* sg1 is able to multiply inside *Echinamoeba exudans* (Fields et al., 1989) and *Vermamoeba vermiformis* (formerly *Hartmannella vermiformis*) (Nahapetian et al., 1991). Likewise, PCR-based examination of potable water also demonstrated the presence of *L. pneumophila* inside *V. vermiformis* (Garcia et al., 2013). Another study used DVC-FISH to detect intracellular *L. pneumophila* inside *Acanthamoeba* and *V. vermiformis* from a potable water supply (Moreno et al., 2019). Other studies (mentioned in the **Table 5.1**) demonstrated the co-existence of free-living *L. pneumophila* and protozoan hosts but did not characterize the specific interaction or fate of internalized bacteria. The systematic literature review identified a more diverse number of potential protozoan hosts from hospitals compared with municipal potable water systems. This could be due to the more diverse dynamics of hospital water distributions systems (**Table 5.2**). The hosts identified in the hospital settings consisted of three phyla, five classes and twelve genera, whereas the hosts isolated from potable water consisted of only two phyla, three classes and five genera. Two genera of Amoebozoa namely, *Vermamoeba* and *Acanthamoeba*, are frequently reported from both types of facilities as potential hosts. Available literature demonstrated that non-cyst forming, and ciliated protozoans can also be potential hosts for *L. pneumophila*. Most of the studies were designed specifically to explore the interactions between *L. pneumophila*-*Vermamoeba*/*Acanthamoeba*, and the diversity and the role of other possible protozoans were not investigated.

In the studies identified, diverse physical and chemicals methods were used to disinfect the hospital and municipal potable water systems. Chlorination (< 0.05 to < 4 mg/L) using different chlorine compounds was frequently reported as being used in both settings. Protozoans and *L. pneumophila* could still be isolated from both hospital and municipal potable water systems despite chlorination (< 0.05 to < 4 mg/L), and/or ozonisation and thermal (< 50 to 70°C) disinfection protocols being in place. Importantly, several studies from hospital settings reported regular outbreaks of legionellosis. This represents a failure of existing disinfection protocols. The systematic literature review revealed that *L. pneumophila*-*Acanthamoeba*/*Vermamoeba* were extensively co-isolated from chlorinated and thermally treated water. This demonstrates the potential tolerance of *L. pneumophila* and protozoan hosts to survive under a wide range of disinfection conditions.

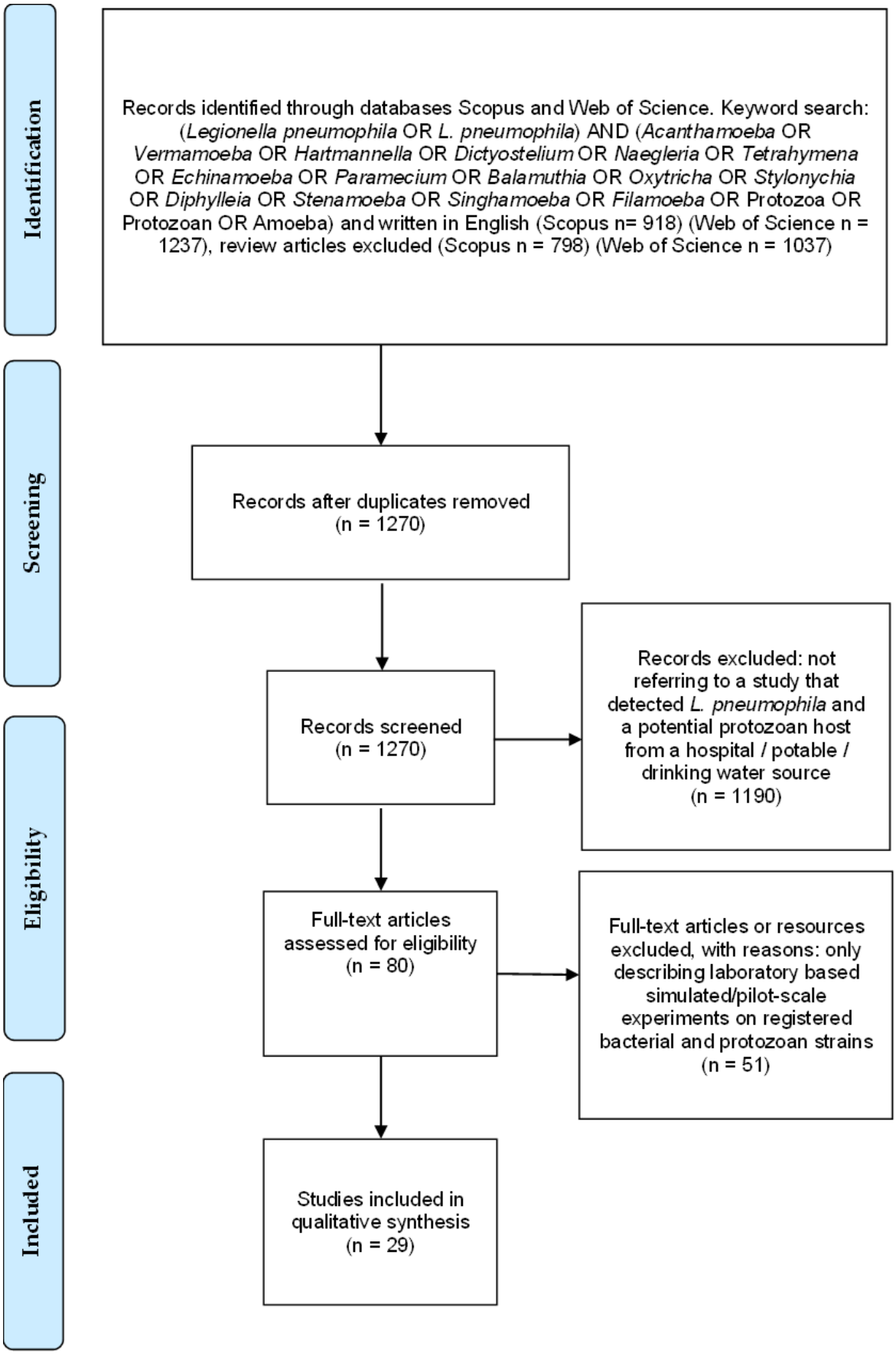

Figure 5.1: Overview of search methods and articles inclusion and exclusion criteria**.**

Table 5.1: Potential protozoan hosts of *Legionella pneumophila* isolated from and hospital and potable water systems.

Isolation Source (Temperature at time of sampling)	Water Treatment Method	*L. pneumophila*		Potential Protozoan Host		Comments	Country of Origin (Sampling site)	Reference
		Identification Method	Serogroup Sequence-Type	Genus/ Species	Identification Method			
Hospital Settings								
Hot (45 – 52°C) water tanks	-	Culturing, coculture assay and serological identification	sg1	*Hartmannella cantabrigiensis* *Vermamoeba vermiformis* *Echinamoeba exudans*	Culturing, light and transmission electron microscopy	Nosocomial legionellosis investigation	USA	(Fields et al., 1989)
Potable water sites (39 – 40°C)	-	Culturing and monoclonal antibody-based serotyping	sg1	*Acanthamoeba hatchetti* *Hartmannella cantabrigiensis* *Vermamoeba vermiformis* *Vahlkampfia* *Filamoeba nolandi* *Comandonia operculata* *Paravahlkampfia ustiana*	Culturing and light microscopy	Nosocomial legionellosis investigation Thermal treatment (70°C) and chlorination (1.5 – 2.0 mg/L) controlled the bacteria for 6 months but not amoebae. The treatment reduced incidence of legionellosis	South Dakota, USA	(Breiman et al., 1990)
Cooling tower, humidifier, hot water tank and supply	-	Culturing and coculture assay	-	*Vermamoeba vermiformis* *Naegleria*	Culturing, light and transmission electron microscopy	-	Paris, France	(Nahapetian et al., 1991)
Hot (39 – 60°C) and cold-water supply	-	Culturing (observed detection range: 1 x 10^3 – 9.7 x 10^4 CFU/L), direct fluorescent antibody and monoclonal antibody-based serotyping	sg1 sg5	*Hartmannella* (Hartmannellidae / limax amoebae)	Culturing and light microscopy	Post nosocomial outbreak surveillance	Halifax, Nova Scotia, Canada	(Bezanson et al., 1992)
Organ transplant unit hot (mean 56.2°C) and cold water (mean 16.6°C) supplies	-	Culturing and serological assay	sg1	*Acanthamoeba* *Hartmannella* *Echinamoeba* *Vahlkampfia* *Tetrahymena* *Vannella*	Culturing and light microscopy	Population density of amoebae was greater in hot water supplies than cold water supplies Along amoebae other diverse eukaryotic microbes were detected as well	UK	(Patterson et al., 1997)
Water supplies	Thermal treatment (60 and 70°C)	Culturing (*Legionella* observed detection range: 2.89 – 6.74 x 10^5 CFU/L), coculture, latex agglutination, indirect and immunofluorescence assays, and PFGE	sg1 sg2	*Acanthamoeba* *Vahlkampfia* *Mayorella*	Culturing and light microscopy	Thermal treatment (70°C) only controlled bacterial contamination for 3 months SG1 is more thermotolerant than SG2 at 60°C	Germany	(Steinert et al., 1998)
Water network system (mean 56°C)	-	Amoebae coculture assay, PCR, and sequencing	-	*Vermamoeba vermiformis*	Culturing, PCR and sequencing	Detection of thermotolerant *Vermamoeba vermiformis*	Lausanne, Switzerland	(Thomas et al., 2006)
Water distribution system (18.9 – 32.6°C)	Chlorine dioxide treatment Thermal treatment (< 50°C)	Culturing (observed detection range*: L. pneumophila* SG1: 1 x 10^2 – 3.5 x 10^4 CFU/L and *L. pneumophila* SG2-14: 1 x	sg1 sg2-14	*Acanthamoeba* *Hartmannella*	Culturing and light microscopy	-	Messina, Italy	(Lagana et al., 2014)

Isolation Source (Temperature at time of sampling)	Water Treatment Method	*L. pneumophila*		Potential Protozoan Host		Comments	Country of Origin (Sampling site)	Reference
		Identification Method	Serogroup Sequence-Type	Genus/ Species	Identification Method			
		10^2 – 4 x 10^4 CFU/L) and latex agglutination assay						
Tap water	Chloramine (1.93 ± 1.04 mg/L) treatment	Culturing (protocol: ISO 11731-2:2004, LOQ: 10 – 300 CFU/plate, observed detection range: 100 – 1.4 x 10^5 ± 1.3 x 10^5 CFU/L), qPCR (LOD: 5 GU, LOQ: 25 GU, *Legionella* observed detection range: 100 – 10^9 gu/L) and EMA-qPCR	ST269	*Acanthamoeba polyphaga*	Culturing, light microscopy and PCR		Italy	(Casini et al., 2018)
Cold (14.9°C) and warm (45.1°C) potable water	Thermal treatment, chlorination (hypochlorates, chloramine), bacterial filters and chlorine dioxide treatment	Culturing (protocols: ISO 11731:1998 and ISO 11731-2:2004, LOQ: 10 – 300 CFU/plate, observed detection range: 0 – 3 x 10^3 CFU/100 mL) and MALDI-TOF MS	-	*Acanthamoeba* *Vermamoeba vermiformis*	Culturing and light microscopy	-	Bratislava, Slovakia	(Trnkova et al., 2018)
Cold water system (20 – 27.3°C)	Chlorine contents 0.01-0.32 mg/L	qPCR (protocol: ISO/TS 12869:2012, LOD: 5 GU, LOQ: 25 GU, observed detection range: 2.7 – 3.8 x 10^2 gu/L)	-	*Acanthamoeba* *Vermamoeba vermiformis*	Culturing and light microscopy		Johannesburg, South Africa	(Muchesa et al., 2018)
Dental unit waterlines	H_2O_2 treatment (occasionally)	Heterotrophic plate counts, culturing (protocol: ISO 11731-2:2004, LOQ: 10 – 300 CFU/plate, observed detection range: 0 – 2700 CFU/L) and agglutination test	-	*Vermamoeba vermiformis*	Culturing, light microscopy, PCR and sequencing		Italy	(Spagnolo et al., 2019)
Potable Water System								
Unchlorinated water supplies (9.5 – 13.5°C)	-	qPCR	-	*Acanthamoeba* *Acanthamoeba polyphaga* *Vermamoeba vermiformis*	qPCR (LOD: 1 cell/reaction), T-RFLP, cloning and sequencing	Along amoebae other diverse eukaryotic microbes were detected as well	Netherlands	(Valster et al., 2009)
Ground water supplies (5 – 39°C)	Aeration, limestone, granular activated carbon slow sand and rapid sand filtration, ozonisation, and pellet softening	Culturing, biofilm batch test and qPCR	-	*Acanthamoeba* *Vermamoeba vermiformis*	18S rDNA sequencing, PCR, T-RFLP and sequencing	Along amoebae other eukaryotic microbes were detected as well	Netherlands	(Valster et al., 2010)
Water supplies (mean 30°C)	Reverse osmosis, distillation (82%), chlorination (<0.005 - 0.2 mg/L), dolomite, limestone	Culturing (LOD: 250 CFU/L, *Legionella* observed detection range: 2.5 x 10^2 – 2.5 x 10^5 CFU/L) and latex agglutination assay	-	*Acanthamoeba* *Vermamoeba vermiformis* *Echinamoeba exundans* *Echinamoeba thermarum* *Neoparamoeba*	qPCR (LOD: 2 C/L, observed detection range: *Acanthamoeba* < 2 – 56 C/L and *V. vermiformis* < 2 – 1670 C/L)	-	Caribbean islands, Leeward Antilles	(Valster et al., 2011)

Isolation Source (Temperature at time of sampling)	Water Treatment Method	*L. pneumophila*		Potential Protozoan Host		Comments	Country of Origin (Sampling site)	Reference
		Identification Method	Serogroup Sequence-Type	Genus/ Species	Identification Method			
	and granular activated carbon filtration, fluoride addition (0.3 - 0.7 mg/L), UV treatment (7.5 - 35.99 mJ/cm^2)							
Water distribution systems (mean 37.3 ± 8.4°C)	Chloramine treatment (Chlorine contents 1.8 mg/L), flocculation, sedimentation, and dual-medium filtration	Culturing, qPCR (LOQ: 1 – 10 copies/reaction, maximum observed detection limit: 13.7 ± 5.1 gc/mL) and T-RFLP	-	*Acanthamoeba* *Vermamoeba vermiformis*	qPCR (LOQ: 1 – 10 copies/reaction, maximum detection limit: *Acanthamoeba* 6.8 ± 2.9 gc/mL and *V. vermiformis* 7.1 x 10^4 ± 4.4 x 10^3 gc/mL)	High concentration of chloramine is unable to disinfect water	Southwest Virginia, USA	(Wang et al., 2012a)
Water treatment plant (7 – 21°C)	-	Multiplex PCR	-	*Vermamoeba vermiformis*	Culturing, light microscopy, PCR and sequencing	Amoebae were frequently detected at 17°C	Aragon, Spain	(Garcia et al., 2013)
Water treatment facility (25 ± 3.4 – 28.2 ± 1.1°C)	-	PCR (*Legionella* observed detection range: 1.2 x 10^4 – 2.4 x 10^5 gc/L) and sequencing	-	*Acanthamoeba* *Vermamoeba vermiformis* *Naegleria*	Culturing, PCR, qPCR (observed detection range: *Acanthamoeba* 2.1 x 10^2 – 7.7 x 10^2 gc/L and *Naegleria* 7.6 x 10^2 – 9.4 x 10^2 gc/L) and sequencing	-	Kaoping River, Taiwan	(Ji et al., 2014)
Sediments of municipal water storage tank (2.2 – 28.9°C)	Chlorination (< 4 mg/L)	qPCR (LOD: 2 CE/reaction, *Legionella* observed detection range: 51 ± 114 – 7.98 x 10^4 ± 2.49 x 10^4 CE/g), cloning and sequencing	sg1	*Acanthamoeba* *Vermamoeba vermiformis*	qPCR (LOD: 2 CE/reaction, observed detection range: *Acanthamoeba* 22 ± 50 – 391 ± 243 CE/g and *V. vermiformis* 17 ± 23 CE/g), cloning and sequencing	-	Northeast, East Coast, Midwest, South and West Coast, USA	(Lu et al., 2015)
Water distribution system	-	qPCR (LOD: 2 CE/reaction *Legionella* observed detection range: 2 ± 4 – 391 ± 17 CE/L), cloning and sequencing	-	*Acanthamoeba* *Acanthamoeba castellanii* *Vermamoeba vermiformis*	qPCR (LOD: 2 CE/reaction, observed detection range: *Acanthamoeba* 1 ± 2 – 16 ± 2* CE/L and *V. vermiformis* 1 ± 1 – 9 ± 11* CE/L), cloning and sequencing	-	USA	(Lu et al., 2016)
Domestic water systems (mean 20.6 ± 3.8°C)	-	Culturing, coculture assay, PCR, and sequencing	-	*Vermamoeba vermiformis*	Culturing, light microscopy, PCR, and sequencing	-	Geneva, Lausanne and Sion, Switzerland	(Lienard et al., 2017)
Sediments of water storage tank	-	qPCR (observed detection range: 25 ± 51 – 300 ± 38 gn/g) and NGS	-	*Acanthamoeba* *Vermamoeba vermiformis*	qPCR (observed detection average: *Acanthamoeba* 3 – 7 gn/g, *V. vermiformis* 99 ± 43 – 120 ± 60 gn/g) and NGS	-	Ohio, West Virginia and Texas, USA	(Qin et al., 2017)
Potable water	Polyaluminium chloride coagulation, sedimentation, sand and biologically	qPCR (LOQ: 1 – 10 copy/reaction, observed minimum detected quantity: 3.5 log(gc)/mL)	-	*Acanthamoeba* *Vermamoeba vermiformis*	qPCR (LOD: 1 – 10 copy/reaction, minimum detection limit: 2 log(gc)/mL for *V. vermiformis* and 4	Antibiotics (sulfadiazine and ciprofloxacin) promote growth of both bacterium and amoebae	Northern China	(Wang et al., 2018b)

Isolation Source (Temperature at time of sampling)	Water Treatment Method	*L. pneumophila*		Potential Protozoan Host		Comments	Country of Origin (Sampling site)	Reference
		Identification Method	Serogroup Sequence-Type	Genus/ Species	Identification Method			
	activated carbon filtration and chlorination				log(gc)/mL for *Acanthamoeba*) and sequencing			
Potable water	Polyaluminium chloride coagulation, sedimentation, sand and biologically activated carbon filtration, chlorination, and ozonisation	qPCR (LOQ: 1 – 10 copies/reaction, observed minimum detection limit ≈ 1 log(gc)/g)	-	*Acanthamoeba* *Naegleria*	qPCR (LOQ: 1 – 10 copies/reaction, observed minimum detection limit: ≈ 0.5 log(gc)/g for *Naegleria* and ≈ 1 log(gc)/g for *Acanthamoeba*)	Combined chlorination and ozonisation are effective than chlorination only	Northern China	(Wang et al., 2018a)
Potable water	Coagulation, ozonisation, pellet softening, granular activated carbon filtration, rapid and slow sand filtration	Heterotrophic plate counts, culturing (protocol: NEN 6275, LOD: 1 log(CFU)/cm^2) epifluorescence microscopy, bioluminescence assay, PCR and sequencing	-	*Vermamoeba vermiformis*	qPCR (observed detection range: 0.7 – 384 CE/cm^2)	-	Netherlands	(van der Kooij et al., 2018)
Residential secondary water supply systems (13.9 ± 4.0 – 17.4 ± 2.9°C)	Chloramine treatment (Chlorine contents 0.19-0.89 mg/L)	qPCR (LOQ: 10 copies/reaction, observed maximum detection limit: ≈ 10^2 gc/mL) and sequencing	-	*Acanthamoeba* *Vermamoeba vermiformis*	qPCR (LOQ: 10 copies/reaction, observed detection range: 10^1 – 10^3 gc/mL for both *Acanthamoeba* and *V. vermiformis*) and sequencing	-	Shanghai, China	(Li et al., 2018)
Water treatment facility	Coagulation, sedimentation, chlorination, ozonisation, granular activated carbon, and sand filtration	qPCR (LOQ: 10 copies/reaction, observed minimum detection limit: 10^2 log(gc)/mL) and sequencing	-	*Vermamoeba vermiformis*	qPCR (LOQ: 10 copies/reaction) and sequencing	Sand filtration after granular activated carbon treatment improves water quality	Southeast China	(Wang et al., 2019)
Water from private wells after flood	-	Culturing (protocol: ISO 11731, LOQ: 10 – 300 CFU/plate) and qPCR (LOQ: 9.5 gc/mL, observed maximum detection limit: 52.4 gc/mL)	-	*Naegleria fowleri*	qPCR (observed detection range: 11 – 610 gc/mL)	-	Louisiana, USA	(Dai et al., 2019)
Potable water	-	Culturing and DVC-FISH	-	*Acanthamoeba* *Vermamoeba vermiformis*	Culturing and PCR	-	Valencia, Spain	(Moreno et al., 2019)

Vermamoeba vermiformis was previously known as *Hartmannella vermiform*; *Paravahlkampfia ustiana* was previously known as *Vahlkampfia ustiana*; Observed (minimum/maximum) detection range/quantity: amount of bacteria/amoebae experimentally determined from the samples

Table Abbreviations: CFU/L: colony forming unit/liter; PFGE: pulsed-field gel electrophoresis; PCR: polymerase chain reaction; ISO: International organization for standardization; MALDI-TOF MS: matrix assisted laser desorption ionization-time of flight mass spectrometry; qPCR: quantitative PCR; gu/L: genome unit/litre; LOQ: limit of quantification; LOD: limit of detection; EMA-qPCR: ethidium monoazide-qPCR; T-RFLP: terminal-restriction fragment length polymorphism; C/L: cells/litre; gc/mL: gene copy/millilitre; gc/L: gene copy/litre; CE/reaction: cell equivalent/reaction; CE/g: cell equivalent/gram; CE/L: cell equivalent/litre; * CE/L: cyst equivalent/litre; gn/g: genome copy number/gram; gc/g: gene copy/gram; NGS: next generation sequencing; NEN: Nederlands normalisatie instituut; CE/cm^2: cell equivalent/cm^2; DVC-FISH: direct viable count combined with fluorescence *in situ* hybridization

Table 5.2: Taxonomic description of potential protozoan hosts.

Hospital Settings	Potable Water System
Phylum: Amoebozoa	**Phylum: Amoebozoa**
Class: Tubulinea Genera: *Vermamoeba*, *Echinamoeba*, *Hartmannella*, *Filamoeba*	**Class: Tubulinea** Genera: *Vermamoeba*, *Echinamoeba*
Class: Discosea Genera: *Acanthamoeba*, *Comandonia*, *Mayorella*, *Vannella*	**Class: Discosea** Genera: *Acanthamoeba*, *Neoparamoeba*
Class: Heterolobosea Genera: *Vahlkampfia*, *Paravahlkampfia*	
Phylum: Percolozoa	**Phylum: Percolozoa**
Class: Heterolobosea Genus: *Naegleria*	**Class: Heterolobosea** Genus: *Naegleria*
Phylum: Ciliophora	
Class: Oligohymenophorea Genus: *Tetrahymena*	

Table 5.3: Efficacy of available potable water disinfection protocols on *Legionella pneumophila* and host protozoans.

Organisms	Disinfectant dose					
	Temperature (°C)	**Chlorine (mg-min/L)**	**Monochloramine (mg-min/L)**	**Chlorine dioxide (mg-min/L)**	**Ozone (mg-min/L)**	**UV rays (mJ/cm^2)**
***Legionella pneumophila* studies**						
Legionella pneumophila [1]	70°C (Steinert et al., 1998, Breiman et al., 1990)	6 mg/L/6 hours (5 log reduction) (Muraca et al., 1987)	17 (3 log reduction) (Dupuy et al., 2011)	0.4 (3 log reduction) (Dupuy et al., 2011)	1 – 2 mg/L/5 hours (5 log reduction) (Muraca et al., 1987)	30 (5 log reduction) [2] (Muraca et al., 1987)
***Legionella pneumophila*-potential host protozoan coculture studies**						
Legionella pneumophila Acanthamoeba coculture	93°C [3] (Dobrowsky et al., 2016)	> 50 mg/L (Kilvington and Price, 1990)	23 (3 log reduction) (Dupuy et al., 2011)	2.8 (3 log reduction) (Dupuy et al., 2011)	-	10.8 (4 log reduction) (Cervero-Arago et al., 2014b)
Legionella pneumophila Vermamoeba coculture	58°C (Rhoads et al., 2015)	-	-	-	-	-
Potential host protozoan studies						
Acanthamoeba (trophozoite)	65°C/10 minutes (inactivation) (Coulon et al., 2010)	28 (2 log reduction)	40 (2 log reduction) (Dupuy et al., 2011)	> 5 (2 log reduction)	10 (3 log reduction) (Loret et al., 2008)	72.2 (3 log reduction) (Cervero-Arago et al., 2014b)
Acanthamoeba (cyst)	80°C/10 minutes (Storey et al., 2004)	3500 (4 log reduction) (Loret et al., 2008)	352 (2 log reduction) (Dupuy et al., 2014)	80 (4 log reduction) (Loret et al., 2008)	15 (4 log reduction) (Loret et al., 2008)	800 (Aksozek et al., 2002)
Vermamoeba (trophozoite)	60°C/5 minutes (4 log reduction) (Cervero-Arago et al., 2014a)	2 – 4 mg/L/30 minutes (inactivation) (Kuchta et al., 1993)	-	-	-	26 (3 log reduction) (Cervero-Arago et al., 2014b)
Vermamoeba (cyst)	60°C/5 minutes (2 log reduction) (Cervero-Arago et al., 2014a)	15 mg/L/10 minutes (inactivation) (Fouque et al., 2015)	-	-	-	76.2 (3 log reduction) (Cervero-Arago et al., 2014b)
Hartmannella (trophozoite)	53°C (Rohr et al., 1998)	15 (2 log reduction) (Dupuy et al., 2014)	12 (2 log reduction) (Dupuy et al., 2014)	5 (2 log reduction) (Dupuy et al., 2014)	-	-
Hartmannella (cyst)	-	156 (2 log reduction) (Dupuy et al., 2014)	34 (2 log reduction) (Dupuy et al., 2014)	1 (2 log reduction) (Dupuy et al., 2014)	-	-
Naegleria (trophozoite)	55°C/15 minutes (Chang, 1978)	5 (2 log reduction) (Dupuy et al., 2014)	4-17 (2 log reduction) (Goudot et al., 2014)	1 (2 log reduction) (Dupuy et al., 2014)	6.75 mg/L 30 minutes (3 log reduction) (Cursons et al., 1980)	24 (4 log reduction) (Sarkar and Gerba, 2012)
Naegleria (cyst)	65°C/3 minutes (Chang, 1978)	29 (2 log reduction)	13 (2 log reduction)	5.5 (2 log reduction)	-	121 (4 log reduction)

Organisms	Disinfectant dose					
	Temperature (°C)	Chlorine (mg-min/L)	Monochloramine (mg-min/L)	Chlorine dioxide (mg-min/L)	Ozone (mg-min/L)	UV rays (mJ/cm^2)
		(Dupuy et al., 2014)	(Dupuy et al., 2014)	(Chen et al., 1985)		(Sarkar and Gerba, 2012)
Vahlkampfia (trophozoite)	-	1 mg/L (inactivation) (Critchley and Bentham, 2009)	-	-	-	-
Vahlkampfia (cyst)	-	2 mg/L/2 hours (3 log reduction) (Critchley and Bentham, 2009)	-	-	-	-

[1] Most of the studies focus on culturable bacteria, and non-culturable cells are not estimated.
[2] No further bacterial inactivation possible, 1 to 2 x10^2 CFU/mL *L. pneumophila* remain stable.
[3] Experiments conducted on *Legionella* sp.

5.5 Discussion

The studies identified in this review have demonstrated the failure of many common disinfection protocols to achieve long term elimination of *L. pneumophila* from hospital and potable water supplies when protozoan hosts are present (Breiman et al., 1990, Steinert et al., 1998) (as mentioned in **Table 5.1**). This long-term survival could be attributed to association with biofilms, inherent tolerance of *L. pneumophila* to high temperature and chemical disinfectants, and constant reseeding from source water (Lau and Ashbolt, 2009). However, perhaps the most interesting and undervalued relationship is the interactions with protozoan hosts. The studies identified (**Table 5.1**) are from 14 different countries, which demonstrates the need for further research to understand the *L. pneumophila*-protozoan interaction under different environmental conditions found globally. Proper management of legionellosis requires a better understanding of *L. pneumophila*-protozoan interaction, the diversity of protozoan hosts in hospital and potable water systems and the role of the host in bacterial survival under different environmental conditions.

5.5.1 Implications for the control of *L. pneumophila*

Numerous studies have demonstrated the presence of *L. pneumophila* in disinfected water supplies (Lin et al., 1998, Whiley et al., 2017). An important factor enabling *L. pneumophila* survival in the built environment is its interaction with a protozoan host (Dobrowsky et al., 2016, Rhoads et al., 2015, Fields et al., 2002) (as mentioned in **Table 5.3**). Thermal treatment is one of the most common methods used to disinfect hospitals and building water supplies. In the USA (Breiman et al., 1990), Germany (Steinert et al., 1998) and Slovakia (Trnkova et al., 2018), thermal disinfection was adopted for management of nosocomial outbreaks of legionellosis. This strategy was unable to maintain water control for a long period of time (Breiman et al., 1990, Steinert et al., 1998) (as mentioned in **Table 5.1**). Rhoads et al. (2015) reported that *L. pneumophila* associated with *V. vermiformis* can tolerate thermal (58°C) treatment, and this disinfection protocol is unable to reduce microbial load in water. Published evidence suggests *Legionella* associated with *Acanthamoeba* are more thermotolerant and can survive at even higher temperatures ranging from 68 to 93°C (Dobrowsky et al., 2016). According to Steinert et al. (1998) members of *L. pneumophila* sg1 are more thermotolerant than sg2. This is significant given the high number of legionellosis cases associated with *L. pneumophila* sg1.

As per WHO guidelines (2004), 0.2 mg/L of free residual chlorine at point of delivery is recommended in potable water for disinfection. A pilot scale study conducted by Muraca et al. (1987) demonstrated that 4 to 6 mg/L chlorine treatment for 6 hours resulted in 5 to 6 log

reduction of *L. pneumophila*. It was also observed that the efficacy of chlorine against *Legionella* was enhanced at 43°C. However, at high temperatures a continuous flow of chlorine was required to overcome thermal decomposition. *In vitro* studies demonstrated higher level of tolerance to free chlorine (up to > 50 mg/L) when bacteria are associated with host *Acanthamoeba* cysts (Kilvington and Price, 1990). According to Kool et al. (1999), water disinfection with monochloramine resulted in a reduction of nosocomial LD outbreaks in USA. However, other studies have shown that some strains of *L. pneumophila* can tolerate high levels of monochloramine disinfection (17 mg-min/L for 3 log reduction) (Dupuy et al., 2011). Donlan et al. (2005) reported that *L. pneumophila* associated with amoebae in biofilm are less susceptible to chlorine and monochloramine treatment. It is also reported that monochloramine disinfection in hospital settings results in transformation of *L. pneumophila* vegetative cells to VBNC state (Casini et al., 2018).

According to Walker et al. (1995) chlorine dioxide can effectively control *L. pneumophila* from hospital water system. *In vitro* studies demonstrated that 0.4 mg-min/L residual chlorine dioxide achieved a 3-log reduction of *L. pneumophila*. However, this procedure was not effective for amoebae associated *L. pneumophila* (Dupuy et al., 2011). According to Schwartz et al. (2003) *Legionella* biofilms on polyvinyl chloride, polyethylene and stainless-steel materials can tolerate chlorine dioxide treatment. Muraca et al. (1987) conducted a pilot scale study and reported that 1 to 2 mg/L residual concentration O_3 treatment for 5 hours resulted in 5 log reduction of *L. pneumophila*. However, half-life of ozone in water is very short, so it is difficult to maintain residual concentration in water supplies. According to Wang et al. (2018a), if chlorination and ozonisation is used in combination, it can target both *L. pneumophila* and its host protozoans effectively. In combination both treatments effectively eliminated planktonic *L. pneumophila* and free living *Naegleria* from water, whereas this combination could only reduce the population of *Acanthamoeba* (≈ 0.9 $\log_{10}$ gene copies/g). In comparison to chlorination alone, this combination method significantly reduced the population of *L. pneumophila* (≈ 3 $\log_{10}$ gene copies/g) and host amoebae (≈ 3 $\log_{10}$ *Naegleria* gene copies/g and ≈ 6.1 $\log_{10}$ *Acanthamoeba* gene copies/g) co-existing in biofilms.

UV irradiation is another method of disinfection. These radiations harbor strong genotoxic attributes. Cervero-Arago et al., (2014b) demonstrated that 5 to 6 mJ/cm^2 UV dose was sufficient to achieve 4 log reduction *L. pneumophila* population. According to Muraca et al. (1987) 30 mJ/cm^2 UV ray treatment for 20 minutes resulted in 5 log reduction of *L. pneumophila*. However, continued exposure to same fluence rate for 6 hours unable to eliminate all culturable *L. pneumophila* (1 to 2 x 10^2 CFU/ml). Schwartz et al. (2003) reported that *Legionella* biofilms on stainless steel, polyvinyl chloride and polyethylene surfaces can

tolerate UV treatment. It was also reported that amoebae associated *L. pneumophila* can tolerate much higher doses of UV rays (Cervero-Arago et al., 2014b).

5.5.2 Protozoan host control strategies

Protozoans present in water supplies play an important role in *L. pneumophila* survival and resistance against disinfection protocols. Interesting, it has also been suggested that some protozoans infected by *L. pneumophila* have increased resistance to disinfection procedures compared to those uninfected (Garcia et al., 2007). As such, an understanding of protozoan disinfectant resistance and *L. pneumophila*-protozoan interactions is essential for the improved management of manufactured water systems. According to Loret et al. (2005), common water chemical disinfection protocols, i.e., ozonisation (0.5 mg/L), chlorination (free chlorine 2 mg/L), electro-chlorination (free chlorine 2 mg/L), monochloramine (free chlorine 2 mg/L), chlorine dioxide (0.5 mg/L) and Cu^{+}/Ag^{+} ions (0.5/0.001 mg/L) treatments, are unable to completely eliminate amoebae cysts hosting *Legionella* from water supplies (**Table 5.3**). These methods appear to be only effective against the free-living amoebae population, as they are not feasible for targeting biofilm-associated amoebae (Thomas et al., 2004). The non-standardized approach to evaluating disinfection limits is one of the gaps in knowledge raised in the discussion section.

In vitro studies have shown 1 mg/L chlorine is sufficient to inhibit the growth of *Acanthamoeba*, *Vermamoeba* and *Vahlkampfia* trophozoites. Importantly, after two hours exposure, chlorine produced complete die-off of trophozoites (Critchley and Bentham, 2009). According to Kuchta et al. (1993) 2 to 4 mg/L chlorine treatment for 30 minutes can completely inactivate *Vermamoeba* trophozoites. Whereas trophozoites of some strains of *Hartmannella* required 15 mg-min/L chlorine treatment for only 2 log reduction (Dupuy et al., 2014). Mogoa et al. (2010) reported that *Acanthamoeba* trophozoites exposed to 5 mg/L chlorine for 30 seconds resulted in a 3-log population reduction. It was also demonstrated that in *Acanthamoeba*, chlorination induces various cellular changes including reduction in cell size and alterations in cellular permeability. Dupuy et al. (2014) noticed that *Acanthamoeba* trophozoites treated with 28 mg/L chlorine for 1 minute only resulted in a 2-log reduction. In comparison with uninfected *Acanthamoeba* trophozoites, *L. pneumophila* infected *Acanthamoeba* trophozoites were more resistant against sodium hypochlorite (1024 mg/L) treatment (Garcia et al., 2007).

Generally, inactivation of *Acanthamoeba* and *Vermamoeba* cysts required 5 mg/L chlorine, whereas for *Vahlkampfia* 2 mg/L chlorine treatment. It is important to note that cysts of *Acanthamoeba* were found highly resistant and only a 2 log reduction was noticed after eight hours exposure (Critchley and Bentham, 2009). It was also reported that *Acanthamoeba* cysts can tolerate 100 mg/L of chlorine for 10 minutes (Storey et al., 2004). According to Dupuy et

al. (2014) treatment of *Acanthamoeba* cysts with 856 mg-min/L results in only 2 log reduction. Loret et al. (2008) reported that to achieve 4 log reduction for *Acanthamoeba polyphaga* cysts 3500 mg-min/L chlorine treatment is required. Likewise certain strains of *Hartmannella* cysts can tolerate high dose of chlorine (2 log reduction by 156 mg-min/L) (Dupuy et al., 2014). Exposure of *Vermamoeba* cysts to 15 mg/L of chlorine for 10 minutes was lethal and resulted in complete inactivation (Fouque et al., 2015).

Unlike *Acanthamoeba* and *Vermamoeba*, trophozoites and cysts of *Naegleria* were found sensitive to available disinfection protocols. *Naegleria* trophozoites were sensitive to 0.79 mg/L chlorine treatment for 30 minutes (Cursons et al., 1980), whereas cysts were inactivated after exposure to 1.5 mg/L chlorine for 1 hour (De Jonckheere and van de Voorde, 1976). Dupuy et al. (2014) reported that chlorine treatment of *Naegleria* trophozoites with 5 mg-min/L resulted in only 2 log reduction and cysts can tolerate much higher levels of chlorine (29 mg-min/L for 2 log reduction). In potable water *Naegleria fowleri* associated with biofilms was able to tolerate 20 mg/L chlorine for 3 hours (Miller et al., 2015).

In comparison to chlorine, chloramine is regarded as more stable disinfectant and capable to penetrate complex biofilms (Kool et al., 1999). Dupuy et al. (2014) suggested that instead of chlorine, monochloramine is effective chemical disinfectants against trophozoites and cysts of *Acanthamoeba*, *Vermamoeba* and *Naegleria*. It is possible that monochloramine harbors greater penetrating power than chlorine and easily enter in trophozoites and cysts. According to Mogoa et al. (2011) monochloramine specifically targets the cell surface of *Acanthamoeba*. Dupuy et al. (2014) identified that 352 mg-min/L monochloramine exposure resulted in 2 log reduction of *Acanthamoeba* cysts. Goudot et al. (2014) noticed that 4 to 17 mg/L monochloramine exposure for 1 minute only resulted in 2 log reduction of both planktonic and biofilm associated *Naegleria*. According to Dupuy et al. (2014) to achieve 2 log reduction of *Hartmannella* trophozoites and cysts 12 mg-min/L and 34 mg-min/L monochloramine dose is required, respectively. Although *in vitro* studies demonstrate that higher concentration of chlorine-based disinfectants can inhibit the proliferation of protozoans; however, it can corrode the plumbing system pipes.

Chlorine dioxide has been shown to easily penetrate into amoeba trophozoites and cysts and specifically promotes cytoplasmic vacuolization in *Acanthamoeba* (Mogoa et al., 2011). However, the efficacy of chlorine dioxide varies from amoeba strains. The cyst form of some *Acanthamoeba* strains have been demonstrated to be highly tolerant to chlorine dioxide (35 mg-min/L for 2 log reduction) (Dupuy et al., 2014). Loret et al. (2008) demonstrated that an 80 mg-min/L dose of chlorine dioxide is required to achieve 4 log reduction of *Acanthamoeba polyphaga* cysts. Importantly, most studies were designed to investigate the effect of

disinfection procedures on amoeba and there are limited studies on *L. pneumophila*-amoebae interactions during disinfection.

Ozonisation is an effective method of water disinfection. According to Cursons et al. (1980), a dose of ozone 6.75 mg/L (0.08 mg/L residual level after 30 minutes) was sufficient to kill 99.9% (3 log reduction) trophozoites of *Acanthamoeba* and *Naegleria*. However, biofilm associated *Acanthamoeba*, *Hartmannella*, and *Vahlkampfia* were always found resistant to such treatments (Thomas et al., 2004). Loret et al. (2008) demonstrated that 10 mg-min/L ozone dose resulted in 3 log reduction of *Acanthamoeba* trophozoites, however cysts retained viability.

Thermal treatment is a common physical disinfection protocol used for potable water supplies. According to Chang (1978) trophozoites of *Naegleria* can survive at 55°C for 15 minutes, whereas cysts can tolerate 65°C for 3 minutes. *Vermamoeba* trophozoites and cysts have been shown to be completely inactivated by exposure to 60°C for 30 minutes (Fouque et al., 2015, Kuchta et al., 1993). Thermal treatment of *Acanthamoeba* trophozoites and cysts at 65°C for 10 minutes resulted in full inactivation (Coulon et al., 2010). Loret et al. (2008) demonstrated that thermal treatment of *Acanthamoeba polyphaga* cysts at 65°C for 120 minutes resulted in 5 log reduction. However, Storey et al. (2004) reported that *Acanthamoeba castellanii* cysts are thermally stable and retain viability at 80°C for 10 minutes. It has also been reported that thermal treatment can enhance the efficiency of chlorination. Although at high temperature (50°C) the solubility of chlorine gas in water decreases significantly and very corrosive to pipe work, but its amoebicidal activity increases slightly (Dupuy et al., 2011).

UV treatment is another method of disinfection recommended by WHO. As per recommendation in 10 mJ/cm^2 dose is sufficient for 99.9% (3 log) inactivation of protozoans like *Giardia* and *Cryptosporidium* (World Health Organization, 2004). According to Cervero-Arago et al. (2014b) to achieve 3 log reduction of *V. vermiformis* trophozoites 26 mJ/cm^2 UV dose was required, whereas 76.2 mJ/cm^2 for cysts. It was also noticed that exposure to 72.2 mJ/cm^2 irradiance resulted in 3 log reduction of *Acanthamoeba* trophozoites (Cervero-Arago et al., 2014b). Aksozek et al. (2002) reported viability of *Acanthamoeba castellanii* cysts after exposure to high doses of UV rays (800 mJ/cm^2). According to Sarkar and Gerba (2012) to achieve 4 log reduction of *Naegleria fowleri* trophozoites and cysts 24 mJ/cm^2 and 121 mJ/cm^2 UV irradiance is required, respectively. A pilot scale study conducted by Langmark et al. (2007) demonstrated inability of UV irradiation to reduce biofilm associated amoebae. In contrast with other protozoans, members of the *Acanthamoeba* genera are more resistant to both chemical and physical disinfection protocols.

As per water quality guidelines of WHO (2004), 41 mg-min/L chlorine at 25°C OR 1000 mg-min/L monochloramine at 15°C OR 7.3 mg-min/L chlorine dioxide 25°C OR 0.63 mg-min/L O_3 at 15°C OR 10 mJ/cm^2 UV rays, treatments are required for inactivation of pathogenic protozoan (reference protozoa *Giardia*), as mentioned earlier in this section protozoans facilitating growth of *L. pneumophila* can thrive in these conditions (**Table 5.3**).

So far, studies have investigated the efficacy of water disinfection protocols against *Acanthamoeba*, *Hartmannella*, *Naegleria* and *Vermamoeba*. However, there are numerous other waterborne cyst forming, non-cyst forming and ciliated protozoans which support the proliferation of *L. pneumophila*. Therefore, there is a need for more research and a standardized approach to evaluating disinfection protocol(s) that target both *L. pneumophila* and potential protozoan hosts. According to our literature survey, the effectiveness of available disinfection protocols depends upon the species, strain, and cellular state of protozoans, as well as the type of disinfection technique and exposure time.

5.5.3 Detection methods

The most commonly used methods to investigate potential *L. pneumophila* protozoan hosts are coculture and co-isolation assays (Boamah et al., 2017). The coculture assay is widely used in the laboratory to study *Legionella*-protozoan interactions. In this method, *Legionella* is allowed to grow in a protozoan host and fate of bacterium is determined microscopically (Dietersdorfer et al., 2016). *In vitro* laboratory studies showed that *Acanthamoeba* (Berk et al., 1998) and *Tetrahymena* (Berk et al., 2008) allow intracellular replication and packaging of live *L. pneumophila* into export vesicles. Other protozoan genera; *Balamuthia* (Shadrach et al., 2005), *Dictyostelium* (Solomon et al., 2000), *Echinamoeba* (Fields et al., 1989), *Naegleria* (Newsome et al., 1985), *Paramecium* (Watanabe et al., 2016), and *Vermamoeba* (Nahapetian et al., 1991), facilitate intracellular replication of *L. pneumophila*. The second method is used to detect naturally co-existing *Legionella*-protozoans from environment, but microscopically it is very difficult to find protozoans containing *Legionella* in the natural environment (Kao et al., 2013). As an alternative approach, a sample is screened for the presence of both *Legionella* and protozoan hosts. Generally, samples are screened by PCR (International Organization for Standardization, 2019, Qvarnstrom et al., 2006), fluorescence *in situ* hybridization (Whiley et al., 2011), classical culturing techniques and microscopy (Standards Australia, 2017, Health Protection Agency UK, 2004). These methods are good for screening environmental samples but are unable to delineate the underlying interactions between *Legionella* and host protozoans. Nowadays, PCR based protocols are widely used to detect *L. pneumophila* and protozoan hosts from engineered water systems. In comparison to classical culturing methods, these protocols are rapid and highly sensitive. However, most of the nucleic acid-based

protocols are unable to differentiate viable and dead organisms. Propidium monoazide-PCR or ethidium monoazide-PCR are modified nucleic acid detection protocols to enumerate the live bacteria (Nocker et al., 2006, Yanez et al., 2011) and protozoan hosts (Brescia et al., 2009, Fittipaldi et al., 2011). To estimate burden of *L. pneumophila* and protozoan hosts in water distribution system, it is necessary to measure the quantity of alive and dead organisms regularly. This literature review demonstrates that *Vermamoeba* and *Acanthamoeba* are predominant hosts of *L. pneumophila* in the context of hospital and potable water systems. Many cyst forming, non-cyst forming and ciliated protozoans have been found associated with *L. pneumophila* and are identified as potential hosts; however, *in vitro* coculture assays and microscopic studies are required for confirmation and characterization of this interaction.

During stress (i.e., thermal, nutrient, chemical and radiation), *L. pneumophila* can enter into a VBNC state. After the end of such a stress period, in presence of a suitable host or favourable environmental conditions, the VBNC state can transform back into metabolically active cellular state (Dietersdorfer et al., 2018). Importantly, the underlying mechanisms of resuscitation from VBNC are not yet well understood. However, as the VBNC form is by definition a non-culturable state, classical microbiology culturing techniques cannot be used to monitor viability. Thus, *in vitro* coculture assays can be used to resuscitate VBNC in the laboratory (Garcia et al., 2007). Alternative approaches to analyse VBNC are the analysis of membrane integrity and molecular screening (Kirschner, 2016). There are also studies that have shown that intracellular replication of *L. pneumophila* induces VBNC state. According to Buse et al. (2013) transformation of *V. vermiformis* trophozoites into cysts promotes biogenesis of VBNC *L. pneumophila*. Therefore, the interaction with protozoan hosts may also affect the ability to monitor the efficacy of disinfection protocols against *L. pneumophila*, because the bacteria may be in the VBNC form. Available literature only discusses disinfection protocols, which target culturable *L. pneumophila*. To our knowledge, there are limited studies investigating the effectiveness of disinfection protocols to eliminate VBNC *L. pneumophila*. It is our suggestion to adopt membrane integrity and *in vitro* coculture assays to evaluate the disinfection procedure against VBNC *L. pneumophila*.

5.6 Conclusions

Protozoans present in potable water play an important role in *L. pneumophila* survival. Further research is needed to better understand *L. pneumophila*-protozoan interactions and the implications for the prevention of Legionnaires' disease. To achieve long term disinfection of a water system the control protocols need to be effective against potential hosts harbouring *L. pneumophila*. Additionally, an understanding of the mechanisms of VBNC state transformation,

and the role of protozoans in this, is needed to effectively evaluate the efficacy of disinfection techniques.

Author contributions

MAN and HW drafted and edited the manuscript, HW, KER, MHB. and RB corrected and contributed to the manuscript. All authors approved of the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

6 Molecular screening and characterization of *Legionella pneumophila* associated free-living amoebae in domestic and hospital water systems

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6.1 Abstract

Free-living amoebae are ubiquitous in the environment and cause both opportunistic and non-opportunistic infections in humans. Some genera of amoebae are natural reservoirs of opportunistic plumbing pathogens, such as *Legionella pneumophila*. In this study, the presence of free-living amoebae and *Legionella* was investigated in 140 water and biofilm samples collected from Australian domestic (n = 68) and hospital water systems (n = 72). Each sample was screened in parallel using molecular and culture-based methods. Direct quantitative polymerase chain reaction (qPCR) assays showed that 41% samples were positive for *Legionella*, 33% for *L. pneumophila*, 11% for *Acanthamoeba*, and 55% for *Vermamoeba vermiformis* gene markers. Only 7% of samples contained culturable *L. pneumophila* serogroup (sg)1, *L. pneumophila* sg2-14, and non-pneumophila *Legionella*. Total 69% of samples were positive for free-living amoebae using any method. Standard culturing found that 41% of the samples were positive for amoeba (either *Acanthamoeba*, *Allovahlkampfia*, *Stenamoeba*, or *V. vermiformis*). *V. vermiformis* showed the highest overall frequency of occurrence. *Acanthamoeba* and *V. vermiformis* isolates demonstrated high thermotolerance and osmotolerance and strong broad spectrum bacteriogenic activity against Gram-negative and Gram-positive bacteria. Importantly, all *Legionella* positive samples were also positive for amoeba, and this co-occurrence was statistically significant ($p < 0.05$). According to qPCR and fluorescence *in situ* hybridization, *V. vermiformis* and *Allovahlkampfia* harboured intracellular *L. pneumophila*. To our knowledge, this is the first time *Allovahlkampfia* and *Stenamoeba* have been demonstrated as hosts of *L. pneumophila* in potable water. These results demonstrate the importance of amoebae in engineered water systems, both as a pathogen and as a reservoir of *Legionella*. The high frequency of gymnamoebae detected in this study from Australian engineered water systems identifies an issue of significant public health concern. Future water management protocols should incorporate treatments strategies to control amoebae to reduce the risk to end users.

6.2 Introduction

Gymnamoebae, or naked amoebae, are a group of free-living aerobic amoeboid microorganisms, which develop broad and smooth cytoplasmic projections known as pseudopodia (Page, 1988). They are abundantly distributed in all types of environments. These free-living amoebae exist in two different physio-morphological forms: a metabolically active vegetative state called a trophozoite and a dormant state known as a cyst. Double-layered cysts tolerate and resist environmental stresses including chemical and physical disinfection treatments (Dupuy et al., 2014). *Acanthamoeba*, *Balamuthia*, *Naegleria*, *Vahlkampfia*, and *Vermamoeba vermiformis* are important free-living amoebae in terms of their

effect on human health (Visvesvara et al., 2007). These amoebae are etiological agents of human diseases and act like carriers of various pathogenic bacteria. *Acanthamoeba* is widely dispersed in nature and associated with different opportunistic and non-opportunistic infections (Marciano-Cabral and Cabral, 2003). For example, it has been identified as a causative agent of amoebic keratitis, granulomatous amoebic encephalitis, cutaneous and nasopharyngeal acanthamoebiasis in immunocompromised persons (Khan, 2006). *Balamuthia mandrillaris* is associated with fatal granulomatous amoebic encephalitis, cutaneous and pulmonary infection in immunocompetent individuals (Matin et al., 2008). *Naegleria fowleri*, the only known pathogenic species of the genus *Naegleria*, is a free-living ameoboflagellate responsible for primary amoebic meningoencephalitis in healthy individuals (Visvesvara et al., 2007). *V. vermiformis* (formerly known as *Hartmannella vermiformis*) is widely distributed in fresh surface water and described as an etiological agent of amoebic keratitis and meningoencephalitis (Siddiqui et al., 2021, Centeno et al., 1996). Free-living amoebae are reservoirs and transmission vehicles of opportunistic premise plumbing pathogens (OPPPs) such as *Pseudomonas*, *Mycobacterium* and *Legionella* (Thomas and Ashbolt, 2011). These amoebae hosts protect OPPPs from physical and chemical disinfection procedures, enabling long term contamination of manufactured water systems.

Legionella is a medically important OPPP with widespread distribution in natural and artificial aquatic environments (Bartram et al., 2007). Humans are an accidental host of *Legionella* while the protozoan predators are its natural host (Boamah et al., 2017). *L. pneumophila* is the primary causative agent of legionellosis (Legionnaires' disease and Pontiac fever). To date, 80 distinct serogroups (sg) of this bacterium have been identified (Miyashita et al., 2020). In the USA and Europe 70% to 92% of the identified cases of legionellosis are associated with *L. pneumophila* sg1 (Mercante and Winchell, 2015). The European Legionnaires' Disease Surveillance Network has classified Legionnaires' disease into community, hospital and travel acquired infections. According to data collected in 2020, 5.1% cases were associated with healthcare settings, 7.8% were acquired via travelling and 66.9% cases were community acquired (The European Legionnaires' disease Surveillance Network, 2022). Cooling towers, shower heads, tap faucets, spas and hot water systems are major sources of *L. pneumophila* contamination (Bartram et al., 2007), as it is transmitted via inhalation or aspiration of *L. pneumophila* contaminated aerosols or water (Prussin et al., 2017). In hospital and domestic water systems, *Acanthamoeba* and *V. vermiformis* are major reservoirs and vehicles of *L. pneumophila* (Nisar et al., 2020a). Symbiotic eukaryotic microbes, biofilms and intrinsic resistance to disinfection treatment, are the main biological factors influencing its survival and persistence (Nisar et al., 2020a). Other abiotic factors such as flow dynamics, stagnation, dead

end, dead legs, and plumbing material also influence the growth of *Legionella* in engineered water systems (Nisar et al., 2020b).

To our knowledge, there are limited studies investigating free-living amoebae in Australian potable water, and these mainly deal with the clinically important species *Acanthamoeba* (Höllhumer et al., 2020) and *N. fowleri* (Puzon et al., 2020). More broadly, there are limited studies that examine the relationship between amoeba and *Legionella* using both molecular and culture-based methods concurrently (Nisar et al., 2020a). In this present study, water and biofilm samples collected from different engineered water systems were screened for the presence of *Legionella* and free-living amoebae. The culturable amoebae were characterized based on their physio-morphologic, stress tolerance and bacteriogenic features. The presence of intracellular *Legionella* was examined using qPCR and florescence *in situ* hybridization (FISH) with rRNA based oligonucleotide probes. This approach enabled potentially new *Legionella* hosts to be identified. This is the first comprehensive study which used culturing and molecular tools for the characterization of free-living amoebae and their association with *Legionella* in Australian hospital and domestic water systems.

6.3 Materials and Methods

6.3.1 Sample collection and processing

The study was approved by the Flinders University Social and Behavioural Research Ethics Committee (SBREC Project Number 7291) as per the recommendations of the National Statement on Ethical Conduct in Human Research, Australia. From February 2019 to November 2021, 140 water and biofilm samples were collected from showers, taps, and tap faucets of domestic and hospital water systems. These samples were collected from 35 different hospital and domestic buildings located in different municipalities of New South Wales and South Australia. Due to strict ethical policies authors cannot disclose the geographic location of the buildings. When possible, physical, and environmental parameters were recorded during sampling. This data, along with climatic data from the Australian Bureau of Meteorology (2022), is presented in **Table 13.2** (Appendix-2). Water and biofilm samples were collected and transported according to the guidelines of the Centres for Disease Control (Centers for Disease Control and Prevention, 2019) and ISO 5667-3:2018 (International Organization for Standardization, 2018). Briefly, 1 L of shower or tap water and 5 to 10 mL of visible biofilm containing water samples were collected in sterile screw capped wide-mouth plastic bottles (2105-0032, Nalgene™) and 15 mL sterile screw capped vials, respectively. Sterile polyurethane-tipped swabs (CleanFoam® TX751B, Texwipe®) were used to harvest visible biofilms. To neutralize residual chlorine-based disinfectants, 0.1 to 0.5 mL of 0.1 N $Na_2S_2O_3$ (124270010, ACROS Organics™) was added to the collected water samples. All

samples were stored at 5°C and analysed within 72 h. Collected samples were vacuum filtered onto a 47 mm diameter 0.2 µm polycarbonate membrane (GTTP04700, Isopore™), and the membrane was transferred to a sterile vial containing 1 mL of sterile water and vortexed vigorously. This suspension was used for microbiological culturing and DNA extraction.

6.3.2 Molecular screening of *Legionella*, *L. pneumophila*, *Acanthamoeba* and *Vermamoeba vermiformis*

Extraction of total genomic DNA from the water and biofilm samples was performed using the Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.) as per the manufacturer's instructions and used for the detection and quantification of *Legionella*, *L. pneumophila*, *Acanthamoeba* and *V. vermiformis* molecular markers. Using the ISO/TS12869:2019 standard qPCR assay, *Legionella* and *L. pneumophila* were enumerated by amplification of the 16 rDNA and *mip* genes, respectively (International Organization for Standardization, 2019). The 18S rDNA gene was amplified to quantify *Acanthamoeba* and *V. vermiformis* (Qvarnstrom et al., 2006, Scheikl et al., 2016). *Legionella* (GenBank Acc CP021281), *L. pneumophila* (GenBank Acc KR902705), *Acanthamoeba castellanii* (GenBank Acc U07413) and *V. vermiformis* (GenBank Acc KT185625) gBlocks gene fragments (IDT™) were used to create a standard curve using 10-fold serial dilutions. Reaction mixes consisting of microbe-specific oligos (BIO-RAD Laboratories Ltd.), 2X SsoAdvanced™ universal probes supermix (172-5281, BIO-RAD Laboratories Ltd.) and template DNA were subjected to a Rotor-Gene Q thermal cycler (QIAGEN Ltd.) for quantitative gene amplification. Sequences of oligos and qPCR assay conditions are described in **Table 13.1** (Appendix-2). In all qPCR assays, signals of 6-carboxyfluorescein (6 FAM: $\lambda_{(excitation/emission)}$ 495/520 nm) and Iowa Black® FQ quencher labelled probes were detected with $\lambda_{(source/detector)}$ 470/510 nm channel. All assays were done in triplicate and mean C_T (cycle threshold) values were used for estimation of the genomic unit per litre (GU/L) and genomic unit per millilitre (GU/mL). To determine the presence of any PCR inhibitors in the extracted DNA, qPCR was performed on neat, and 10-times diluted extracted DNA. If the mean C_T value for the diluted sample was less than ≈ 3.3 than the extracted DNA, it was considered that the sample contained reaction inhibitors, and the diluted sample (1:10) was used to estimate GU/mL (for biofilm samples) or GU/L (for water samples) (Hayes-Phillips et al., 2019). The qPCR assays for both *Legionella* and selected amoebae yielded a linear relationship between the C_T and log gBlock DNA concentration. The standard curves of *Legionella* and *L. pneumophila* revealed an 89% and 99% efficiency of the qPCR assay, respectively. The limit of detection for both *Legionella* and *L. pneumophila* was 35 GU/reaction. The standard curves of *Acanthamoeba* and *V. vermiformis* revealed an 81% and 75% efficiency of the qPCR assay, respectively. The limit of detection was 40 GU/reaction for *Acanthamoeba* and 44 GU/reaction for *V. vermiformis*. The quantity of the *Legionella*, *L.*

pneumophila, *Acanthamoeba* and *V. vermiformis* gene markers was subsequently estimated from shower water (n = 90), basin water (n = 27) and biofilm (n = 23) samples.

6.3.3 Isolation and characterization of culturable *Legionella* and *L. pneumophila*

Presence of culturable *Legionella* and *L. pneumophila* was confirmed by isolation and culturing using ISO11731:2017-05 and AS5132:2017 protocols (International Organization for Standardization, 2017, Standards Australia, 2017). Samples were concentrated by filtration and heated at 50 ± 1°C for 30 ± 2 minutes before being cultured on BCYE (buffered charcoal yeast extract: CM0655, Oxoid Ltd.) agar supplemented with GVPC (glycine, vancomycin, polymyxin B and cycloheximide: SR0152, Oxoid Ltd.) and *Legionella* growth supplement (α-ketoglutarate, buffer/potassium hydroxide, ferric pyrophosphate, and $_{L}$-cysteine: SR0110C, Oxoid Ltd.); and incubated at 37 ± 1°C for 3 to 5 days. Colonies showing *Legionella*-like morphology (circular, off-white, ground-glass, or opalescent appearance) were sub-cultured and identified by a latex agglutination assay. This assay kit (*Legionella* Latex Test DR0800, Oxoid Ltd.) identifies *L. pneumophila* sg1, *L. pneumophila* sg2-14, *L. anisa*, L. *bozemanii*, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. longbeachae*, and *L. micdadei* species and serogroups. The colonies having a positive latex agglutination assay reaction or showing morphological features similar to *Legionella* were tested by PCR. *Legionella* and *L. pneumophila* isolates were preserved in 50% glycerol solution supplemented with *Legionella* growth supplement (SR0110A, Oxoid Ltd.) and stored at –80°C.

6.3.4 Isolation and culturing of amoebae

For isolation of amoebae, 0.1 mL of filtrate was spread onto a lawn of heat-killed (57°C for 45 min) *Escherichia coli* American Type Culture Collection (ATCC®) 700891™ on a 1.5% non-nutrient agar (Eco-NNA: CM0003, Oxoid Ltd.) plate. Plates were incubated under aerobic conditions at 25 ± 1°C and examined daily for 14 days under an inverted light microscope (AMEFC4300, EVOS™ FL, ThermoFisher Scientific). To subculture isolates, a section showing amoebae growth was excised (< 10 mm) and placed on a freshly prepared Eco-NNA plate and incubated under aerobic conditions at 25 ± 1°C for 5 days. Development of a zone of clearance indicated amoebae growth and migration. This subculturing procedure was repeated until an amoebae monoxenic culture was obtained (Page, 1988, Health Protection Agency UK, 2004). Amoebae were preserved on an Eco-NNA slant at 4°C for 6 months and for long term storage in 15% dimethyl sulfoxide stocks and stored at –80°C (Menrath et al., 1995).

6.3.5 Microscopic examination of amoebae isolates

Live cells were examined to study cellular shape, locomotive morphology, nucleus, pseudopodia, granuloplasm, hyaloplasm orientation, and uroid characteristics. These morphodynamic features were used for the identification of morphotypes (Smirnov et al., 2011, Smirnov and Goodkov, 1999). Acanthopodial morphotype amoebae were further characterized based on morphological features of cysts (Page, 1988). All digital micrographs were captured using a BX43 upright light microscope (Olympus) equipped with a U-TV1X-2 magnification adapter (Olympus) and a U-CMAD3 camera (Olympus) powered by cellSens version 1.18 (Olympus).

6.3.6 Molecular identification of amoebae isolates

Genomic DNA of each amoebae isolate was extracted using the Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.) according to recommendations of the manufacturer. Extracted DNA was dispatched to Australian Genome Research Facility (AGRF Ltd) for amplification and sequencing of the 18S rDNA gene using degenerate primes (Appendix-2, **Table 13.1**) (Moreno et al., 2018). The quality of sequencing data was analysed with ChromasPro software (Technelysium Pty Ltd.), then Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify closely related sequences available in the GenBank® database.

6.3.7 Physiological tolerance assay

A thermotolerance assay was performed to determine the ability of amoebae isolates to grow at different temperatures and to identify the optimum growth temperature. In this assay, all isolates were cultured on Eco-NNA plates and incubated at 25°C, 30°C, 35°C, 40°C and 45°C for 5 to 14 days. Similarly, to study osmotolerance of isolates, trophozoites of each amoeba were cultured on Eco-NNA plates supplemented with 250 mM, 500 mM, 750 mM, and 1000 mM of $_{D}$-mannitol and incubated at 25 ± 1°C for 5 to 14 days. In both assays, each plate was examined microscopically to record amoebic growth and migration (Caumo et al., 2009).

6.3.8 Bacterial predation assay

Amoebal predation activities were tested against *Enterococcus faecium* ATCC® 19434™, *Staphylococcus aureus* ATCC® 6538P™, *E. coli* ATCC® 700891™, *Acinetobacter baumannii* ATCC® 17978™, *Klebsiella pneumoniae* Flinders Culture Collection (FCC) 575 (environmental isolate), and *Pseudomonas aeruginosa* (environmental isolate). *E. faecium*, *S. aureus*, *E. coli* and *K. pneumoniae* were cultured on tryptone soya agar (CM0131B, Oxoid Ltd.) at 37°C for 24 h. *A. baumannii* and *P. aeruginosa* were cultured on MacConkey (CM0007, Oxoid Ltd.) and cetrimide agar (CM059, Oxoid Ltd.), respectively and incubated at 37°C for 24 h. Prior to this

assay it was confirmed that these bacteria could survive on NNA plates for 10 days. For the predation assay, one or two crescentic streaks of freshly grown bacteria (diameter 15 mm) were made on NNA plates. An amoebae agar block (< 10 mm) was placed on the bacterial streak and incubated under aerobic conditions at 25 ± 1°C for 5 to 10 days (Page, 1988). To monitor amoebic growth and migration, each plate was examined daily under a microscope. After 24 h of incubation, the clearance zone was measured to estimate the speed of predations.

6.3.9 Screening for intracellular *Legionella*

Amoebae isolates were screened for the presence of intracellular *Legionella* and *L. pneumophila* by using two methods: genus and species-specific qPCR and FISH assays. In the qPCR assay, according to the recommendations of ISO/TS12869:2019, the 16S rDNA and *mip* genes were amplified to detect intracellular *Legionella* and *L. pneumophila*, respectively (International Organization for Standardization, 2019). In the FISH assay, Alexa Fluor 647 labelled universal eukaryotic EUK1209 and Alexa Fluor 488 labelled *Legionella* LEG705 fluorogenic probes (Invitrogen™, Appendix-2, **Table 13.1**) were used to visualize intra-amoebic *Legionella* (Whiley et al., 2011, Manz et al., 1995, Amann et al., 1990). Briefly, paraformaldehyde fixed cells were dehydrated in a 50%, 80% and 96% ethanol series. To these cells, 200 µL hybridization buffer (0.01% SDS, 900 mM NaCl and 20 mM Tris-HCl pH 7.6) containing 100 ng of each fluorogenic probe was applied and incubated at 55 ± 1°C for 100 min. After washing and drying, CitiFluor™ AF1 (17970-25, Electron Microscopy Sciences) was used for mounting, and images were captured using confocal microscopy (LSM 880 fast airyscan confocal, Zeiss).

6.3.10 Statistical analysis

Data analyses were performed using SPSS software and an online calculator (https://www.socscistatistics.com). Pearson's chi-squared test was used to determine the association between co-occurrence of *Legionella* and free-living amoebae. The results were interpreted at the level of significance $p < 0.05$.

6.4 Results

6.4.1 Prevalence of *Legionella*, *L. pneumophila*, *Acanthamoeba* and *Vermamoeba vermiformis* gene markers

Legionella was present in 41% (n = 58) of samples within the range of: shower water, 1×10^2 to 2.8×10^6 GU/L; basin water, 1.2×10^3 to 1.2×10^6 GU/L; and biofilm, 40 to 7.4×10^4 GU/mL. *L. pneumophila* was identified in 33% (n = 46) samples within the range of: shower water, 40 to 1.5×10^5 GU/L; basin water, 4.7×10^2 to 1.8×10^4 GU/L; and biofilm, 1.24×10^2 to 3.2×10^3 GU/mL. *Acanthamoeba* and *V. vermiformis* gene markers were detected in 11% (n = 15) and

55% (n = 77) of 140 hospital and domestic water samples that were analysed for these free-living amoebae, respectively. In 10% of domestic (n = 10) and hospital (n = 4) samples *V. vermiformis* co-occurred with *Acanthamoeba*. The total gene marker count of *Acanthamoeba* and *V. vermiformis* was found to be significantly different in the sample types. Numbers of *Acanthamoeba* varied from 40 to 9.4×10^2 GU/mL and 2.1×10^2 to 1.7×10^4 GU/L in the biofilm and shower water samples, respectively. The gene copy numbers of *V. vermiformis* were 50 to 2.6×10^5 GU/mL and 2.7×10^2 to 3.8×10^7 GU/L in biofilm and shower water samples, respectively. In the basin water samples, *V. vermiformis* was found to be more abundant, ranging from 2.7×10^4 to 7.4×10^7 GU/L, whereas *Acanthamoeba* varied from 1.1×10^3 to 2.8×10^3 GU/L. **Table 6.1** presents the proportion of *Legionella*, *L. pneumophila*, *Acanthamoeba* and *V. vermiformis* contamination from hospital and domestic water systems.

6.4.2 Identification of culturable *Legionella*

From 59 domestic shower water samples, eight were positive for culturable *Legionella* of which six were identified as *L. pneumophila* sg1 and two were *L. pneumophila* sg2-14. In serological identification assays, the domestic shower water isolates were found to be negative for *Legionella sp.*, however they reacted positively in a serogrouping assay as either *L. pneumophila* sg1 or *L. pneumophila* sg2-14. This implies that the commercially available *Legionella* latex agglutination assay does not always identify environmental *Legionella* isolates. Of 58 hospital basin and shower water samples, only two were positive for culturable *Legionella*, which were identified as non-pneumophila *Legionella*. No culturable *Legionella* was detected in the domestic and hospital biofilm samples. Finally, through the qPCR assay all domestic shower water isolates were confirmed as harbouring *L. pneumophila* whereas the *Legionella* from both hospital water isolates were confirmed as non-pneumophila *Legionella*.

6.4.3 Culturable free-living amoebae and morphotypes

Of the 140 samples, 58 (41%) were found to be positive for culturable amoebae **(Table 6.1)**. Due to fungal overgrowth, 22 were unable to develop monoxenic cultures. Of these seven showed acrasid amoebae-like morphology. Therefore, 36 monoxenic isolates were selected and processed for further studies **(Figure 6.1)**. Light microscopy of trophozoites revealed four distinct morphotypes.

1. Acanthopodial morphotypes: Locomotive form showing an acanthopodial morphotype (n = 1) that harboured acanthopodia (flexible, furcate and slender projections emerging from hyaloplasm) for movement. Trophozoites (20 to 40 μm) contained a single vesicular nucleus (single and central nucleolus containing nucleus) and one or two prominent contractile vacuoles. The cytoplasm consisted of two distinct regions, a

granuloplasm (dense granular region) and a hyaloplasm (translucent peripheral region) (**Figure 6.2 A** and **B**). In aqueous solution (growth medium), some motile trophozoites developed “membrane bleb” like protrusions. In cysts (10 to 20 μm), the intine (inner wall) and exine (outer wall) were well separated. The intine consisted of 6 to 10 rays joined to exine at the end of rays (**Figure 6.2 C**). These morphological characteristics are compatible with members of *Acanthamoeba* group I (Page, 1988).

2. Monotactic morphotype: Locomotive forms exhibiting a monotactic morphotype (n = 30) were elongated cylindrical, non-vahlkampfiid and worm-like “limax” amoebae. Amoebae were predominantly monopodial (single prominent tick and blunt pseudopodia) (**Figure 6.2 D**). Only when a cell started changing direction of movement, it developed discrete protruding lateral branches and/or two or more pseudopodia (**Figure 6.2 E**). During continuous locomotion, they had a stable anterior hyaline cap (clear and semi-fluid portion front of the cytoplasm) and maintained a clear fountain like flow of granuloplasm (hyaloplasm-granuloplasm transformation). Some motile cells harboured a posterior bulbous, villous-bulbous, or an adhesive uroid (mass of cytoplasm at posterior end acting like point of adhesion). In some actively moving amoebae, the length of the uroidal filament extended up to the cell length. All trophozoites (15 to 45 μm) were uninucleate and possessed a contractile vacuole situated at the posterior end. The cysts (7 to 15 μm) were spherical and consisted of distinctly separated inner and outer walls (**Figure 6.2 F**).
3. Lingulate morphotype: Locomotive forms demonstrating a lingulate morphotype (n = 2) were linguiform or oblong shaped amoebae (**Figure 6.3 B**), occasionally adopting a flattened shape. During “non-directed movements” amoebae developed variable and irregular shapes and produced dactylopodia like structures (finger-like hyaline sub-pseudopodia) (**Figure 6.3 C**). These motile cells did not harbour any prominent uroidal filaments. Non-adherent floating cells formed multiple uneven pseudopods, radiating from a central cell mass (**Figure 6.3 A**). This state was very brief, and it immediately settled down and continued locomotion. Trophozoites (15 to 35 μm) contained a single vesicular nucleus and a prominent contractile vacuole at the posterior end. The anterior part was broad and round, whereas the posterior part was more granular. The cysts (9 to 13 μm) were double-walled and spherical in shape (**Figure 6.3 D**). Two types of cyst walls were observed. The inner wall closely attached to the outer wall and the inner wall were well separated, in cysts of same clonal culture. Unlike other morphotypes, in agar cultures during cyst formation, trophozoites started active movement and developed a highly organized aggregate of cysts (**Figure 6.1 E** to **H**).
4. Eruptive morphotype: Locomotive form depicting an eruptive morphotype (n = 3) harboured typical vahlkampfiid morphology with eruptive lobopodia. During locomotion

an eruptive limax shape was predominant (**Figure 6.3 E**), although a flabellate shape (fan-shaped translucent lobes) developed regularly (**Figure 6.3 G**). Posterior end of the locomotive form also developed bulbous or adhesive uroidal structures (**Figure 6.3 F**). “Non-directionally” moving amoebae developed a more compact shape and formed multiple eruptive lobopodia in different directions. In these stationary cells, the hyaloplasm developed a uniform layer at the peripheral region, whereas the dorsal surface of the granuloplasm formed ridges and folds (**Figure 6.3 G**). Trophozoites (20 to 35 μm) were mononucleated with vesicular nucleus and harboured a contractile vacuole located at the posterior terminus. None of the cells developed a flagellate stage. The cysts (7 to 15 μm) were circular in shape and the inner cyst wall was closely attached to the outer wall (**Figure 6.3 H**). In agar cultures, amoebae formed two different types of aggregates. Trophozoites actively migrated and arranged themselves into loose mounds or multilayered amoebae aggregates (**Figure 6.1 I** and **J**). During depletion of nutrients within aggregates the amoebae started forming cysts. These cyst aggregates were not entrapped in any fruiting structure or sorocarp (**Figure 6.1 K** and **L**).

Comprehensive microscopic examination of amoebae demonstrated that monotactic morphotype (n = 30) was widely distributed in engineered water systems.

6.4.4 Molecular identification of amoebae

Of the 36 monoxenic cultures, 33 isolates successfully had their 18S rDNA region sequenced (**Table 6.2**) identifying 29 as *V. vermiformis*, two as *Stenamoeba* (formerly known as *Platyamoeba*), one *Allovahlkampfia* and one as *Acanthamoeba*. All hospital water system isolates were characterized as *V. vermiformis*. The percentage identity between the amoebae sequences from the present study and those taken as reference sequences from GenBank ranged from 96 to 100%. Based on sequence analysis, all *V. vermiformis* isolates (n = 29) were closely related to each other regardless of the sampling point.

Table 6.1: Prevalence of *Legionella* and gymnamoebae in domestic and hospital water systems.

Samples (n = 140)	***Legionella*/*L. pneumophila* qPCR & Culturing**				***Acanthamoeba*/*Vermamoeba vermiformis* qPCR & Culturing**			
	***Legionella* & *L. pneumophila* qPCR**		**Culturing**		***Acanthamoeba* & *Vermamoeba vermiformis* 18S rDNA qPCR**			**Culturing**
	***Legionella* 16S rDNA**	***L. pneumophila mip***	***Legionella***	***L. pneumophila***	***Acanthamoeba* & *V. vermiformis***	***V. vermiformis***	***Acanthamoeba***	
Domestic water samples (n = 68)								
Tap faucet biofilm (n = 9)	2	1	-	-	4	2	-	4
Shower water (n = 59)	35	24	8	8	6	17	1	28
Hospital water samples (n = 72)								
Basin water (n = 27)	10	10	1	-	3	18	-	17
Tap faucet biofilm (n = 14)	3	3	-	-	1	6	-	1
Shower water (n = 31)	8	8	1	-	-	20	-	8
Total	**58**	**46**	**10**	**8**	**14**	**63**	**1**	**58**

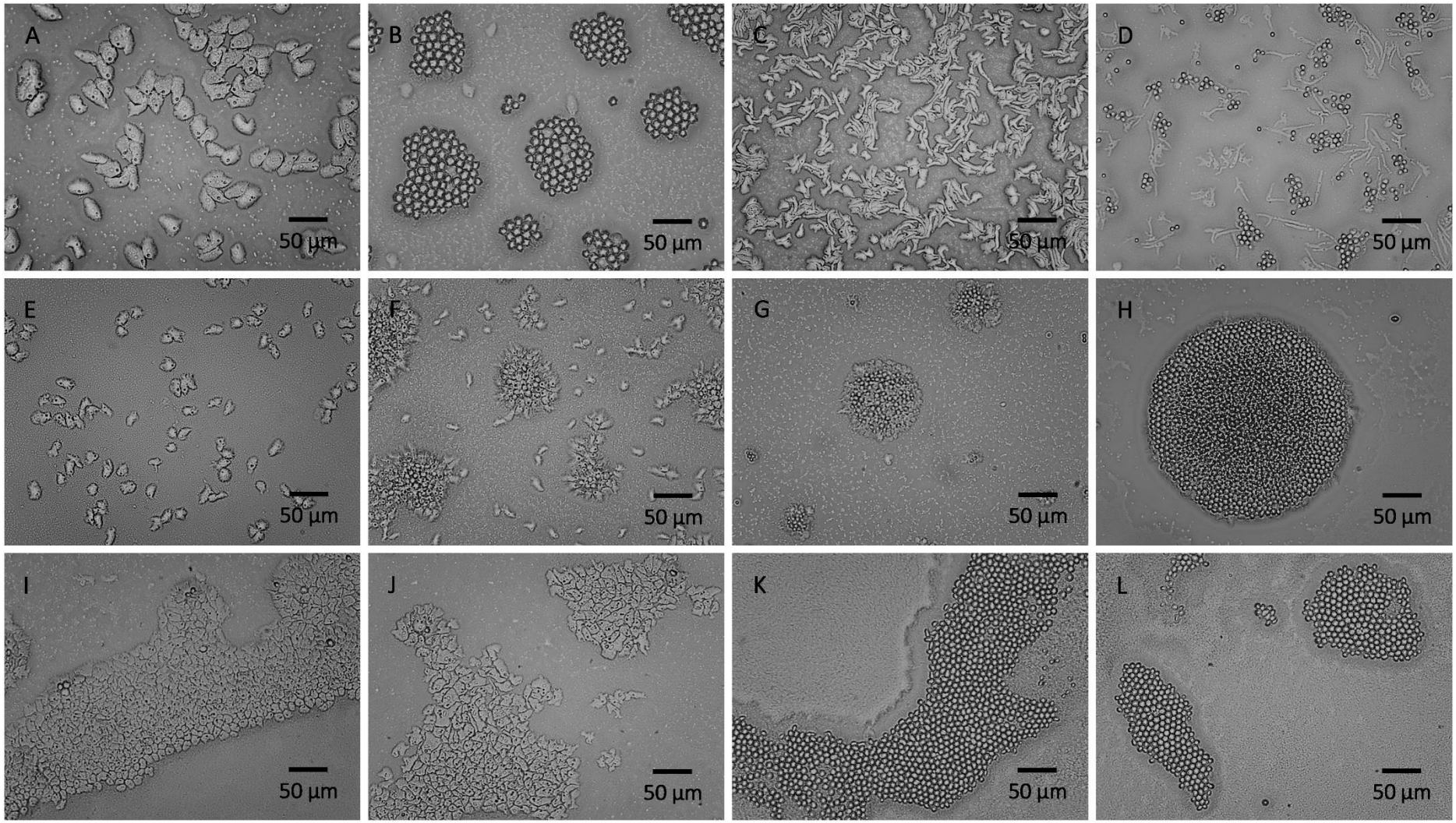

Figure 6.1: Light microscopic examination of *Acanthamoeba* (A and B), *V. vermiformis* (C and D), *Stenamoeba* (E to H) and *Allovahlkampfia* (I to L) growing on heat-killed *Escherichia coli* supplemented non-nutrient agar. Scale bar = 50 µm.

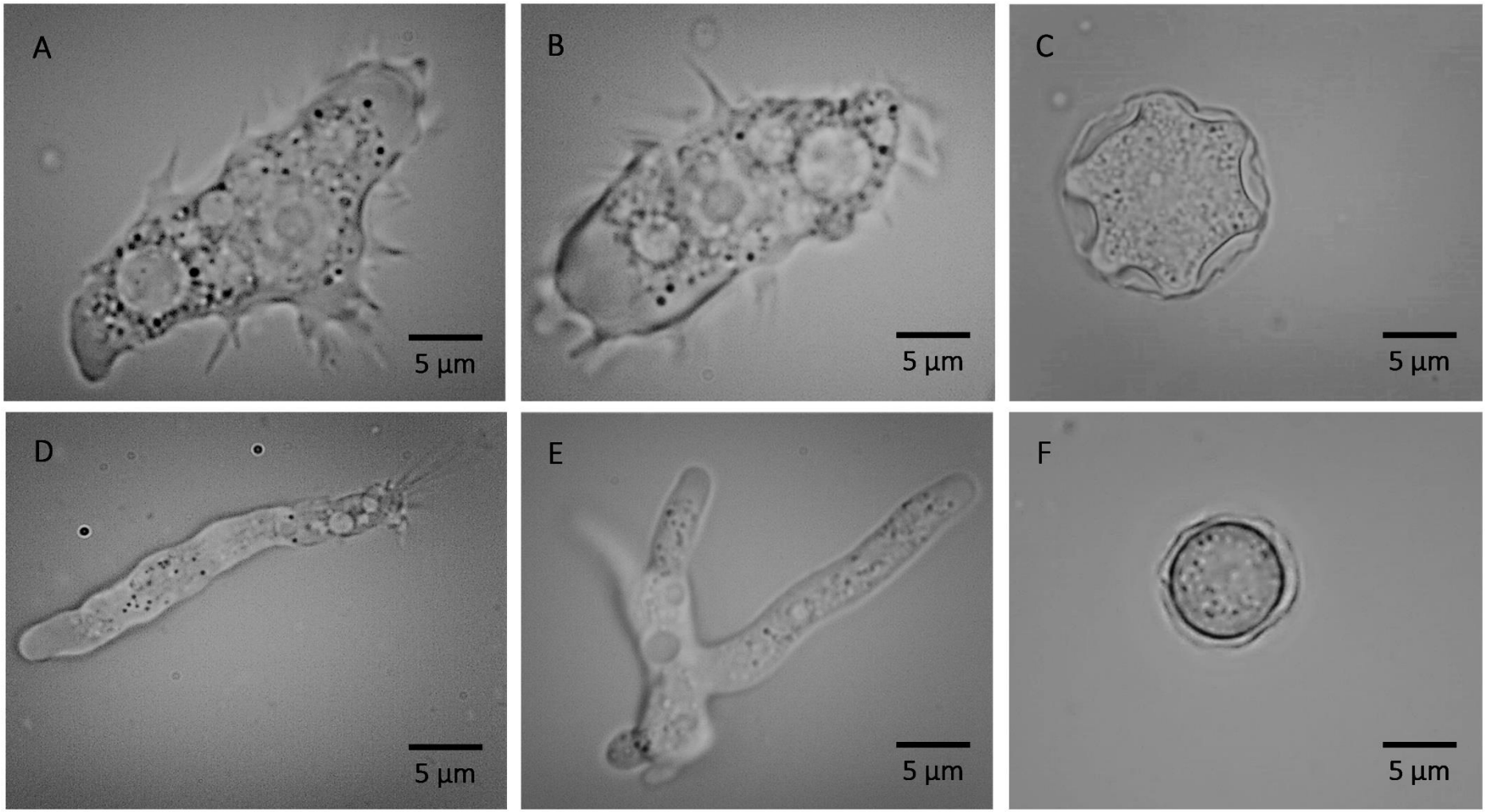

Figure 6.2: Light microscopic images of *Acanthamoeba* and *V. vermiformis*. Locomotive form (A and B) of *Acanthamoeba* developed acanthopodia and cysts (C) showed morphology compatible to members of *Acanthamoeba* group I. Locomotive form (D and E) of *V. vermiformis* were predominantly monopodial and two distinct walls could be visualized in the mature cysts (F). Scale bar = 5 µm.

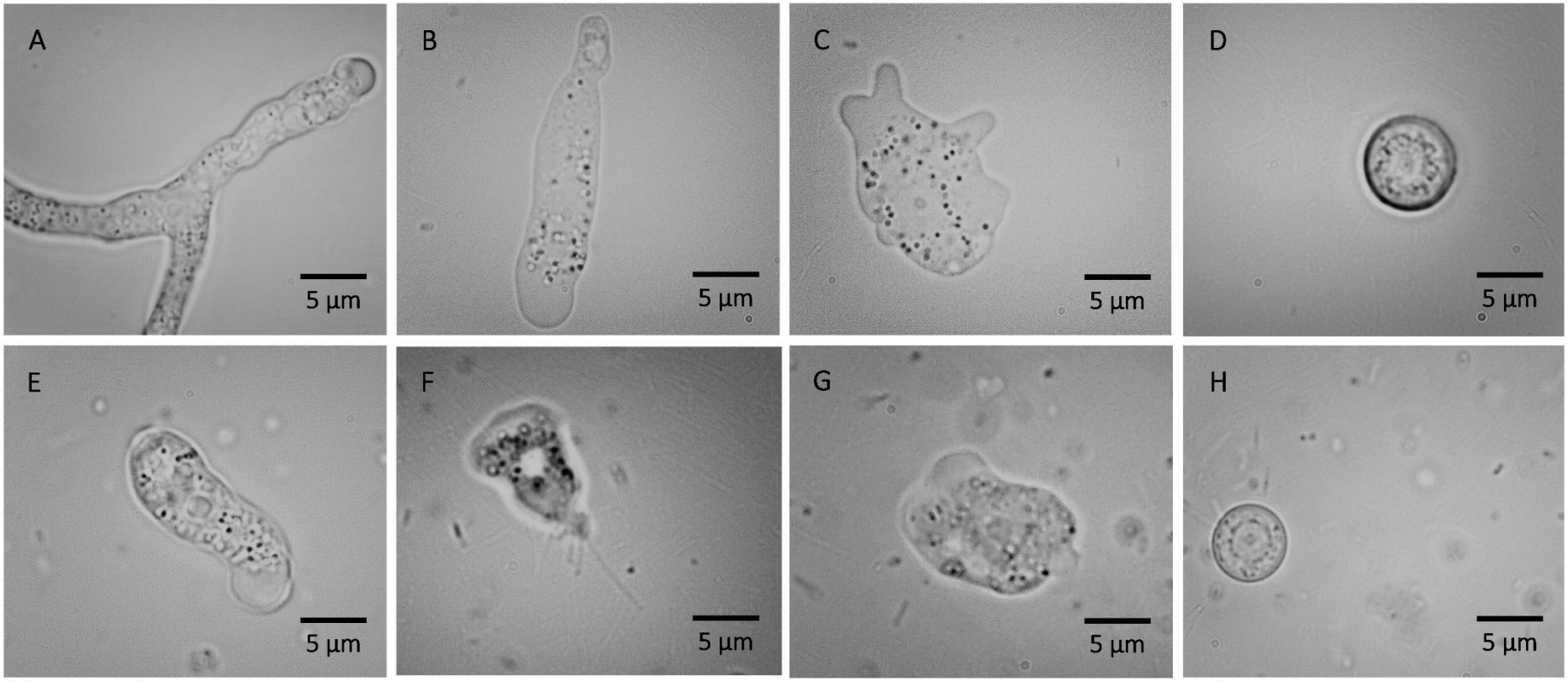

Figure 6.3: Light microscopic images of *Stenamoeba* and *Allovahlkampfia*. Locomotive form developed linguiform (B) whereas during “non-directed movements” dactylopodia like structures appeared (C). Non-adherent floating cells (A) produced multiple pseudopodia. Motile *Allovahlkampfia* developed eruptive limax shape (E) and some cells developed uroidal filaments (F). Unlike *Stenamoeba*, “non-directed movements” produced flabellate shaped trophozoites (G). Both *Stenamoeba* (D) and *Allovahlkampfia* (H) developed mononucleated cysts. Scale bar = 5 µm.

6.4.5 Thermotolerance and osmotolerance of amoebae

Growth kinetics of the isolates were studied at temperatures ranging from 25°C to 45°C (**Table 6.2**). The results indicated that *Allovahlkampfia* was able to grow at 25°C but at 30°C it encysted. All the *V. vermiformis* isolates were able to grow at 30°C but unexpectedly five domestic and two hospital water system isolates were also able to grow at 45°C, which explains their ability to proliferate in hot water systems. For all amoebae isolates, ≥ 35°C temperature significantly increased the doubling time and promoted encystment. The ability of amoebae to encyst had been associated with their survival capacity (Page, 1988). According to osmotolerance assays, all isolates were able to grow on Eco-NNA agar supplemented with 250 mM $_{D}$-mannitol except for *Allovahlkampfia*. Using 500 mM $_{D}$-mannitol, growth of *Stenamoeba* and *V. vermiformis* was reduced significantly and a large quantity of cysts was produced within four days. Interestingly, three *V. vermiformis* isolates not only grew at ≥ 40°C but also in the presence of 750 mM $_{D}$-mannitol indicating the pathogenic potential of these amoebae isolates. More importantly, *Acanthamoeba* displayed growth in presence of 1 M $_{D}$-mannitol. Microscopic examination demonstrated that trophozoites growing in presence of ≥ 500 mM $_{D}$-mannitol were unable to maintain their typical cell shape and developed numerous vacuoles that occupied the entire granuloplasm.

6.4.6 Amoebae predation on pathogenic bacteria

In this study, 33 amoebae belonging to four different genera, *Vermamoeba vermiformis*, *Stenamoeba*, *Allovahlkampfia* and *Acanthamoeba*, were tested for their ability to feed on six different pathogenic Gram-positive and Gram-negative bacteria (**Table 6.2**). *Acanthamoeba* (n = 1) and most of the *V. vermiformis* (n = 21) isolates showed strong bacteriogenic activity against all test bacteria. Importantly, these amoeba isolates showed similar bacteriogenic activities against both Gram-negative and Gram-positive bacteria. Overall, their bactericidal effects were visible after 36 h. However, it was found that patterns of predation of *V. vermiformis* isolated from hospital and domestic water systems differed. *V. vermiformis* from the domestic water system had the highest predation speed and formed large sized clearance zones after 72 h, as compared to hospital water system isolates. This showed that these *Acanthamoeba* and *V. vermiformis* isolates had a broad spectrum bacteriogenic activity and could consume different pathogenic bacteria. Similarly, one of the *Stenamoeba* isolates (DS1) was more effective and able to consume *A. baumannii* and *P. aeruginosa* steadily, but after 96 h further predation was halted. *Allovahlkampfia* did not consume Gram-negative bacteria during first 36 h. However, it was able to consume them during next 60 h. *Allovahlkampfia* and one of the *Stenamoeba* isolate (DS2) did not consume *P. aeruginosa* and persisted in a cyst state, even after 14 days of incubation. **Table 6.2** shows the bacteriogenic activity of all amoebae isolates.

6.4.7 *Legionella*-amoebae interactions

A strong positive correlation was identified between the presence of *Legionella* and amoebae **(Table 6.3)**. All water and biofilm samples classified as positive for the *Legionella*/*L. pneumophila* gene markers or culturable *Legionella*/*L. pneumophila*, were also positive for *Acanthamoeba*/*V. vermiformis* gene marker or culturable amoebae. Analysis of these data using a Pearson's chi-squared test showed a significant positive association between *Legionella* and amoebae: *Acanthamoeba* (qPCR positive) and *Legionella* (culture or qPCR positive) (χ^2 = 7.047, p = 0.008), *V. vermiformis* (qPCR positive) and *Legionella* (culture or qPCR positive) (χ^2 = 9.849, p = 0.002), and culturable amoebae and *Legionella* (culture or qPCR positive) (χ^2 = 9.7639, p = 0.002).

Table 6.2: Co-existence/co-absence of *Legionella* and gymnamoebae in domestic and hospital water systems.

18S rDNA *Acanthamoeba* and 16S rDNA *Legionella*		
χ^2 = 7.047, p = 0.007938	*Legionella* Positive (n)	*Legionella* Negative (n)
Acanthamoeba Positive (n)	11	4
Acanthamoeba Negative (n)	47	78
18S rDNA *V. vermiformis* and 16S rDNA *Legionella*		
χ^2 = 9.849, p = 0.001699	*Legionella* Positive (n)	*Legionella* Negative (n)
V. vermiformis Positive (n)	41	36
V. vermiformis Negative (n)	17	46
Culturable Amoebae and 16S rDNA *Legionella*		
χ^2 = 9.7639, p = 0.00178	*Legionella* Positive (n)	*Legionella* Negative (n)
Culturable Amoebae Positive (n)	33	25
Culturable Amoebae Negative (n)	25	57

Positive: Sample positive through qPCR or standard culturing assay
Negative: Sample negative through qPCR or standard culturing assay

6.4.8 Detection of intracellular *L. pneumophila*

Thirty-three amoebae isolates were analysed for the presence of intracellular *Legionella* and *L. pneumophila*. Three *V. vermiformis* and one *Allovahlkampfia* from domestic water systems showed the presence of intracellular *L. pneumophila* using qPCR **(Table 6.2)**. *L. pneumophila* specific PCR positive amoebae cultures were subjected to FISH assays and confocal microscopy. In FISH micrographs, *Legionella* appeared as green- or yellow-coloured cells localized inside red-coloured *V. vermiformis* and *Allovahlkampfia* **(Figure 6.4)**. These micrographs clearly demonstrated that *Legionella* cells were closely clustered at the periphery as well as situated inside the cytoplasmic regions of the amoebae cells.

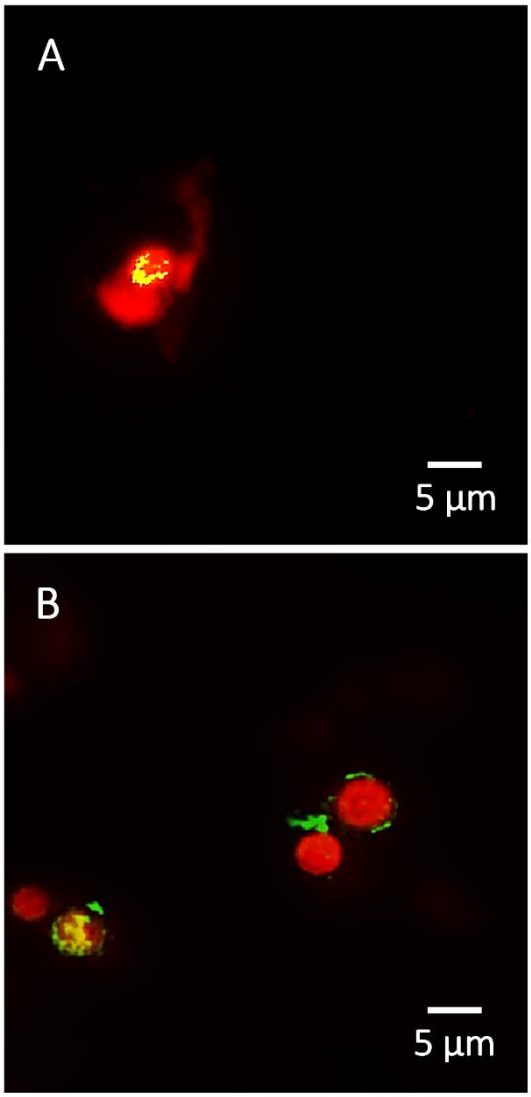

Figure 6.4: Fluorescence *in situ* hybridisation of *V. vermiformis* (A) and *Allovahlkampfia* (B) with Alexa Fluor 647 labelled EUK1209 (red) and *Legionella* with Alexa Fluor 488 labelled LEG705 (green or yellow) probes. These micrographs highlight presence of intra-amoebic *Legionella*. Scale bar = 5 µm.

Table 6.3: Characterization of amoebae isolated from domestic and hospital water systems.

Isolates (n = 33)	Thermaltolerance Temperature (°C)		Osmotolerance	Bacterial predation assay						Intracellular *Legionella*	
				Gram positive cocci		Gram negative bacilli					
	Optimum	Maximum	D-Mannitol (mM)	*Enterococcus faecium*	*Staphylococcus aureus*	*Acinetobacter baumannii*	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Legionella* 16S rDNA	*Legionella pneumophila mip*
Domestic water system: Tap faucet biofilm (n = 4)											
Acanthamoeba DB1	25	35	1000	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis DB1	25	35	250	+++	+++	+++	+++	+++	++	-	-
V. vermiformis DB2	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis DB3	25	35	250	+++	+++	+++	+++	+++	+++	-	-
Domestic water system: Shower water (n = 12)											
Allovahlkampfia DS1	25	30	< 250	+	++	+	+	++	-	Positive	Positive
Stenamoeba DS1	25	40	500	++	++	+++	+++	+++	+++	-	-
Stenamoeba DS2	25	35	500	+	+	+	-	++	-	-	-
V. vermiformis DS1	30	45	250	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis DS2	25	35	250	++	+++	+++	+++	+++	++	-	-
V. vermiformis DS3	30	45	250	+++	+++	+++	+++	+++	+++	Positive	Positive
V. vermiformis DS4	30	45	500	++	+++	+++	+++	+++	+++	Positive	Positive
V. vermiformis DS5	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis DS6	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis DS7	25	40	500	+++	+++	+++	+++	+++	+++	Positive	Positive
V. vermiformis DS8	25	45	500	++	+++	+++	+++	+++	+++	-	-
V. vermiformis DS9	25	45	500	+++	+++	+++	+++	+++	+++	-	-
Hospital water system: Basin water (n = 11)											
V. vermiformis HB1	25	40	250	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB2	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB3	25	40	250	+++	+++	+++	+++	+++	+++	-	-

Isolates (n = 33)	Thermaltolerance Temperature (°C)		Osmotolerance D-Mannitol (mM)	Bacterial predation assay						Intracellular *Legionella*	
				Gram positive cocci		Gram negative bacilli					
	Optimum	Maximum		*Enterococcus faecium*	*Staphylococcus aureus*	*Acinetobacter baumannii*	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Legionella* 16S rDNA	*Legionella pneumophila mip*
V. vermiformis HB4	25	40	250	+++	+++	+++	++	+++	+++	-	-
V. vermiformis HB5	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB6	25	45	750	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB7	25	45	750	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB8	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB9	25	35	250	++	++	+++	++	+++	+++	-	-
V. vermiformis HB10	25	40	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HB11	25	40	750	+++	+++	+++	+++	+++	+++	-	-
Hospital water system: Shower water (n = 6)											
V. vermiformis HS1	25	35	250	+++	+++	+++	-	+++	+++	-	-
V. vermiformis HS2	25	35	250	+++	++	+++	+++	+++	++	-	-
V. vermiformis HS3	25	35	500	++	++	+	++	+++	+	-	-
V. vermiformis HS4	25	35	500	+++	+++	+++	+++	+++	+++	-	-
V. vermiformis HS5	25	35	250	++	++	+++	-	+++	+++	-	-
V. vermiformis HS6	25	40	250	+++	+++	+++	+++	+++	++	-	-

+++ Very strong growth; ++ Strong growth; + Weak growth; - Negative result

6.5 Discussion

Free-living amoebae have direct and indirect public health significance. Not only are they associated with various deadly human diseases (Marciano-Cabral and Cabral, 2003, Khan, 2006, Matin et al., 2008, Visvesvara et al., 2007, Siddiqui et al., 2021, Centeno et al., 1996), they can also play a role as reservoirs of opportunistic pathogenic bacteria (Thomas and Ashbolt, 2011). To our knowledge, this is the first comprehensive study conducted in Australia that investigates the presence of free-living amoebae in domestic and hospital water systems. This revealed that the culture-based amoebae detection method showed lower sensitivity, with only 7% and 38% of qPCR positive *Acanthamoeba* and *V. vermiformis* samples able to grow on agar, respectively. Unfortunately, due to persistent fungal contamination, 22 culturable amoebae were lost. Regardless, the major advantage of culturing is to find and detect viable culturable amoebae. A second advantage is that these amoebae isolates can be further characterized at the molecular level and screened for the presence of endosymbiotic microbes. Despite the availability of molecular techniques, microscopic analysis of trophozoites and cysts is an effective method to identify amoebae morphotypes. However, morphological examination of trophozoites and cysts depends upon culturing conditions and varies within the same species (Visvesvara et al., 2007). From this study, it can be seen that application of all three techniques, classical culturing, microscopy, and molecular profiling, in combination is the most appropriate way to study freshwater free-living amoebae.

Morphological characterization demonstrated the presence of four distinct morphotypes: acanthopodial, monotactic, lingulate and eruptive shown by *Acanthamoeba*, *Vermamoeba vermiformis*, *Stenamoeba* and *Allovahlkampfia*, respectively (**Figure 6.2** and **6.3**). Microscopic analysis of *Stenamoeba* cultures delineated that as have been seen with *Vannella pentlandii*, on an agar plate a trophozoite climbed on the top of a cyst, encysted itself, and finally developed a three-dimensional structure (Maciver et al., 2017). Similarly, *Allovahlkampfia* trophozoites clumped into multilayered or lose aggregates (**Figure 6.1 I** and **J**). In the literature, only loose aggregates of *Allovahlkampfia* are mentioned (De Obeso Fernández Del Valle and Maciver, 2017). So far, the purpose of these types of aggregations in both *Stenamoeba* and *Allovahlkampfia* is not known. Clustering of cysts was also observed in the *Acanthamoeba* culture, but it was associated with some passive mechanism after cyst formation (**Figure 6.1 B**).

This study found that the genus *Vermamoeba vermiformis* was more prevalent in domestic and Australian hospital water systems than *Acanthamoeba*. This could be attributed to continuous decay and lower levels of residual disinfectants present at the point of water delivery (tap or shower heads). Generally, *V. vermiformis* is more sensitive to disinfection

procedures compared with *Acanthamoeba* (Nisar et al., 2020a). A high percentage of *V. vermiformis* and *Acanthamoeba* in engineered water systems presents a public health risk for immunosuppressed individuals and contact lens users (Khan, 2006). It has previously been seen that *V. vermiformis* is present in sources of potable water (rivers and lakes) at concentrations ranging from 5 to 75 cells/L (Kuiper et al., 2006), whereas *Acanthamoeba* is more prevalent in water treatment plants (43%) and recreational hot water springs (47.5%) (Ji et al., 2014). Garcia et al., (2013b) demonstrated that *Acanthamoeba* (31.9%) is more prevalent in those water reservoirs that have high levels of organic matter. A comprehensive study conducted in two counties in Ohio detected high prevalence of *Hartmannella* (53%), *Acanthamoeba* (51%), *Vahlkampfia* (32%) and *Naegleria* (3%) from household water samples (Stockman et al., 2011). However, in a study in the Canary Islands, 59.5% of tap water samples were found to be contaminated with *Acanthamoeba* (Lorenzo-Morales et al., 2005). In another study conducted in Australia within the greater Sydney region, based on morphological characteristics, 29% of bathroom taps were positive for *Acanthamoeba* (Carnt et al., 2020). In this present study, only 11% engineered water system samples were positive for *Acanthamoeba*, whereas 55% were positive for *V. vermiformis*. Initially morphological analysis and PCR-based screening studies were specifically designed to detect *Naegleria*, *N. fowleri* and *Acanthamoeba* from engineered water systems, which may explain this discrepancy. Recently, metagenomic analysis of faucet biofilm samples demonstrated that the most abundant eukaryotic microorganism is *V. vermiformis*, followed by *Acanthamoeba* and *Echinamoeba* (Liu et al., 2012). Similarly, microbiome analysis of drinking water also showed *V. vermiformis* as the most prominent eukaryotic microbe (Delafont et al., 2016, Delafont et al., 2013). In our study, *V. vermiformis* is identified as the most abundant free-living amoebae of hospital and domestic water systems (**Table 6.1** and **6.2**). Amoebae can also serve as an indicator for microbial water quality with a previous study showing that the total number of amoebae in a sample is directly related to the total number of bacteria (Anderson et al., 2001). In conclusion, our study demonstrates that hospital and domestic water systems in Australia are also colonized by other plumbing system bacteria.

Thermotolerance and osmotolerance assays are often used as indicators of pathogenic potential of free-living amoebae. Generally, amoebae capable of tolerating and proliferating at high temperatures and in hyperosmolar media are considered highly pathogenic (Khan et al., 2001). The thermotolerance assay demonstrated that all isolates except *Allovahlkampfia* remained viable and could reproduce at a temperature near human body temperature. Despite the high level of 18S rDNA sequence similarity, difference in thermotolerance and osmotolerance of *V. vermiformis* isolates provided evidence of intraspecies variations.

The relationship between amoebae and bacteria is very complex, and consists of a range of interactions including predation, mutualism, and parasitism (Shi et al., 2021). Naturally, amoebae are predators that regulate the population of environmental microbes (Rodriguez-Zaragoza et al., 2005). In predation, amoebae track bacteria through chemotaxis and consume them via phagocytosis. The main purpose of the bacterial predation assay was to determine whether domestic and hospital water amoebae could phagocytose medically important pathogenic bacteria. Phagocytosis is a complex phenomenon affected by multiple factors related to amoebae and bacteria. Generally, amoebae prefer and migrate towards Gram-negative bacteria and non-capsular bacteria (Rashidi and Ostrowski, 2019, Bornier et al., 2021). Differences in the predatory kinetics of all studied four genera were noticeable. Twenty-one *V. vermiformis* and one *Acanthamoeba* displayed strong bacteriogenic activity and ability to consume all test bacteria, while *Allovahlkampfia* did not show such activity. Initially *Stenamoeba* showed very strong bacteriogenic activity against *A. baumannii* and *P. aeruginosa* but after 96 h it declined, suggesting that further predation was stopped due to establishing an equilibrium. This can be explained through the ability of *A. baumannii* and *P. aeruginosa* to promote encystment and cell death in amoebae hosts (Matz et al., 2008, Tamang et al., 2011). Naturally, *A. baumannii*, *K*. *pneumoniae*, and *S. aureus* synthesize protective polysaccharide layers (Bornier et al., 2021). However, predation kinetics suggested that this polysaccharide layer did not impact the bacteriogenic activity. This study demonstrated that amoebae from domestic and hospital water systems can predate bacteria pathogenic to humans; however, further studies are required to explore these interactions in the natural environment.

Legionella is an important premise plumbing pathogen and considered as indicator of water quality in engineered water systems (Zhang and Lu, 2021). Surveys of *Legionella* colonization of engineered water systems have been conducted in different parts of the world (Stout et al., 2007, Sikora et al., 2015, Hayes-Phillips et al., 2019). In Spain, 37.2% water samples from 20 hospitals were contaminated with *Legionella* and serotyping suggested coexistence of *L. pneumophila* sg1 and *L. pneumophila* sg2-14 (Sabrià et al., 2004). In America, national surveillance studies conducted in 13 different states demonstrated that 70% of hospital water systems were colonized with *L. pneumophila* and *L. anisa*. Additionally, 30% of the water outlets in hospitals documenting hospital acquired legionellosis were contaminated with *L. pneumophila* sg1 (Stout et al., 2007). In Taiwan, 63% of hospital water systems were contaminated with *Legionella* and *L. pneumophila* sg1 was the most frequently recovered serogroup (Yu et al., 2008). A study conducted in Poland examined water samples from hospitals, hotels, industrial plants, military barracks, shopping centres and single mother homes showed the presence of *L. pneumophila* in 74.77% of hot water samples. In all of these

tested samples, *L. pneumophila* sg2-14 was the more frequently detected serogroup (Sikora et al., 2015). In a study conducted in Hungary, 60% of water samples taken from residential sites, healthcare facilities, educational and industrial buildings were colonized with *Legionella* and *L. pneumophila* sg2-14 was most prevalent serogroup (Barna et al., 2016). A study conducted in Italy found that 36.8% of water samples from sites in retirement homes and 10.3% in group homes were contaminated with *Legionella* and *L. pneumophila* sg1 was the most abundant serogroup (De Filippis et al., 2018). In a recent study conducted in USA, PCR assay demonstrated that 93.8% samples collected from chloraminated municipal drinking water distribution system were positive for *Legionella* (Zhang and Lu, 2021). A recent study conducted in South Australia detected *Legionella* and *L. pneumophila* gene markers in 74.6% and 64.2% domestic shower water samples, respectively (Hayes-Phillips et al., 2019). In this study, qPCR assays showed that 41% samples were positive for *Legionella* and 33% for *L. pneumophila* gene markers (**Table 6.1**). Only 7% samples were positive for culturable *Legionella*. Obtained data suggests that both standard qPCR and culturing methods portray an incomplete picture and ignore viable but non-culturable (VBNC) *Legionella* and *L. pneumophila*.

In engineered water systems, amoebae exist and proliferates in both trophozoite and cyst forms (Zhang and Lu, 2021). The latter state protects intracellular *Legionella* from adverse environmental conditions and prolonged disinfection treatments (Boamah et al., 2017, Dobrowsky et al., 2016, Kilvington and Price, 1990). Inside amoebae, *Legionella* survive and proliferate within cytoplasmic vesicles. *Acanthamoeba*, *Balamuthia*, *Dictyostelium*, *Echinamoeba*, *Naegleria*, *Paramecium*, *Tetrahymena* and *V. vermiformis* support intracellular multiplication of *L. pneumophila* (Shadrach et al., 2005, Solomon et al., 2000, Fields et al., 1989, Watanabe et al., 2016, Nahapetian et al., 1991, Boamah et al., 2017, Berk et al., 2008, Berk et al., 1998). Trophozoites facilitate intracytoplasmic replication and biogenesis of potentially infectious and stress tolerant VBNC *L. pneumophila* (Bouyer et al., 2007, Koubar et al., 2011, Nisar et al., 2020a). More importantly, under favourable conditions these trophozoites permit transformation of VBNC cells into culturable *L. pneumophila* (Epalle et al., 2015, Garcia et al., 2007, Steinert et al., 1997). In return, intracellular *Legionella* increases the tolerance of its amoebae host against chemical and physical disinfection procedures (Nisar et al., 2020a). *In vitro* studies demonstrated that *Acanthamoeba polyphaga* harbouring intracellular *L. pneumophila* are more resistant against sodium hypochlorite treatment compared to uninfected protozoans (Garcia et al., 2007). According to available literature, *Allovahlkampfia spelaea* supports intracellular proliferation of *Aeromonas hydrophila*, methicillin resistant *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Mohamed et al., 2016). In this study, we directly detected intracellular *L. pneumophila* from *Allovahlkampfia* and

V. vermiformis (**Figure 6.4**). To our knowledge, this is the first study documenting *Allovahlkampfia* as a host of *L. pneumophila*. Future research is needed to explore the use of these hosts in amoeba co-culture assays used to detect VBNC *Legionella* (Dey et al., 2019, Conza et al., 2013). The chi-squared test results on *Legionella* and amoebae clearly demonstrated that in hospital and domestic water system *Legionella* or *L. pneumophila* always co-occur with *Acanthamoeba* or *V. vermiformis* or other free-living amoebae (**Table 6.3**). It also demonstrated that in engineered water system *V. vermiformis* is host and carrier of *Legionella* or *L. pneumophila*.

This study has shown that in engineered water systems *Legionella* co-occurs with free-living amoebae suggesting that colonisation of systems by free-living amoebae may be a prerequisite for *Legionella* colonisation. Based on their opportunistic nature and role as reservoirs of infectious bacteria, gymnamoebae could be used as a water quality parameter in domestic and hospital water system. These findings advocate the need to investigate the prevalence of free-living amoebae in engineered water system globally and to design guidelines to safeguard the public health. Moreover, there is a necessity to develop effective disinfection procedures to manage the amoebae contamination in engineered water systems.

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Author contributions

MAN and HW conceived and designed research. MAN performed the experiments. JH assisted in sampling and data collection. KER, MHB, HW and RB provided technical assistance. MAN and HW drafted and edited the manuscript, HW, KER, MHB, RB and JH corrected and contributed to the manuscript. All authors approved of the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Legionella and flow dynamics

In this chapter the relationship between *Legionella* and flow dynamics/water stagnation within building water distributions is explored. It addresses objectives 6 to 8 and consists of three manuscripts, one published and two that are currently under review. The first is a systematic literature review investigating the relationship between *Legionella* and flow dynamics/water stagnation in hospital and domestic water systems (objective 6). The second manuscript (objective 7) is a research article that uses a model biofilm system to investigate the effect of flow dynamics/water stagnation on *Legionella* and the final article (objective 8) investigates the effect of flow dynamics/water stagnation on the persistence and survival of *Legionella* in Australian hospital water distribution system.

7 Water stagnation and flow obstruction reduces the quality of potable water and increases the risk of legionelloses

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7.1 Abstract

Legionella is an opportunistic waterborne pathogen associated with Legionnaires' disease and Pontiac fever. Despite improved public awareness, the incidence of *Legionella* associated infections has been increasing. Aerosols generated from engineered potable water systems are a demonstrated cause of both nosocomial and community-acquired legionellosis. The ecology of *Legionella* in these systems is complex with multiple factors impacting their colonization and persistence. Flow dynamics has been identified as an important factor and stagnation in cooling towers is an accepted risk for increased *Legionella* growth; however, less is known about the impact of flow dynamic on *Legionella* in potable water systems. This is especially complex due to the inherent intermittent and variable usage observed within outlets of a potable water system. This systematic literature review examines the role of fluid dynamics and stagnation on the colonization and growth of *Legionella* in potable water systems. Twenty two of 24 identified studies show a positive association between stagnation zones and increased colonization of *Legionella*. These zones included dead legs, dead ends, storage tanks and obstructed water flow (such as intermittent usage or flow restriction). Prolonged stagnation in building plumbing systems also deteriorates the quality of thermally or chemically treated potable water. This stimulates the colonisation of *Legionella* established biofilms. Such biofilms are intrinsically resistant to disinfection procedures and accelerate the rate of decay of chemical disinfectants. Sub-lethal doses of disinfectants and the presence of protozoan hosts in stationary water promote generation of viable but non-culturable *Legionella* cells. This results in false negatives in surveillance methods that use culture methodology. In conclusion, elimination of temporal and permanent stagnation points can improve the quality of potable water, efficacy of disinfectants and reduce the risk of legionellosis. Current guidelines and water safety plans recognise the risks associated with permanent stagnation point (dead ends and dead legs); however, there is a need for greater emphasis on controlling temporal stagnation arising from intermittent usage.

7.2 Introduction

The importance of *Legionella* as an opportunistic waterborne intracellular human pathogen is well documented (Berjeaud et al., 2016). It is frequently associated with nosocomial and community-acquired legionellosis (Pontiac fever and Legionnaires' disease [LD]) in immunocompromised and immunosuppressed individuals (Fields et al., 2002). *Legionella* is ubiquitously present in constructed water systems. It is frequently detected in domestic and hospital water systems, cooling towers, humidifiers, fountains, and pools, (Bartram et al., 2007, Fitzhenry et al., 2017).

Despite recent advancements in disease surveillance and management systems, the legionellosis incidence and outbreak rate remains high. In the USA, there were 290 reported outbreaks of legionellosis from 2009 to 2017 (Centers for Disease Control and Prevention, 2020) and in 2017, 28 outbreaks were reported in 9 European Union countries. In Europe, the legionellosis notification rate also increased from 12 cases/million in 2013 to 18 cases/million in 2017 (European Centre for Disease Prevention and Control, 2019).

According to the USA Centres for Disease Control and Prevention, from 2013 to 2015 approximately 67% potable waterborne outbreaks were caused by *Legionella pneumophila* (Shah et al., 2018). Plumbing networks and potable water distribution systems of hotels, healthcare facilities and residential buildings were identified as *Legionella* hotspots (Benedict et al., 2017). Clearly, understanding the environmental factors that affect the survival and growth of *Legionella* in potable water is essential to inform improved management and control strategies.

The growth of *Legionella* in potable water systems is primarily associated with biofilms and the free-living amoeba hosts feeding on biofilms (Ashbolt, 2015). This provides *Legionella* protection from severe physiochemical stresses (Thomas et al., 2004, Dupuy et al., 2011). The environmental conditions that influence the formation of biofilms are essential for the control of *Legionella.* These include stagnation and the presence of plumbing dead legs and dead ends that provide favourable conditions for microbial growth (Bartram et al., 2007). Dead legs (isolated pipes with limited or no water flow) and dead ends (redundant capped pipe which completely obstruct water flow) are well established as factors contributing to stagnation (National Academies of Sciences Engineering and Medicine, 2019). Stagnation or areas with inappropriate hydraulic dynamics are likely to result in the failure of disinfection procedures (Ling et al., 2018). Due to the complex relationship between different environmental variables, it can be challenging to study role of stagnation in *Legionella* growth. This systematic literature review examines the specific role that water stagnation plays in *Legionella* colonization and growth within potable water systems. Studies on potable water systems including surveillance and laboratory-based studies that examine the relationship between stagnation and fluid dynamics on water quality and *Legionella* colonization are explored.

7.3 Materials and Methods

The protocol of this systematic literature review was designed according to the instructions of PRISMA statement (**Figure 7.1**). The databases Scopus and Web of Science were searched for all articles written in English and published prior to April 2020. In the databases the search terms ("*Legionella pneumophila*" OR "*L. pneumophila*" OR *Legionella* OR legionellosis OR

"Legionnaires' disease" OR "Pontiac fever") AND (flow* OR "dead end*" OR "dead leg*" OR "water circulation" OR "water recirculation" OR "Reynolds number" OR stagnation OR stagnant OR "stationary water" OR turbid OR turbidity OR usage*) AND (plumbing OR pipework OR pipe OR artificial OR piping OR building OR manufactured OR engineered OR potable OR manmade* OR biofilm OR water) were applied. Initially, all identified records were combined and duplicates removed. Subsequently, articles were manually screened by reading the titles and abstracts and excluding those that discussed engineering works, bacterial colonization, or biofilms without specifically referring to *Legionella*. Papers were also excluded that referred to stagnation in municipal water supplies as this is a different issue compared with building water supplies. Finally, the remaining articles were analysed in full and excluded if they did not examine or describe the relationship between *Legionella* colonization, growth, survival and water stagnation, or recirculation in building water systems.

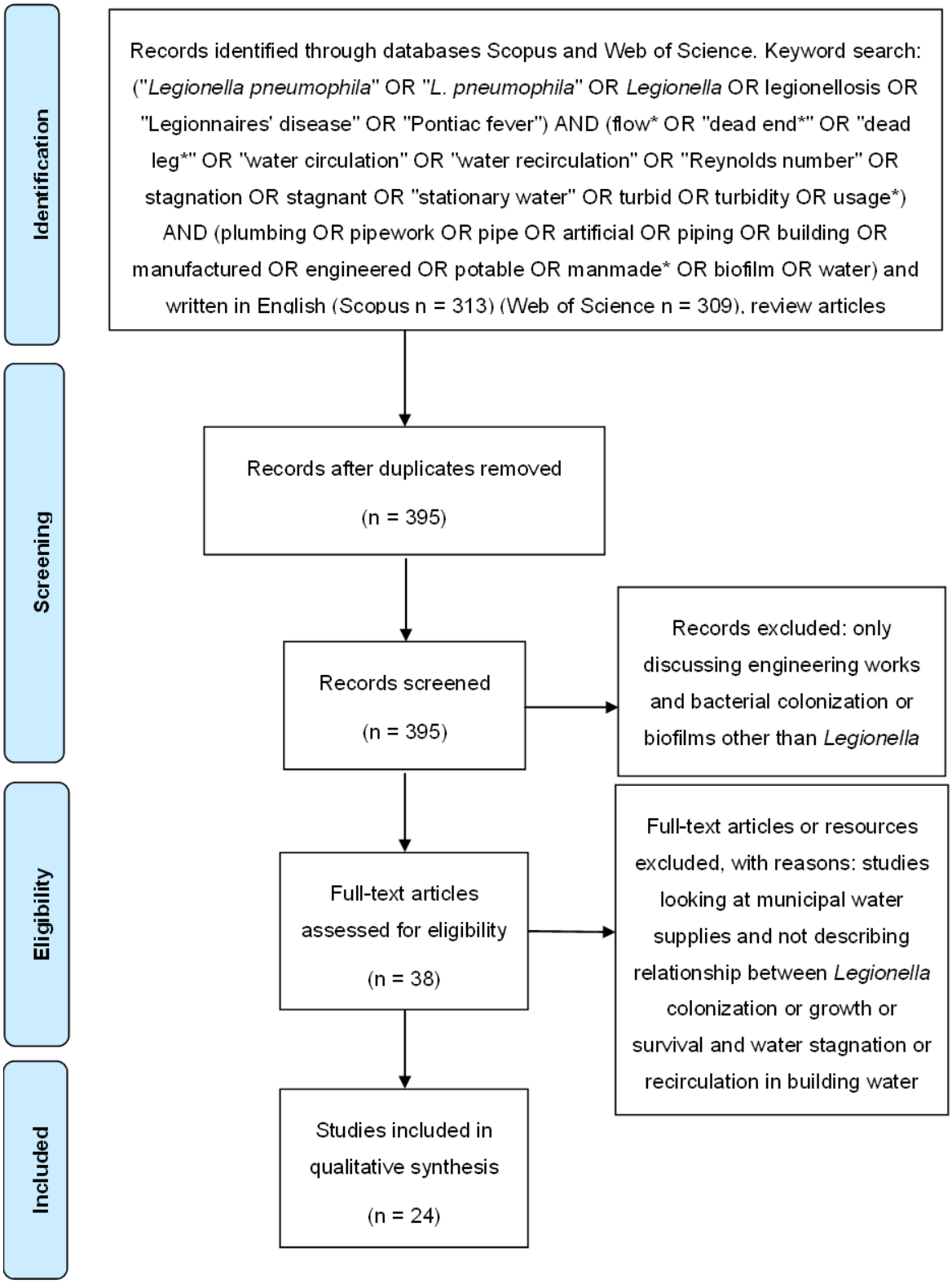

Figure 7.1: Overview of the PRISMA strategy used for selection of articles.

7.4 Results

A total of 395 abstracts were obtained from the Web of Science and Scopus. After applying the described inclusion and exclusion criteria (as presented in **Figure 7.1**), 24 research articles describing relationship between water stagnation and *Legionella* or *L. pneumophila* survival and colonization were identified as suitable for inclusion in this review (**Table 7.1**).

7.4.1 Study sites

Seventeen of 24 studies (**Table 7.1**) investigated hot or cold-water storage tanks and building water distribution systems; 7/24 studies were *in vitro* modelling experiments. The majority of the real-world studies were from investigations of hospital water systems (12/17), and almost half of these were conducted in response to a legionellosis outbreak (5/12). Half of the studies (14/24) discussed the relationship between *Legionella* contamination or colonization and permanent stagnation points (dead ends 6/24, dead legs 8/24). In the majority of studies, there was limited information provided describing fluid or water dynamics.

7.4.2 Building and plumbing system studies

According to **Table 7.1**, *L. pneumophila* serogroup (sg) 1 frequently contaminates water and plumbing system of hospitals and municipal buildings. *L. pneumophila* sg1 is the most common serogroup associated with legionellosis. Few studies reported contamination of other *L. pneumophila* serotypes (sg2-4, sg2-14, sg2-16, sg3, sg4, sg6, sg10 and sg10-14) and *Legionella* species (*L. anisa*, *L. steigerwaltii*, *L. feelii*, *L. longbeachae*, *L. micdadei* and *L. rubrilucens*).

Sixteen out of seventeen studies examining building plumbing systems showed a positive association between stagnation and increased *Legionella* colonization/persistence. Ten out of 17 studies of the building plumbing systems demonstrated that permanent stagnation points (dead ends 5/17, dead legs 5/17) were positively associated with increased *Legionella* contamination. Three studies presented in **Table 7.1** specifically demonstrated that restricted water circulation resulted in persistence of *Legionella* in hospital hot water storage tanks with capacity of ≈ 7,600 to 15,000 L (Ciesielski et al., 1984, Darelid et al., 2002, Ezzeddine et al., 1989).

Ten building water system studies reported a reduction in *Legionella* numbers after increasing water flow rates and removal of dead legs or dead ends. Some studies specifically used hot water recirculation (Gavalda et al., 2019, Darelid et al., 2002) or an increase in the flow rate of hot water (Boppe et al., 2016, Ezzeddine et al., 1989) to reduce the *Legionella* contamination. In Italy (2018 to 2020), installation of time flow taps with 64 to 192 L/day flow rate in the vicinity

of dead legs and dead ends effectively reduced *L. pneumophila* contamination from hot water supplies of hospitals (Totaro et al., 2018, Totaro et al., 2020).

The one study that did not find any positive association between stagnation and *Legionella* growth was by Sidari et al. (2004). This study examined water storage and distribution in a 437-bed hospital in Pennsylvania after numerous cases of nosocomial LD over a five-year period (1994 to 1999). Flushing of hot water temporarily reduced the concentration of *Legionella*; however, this study found that removal of dead legs and routine chlorination was not sufficient to reduce *Legionella* contamination. Finally, ClO_2 (0.3 to 0.5 mg/L) treatment for 21 months resulted in complete removal of *Legionella* (culture negative).

7.4.3 *In vitro* model plumbing studies

As with the real-world studies, in 6/7 *in vitro* model plumbing studies a positive relationship between water stagnation and *Legionella* colonization was identified (**Table 7.1**). Several model systems demonstrated that stagnation promoted genesis of stable *Legionella*-eukaryotic microbe biofilms (Biyela et al., 2012, Vervaeren et al., 2006). Farhat et al. (2010) constructed a pilot scale hot water system consisting of both dynamic water loops (flow rate: 15 L/min) and dead leg stagnation areas. They estimated the *Legionella* concentration (using culture, qPCR and epifluorescent microscopic counts) was 1 to 2 log greater in stagnant points. Two studies also investigated the role of ClO_2 treated water in elimination of *Legionella* contamination from dead legs (Thomas et al., 2004, Loret et al., 2005). It was found that 20% replacement of dead leg water with ClO_2 treated water was not sufficient to completely eliminate culturable *Legionella*. However, regular complete replacement of ClO_2 treated water in dead legs was able to eliminate culturable *Legionella* within seven days (Loret et al., 2005). Another pilot scale domestic water model consisting of both dynamic and stagnant water channels demonstrated that continuous flow of ClO_2 treated water for 7 days significantly reduced *L. pneumophila* from copper dead legs (Thomas et al., 2004).

The one *in situ* model study that did not find any positive association with stagnation and *Legionella* colonisation was conducted by Liu et al. (2006). This model consisted of three parallel pipes within a partially open system (5% of water continuously flowed through the system while 95% of the water was recirculated). The water temperature was maintained at 24°C, one pipe had laminar flow (Re: 355 to 2000), one turbulent flow (Re: 10,000 to 40,000) and one was stagnant. Significantly higher concentrations of culturable *Legionella* were recovered from the biofilm in the pipe with turbulent flow followed by laminar flow with the lowest *Legionella* concentrations observed in the biofilm in the pipe with stagnant flow. However, a significant difference in *Legionella* concentrations in biofilm was observed after one week. These concentrations remained static for the remaining five weeks of sampling. The

authors attributed this result to the turbulent flow increasing the mass transfer of the nutrients and oxygen from the water in the seeding tank. They also noted that the turbulence created may have been insufficient to detach the biofilm. There was no significant difference in planktonic *Legionella* between any of the treatments. This study concluded that intermittently used pipe work (dead legs) sustained aerobic microbial populations than dead ends (where nutrient depletion is more likely).

Table 7.1: Relationship between water stagnation and *Legionella* colonization.

Settings	Species/Serogroup/ Sequence type/Type culture	Comments	Geographic location	References
Building and Plumbing System Studies				
Hospital hot water storage tanks (capacity 7,600 L each)	*L. pneumophila* sg1	Post nosocomial LD outbreak prevention measures adopted. Prevention of stagnation in hot water (45.5 to 47.5°C) tanks reduced *L. pneumophila* population.	Colorado, USA	(Ciesielski et al., 1984)
Residential and institutional cold and hot water distribution system	*L. pneumophila* sg1	Stagnation, low hydraulic flow, and low chlorine concentration (0 to 0.7 mg/L) resulted in *L. pneumophila* contamination.	Ohio, USA	(Voss et al., 1985)
Hospital hot water storage tanks (capacity 10,000 L) and distribution system	*L. pneumophila* sg1 (0.7%), sg6 (87%) and sg10 (7%) *L. longbeachae* (4.3%) *L. micdadei* (1.3%)	Post nosocomial legionellosis infections in immunocompromised patients control measures adopted. Increase in flow rate, maintaining temperature at 60°C and elimination of dead ends reduced bacterial population in hot water.	Brussels, Belgium	(Ezzeddine et al., 1989)
Hospital hot and cold-water storage distribution system	*L. pneumophila* sg1 *L. anisa* *L. steigerwaltii* *L. feelii*	Post fatal nosocomial LD outbreak prevention measures adopted. Removal of fire hydrant spurs (dead legs) connected to storage tanks reduced the bacterial contamination.	Glasgow, Scotland	(Patterson et al., 1994)
Hospital hot water storage (15,000 L) and distribution system	*L. pneumophila* sg1	10-year surveillance program designed to control nosocomial LD. Circulation of hot water (> 55°C) identified as the best way to reduce risk of nosocomial LD.	Jönköping, Sweden	(Darelid et al., 2002)
Hospital water storage (capacity ≈ 19,68,000 L) and water (hot and cold) distribution system	*Legionella*	Removal of dead legs and plumbing system repair unable to produce any profound effect on *Legionella* control. Disinfection of water with ClO_2 (0.3 to 0.5 mg/L) best way to control *Legionella*.	Pennsylvania, USA	(Sidari et al., 2004)
Hospital water distribution system	*L. pneumophila* sg1	Probing of nosocomial legionellosis infections demonstrated that dead end pipe source of *Legionella*.	Jesenice, Slovenia	(Tercelj-Zorman et al., 2004)
Hotel, office, school, store and assembly hall hot water distribution system	*L. pneumophila* sg1 and sg4 *L. anisa* Non-culturable *Legionella*	Hot water system of 40% buildings found contaminated with *Legionella*. Buildings with central hot water storage system (66.7%) showed higher prevalence of *Legionella* than buildings with central hot water circulation system (38.5%) or local instantaneous hot water production system (20%).	Osaka, Japan	(Edagawa et al., 2008)
Hospital hot water distribution system	*L. pneumophila* sg1 (18.8%), sg2-4 (68.3%) and other sg (12.9%)	Two-year hyperchlorination, regular maintenance of boiler and storage tanks, replacement of showerheads and increase in boiler outlet temperature (60°C) were unable to eliminate *Legionella* contamination for longer period of time. Dead legs suspected as reason of recolonization of *Legionella* in hot water system. High temperature (50 to 60°C), ClO_2 (0.2 to 0.7 mg/L)	Milan, Italy	(Tesauro et al., 2010)

Settings	Species/Serogroup/ Sequence type/Type culture	Comments	Geographic location	References
		disinfection and removal of dead legs reduced *Legionella* contamination.		
Nursing home hot and cold-water distribution system	*L. pneumophila* sg1 (ST23)	Probing of nosocomial legionellosis outbreak demonstrated that closed pipes and disturbance in water flow sites promoted bacterial contamination.	Ljubljana, Slovenia	(Trop Skaza et al., 2012)
Hospital hot water distribution system	*L. pneumophila*	By increasing flow rate (> 0.2 m/s) and maintaining 55°C temperature in tap resulted in 93.1 to 46.1% reduction of *L. pneumophila* population within 4 weeks in tap water.	Québec, Canada	(Boppe et al., 2016)
Residential building, nursing home, sports facilities, hotels, and kitchen water distribution system	*Legionella*	Comprehensive surveillance study demonstrated that temperature, pipe length measures, and stagnation are three parameters to predict *Legionella* contamination in drinking waters system. Stagnant hot water (45°C) can easily get contaminate with *Legionella* (predicted risk 77.2%).	Cologne, Germany	(Volker et al., 2016)
Hospital hot water distribution system	*L. pneumophila* sg3, sg2-4, sg6 and sg10–14	Installation of time flow taps (flow rate 64 to 192 L/day) in proximity of dead legs reduced the bacterial contamination in hot water system (temperature: 38.2 ± 2.1 to 46.8 ± 2.1°C and free chlorine: 0.05 ± 0.007 to 0.30 ± 0.01 mg/L).	North-western Tuscany, Italy	(Totaro et al., 2018)
Hospital hot water distribution system	*L. pneumophila* sg2-4 (predominant) and sg1	Dead legs and low-use taps identified as sites of *Legionella* colonization in hot water system. Maintaining temperature at 55°C and water recirculation managed bacterial colonization.	Catalonia, Spain	(Gavalda et al., 2019)
Hospital hot water distribution system	*L. pneumophila* sg1 (ST1 and ST104) *L. rubrilucens* *L. anisa*	WTP828 (water team process 828: 34% wt/wt H_2O_2 and 0.003% wt/wt Ag^+ salt) disinfectant efficiently reduced bacterial load. Efficacy of disinfectant increased by plumbing repairs i.e., removal of dead ends and management of water stagnation.	Cotignola, Italy	(Girolamini et al., 2019)
Hotel water distribution system	*L. pneumophila* sg1 (ST763)	Probing of LD infections demonstrated that potable water (temperature: 60.4 to 122.9°F, chlorine: 0.4 to 2 mg/L, bromine: 2 to 4 ppm) system was contaminated due to dead end and stagnation.	Missouri, USA	(Ahmed et al., 2019)
Hospital hot water distribution system	*L. pneumophila* sg1, sg2-14 and sg2-16	Installation of time flow taps (flow rate: 192 L/day) in correspondence to dead ends, proper hot water recirculation (44.8 to 53.2°C) and chlorination (0.23 to 0.36 mg/L) effectively reduced the bacterial population within 24 hours to 15 days.	Pisa, Italy	(Totaro et al., 2020)
***In Vitro* Model Plumbing Studies**				
Pilot scale domestic water system of loops and dead legs	*L. pneumophila* sg1	Continuous flow of ClO_2 (0.5 mg/L) and chlorine (2.5 mg/L) treated water reduces the bacterial population in loops, whereas continuous flow (7 days) of ClO_2 treated water is only effective way to reduce bacterial population in dead legs. Copper dead legs possess intrinsic antibacterial property and inhibits proper colonization and growth of *Legionella*.	France	(Thomas et al., 2004)

Settings	Species/Serogroup/ Sequence type/Type culture	Comments	Geographic location	References
Pilot scale domestic water distribution model	*Legionella*	Regular 20% renewal of dead legs water unable to produce any impact on *Legionella* population. However, regular complete replacement of dead legs water eliminates culturable *Legionella* within 7 days.	France	(Loret et al., 2005)
Stagnant water model	*L. pneumophila* sg1 (ATCC 33152)	Heat treated (60°C) stagnant tap water in dead ends promotes growth of *L. pneumophila* in biofilms. Moreover, it also increases the diversity of eukaryotic microbes in the biofilm.	Belgium	(Vervaeren et al., 2006)
Plumbing model of three parallel pipe	*L. pneumophila* sg1 and sg6	Bacteria survived and proliferated in turbulent flowing (Re: 10,000 to 40,000), laminar flowing (Re: 355 to 2000) and stagnant water. Stagnation did not promote *Legionella* colonization.	USA	(Liu et al., 2006)
Pilot scale 1 stainless steel hot water system (volume: 316 L)	*L. pneumophila* *Legionella*	Dead legs (stagnant point) water harboured 1 to 2 log higher concentration of bacteria than loops (flow rate 15 L/min)	France	(Farhat et al., 2010)
Dual pipe loop system (50 L volume)	*Legionella*	Biofilm of *Legionella* and *Naegleria fowleri* ATCC 30894 developed in stagnant water (dead-end, reservoirs and hydrant source).	USA	(Biyela et al., 2012)
Pilot scale household hot water system (volume: 71.9 L)	*L. pneumophila*	*L. pneumophila* population persisted at a high density in stagnant/low frequency usage taps than dynamic water. Hot stagnant water (temperature 51°C) supported selection of *L. pneumophila* (125 times more) than dynamic hot water.	USA	(Rhoads et al., 2015)

LD: Legionnaires' disease; ClO2: Chlorine dioxide gas; wt/wt: weight/weight; Re: Reynolds number

Table 7.2: Methods available for screening and detection of *Legionella* contamination from potable water and building plumbing system.

Techniques	Description	Limitations	References
Microbiological culturing and isolation	Gold standard to detect and identify *Legionella* contamination. Processed water sample/biomass is cultured on buffered charcoal yeast extract (BCYE) agar. Isolated bacterial colonies are further identified by serological/molecular assays. Its detection limit is 35 CFU/L.	Two weeks required to grow *Legionella* from potable water samples. Only detects culturable *Legionella* and is unable to screen any VBNC *Legionella*.	(International Organization for Standardization, 2017, Standards Australia, 2017, Volker et al., 2016)
Legionella–amoebae co–culture assay	Most appropriate method to identify VBNC *Legionella*. Processed samples are inoculated on amoebae (i.e., *Acanthamoeba*) culture plate. Then plates are regularly examined microscopically to identify any cytological and morphological modifications in amoebae cells.	Longer periods of incubation required to resuscitate VBNC *Legionella*. Difficult to quantify density of VBNC *Legionella*.	(Conza et al., 2013, Garcia et al., 2007, Epalle et al., 2015)
Fluorescent *in situ* hybridization	It is a whole cell-based screening method. Nucleic acid (16S rRNA) or antibody-based probes are used for visual detection of cells. It can be modified to detect and estimate VBNC *Legionella*.	Probes can interact with 16S rRNA of dead cells. Probes can cross react with background environmental bacteria.	(Delgado-Viscogliosi et al., 2005, Kirschner et al., 2012, Declerck et al., 2003)
Flow cytometry	It is a membrane integrity-based assay to identify VBNC *Legionella*. In this method differential live/dead stains (SYBR green/propidium iodide, Syto9/propidium iodide and thiazole orange/ propidium iodide) and/or labelled probes are used to characterize VBNC and dead cells of *Legionella*. Its detection limit is 45 to 150 cells/L.	Only detects specific *Legionella* species/strains/serogroups/serotypes, so effective for controlled *in vitro* studies. Universal probes which cover entire *Legionella* complex are not available.	(Allegra et al., 2008, Füchslin et al., 2010, Keserue et al., 2012)
PCR detection and enumeration	Rapid and efficient method to detect and quantify *Legionella* contamination. Processed water sample/biomass is subject to 16S rDNA (*Legionella*) and *mip* (*L. pneumophila*) genes qPCR assay. Its detection limit is 500 GU/L.	Only detects DNA of *Legionella* and unable to differentiate culturable, VBNC and dead cells of *Legionella*.	(International Organization for Standardization, 2019, Wellinghausen et al., 2001)
Viability-qPCR	In this method, prior to nucleic acid extraction and qPCR the sample is processed with cell membrane impermeable nucleic acid intercalating dyes (ethidium monoazide or propidium monoazide). It is a good method to detect and quantify VBNC *Legionella*.	Presence of background bacteria in high density (common in environmental samples) challenges validity. Not suitable for *Legionella* quantification from biofilm samples.	(Delgado-Viscogliosi et al., 2009, Ditommaso et al., 2014, Ditommaso et al., 2015, Taylor et al., 2014)

CFU/L: colony–forming unit/litre; GU/L: genomic unit/litre

7.5 Discussion

Legionella is a typical premise plumbing pathogen that can tolerate disinfectants, develop biofilms, survive in waterborne protozoa, and thrive in low levels of nutrients (Fields et al., 2002). Prolonged water stagnation can result in accumulation of nutrients and compromises disinfection, promoting colonization of premise plumbing pathogens in potable water systems (Rhoads et al., 2016, Ling et al., 2018, Xu et al., 2018, Gauthier et al., 1999).

7.5.1 Influence of water stagnation on bacterial colonization

Microbial ecology of potable water is very complex. From water source to point of use, the varying environmental conditions modulate growth and composition of microbial communities. In building plumbing systems, water can stagnate permanently or temporarily (Prévost et al., 1997). Dead ends permanently stagnate water (Tercelj-Zorman et al., 2004); whereas, water storage tanks and temporal water usage can result in intermittent stagnation (Peter and Routledge, 2018). Prior to consumption, water can stagnate from a few hours to weeks in a building piping system (Manuel et al., 2009). This may result in the deterioration of water quality and promote accumulation of biomass thereby increasing the concentration of intact and cultivable cells.

7.5.1.1 Temporary stagnation

Water in municipal distribution pipelines rarely gets obstructed. As it enters domestic building plumbing systems it stagnates and changes the diversity of microbial communities (Pepper et al., 2004). It has been observed that overnight stagnation resulted in a 4 to 580 fold increase in microbial load (using heterotrophic plate counts), a 2 to 3 fold increase in total cell concentration (using flow cytometry) and a 2 to 8 fold increase in microbial activity (ATP analysis) (Lautenschlager et al., 2010). This was supported by a study which showed that a two week stagnation of a domestic building plumbing systems resulted in a 2 log increase in microbial load (using flow cytometry) (Lipphaus et al., 2014).

Building design also impacts on stagnation and microbial water quality. A recent study demonstrated that water from the plumbing system of a net-zero energy residential building contained 5 log higher amount of bacterial 16S rDNA (6.46×10^7 gene unit/mL) and *Legionella* 16S rDNA (8.91×10^4 gene copies/mL) compared with a typical residential building plumbing system (400 gene unit/mL and 100 to 2.3×10^3 gene copies/mL respectively). This variation, due to building structure and design, could be attributed to increased stagnation prior to consumption as the average stagnation time for a net-zero energy residential building is 2.7 days compared with 1 day in a typical residential building (Rhoads et al., 2016). *In vitro* green building hot water plumbing model studies also suggested limited water flushing and

inadequate temperature (51°C) support colonization of *Legionella* and host protozoa (Rhoads et al., 2015). In conclusion, stagnation, and aging of water in plumbing system of green energy buildings resulted in persistence of microbial contamination.

7.5.1.2 Permanent stagnation

Dead legs and dead ends have been implicated in several nosocomial outbreaks of LD (Patterson et al., 1994, Tercelj-Zorman et al., 2004, Bartley et al., 2016). These permanent stagnation points should be avoided or managed during building construction or modifications (Bartram et al., 2007). However, it is impractical to remove them all. Recently, two studies of Italian hospitals demonstrated that installation of time flow taps (flow rate 64 to 192 L/day) in the vicinity of dead legs was successful in reducing *L. pneumophila* contamination in the hot water system (Totaro et al., 2018, Totaro et al., 2020). In the first study, *L. pneumophila* from various serogroups including sg3, sg2-4, sg6 and sg10-14, with concentrations ranging from 1 x 10^2 to 1.3 x 10^5 CFU/L, was present in hospital hot water (temperature: 38.2 ± 2.1 to 46.8 ± 2.1°C and free chlorine: 0.05 ± 0.007 to 0.30 ± 0.01 mg/L). Instead of removing dead legs, time flow taps were installed in the outlet closest to each identified dead leg. This strategy successfully eliminated all culturable *L. pneumophila* (Totaro et al., 2018). In the second study, it was found that within 15 days of installation of time flow taps (flow rates 192 L/day) in the proximity of dead ends, *L. pneumophila* sg1, sg2-14 and sg2-16 contamination in hospital hot water (temperature: 44.8 to 53.2°C and free chlorine: 0.23 to 0.36 mg/L) was reduced from 2 x 10^2 to 1.4 x 10^5 CFU/L to no culturability (Totaro et al., 2020). These studies demonstrate that reducing temporary stagnation and increasing flow, even in the presence of permanent stagnation points, reduces the risk of *Legionella* contamination.

Several model studies have explored the impact of permanent stagnation on *Legionella* colonization. In a pilot scale model, bacterial biofilms in dead legs (pre-treatment population density 10^7 CFU/L and 10^8 genomic unit/L) survived thermal shock treatment and promoted rapid recolonization within 48 hours (Farhat et al., 2010). Thus, permanent stagnation sites act as a reservoir of *Legionella* biofilms that play an important role in re-contamination of water.

7.5.2 Water stagnation and failure of disinfection procedures

The accelerated decay of residual disinfectant significantly increases the risk of LD (Voss et al., 1985). Prolonged water stagnation, microbial communities and organic nutrients accelerate the decay of disinfectants (Rhoads et al., 2016, Ling et al., 2018, Xu et al., 2018). According to the US CDC, from 2000 to 2014 70% of LD outbreaks were due to inadequate disinfectant concentrations in water supplies (Garrison et al., 2016). Currently in the USA, six disinfection procedures: Cu-Ag ionization, chlorine, chlorine dioxide, monochloramine, ozonisation and ultraviolet disinfection, are used to control *Legionella* (United States Environmental Protection

Agency, 2016b). In the USA, the concentration of chemical disinfectant is maintained in 95% potable water delivered to consumers (United States Environmental Protection Agency, 2016a). In studies from Austria, Germany, Netherlands and Switzerland, the concentration of residual disinfectant in potable water (delivered to consumers) is not maintained (Rosario-Ortiz et al., 2016).

7.5.2.1 Decay of disinfectant

Different chemical disinfectants are widely used to disinfectant potable water supplies (World Health Organization, 2004, Pontius, 1990, Kim et al., 2002). The residual disinfectant concentration in treated water does not remain constant and it gradually decreases within building plumbing systems. This continuous process of decay may result in complete degradation of chemical disinfectants, thereby increasing the chances of persistence of microbial contamination in building plumbing systems (Vieira et al., 2004). Rates of decay have been associated with temporary or permanent stagnation, aging of plumbing systems, nature of plumbing material and microbial biomass (Vieira et al., 2004, Patrick et al., 2012, Ling et al., 2018). Goyal and Patel (2015) reported continuous temporal stagnation in storage tanks for 22 hours decreased residual chlorine concentration (from 0.2 to ≈ 0.12 mg/L) in domestic buildings. Similarly, Barbeau et al. (2005) reported that 24 hours of temporary stagnation decreased chlorine content of water (from 0.6 to ≈ 0.3 mg/L in cement line ductile and 0.4 to 0.05 mg/L in grey cast iron pipe dead end) and increased the microbial count. Galvin (2011) demonstrated that the lower flow velocity observed in dead ends can result in a decrease in concentration of both chlorine and chloramine within 200 hours. Laboratory model based experiments have also demonstrated that an increase in residence time of water results in decreased concentrations of chlorine, chloramine and ozone and increased microbial contamination (Clark et al., 1994). Another model system showed that complete renewal of ClO_2 (0.5 mg/L) disinfected water in dead legs eliminated culturable *Legionella* (Loret et al., 2005).

7.5.2.2 Persistence of intrinsically disinfectant resistant *Legionella*

Intrinsic resistance is a natural tolerance and resistance attributed to *Legionella* against physical and chemical water treatments (Steinert et al., 1998, Cooper and Hanlon, 2010). Biofilm in areas of stagnation within plumbing systems may harbour resistant populations and act as a continuous source of microbial contamination (Bartley et al., 2016) . Studies have shown that currently available water disinfection methods (i.e., chlorine, chlorine dioxide, chloramines, hydrogen peroxide, ozone, copper, and silver ions) are only successful in reducing or eliminating *Legionella* populations transiently. Sidari III et al. (2004) and Totaro et al. (2018) (see **Table 7.1**) demonstrated the inability of chlorine to eliminate all culturable *Legionella*. According to Totaro et al. (2018), circulation of hot chlorinated water in dead legs

eliminated all chlorine sensitive culturable *L. pneumophila* sg2-4 and sg10-14 serotypes, though chlorine resistant and thermostable *L. pneumophila* sg3 and sg6 serotypes persisted in low concentrations.

A study conducted in a century old hospital in Italy found that continuous hyperchlorination (0.5 to 1 mg/L) for five years was not sufficient to completely eliminate *Legionella* contamination. Multiple factors including outdated piping, dead legs, improper water circulation and corrosion of plumbing materials were proposed as causes of *Legionella* persistence (Orsi et al., 2014). Similarly, a study conducted in another Italian hospital plumbing system demonstrated persistence of the same strain of *L. pneumophila* for a period of 15 years (1990 to 2004) despite thermal treatment, chlorination, and chlorine dioxide treatment (Scaturro et al., 2007). This demonstrates the role permanent stagnation (dead ends and dead legs) has in harbouring and potentially selecting for more resistant strains through constant exposure to sub-lethal concentration of disinfectants (Cooper and Hanlon, 2010, Dupuy et al., 2011).

7.5.2.3 Stagnation and microbial biofilms

In water storage and distribution systems, 95% of the microbial population exists as biofilms attached to the inner surfaces and only 5% in the water (Flemming et al., 2002). According to an *in vitro* simulation experiment, plumbing coated with *Legionella* biofilms decayed free chlorine in stagnant water (48 hours stagnation period) and increased the risk of legionellosis up to six times (Huang et al., 2020). Available literature shows that in potable water and building plumbing systems complex biofilms and amoebae hosts protect *Legionella* (Thomas et al., 2004, Kilvington and Price, 1990). Cargill et al. (1992) reported that in contrast to free living cells, *L. pneumophila* existing in complex biofilms can tolerate high doses of disinfectant. Specifically, the amoeba *Acanthamoeba* and *Vermamoeba* provide additional protection from prolonged and persistent water treatment processes (Dobrowsky et al., 2016, Cervero-Arago et al., 2014b, Dupuy et al., 2011, Kilvington and Price, 1990). Donlan et al. (2005) observed that for *L. pneumophila*–amoebae complex biofilms, monochloramine (0.5 mg/L) was a more effective disinfectant compared to free chlorine (0.5 mg/L). Stagnant potable water allows formation of thick, dense, complex and adherent biofilms, which accelerate decay of disinfectants (Tsagkari and Sloan, 2018). More importantly, chemical disinfectants are unable to penetrate such multispecies biofilms (Bridier et al., 2011).

7.5.3 Stagnation in building hot water system

The studies presented in **Table 7.1** demonstrate that due to stagnation points and low water consumption, building hot water systems will develop *Legionella* contamination. Unlike cold water, hot water systems contained diverse species and serotypes of *Legionella* (Voss et al., 1985, Trop Skaza et al., 2012, Totaro et al., 2018, Girolamini et al., 2019). An *in vitro* study

demonstrated colonization of diverse species of *Legionella* i.e., *L. pneumophila*, *L. anisa*, *L. taurinensis* and *L. drancourtii*, and protozoan hosts i.e., Alveolata members, *Bodonidae*, *Euglenozoa*, *Neobodo curvifilu*, *Thecamoebae*, *Vannella*, and *Vermamoeba vermiformis* in a hot water plumbing model. Moreover, it was also noticed that pathogenic *L. pneumophila* and *L. anisa* developed stable biofilms with protozoa (*Thecamoebae*, *Vannella*, and *V. vermiformis*) in storage tanks and survived during thermal (70°C/30 minutes) and chemical (biodispersant: tensio-active Ferrofos® 5260 and biocide: H_2O_2-peracetic acid) treatments. After such treatments, these stable biofilms re-contaminated hot water within the entire plumbing model (Farhat et al., 2012). Saby et al. (2005) analysed hot water plumbing system models (materials: steel, galvanized steel, and chlorinated polyvinyl chloride) and concluded that chemical disinfection procedures (H_2O_2, continuous chlorination, hyperchlorination and peracetic acid treatment) temporarily eradicated established *Legionella* biofilm. The only solution to eradicate *Legionella* biofilms was to maintain water temperature at > 55°C at all points, which required continuous circulation of hot water. In another pilot study (material: stainless steel), it was noticed that thermal treatment (70°C/30 minutes) of the plumbing system containing a well-established *Legionella* (10^3 CFU/cm^2) biofilm decreased culturable *Legionella*. It was also observed that existence of dead legs in plumbing system promoted rapid recontamination of remaining water (Farhat et al., 2010). All studies discussing hot water supplies to buildings showed that stagnation of hot water in storage tanks and dead legs or dead ends led to a reduction in water temperature (< 45°C), and rapid decay of disinfectants promoting colonization of *Legionella*. This was exacerbated in warm water systems as dissipation of the residual chemical disinfectant was further accelerated at high temperature (Vasconcelos et al., 1997, Ndiongue et al., 2005).

7.5.4 Nutrient and oxygen supply hypothesis

One argument presented by Lui et al (2006) that counters the positive association of stagnation with *Legionella* contamination of biofilms is the reduced oxygen and nutrient content present in areas of stagnation compared with areas of turbulent flow. However, *L. pneumophila* sg1 has been shown to survive in drinking water under low nutrient availability for more than 2 years (Paszko-Kolva et al., 1992). *L. pneumophila* can also proliferate efficiently at 6 to 6.7 mg/L concentration of dissolved oxygen. However, at oxygen concentration less than 2.2 mg/L it can survive but stops multiplying (Wadowsky et al., 1985). *L. pneumophila* (sg1, sg2, sg3, sg4 and sg6) has also been isolated from different water bodies (i.e. lakes and rivers) with dissolved oxygen concentrations of 0.3 to 9.6 mg/L (Fliermans et al., 1981). In water at temperatures of 20 to 45°C, the concentration of dissolved oxygen ranges from 9.06 to 5.94 mg/L (United States Environmental Protection Agency, 1989), which is sufficient for *Legionella* survival and growth. Furthermore, upon environmental stresses (nutrient starvation, low

oxygen, osmolarity alterations, pH and temperature fluctuations) bacteria in biofilms activate stress tolerance mechanisms, which can lead to genesis of viable but non-culturable (VBNC) cells (Fux et al., 2005, Yamamoto et al., 1996). It is well known that protozoan hosts (Buse et al., 2013) and application of disinfectants (Casini et al., 2018, Turetgen, 2008, Mustapha et al., 2015, Allegra et al., 2008, Whiley et al., 2017) promote transformation of vegetative *Legionella* into VBNC cells. As such, studies that rely solely on culture methods of detection (such as the study by Lui et al (2006)) will not detect VBNC *Legionella* and underestimate the numbers present (Whiley, 2016).

7.5.5 Current methods of *Legionella* screening

In stagnation areas, limited nutrient availability and sub-lethal doses of disinfectant promote generation of VBNC *Legionella* (Li et al., 2014). According to Farhat et al. (2010) thermal treatment of water decreased the numbers of culturable *Legionella* temporarily, but increased concentration of VBNC cells. A one year pilot study demonstrated that chemical disinfection (Ferrocid® 8591, Ferrofos® 5260, H_2O_2 and peracetic acid) decreased culturable *Legionella* and *L. pneumophila* from 0.5 to 2 log, however application of a PCR analysis showed existence of VBNC in high density (Farhat et al., 2011). As mentioned earlier, at stagnation points *Legionella* also exists in complex biofilms, and amoebae hosts dwell in such microbial communities as well. It is well known that amoebae host (*V. vermiformis*) are capable to transform culturable *Legionella* into VBNC (Buse et al., 2013). VBNC are pathogenic in nature and infect amoebae and human cell lines (Cervero-Arago et al., 2019). Proteomic profiling suggests VBNC are able to synthesize some virulence factors and proteins involved in different metabolic pathways (Alleron et al., 2013). *Acanthamoeba castellanii* and *A. polyphaga* resuscitate VBNC synthesized by starvation (Steinert et al., 1997) and disinfectant treatment (Garcia et al., 2007), respectively. *In vitro* studies showed *A. polyphaga* resuscitated *Legionella* are infectious for alveolar epithelial and macrophage like cells (Epalle et al., 2015). So far, the underlying mechanisms of VBNC biogenesis and resuscitation are not yet well understood.

Multiple methods are available to screen and detect *Legionella* contamination in potable water and building plumbing systems (**Table 7.2**). Any method that can identify and estimate both culturable and VBNC *Legionella* is most appropriate to monitor building plumbing system and potable water. Microbiological culturing (International Organization for Standardization, 2017) and qPCR (International Organization for Standardization, 2019) are approaches recommended by ISO (international organization for standardization), however both methods are unable to provide information about VBNC *Legionella*. Fluorescence *in situ* hybridization (Delgado-Viscogliosi et al., 2005) and viability qPCR (Ditommaso et al., 2014) are two techniques that can be used to estimate populations of VBNC *Legionella*, however validity of

both assays is challenged by high density of sample background bacteria other than *Legionella*. Differential live/dead stain flow cytometry is widely used for *in vitro* disinfectant efficacy and plumbing model experiments to estimate the population of VBNC *Legionella* (Mustapha et al., 2015, Wang et al., 2010, Allegra et al., 2008, Allegra et al., 2011). Some researchers have tried to develop and use dye labelled *Legionella* specific antibodies for detection of VBNC, however these antibodies are highly specific and can only detect specific strain/serogroup/serotypes (Keserue et al., 2012, Füchslin et al., 2010). A universal probe, which can cover all members of genus *Legionella*, is required to be effective to detect and estimate VBNC contamination from building plumbing systems. *Legionella*-amoebae coculturing is one of the best techniques to identify VBNC contamination. In this assay, suspected samples are cultivated on amoebae hosts (preferably *Acanthamoeba*) and VBNC resuscitation is monitored microscopically (Conza et al., 2013). Garcia et al. (2007) artificially generated VBNC and then resuscitated them back into culturable *Legionella* using an *Acanthamoeba* host. Using an *Acanthamoeba* co–culture assay, Conza et al. (2013) estimated the quantity of VBNC *Legionella* (10^2 to 10^6 cells/g) from a composting facility. A major drawback of *Legionella*-amoebae coculturing is the requirement of incubation for prolonged time. However, to our knowledge available literature has not discussed the application of co–culture assays to estimate VBNC contamination from building plumbing system.

7.6 Conclusion

Restricted water circulation and temporary or permanent water stagnation allows *Legionella* to colonize in building plumbing systems and water storage facilities. Aging of water, stagnation and microbial biofilms accelerate decay of residual disinfectants and deteriorates water quality in buildings. Further research is required to better understand role of complex *Legionella*-protozoa biofilms in degradation of disinfectant in stationary water. To achieve long term disinfection of potable water continuous circulation of thermally or chemically treated water in buildings is the only solution to prevent outbreaks of legionellosis.

Author contributions

MAN and HW drafted and edited the manuscript. HW, KER, MHB, and RB corrected and contributed to the manuscript. All authors approved of the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Once-a-day flushing of a model plumbing system significantly reduced colonisation of *Legionella*

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8.1 Abstract

Legionella is the causative agent of Legionnaires' disease and its prevalence in potable water is a significant public health issue. Water stagnation within buildings has been shown to increase the risk of *Legionella.* However, there are limited studies investigating the effect of short-term stagnation arising through intermittent usage on *Legionella* proliferation and the studies available do not consider viable but non culturable (VBNC) *Legionella*. This study used a model plumbing system to examine the effect of intermittent water stagnation on both VBNC and culturable *Legionella*. The model plumbing system contained a water tank supplying two biofilm reactors. The model was initially left stagnant for 147 days, after which one reactor was flushed daily, and the other weekly. Biofilm coupons and water samples were collected for analysis at days 0, 14 and 28. These samples analysed for culturable and VBNC *Legionella*, free-living amoebae, and heterotrophic bacteria. After 28 days, once-a-day flushing significantly ($p < 0.001$) reduced the amount of biofilm-associated culturable *Legionella* (1.5 $\log_{10}$ reduction) compared with once-a-week flushing. However, higher counts of biofilm-associated VBNC *Legionella* (1 $\log_{10}$ higher) were recovered from the reactor with once-a-day flushing compared with once-a-week. Likewise, once-a-day flushing increased the population of biofilm-associated *V. vermiformis* (≈3 $\log_{10}$ higher) compared with once-a-week, which indicated a positive relationship between VBNC *Legionella* and *V. vermiformis*. This is the first study to investigate the influence stagnation on VBNC *Legionella* under environmental conditions. Overall, this study showed that a reduction in water stagnation decreased culturable *Legionella* but not VBNC *Legionella*.

8.2 Introduction

Legionella pneumophila is an opportunistic premise plumbing pathogen (OPPP) associated with hospital and community acquired infections. It is the causative agent of Pontiac Fever, an acute "flu-like" illness and Legionnaires' Disease, a severe atypical pneumonia like infection (Bartram et al., 2007). The genus *Legionella* consists of 60 species and 80 distinct serogroups (*sg*) (Miyashita et al., 2020), with *L*. *pneumophila sg*1 being the primary etiological agent of Legionnaires' Disease and responsible for 70 to 92% of reported cases (Mercante and Winchell, 2015). Globally, the incidence of legionellosis has been increasing (National Notifiable Diseases Surveillance System and Centers for Disease Control and Prevention, 2018). In 2022, the European Legionnaires' Disease Surveillance Network documented 8372 confirmed cases of Legionnaires' Disease, of which 5.1% were hospital acquired and 66.9% were community acquired infections (The European Centre for Disease Prevention and Control, 2022).

Legionella is ubiquitous in natural and manufactured water bodies. Cooling towers, humidifiers, engineered water systems, recreational water, and building plumbing systems are major reservoirs of *Legionella* (Bartram et al., 2007). Intrinsic resistance against commercially available disinfectants, mutualistic and symbiotic relationships with protozoans, and growth within multispecies biofilms, are key biotic factors responsible for the survival and persistence of *Legionella* in manufactured water systems (Falkinham III et al., 2015b, Fields et al., 2002, Nisar et al., 2020a). *Legionella* spp. are intracellular parasites of various freshwater protozoan species, such as amoebae (*Acanthamoeba*, *Naegleria*, *Vahlkampfia*, and *Vermamoeba vermiformis*) and ciliates (*Paramecium* and *Tetrahymena*) (Boamah et al., 2017). Members of gymnamoebae, noticeably *Vermamoeba vermiformis* and *Acanthamoeba*, have been identified as major reservoirs and vehicles of *Legionella* in both hospital and domestic water systems (Nisar et al., 2020a). Amoebae trophozoites support the intracellular division and biogenesis of potentially infectious and highly pathogenic viable but nonculturable (VBNC) *Legionella.* These amoebic cysts have been demonstrated to protect *Legionella* from prolonged disinfection treatments.

Abiotic factors such as hydraulic dynamics, age, plumbing system materials, water stagnation, corrosion, water temperature and inadequate disinfection procedures contribute to the growth and persistence of *Legionella* in engineered water systems (Bartram et al., 2007, Hayes-Phillips et al., 2019). In building plumbing systems, aerators, balancing valves, dead ends, dead legs, diffusers, flow restrictors, and intermitted usage all impact hydraulic dynamics and promote temporary or permanent water stagnation (Nisar et al., 2020b). This is often exacerbated in green building plumbing systems that have been designed deliberately to reduce water flow (Rhoads et al., 2015, Rhoads et al., 2016). Recently, during the COVID-19 pandemic, prolonged periods of lockdown caused extreme water stagnation in complicated plumbing system of commercial buildings (Liang et al., 2021). Both inappropriate hydraulic dynamics and water stagnation are likely to result in the failure of disinfection treatments, corrosion, and accumulation of sediments and nutrients. Previous studies, as well as government regulations from across the globe, recommend the elimination of factors promoting water stagnation within engineered water systems to minimize the risk of *Legionella* (Ciesielski et al., 1984, Nisar et al., 2020b, SA Health, 2013). However, this has been contradicted by several studies that suggest that avoiding conditions promoting water stagnation has no effect on *Legionella* persistence. It has been proposed that water stagnation restricts the delivery of nutrients, whereas recirculation of water provides nutrients to the point of delivery (e.g., taps, showers etc.) ultimately promoting OPPP regrowth in building plumbing system (Sidari et al., 2004, Liu et al., 2006). Many biofilm studies have identified that flow of the bathing medium promotes more robust biofilm formation and attachment (Liu et al., 2006).

Due to the complex relationship between different biotic and abiotic factors, it is always challenging to study the role of water stagnation in *Legionella* and the associated host amoebae survival and persistence in engineered water systems. This is further complicated by the complexities and sensitivities of different *Legionella* detection methods. In engineered water systems, bacteriological culturing and quantitative PCR (qPCR) are the standard methods used to detect *Legionella* contamination (International Organization for Standardization, 2017, International Organization for Standardization, 2019); however, neither assay provides any valuable information about VBNC *Legionella*. Most previous studies conducted to examine the relationship between *Legionella* and water stagnation have focused on culturable *Legionella* (Ciesielski et al., 1984, Liu et al., 2006, Sidari et al., 2004). However, quantification of both culturable and VBNC states is necessary to monitor the survival of viable and potentially infectious *Legionella* populations.

This study used a model plumbing system to investigate the effect of water stagnation and hydraulic dynamics on the survival and persistence of *Legionella* in engineered water systems. VBNC *Legionella* were enumerated using a newly described technique (Nisar et al., 2023), which was conducted concurrently with traditional culture and qPCR methods. The amoebic hosts *Acanthamoeba* and *V. vermiformis* were also enumerated via culture and qPCR and total bacteria were via a heterotrophic plate count. The effect of changing hydraulic conditions on biofilm communities, and the relationship between *Legionella* and amoeba hosts, was examined using fluorescent *in situ* hybridization and confocal scanning microscopy.

8.3 Materials and Methods

8.3.1 Model plumbing system

A simulated building plumbing system was constructed (**Figure 8.1**) that consisted of a 60 L capacity plastic water tank (DR5060, AdMerch) connected to a source of municipal water tap and two Bio-inLine® biofilm reactors (IBR 500, BioSurface Technologies Corporation). Each IBR contained 12 polypropylene disc coupons (diameter = 12.7 mm; RD128-PP, BioSurface Technologies Corporation). To maintain the water temperature at 37 ± 2°C, a 2 m long immersion electric heater with a digital thermostat (SKU BDIH2400W, Scintex®) was fitted in the water tank and measured using a glass thermometer immersed in the water. This optimum growth temperature of *Legionella* was selected to represent the worst-case scenario within a building plumbing system. The IBRs were connected via PE-Xb piping (4950091, SmarteX™) and brass copper push-fit connectors (elbow: 4700328, slip-coupling: 4700354 and tee: 4700360, SmarteX™). To control unidirectional water flow, brass copper push-fit valves (4790383, SmarteX™) were installed. The water tank, piping, connectors, and valves were

disinfected with 80% ethanol before use (EA043, ChemSupply). IBRs and coupons were cleaned by dry heat sterilization using thermal treatment at 121°C for 15 min.

8.3.2 Experimental setup

The experimental sampling was divided into two stages: (1) biofilm establishment and (2) operational. The entire experiment was conducted twice with the model plumbing system disinfected and then reseeded with potable water for the second experiment, as described. Dates of the experiment were March 2021 to August 2021 and December 2021 to May 2022.

8.3.2.1 Colonization phase

Potable water that had been previously found to be positive for culturable *L. pneumophila sg*1, *Acanthamoeba* group I and *V. vermiformis* (Nisar et al., 2022) was collected from a shower located in a commercial building and added to the water tank (**Figure 8.1**) as inoculum. To increase biofilm development, additional microbes and organic matter were added to the inoculum by vacuum filtering [onto 47 mm diameter 0.2 µm polycarbonate membrane (GTTP04700, Isopore™)] an additional 100 L of the same potable water sample. The harvested residue was then added further to the inoculum. The system was then left stagnant, without further addition of any nutrients or microbial inoculum, for a period of five months (147 days) at 37 ± 2°C to allow the biofilm to establish.

8.3.2.2 Operational phase

After 147 days of water stagnation the model entered the operational phase. During this period, the effect of unidirectional hydraulic flow was tested fortnightly for 28 days. One IBR was used to represent low usage and was flushed once-a-week. The other reactor was used to represent high usage and was flushed once-a-day. In each flushing cycle, ≈ 70 L water was drained out from each IBR, which is roughly equal to the average amount of water utilised per shower (Kinver, 22 November 2011).

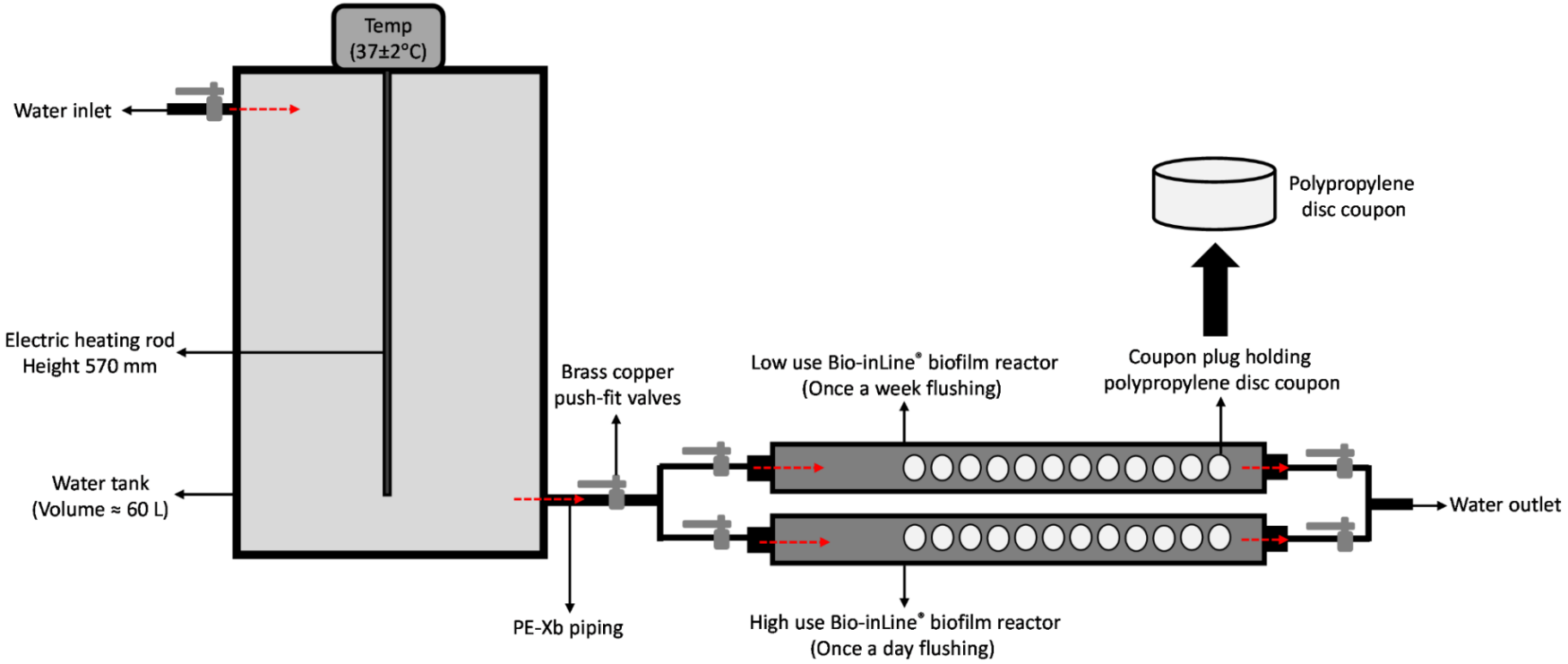

Figure 8.1: Overview of model plumbing system constructed to examine the effect flushing frequency on *Legionella* persistence. Red arrow indicates unidirectional flow of water.

8.3.3 Sample collection and processing

After the biofilm colonization phase (day 147), and at day 14 and day 28 of the operational phase, four coupons were collected from each IBR. Each coupon was placed in a sterile 50 mL tube with 5 mL of 1X phosphate-buffered saline (PBS; P4417, Sigma-Aldrich) then scraped using sterile polyurethane-tipped swabs (CleanFoam® TX751B, Texwipe®) followed by 15 min vigorous shaking (wrist action shaker: 896331, Griffin & George Ltd.) and 5 min of sonication in an ultrasonic water bath (895, Cooper Surgical, Inc.) to detach the biofilms. In case of water (of the tank), sampling was performed after completion of the colonization phase and at day 7, 14, 21 and 28 of the operational phase. A total of 100 mL water sample was collected from each IBR in triplicate prior to flushing. The water sample was vacuum filtered through a 47 mm diameter, 0.2 µm pore size, polycarbonate membrane (GTTP04700, Isopore™). The filter membrane was resuspended in 5 mL of 1X PBS and vortexed for 10 min to dislodge microbes. Both biofilm and water suspensions were further analysed to characterize surface adherent and planktonic microbes, respectively.

8.3.4 Molecular testing

Quantification of *Legionella*, *L. pneumophila*, *Acanthamoeba*, and *V. vermiformis* was conducted using quantitative qPCR. Standard qPCR assays were used to detect and quantify 16 rDNA and *mip* genes of *Legionella* and *L. pneumophila*, respectively (International Organization for Standardization, 2019). To quantify *Acanthamoeba* and *V. vermiformis* the 18S rDNA gene was used for amplification (Qvarnstrom et al., 2006, Scheikl et al., 2016). Briefly, 1 mL biofilm/water suspension was processed for DNA extraction using the Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.). The qPCR reaction mixture contained 1X PCR reaction buffer (2X SsoAdvanced™ universal probes supermix:172-5281, Bio-Rad Laboratories Ltd.), microbe-specific oligos (Bio-Rad Laboratories Ltd.) and template DNA. To detect the presence of environmental inhibitors of PCR, both the purified and the 10 times diluted DNA samples were used as template (Nisar et al., 2022, Hayes-Phillips et al., 2019). Each template DNA was amplified in triplicate using Rotor-Gene Q thermal cycler (Qiagen Ltd.). Primers and fluorescence labelled probes used in this study are listed in **Table 14.1** (Appendix-3). Quantification of template DNA was done using a standard curve comprising a concentration range of 10^2 to 10^9 copies per reaction. Synthetic DNA fragments (gBlocks, IDT™) of 16S rDNA *Legionella* (Accession Number CP021281), *mip L. pneumophila* (Accession Number KR902705), 18S rDNA *Acanthamoeba castellanii* (Accession Number U07413) and 18S rDNA *V. vermiformis* (Accession Number KT185625) genes were used as standards and a positive control (Nisar et al., 2022). Gene markers of biofilm-associated microorganisms and planktonic microorganisms were estimated in genomic units per mL (GU/mL) and genomic units per cm^2 (GU/cm^2), respectively. It is estimated that the limit of

detection for both *Legionella* and *L. pneumophila* was 35 GU/reaction, whereas it was 40 GU/reaction for *Acanthamoeba* and 44 GU/reaction for *V. vermiformis*.

8.3.5 Quantification of total viable *Legionella* population

Total viable *Legionella* and *L. pneumophila* (which include both alive potentially culturable and VBNC cells) were quantified using viability based flow cytometry-cell sorting and qPCR (VFC+qPCR) (Nisar et al., 2023). Briefly, 300 µL sample was mixed with 200 µL filter sterilized staining buffer (1 mM EDTA and 0.01% tween-20 in 1X PBS, pH 7.4 ± 0.1). Using the Becton Dickinson (BD™) cell viability kit (349480, BD™), 420 nM of thiazole orange (TO: $\lambda_{(excitation)}/\lambda_{(emission)}$: 512/533 nm) and 48 µM propidium iodide (PI; $\lambda_{(excitation)}/\lambda_{(emission)}$: 537/618 nm) was added and incubated at 5°C for 15 min. Then, cells were analysed on a BD™ FACSAria™ Fusion instrument and segregated into alive (potentially culturable: TO-stained fraction), dead (PI-stained fraction), and injured (potentially VBNC: TO-stained fraction) cell populations. Both alive and injured cell fractions were isolated for further analysis. These fractions were subjected to DNA extraction and quantification of *Legionella* and *L. pneumophila* gene markers as described above.

8.3.6 Microbiological analysis

Standard culture methods were used to detect and quantify biofilm-associated microorganisms (surface adherent) and planktonic (floating in water) culturable bacteria and amoebae. Culturable *Legionella* and *L. pneumophila* were grown and quantified according to standard guidelines (International Organization for Standardization, 2017, Standards Australia, 2017). Briefly, heat treated (50 ± 1°C for 30 ± 2 min), acid treated (HCl-KCl buffer treatment for 5 ± 0.5 min) and untreated samples were plated on *Legionella* agar (CM1203, Oxoid Ltd.) supplemented with GVPC (glycine, vancomycin, polymyxin B and cycloheximide: SR0152, Oxoid Ltd.) and *Legionella* growth supplement (α-ketoglutarate, buffer/potassium hydroxide, ferric pyrophosphate, and $_L$-cysteine: SR0110C, Oxoid Ltd.). *Legionella*-like colonies were enumerated after 3 to 7 days of incubation at 37°C. These presumed *Legionella* were further identified using *Legionella* latex agglutination test kit (DR0800, Oxoid Ltd.). Heterotrophic plate counts (HPC) were obtained by culture on R_2A agar (CM0906, Oxoid Ltd.) after 2, 5 and 7 days of incubation at 35°C. Culturable Gram-negative bacteria and *Pseudomonas* were enumerated after growth on MacConkey (CM0007, Oxoid Ltd.) and cetrimide agar (CM059, Oxoid Ltd.), respectively. Colonies were counted after 2 to 5 days of incubation at 37°C. Detection of culturable amoebae was achieved by growing the samples on heat-inactivated (57°C for 45 min) *Escherichia coli* American Type Culture Collection 700891™ supplemented 1.5% non-nutrient agar (Eco-NNA: CM0003, Oxoid Ltd.) at 25°C for 14 days. The growth of

amoebae was examined daily with the aid of an inverted light microscope (AMEFC4300, EVOS™ FL, ThermoFisher Scientific).

8.3.7 Microscopic analysis

Fluorescence *in situ* hybridization (FISH) with oligonucleotide probes combined with confocal laser scanning microscopy were used to examine the microbial composition of the biofilms. In the FISH assay, Alexa Fluor 488 labelled LEG705 (Manz et al., 1995), Alexa Fluor 546 labelled EUB338 (Amann et al., 1990) and Alexa Fluor 647 labelled EUK1209 (Lim et al., 1993) fluorogenic oligonucleotide probes (Invitrogen™, Appendix-3 **Table 14.1**) were used for the detection of *Legionella*, eubacteria, and eukaryotic microbes, respectively. In this assay, paraformaldehyde fixed biofilm samples were dehydrated in an ethanol series (50%, 80% and 90%), then covered with hybridization buffer (0.9 mM NaCl, 0.01% SDS and 0.02 M Tris-HCl, pH 7.6) containing 100 ng of each fluorogenic oligonucleotide probe and incubated at 55°C under humid conditions for 100 min. After final washing and drying steps, samples were mounted with CitiFluor™ AF1 (17970-25, Electron Microscopy Sciences) and images were acquired by confocal microscopy (LSM 880 fast airyscan confocal, Zeiss) using oil immersion objective (C Plan-Apochromat 63x/1.4 oil DIC M27, Zeiss) (Whiley et al., 2011). Moreover, the same imaging settings were used when comparing the degree of labelling between samples. The processing of captured images was conducted using Fiji software (https://imagej.net/software/fiji/).

8.3.8 Data analysis

The log transformed data are depicted as mean ± standard deviation of six to eighteen independent replicates. Statistical analyses and graphical representation were performed using R language computer program agricolae (version 1.3-5) and ggplot2 (version 3.3.6) packages in R environment (de Mendiburu, 2021, Wickham, 2016). To compare daily and weekly flushing bioreactors (i.e., Coupons at day 0, 14 and 28 and Water at day 0, 7, 14, 21 and 28), data was log transformed and normality was checked by quantile-quantile (q-q) plots and Shapiro-Wilk test. Then, an ANOVA was performed followed by a Tukey's honestly significant difference (HSD) and least significant difference (LSD) tests. Correlation between flushing and other microbiological parameters were computed by comparing the frequency of flushing events (both daily and weekly events are combined) versus microbiological factors (*Legionella* or another microbe). Here flushing events of both daily and weekly events are combined as number of flushing events 1, 2, 3, 4, 7, 14, 21, 28 for water and 2, 4, 7, 14, 28 for coupons. Similarly, microbiology factor of both daily and weekly events is combined it became non-normal and confirmed with quantile-quantile (q-q) plots and Shapiro-Wilk tests as well.

8.4 Results

8.4.1 Biofilm colonization

After 147 days in the colonization phase, visual inspection found that the biofilm depositions were relatively homogenous between and along the coupons. In the stagnant water suspended semi-solid residues were observed. The microbial population developed on coupons contained a diverse mixture of heterotrophic aerobic bacteria, Gram-negative bacteria, and amoebae. Each coupon was colonized by high numbers of *Legionella* (≈ 6.7 $\log_{10}$) and culturable heterotrophic bacteria (≈ 4.7 $\log_{10}$) (**Table 8.1**). The difference between total *Legionella* (VBNC, culturable and dead) estimated by qPCR and culturable *Legionella* counted by classical culturing method was ≈ 2 $\log_{10}$. Culturable *Pseudomonas* was absent from both biofilm and water phases. Unlike *Legionella* and culturable heterotrophic bacteria, the number of planktonic amoebae were significantly higher than biofilm-associated amoebae (**Tables 8.1** and **8.2**).

8.4.2 *Legionella* and flow dynamics

Statistical analysis clearly showed that all four categories of the *Legionella* population screened in this study were significantly affected by flushing frequency (**Tables 8.1** and **8.2**). Interesting, the acid and heat pre-treatment options used in the standard culture method affected the recovery of viable *Legionella.* Either pre-treatment method reduced the culturable *Legionella* to undetectable levels in all cases for day 7, 14, 21 and 28 days, both in the water and biofilm. Therefore, pre-treatment steps recommended by standard culturing methods were omitted and bacterial colonies were characterized by serological and molecular identification assays.

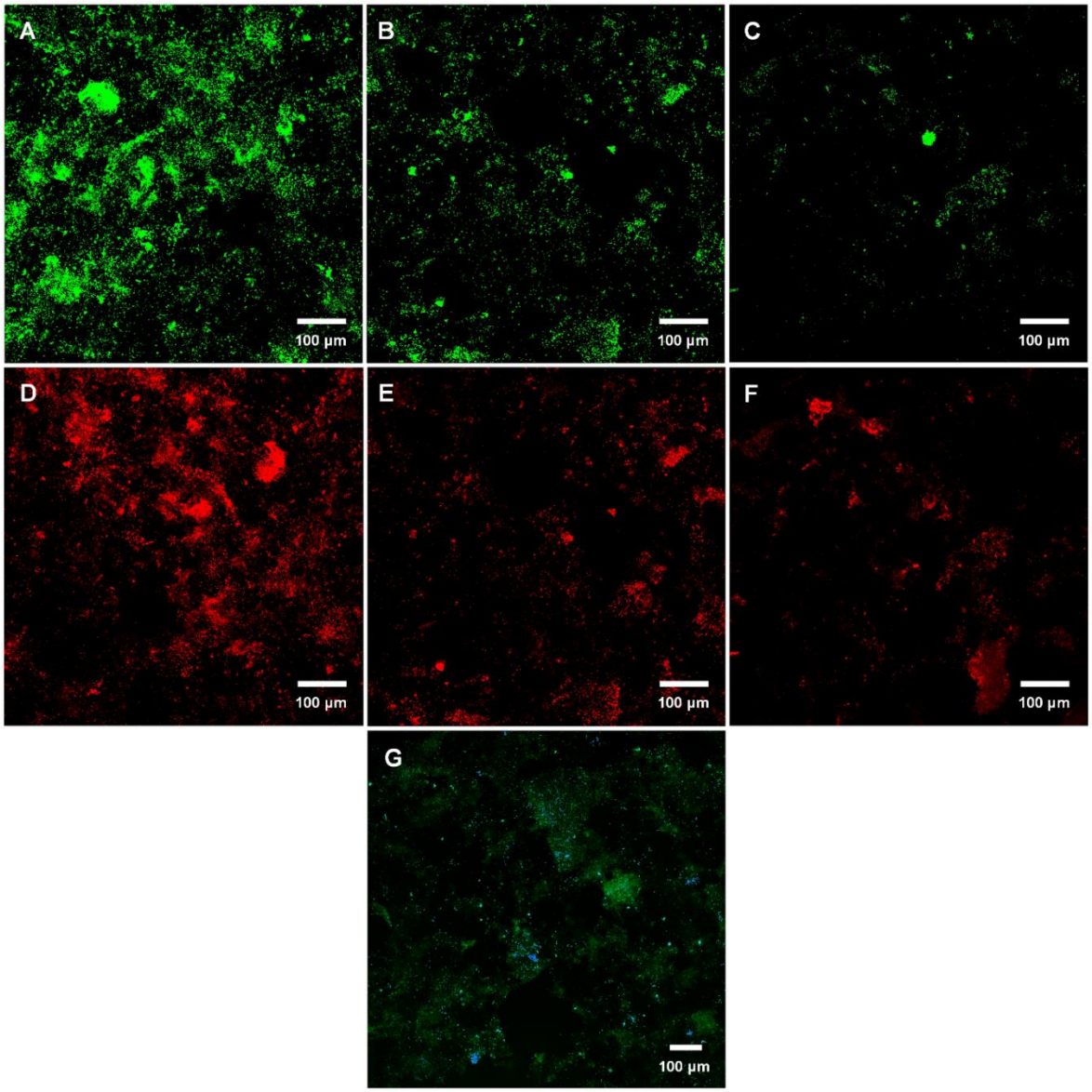

Figure 8.2: Fluorescence *in situ* hybridisation images of biofilms from the model plumbing system. stained with *Legionella* (green coloured), eubacteria (red coloured), and eukaryotic (blue coloured) specific probes. In comparison to once-a-week flushing (B and E), once-a-day flushing (C and F) decreased the population of biofilm-associated *Legionella* and eubacteria. A, D and G: colonization phase; B and E: day 28 of once-a-week flushing; C and F: day 28 of once-a-day flushing. The bar represents 100 µm.

8.4.2.1 Biofilm-associated *Legionella*

The effect of high and low frequency flushing events on biofilm-associated *Legionella* is shown in **Figure 8.3**. The incidence of flushing significantly influenced the populations of total, alive, VBNC and culturable *Legionella*. Total (both viable and dead), alive (potentially culturable) and culturable *Legionella* populations were significantly reduced with increased flushing events. In contrast to weekly flushing, daily flushing significantly ($p < 0.001$) reduced total *Legionella* by 19.4% on day 28 (Appendix-3, **Figure 14.1**). Likewise, daily flushing significantly decreased the population of alive *Legionella* by 17.5% and 36% on day 14 and 28, respectively, as compared to weekly flushing (**Table 8.1** and Appendix-3, **Figure 14.2**). The population of

culturable *Legionella* was also sensitive to flushing events. The daily flushing events resulted in a reduction of culturable *Legionella* by 9.9% on day 14 and 41% on day 28 (**Table 8.1** and Appendix-3, **Figure 14.4**). Similarly, flushing frequency was negatively correlated with total *Legionella* ($\rho = -0.651$, $p < 0.001$), alive *Legionella* ($\rho = -0.955$, $p < 0.001$) and culturable *Legionella* ($\rho = -0.939$, $p < 0.001$). This showed that once-a-day flushing produced a greater reduction than once-a-week flushing. This analysis was supported by the FISH micrographs which at the commencement of the operational phase showed (**Figure 8.2 A**) the formation of well-established biofilm consisting of *Legionella* (green coloured), eubacteria (red coloured) and eukaryotic microorganisms (blue coloured). In **Figure 8.2 A**, the presence of some areas of increased *Legionella* density demonstrated that *Legionella* developed compact and well-structured biofilms. Comparison of weekly (**Figure 8.2 B**) and daily (**Figure 8.2 C**) flushing on day 28, clearly illustrate that daily flushing decreased the amount of biofilm-associated *Legionella*. On the other hand, daily flushing significantly increased VBNC *Legionella* by 27.6% on day 14 and 8.8% on day 28 (**Table 8.1** and Appendix-3, **Figure 14.3**). Likewise, flushing was positively correlated to VBNC *Legionella* ($\rho = 0.696$, $p < 0.001$). Overall, Spearman's analysis demonstrated that high flushing frequency decreased the amount of alive and culturable *Legionella* but increased the quantity of VBNC *Legionella*.

8.4.2.2 Planktonic *Legionella*

Figure 8.4 shows boxplots of flushing events and the amount of planktonic *Legionella* taken from the water samples. The plots show that the populations of total, alive and culturable *Legionella* were higher in the IBR that was flushed weekly compared with the IBR flushed daily. Flushing daily significantly reduced total amount of *Legionella* by 35.4%, 30.8%, 19% and 41% on day 7, 14, 21 and 28, respectively, compared with the samples collected on the same days from the IBR that was flushed weekly (**Table 8.2** and Appendix-3, **Figure 14.5**). In comparison with weekly flushing, daily flushing significantly reduced the population of alive *Legionella* by 19.2% on day 14 and 36.5% on day 28 (**Table 8.2** and Appendix-3, **Figure 14.6**). The daily flushing events resulted in reduction of culturable *Legionella* by 38.6%, 24.4%, 14.9%, and 36.2% on day 7, 14, 21, and 28, as compared to corresponding weekly flushing (**Table 8.2** and Appendix-3, **Figure 14.8**). Likewise, flushing frequency negatively correlated with total *Legionella* ($\rho = -0.947$, $p < 0.001$), alive *Legionella* ($\rho = -0.706$, $p < 0.001$) and culturable *Legionella* ($\rho = -0.816$, $p < 0.001$). It clearly showed that high flushing frequency decreased the amount of alive and culturable *Legionella*. As compared to weekly flushing, daily flushing significantly increased the population of VBNC *Legionella* by 22.8% on day 7, 16.8% on day 14, 42.4% on day 21, and 24% on day 28 (**Table 8.2** and Appendix-3, **Figure 14.7**). Similarly, flushing positively correlated to planktonic VBNC *Legionella* ($\rho = 0.802$, $p < 0.001$). The combination of detection methods demonstrated that overall, the once-a-day flushing resulted

in a statistically significant reduction in the alive *Legionella*, but an increase in VBNC *Legionella* compared with the once-a-week flushing.

8.4.3 Culturable heterotrophic bacterial population and flow dynamics

The effect of flushing frequency on biofilm-associated and planktonic heterotrophic culturable bacteria is represented in **Figure 8.5** and **8.6**, respectively. It was found that over time the once-a-day flushing resulted in a consistent decline in the HPC. In contrast to weekly flushing, daily flushing significantly ($p < 0.001$) reduced biofilm-associated heterotrophic bacteria by 9.9% on day 28 (**Table 8.1** and Appendix-3, **Figure 14.9**). The daily flushing events resulted in a reduction of planktonic heterotrophic bacteria by 9.2% on day 7 and 23.8% on day 28, as compared with weekly flushing (**Table 8.2** and Appendix-3, **Figure 14.10**). Similarly, Spearman's analysis demonstrated that flushing frequency negatively correlated with both the biofilm-associated ($\rho = -0.942$, $p < 0.001$) and planktonic ($\rho = -0.683$, $p < 0.001$) heterotrophic bacteria.

These results revealed that high flushing significantly reduced the heterotrophic bacterial counts. The morphological characteristics of the bacterial colonies were analysed to assess the diversity and community structure of planktonic and biofilm-associated culturable heterotrophic bacteria. The biofilm-associated heterotrophic bacterial population showed a stable and consistent community over time and was apparently not affected by the flushing events, as the diversity remained the same and low throughout the operational phase. However, strong bacterial community shifts within planktonic heterotrophic bacteria were seen in daily flushing IBR. Similarly, the pattern of planktonic Gram-negative bacteria showed striking shifts after daily flushing. As observed in the colonization phase, culturable *Pseudomonas* was absent from both water and biofilm phases as no growth.

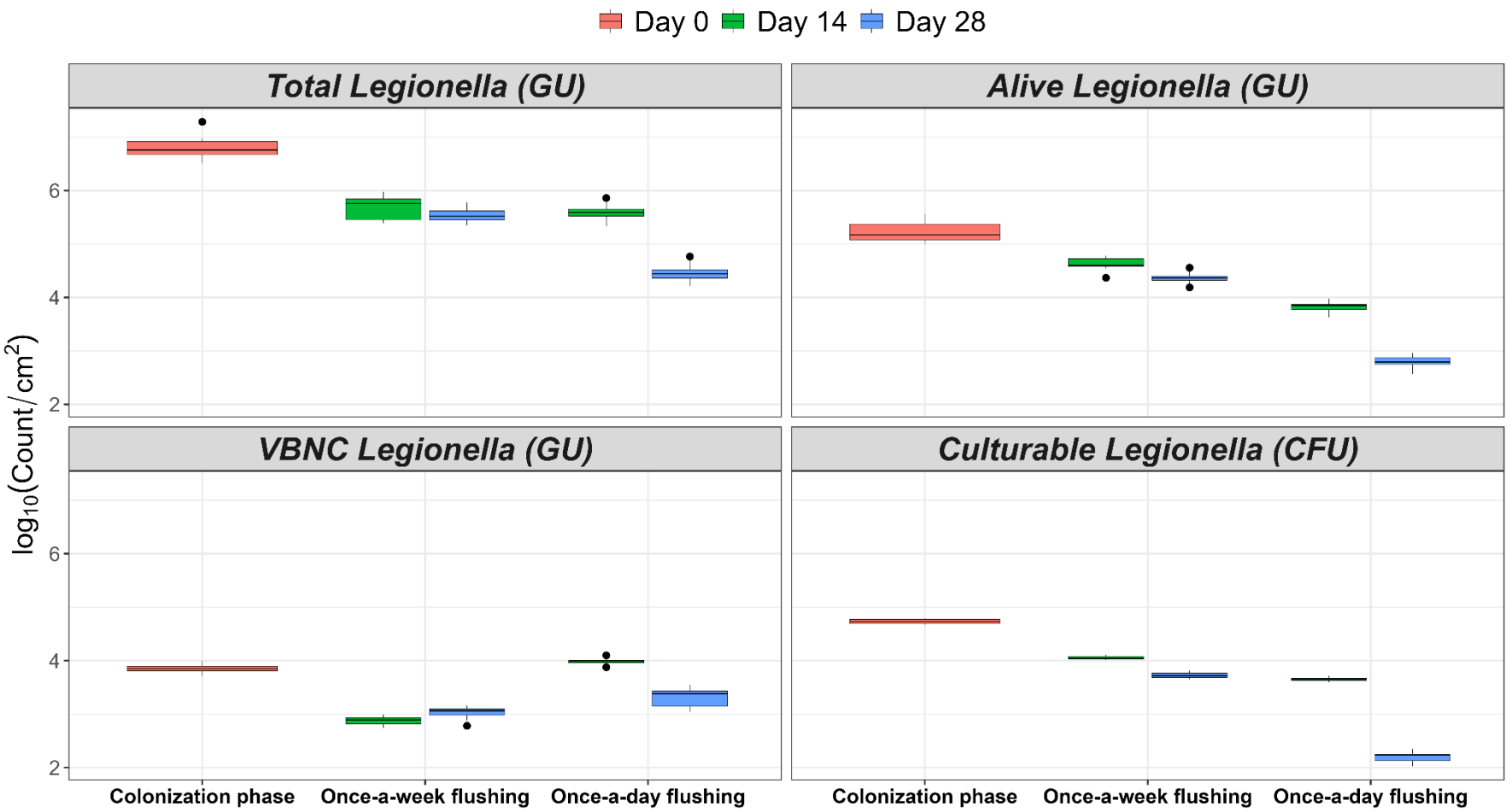

Figure 8.3: The effect of flushing events on the biofilm-associated total, alive (potentially culturable), VBNC and culturable *Legionella*. The log transformed data are shown as mean ± standard deviation of nine to eighteen replicates. GU: genomic unit quantified by qPCR assay; CFU: colony forming unit estimated by standard culturing method. Pink colour: colonization phase, green colour: day 14, and blue colour: day 28. The bar across each box represents standard deviation.

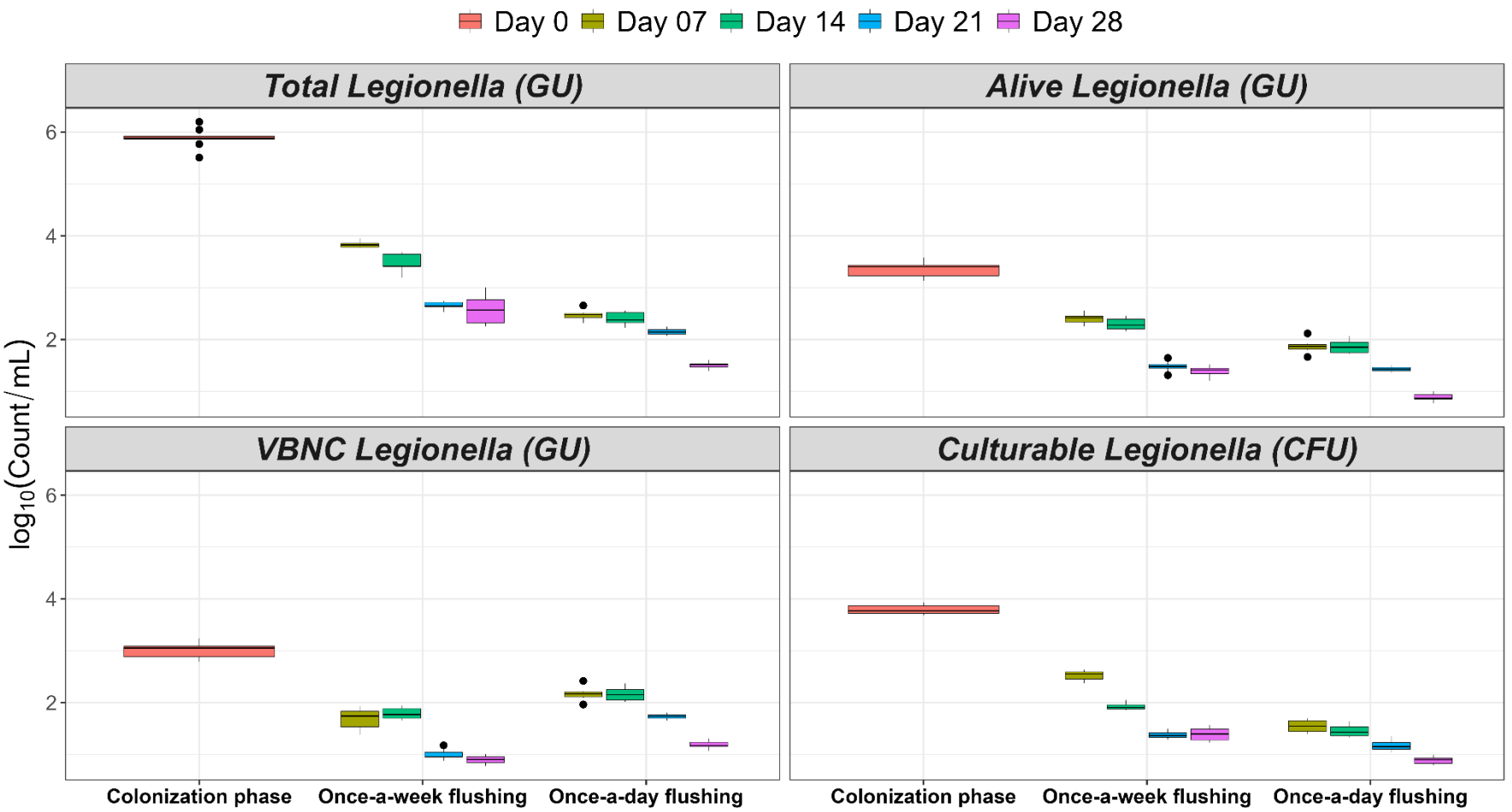

Figure 8.4: The effect of flushing events on the planktonic total, alive (potentially culturable), VBNC and culturable *Legionella*. The log transformed data is shown as mean ± standard deviation of nine replicates. GU: genomic unit quantified by qPCR assay; CFU: colony forming unit estimated by standard culturing method. Pink colour: colonization phase, olive colour: day 7, green colour: day 14, blue colour: day 21, and dark pink colour: day 28. The bar across each box represents standard deviation.

8.4.4 Amoebae and flow dynamics

Both culturing and the qPCR assays successfully detected and quantified planktonic and biofilm-associated *Acanthamoeba* and *V. vermiformis*. These results clearly showed that the amoebae population was significantly affected by the flushing frequency.

8.4.4.1 Acanthamoeba

The effect of flushing frequency on biofilm-associated and planktonic *Acanthamoeba* is depicted in **Figures 8.5** and **8.6**, respectively. Daily flushing significantly reduced biofilm-associated *Acanthamoeba* by 10.8% on day 14 and 18.5% on day 28 (**Table 8.1** and Appendix-3, **Figure 14.11**); and planktonic *Acanthamoeba* by 10.6%, 15.7% and 43.7% on day 14, 21 and 28 (**Table 8.2** and Appendix-3, **Figure 14.12**), respectively, compared with the IBR that was flushed once-a-week. Likewise, flushing frequency was negatively related to both the biofilm-associated ($\rho = -0.949$, $p < 0.001$) and planktonic ($\rho = -0.717$, $p < 0.001$) *Acanthamoeba*. It demonstrated that once-a-day flushing decreased the population of both planktonic and biofilm-associated *Acanthamoeba*.

8.4.4.2 Vermamoeba vermiformis

Figures 8.5 and **8.6** show the effect of flushing frequency on biofilm-associated and planktonic *V. vermiformis*, respectively. Under daily flushing conditions, the population of the biofilm-associated *V. vermiformis* increased by 83.9% on day 14 and 58.3% on day 28, compared with their respective time samples from the once-a-week flushing IBR (**Table 8.1** and Appendix-3, **Figure 14.13**). In the case of planktonic *V. vermiformis*, the amoebae population initially remained unaltered for 14 days; however, the population dropped by 10.8% on day 21 and 37.1% on day 28 in the IBR that was flushed daily compared with the corresponding time samples from the IBR that was flushed weekly (**Table 8.2** and Appendix-3, **Figure 14.14**). Flushing frequency was positively correlated to biofilm-associated *V. vermiformis* ($\rho = 0.706$, $p < 0.001$), whereas negatively correlated to planktonic *V. vermiformis* ($\rho = -0.362$, $p < 0.001$). This highlights that once-a-day flushing increased the quantity of biofilm-associated *V. vermiformis* but decreased planktonic *V. vermiformis*.

8.4.5 Relationship between *Legionella* and microbial flora

Culturable *Legionella* and heterotrophic plate count were positively correlated (biofilm-associated: $\rho = 0.929$, $p < 0.001$ and planktonic: $\rho = 0.802$, $p < 0.001$). Similarly, alive *Legionella* were significantly correlated with *Acanthamoeba* (biofilm-associated: $\rho = 0.917$, $p < 0.001$ and planktonic: $\rho = 0.894$, $p < 0.001$). In addition, biofilm-associated VBNC *Legionella* were positively correlated with biofilm-associated *V. vermiformis* ($\rho = 0.848$, $p < 0.001$), whereas planktonic VBNC *Legionella* were positively correlated with planktonic *Acanthamoeba*

(ρ = 0.526, $p < 0.001$). It demonstrated that VBNC *Legionella* and amoebae populations increased concurrently. It can be hypothesized that the daily flushing had an impact on culturable *Legionella*, which were transformed into VBNC *Legionella* via intracellular replication within host amoebae. Furthermore, the FISH micrographs (**Figure 8.2 G**) demonstrated that *Legionella* appeared in distinct clusters and often associated with protozoa, suggesting a strong association between *Legionella* and protozoa in biofilms.

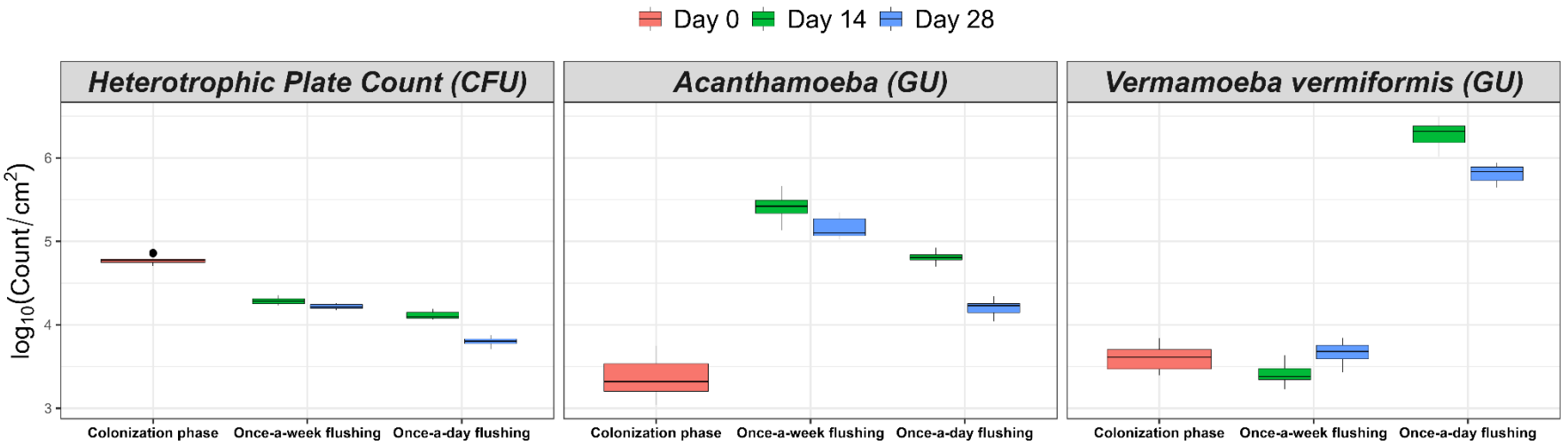

Figure 8.5: The effect of flushing events on the biofilm-associated heterotrophic bacteria and amoebae. The log transformed data is shown as mean ± standard deviation of six to twelve replicates. GU: genomic unit quantified by qPCR assay; CFU: colony forming unit estimated by standard culturing method. Pink colour: colonization phase, green colour: day 14, and blue colour: day 28. The bar across each box represents standard deviation.

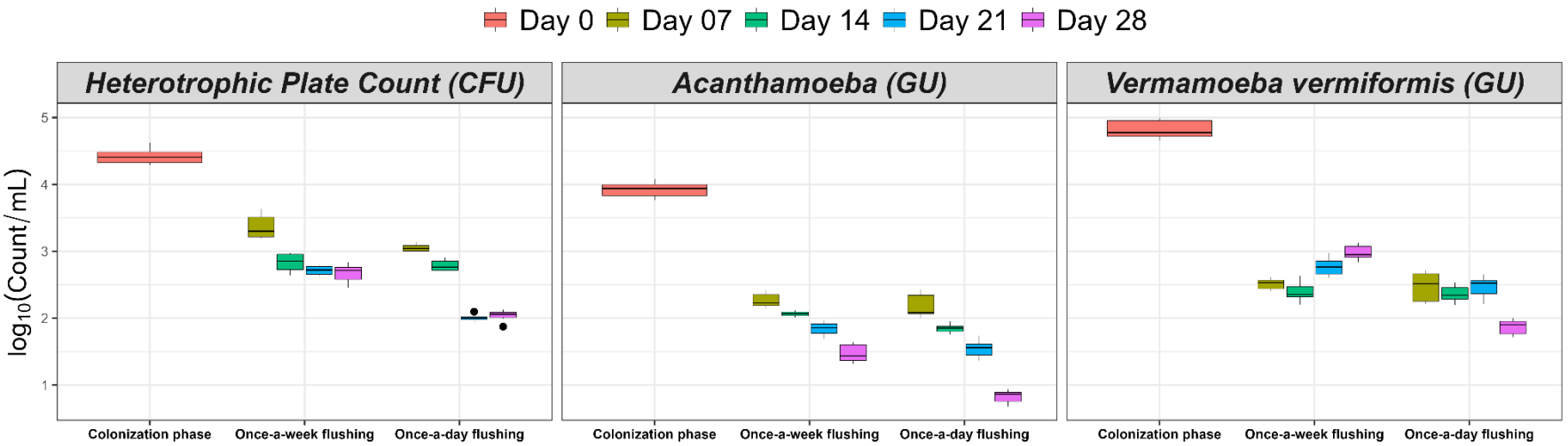

Figure 8.6: The effect of flushing events on the planktonic heterotrophic bacteria and amoebae. The log transformed data is shown as mean ± standard deviation of six replicates. GU: genomic unit quantified by qPCR assay; CFU: colony forming unit estimated by standard culturing method. Pink colour: colonization phase, olive colour: day 7, green colour: day 14, blue colour: day 21, and dark pink colour: day 28. The bar across each box represents standard deviation.

Table 8.1: Influence of flushing events on biofilm-associated bacteria and amoebae

Samples	*Legionella* log (GU/cm^2)			Culturable Bacteria log (CFU/cm^2)		Amoebae log (GU/cm^2)	
	Total *Legionella*	Alive *Legionella*	VBNC *Legionella*	*Legionella*	Heterotrophic plate count	*Acanthamoeba*	*Vermamoeba vermiformis*
Colonization phase (Day 0)	6.796180 ± 0.1794369 (a)	5.224538 ± 0.1876002 (a)	3.851340 ± 0.08402355 (b)	4.731191 ± 0.04288411 (a)	4.775569 ± 0.04727078 (a)	3.362325 ± 0.20252933 (e)	3.597627 ± 0.1300029 (c)
Once-a-week flushing (Day 14)	5.691700 ± 0.2179521 (b)	4.627558 ± 0.1282385 (b)	2.883151 ± 0.08475124 (d)	4.053507 ± 0.03914264 (b)	4.286761 ± 0.04726848 (b)	5.401645 ± 0.16440669 (a)	3.407515 ± 0.1182785 (d)
Once-a-week flushing (Day 28)	5.550057 ± 0.1491928 (b)	4.360268 ± 0.1132404 (c)	3.019045 ± 0.12381313 (d)	3.725728 ± 0.06394488 (c)	4.219076 ± 0.03278179 (b)	5.160420 ± 0.12151434 (b)	3.667699 ± 0.1333505 (c)
Once-a-day flushing (Day 14)	5.596546 ± 0.1719931 (b)	3.816865 ± 0.1109382 (d)	3.986392 ± 0.06342490 (a)	3.651335 ± 0.04406228 (c)	4.114713 ± 0.05115785 (c)	4.814152 ± 0.06742957 (c)	6.267837 ± 0.1636113 (a)
Once-a-day flushing (Day 28)	4.471549 ± 0.1781023 (c)	2.789584 ± 0.1238962 (e)	3.310937 ± 0.18112956 (c)	2.195640 ± 0.11803095 (d)	3.799519 ± 0.05868282 (d)	4.204131 ± 0.09439469 (d)	5.806846 ± 0.1048668 (b)

The log transformed data are represented as mean ± standard deviation.

The same alphabetic letter in a column represents statistical similarities at $p < 0.001$ according to Tukey's HSD test.

Table 8.2: Influence of flushing events on planktonic bacteria and amoebae

Samples	*Legionella* log (GU/mL)			Culturable Bacteria log (CFU/mL)		Amoebae log (GU/mL)	
	Total *Legionella*	Alive *Legionella*	VBNC *Legionella*	*Legionella*	Heterotrophic plate count	*Acanthamoeba*	*Vermamoeba vermiformis*
Colonization phase (Day 0)	5.887211 ± 0.18768987 (a)	3.3641397 ± 0.15057701 (a)	3.0174425 ± 0.15056954 (a)	3.7941842 ± 0.09963189 (a)	4.422965 ± 0.12777241 (a)	3.9280153 ± 0.10749713 (a)	4.826105 ± 0.13078603 (a)
Once-a-week flushing (Day 07)	3.827483 ± 0.05990732 (b)	2.4104544 ± 0.09503362 (b)	1.6698851 ± 0.19305906 (c)	2.5235373 ± 0.10450700 (b)	3.366247 ± 0.19464344 (b)	2.2707260 ± 0.10324332 (b)	2.511237 ± 0.07615361 (d)
Once-a-week flushing (Day 14)	3.470073 ± 0.16680511 (c)	2.3021665 ± 0.11410929 (b)	1.7951168 ± 0.11404832 (c)	1.9276290 ± 0.07206814 (c)	2.831910 ± 0.14084130 (d)	2.0678394 ± 0.03711549 (c)	2.383038 ± 0.13855341 (d)
Once-a-week flushing (Day 21)	2.655938 ± 0.06464352 (d)	1.4723297 ± 0.10113754 (d)	0.9969545 ± 0.10455763 (e)	1.3786453 ± 0.07565612 (d)	2.715404 ± 0.07068983 (d)	1.8381295 ± 0.09582731 (d)	2.766823 ± 0.13200170 (c)
Once-a-week flushing (Day 28)	2.577065 ± 0.26404276 (de)	1.3920179 ± 0.10030007 (d)	0.9004404 ± 0.07877558 (e)	1.3918208 ± 0.13706845 (d)	2.672887 ± 0.14390240 (d)	1.4751593 ± 0.12577191 (e)	2.982381 ± 0.09912277 (b)
Once-a-day flushing (Day 07)	2.470794 ± 0.09215929 (de)	1.8642078 ± 0.12172286 (c)	2.1652378 ± 0.12172286 (b)	1.5477709 ± 0.12762168 (d)	3.053701 ± 0.05729027 (c)	2.1674279 ± 0.16293815 (bc)	2.476077 ± 0.19921452 (d)
Once-a-day flushing (Day 14)	2.401284 ± 0.12812465 (e)	1.8584687 ± 0.12319633 (c)	2.1594987 ± 0.12319633 (b)	1.4558376 ± 0.12407781 (d)	2.788624 ± 0.08596752 (d)	1.8475462 ± 0.06656809 (d)	2.358967 ± 0.12060405 (d)
Once-a-day flushing (Day 21)	2.150556 ± 0.05749267 (f)	1.4323949 ± 0.05308115 (d)	1.7334249 ± 0.05308115 (c)	1.1729664 ± 0.11305746 (e)	2.004026 ± 0.05686202 (e)	1.5484953 ± 0.11716246 (e)	2.466070 ± 0.15611579 (d)
Once-a-day flushing (Day 28)	1.503124 ± 0.06993165 (g)	0.8838509 ± 0.07854649 (e)	1.1848809 ± 0.07854649 (d)	0.8876095 ± 0.07525127 (f)	2.036556 ± 0.09094789 (e)	0.8296887 ± 0.09093314 (f)	1.872947 ± 0.10598911 (e)

The log transformed data are represented as mean ± standard deviation.

The same alphabetic letter in a column represents statistical similarities at $p < 0.001$ according to Tukey's HSD test.

8.5 Discussion

Various chemical and physical water disinfection protocols are designed to control *Legionella* in engineered water systems (Kim et al., 2002). However, in the real-world none of these disinfection procedures can achieve total eradication of *Legionella* (Thomas et al., 2004, Cooper and Hanlon, 2010, Saby et al., 2005). From the water source to the point of utilization, the concentration of chemical disinfectants fluctuates with disinfection decay accelerated by biofilms and water stagnation (Xu et al., 2018). Thermal disinfection is another physical approach used to control *Legionella* in building water systems (Darelid et al., 2002). However, the presence of dead ends and dead legs (which are not reached by hot water used for disinfection treatment), biofilms (which provide protection) and the development/selection of thermotolerant strains results in frequent failure of thermal disinfection (Whiley et al., 2017, Nisar et al., 2020b). Extended water stagnation and water aging in buildings are significant factors influencing *Legionella* proliferation (Nisar et al., 2020b). This is especially topical due to COVID-19 lockdowns, as well as the increased interest in green buildings. Green buildings use available strategies of water conservation including plumbing fixtures that reduce usage and flow of potable water (DeOreo and Mayer, 2012, Von Paumgartten, 2003). These strategies reduce water usage by increasing the hydraulic retention time of the building plumbing system. In summary, these building plumbing systems have higher surface area to volume ratios, water stagnation, variable hydraulic regimes, and water temperature (Springston and Yocavitch, 2017). This increased half-life of water in the building system and permits increased decay of chemical disinfectants as shown by a study conducted in the USA that consistently detected lower concentrations of chlorine in green building plumbing systems (Rhoads et al., 2016).

Previous studies have indicated that in building plumbing systems the removal of water stagnation points reduces the risk of legionellosis (Ciesielski et al., 1984, Darelid et al., 2002). However, there are some studies that did not find any positive relationship between *Legionella* persistence and water stagnation (Sidari et al., 2004, Liu et al., 2006). Some authors suggest that water circulation in piping networks support the colonization of *Legionella* (Liu et al., 2006). This positive association of water circulation with *Legionella* contamination is justified by the “nutrient and oxygen supply hypothesis”, which suggests that circulating water evenly distributes nutrients and microbes which accelerates microbial growth in the building plumbing system (Liu et al., 2006). In the present study, the model plumbing system was designed with a unidirectional water flow to prevent the recirculation and mixing of residual water. This simulates the water stagnation occurring at plumbing outlets.

During water stagnation in building plumbing systems, chemical, physical and biological parameters of potable water are interlinked and affect each other (Proctor et al., 2020). In this study, we only focused on biological quality parameters. We examined how flushing events and water stagnation influenced the growth and persistence of *Legionella* and host free-living amoebae in the building plumbing system. To do this both the routine culturing and qPCR assays were complemented with VFC+qPCR to estimate the real amount of *Legionella* in both biofilm and water phases. The comparison of total *Legionella* (both viable and dead: qPCR counts) and culturable *Legionella* (CFU) illustrated that total *Legionella* were 0.7 to 2.1 $\log_{10}$ greater than culturable *Legionella*. This discrepancy can be explained by the population of VBNC *Legionella* estimated by VFC+qPCR and dead *Legionella*. According to this assay the quantity of alive (potentially culturable) *Legionella* was also estimated, which was close to the amount of *Legionella* estimated by culture.

It is important to note that water and biofilm samples collected during the operational phase were sensitive to both the acid and heat pre-treatment recommended by ISO11731:2017-05 (International Organization for Standardization, 2017). Previous work has shown that sample handling and both thermal and acid treatment steps are responsible for ≈ 30% transformation of culturable *Legionella* to VBNC *Legionella* (Nisar et al., 2023, Leoni and Legnani, 2001). Therefore, these pre-treatment steps were skipped. It also confirms the assertions that previous studies that solely used *Legionella* culture underestimated the real burden of *Legionella*.

Building water systems consist of plumbing pipes, fixtures, and devices from point of entry to point of delivery (Proctor et al., 2020). It is very difficult to simulate such a complex and highly variable system in a laboratory. Our study designed and validated a simplified plumbing system model with a naturally formed biofilm (**Figure 8.1**). This model plumbing system was capable of developing a stable microbial ecosystem in non-supplemented shower water contaminated with *Legionella* and amoebae. The microflora constituting this ecosystem predominantly consisted of *Legionella*, culturable heterotrophic bacteria, *Acanthamoeba* and *V. vermiformis*. The model was shown to be able to harbor very high numbers of *Legionella*. It models natural microbial communities typically present within actual building plumbing systems (Hayes-Phillips et al., 2019, Proctor et al., 2018). Our designed plumbing model system is a valuable tool to study colonization and persistence of *Legionella* in engineered water systems.

Biofilm sloughing is driven by biological and physical factors. To our knowledge, the current study is the first time that (1) biofilm sloughing by water flushing (a physical factor); (2) the effect of water flushing on biofilm-associated microbes and (3) planktonic microflora, have been comprehensively examined using an integrated approach. During the colonization phase

(147 days water stagnation), the microbes slough off from loosely attached biofilm and disperse into surrounding stagnant water. The planktonic bacteria and amoebae growing in the stagnant water primarily detached from biofilm (developed on polypropylene disc coupons) by some active biological processes. Generally, biological factors responsible for this detachment are microbial communication mediated dispersal, seeding dispersal, cell division mediated dispersal and nutrient fluctuations mediated dispersal (Kaplan, 2010).

The results of the operational phase presented in **Tables 8.1** and **8.2** showed that the lowest concentration of alive (potentially culturable) and culturable *Legionella* was always recovered from the biofilms growing in the high-use IBR. In the case of potable water, it is recognised that flushing events are the most important physical factors responsible for biofilm sloughing (Prest et al., 2013, Lautenschlager et al., 2010). It was observed that initially there was little difference in the concentration of total *Legionella* in both high-use and low-use IBRs after 14 days of the operation phase (**Table 8.1**). However, culturing results demonstrated that the *Legionella* population decreased by 1.9 $\log_{10}$ in the high-use IBR (**Figure 8.4**). By the end of operational phase, biofilm-associated culturable *Legionella* were 1.5 $\log_{10}$ greater in the low-use IBR relative to the high-use IBR, suggesting that this difference was induced by flushing events (**Table 8.1** and **Figure 8.4**) and perhaps water stagnation stimulated denser and stronger attachment of biofilm to surfaces. A previous pilot scale study of a plumbing system also suggested that the concentration of *Legionella* increases with water stagnation (Rhoads et al., 2015). This supported the findings from this study that showed once-a-week flushing increased water stagnation time, which resulted in proliferation of both planktonic and biofilm-associated alive and culturable *Legionella*. Alternatively, once-a-day water flushing caused looser attachment (dissociation) of biofilm to surfaces that was then readily removed by flushing. The doubling time of bacteria in potable water is reported to be several days, which supports our argument that during this period of water stagnation bacteria regrow and maintain their population (Prest et al., 2016, Boe-Hansen et al., 2002). Thus, with a high flushing frequency the water efficiently dislodged biofilm and significantly decreased the number of alive and culturable *Legionella*, however the number of VBNC *Legionella* increased (**Table 8.1**, and **Figures 8.1** and **8.3**). Consequently, the growth of VBNC *Legionella* might have been stimulated by flushing events in a more direct way. After daily flushing, a high number of VBNC *Legionella* were recovered from biofilm matrix in the presence of *V. vermiformis*. Similarly, after daily flushing high concentrations of planktonic VBNC *Legionella* were detected in the water which was highly contaminated with *Acanthamoeba*. *In vitro* studies have shown that VBNC *Legionella* proliferates intracellularly in amoebae (Buse et al., 2013). It is also reported that in natural environments, both physiochemical stresses and host protozoans transform culturable *Legionella* into VBNC *Legionella* (Nisar et al., 2020b, Farhat et al., 2010). So, this increase in

VBNC *Legionella* could be explained by the fact that daily flushing impacted and promoted the differentiation of culturable *Legionella* into VBNC *Legionella* by the intracytoplasmic division within host amoebae. It is worth noting that biofilm formation is a 'stress response' to hostile environments e.g., low nutrients and disinfectant treatments. Flushing introduces both these stressors in building water systems and so may induce the VBNC state. Our findings also suggest that elimination of water stagnation does not necessarily fix the problem of viable and potentially infectious *Legionella* which broadly stimulated the synthesis of VBNC *Legionella*.

This study found that culturable heterotrophic bacterial populations negatively correlated with increased incidence of flushing events and positively correlated with increased water stagnation. It showed that water stagnation quickly altered the microbial water quality and substantially increased the number of bacteria. Once-a-day flushing also impacted the structure and diversity of the culturable heterotrophic bacteria population. The municipal water source used for flushing could explain the rapid change in diversity of culturable heterotrophic bacteria. Generally, it is difficult to interpret the public health significance of HPC results because the correlation with OPPPs is debated (Bartram et al., 2003). Secondly, to our knowledge HPC levels have been not associated with any known disease outbreak and public health concern. However, this study found that HPC levels positively correlated to total, alive and culturable *Legionella* concentrations and water stagnation. These results support a previous survey of residential buildings that also demonstrated a positive correlation between heterotrophic bacterial population and *Legionella* concentration (Ley et al., 2020).

Free-living amoebae are an important part of plumbing systems (Thomas and Ashbolt, 2011). *Acanthamoeba* and *V. vermiformis* are the most common and abundant hosts of *Legionella* (Nisar et al., 2020a). Our results illustrated that the population of amoebae was also affected by water stagnation and flushing. Interestingly, increased incidence of flushing was observed to increase the population of biofilm-associated *V. vermiformis* (**Table 8.1** and **Figure 8.3**) but decrease the concentration of *Acanthamoeba* (**Table 8.1** and **Figure 8.3**). This increase in *V. vermiformis* concentration was associated with an increase in VBNC *Legionella*. This is potentially due to the increased transformation of alive *Legionella* into VBNC via *Legionella* intracellular replication within amoeba hosts (Boamah et al., 2017, Bigot et al., 2013). Secondly, it may have benefited from the delivery of carbon and nutrients to the biofilm after daily flushing. There is another possibility: that once-a-day flushing induced stress on biofilm-associated *Legionella* and other bacteria, which chemotactically attracted amoebae for their protection and genesis of VBNC bacterial cells (Shi et al., 2021, Bigot et al., 2013). To our knowledge, effect of water stagnation and water flushing on amoebae growth and proliferation

has not yet been investigated. Further studies are required to properly understand how free-living amoebae behave in building plumbing systems under different hydraulic regimes.

By the time potable water reaches the point of delivery within a building, it can be a few hours to several days old. Extended periods of water stagnation are linked to failures of disinfection procedures and increased microbial populations. This study used a model plumbing system to demonstrate that daily water flushing had a significant effect on *Legionella* prevalence in a building plumbing system compared with once-a-week flushing and an extended period of water stagnation. An increased incidence of flushing was statistically significantly associated with a decrease in *Legionella* concentration. However, it also demonstrated that once *Legionella* had developed and become incorporated into the biofilm matrix it persisted, and regular flushing was unable to eradicate it. As such, multiple strategies are needed for the management and control of building water systems. This should include the prevention of water stagnation, in combination with additional physical or chemical disinfection approaches.

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Authors contributions

MAN and HW conceived and designed research. MAN performed the experiments. GB and NSE assisted in the flow cytometry and microscopic analysis, respectively. KER, MHB, HW and RB provided technical assistance. MAN and HW drafted and edited the manuscript, HW, KER, MHB, RB, GB, NSE, and SCL corrected and contributed to the manuscript. All authors approved of the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

9 Stagnation arising through intermittent usage is associated with increased viable but non culturable *Legionella* and amoeba hosts in a hospital water system

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9.1 Abstract

Hospital water systems are a significant source of *Legionella*, resulting in the potentially fatal Legionnaires' disease. One of the biggest challenges for *Legionella* management within these systems is that under unfavourable conditions *Legionella* transforms itself into a viable but non culturable (VBNC) state that cannot be detected using the standard methods. This study used a novel method (flow cytometry-cell sorting and qPCR [VFC+qPCR] assay) concurrently with the standard detection methods to examine the effect of temporary water stagnation, on *Legionella* spp. and microbial communities present in a hospital water system. Water samples were also analysed for amoebae using culture and *Vermamoeba vermiformis* and *Acanthamoeba* specific qPCR. The water temperature, number, and duration of water flow events for the hand basins and showers sampled was measured using the Enware Smart Flow® monitoring system. qPCR analysis demonstrated that 21.8% samples were positive for *Legionella* spp., 21% for *L. pneumophila*, 40.9% for *V. vermiformis* and 4.2% for *Acanthamoeba*. All samples that were *Legionella* spp. positive using qPCR (22%) were also positive for VBNC *Legionella* spp.*;* however, only 2.5% of samples were positive for culturable *Legionella* spp. 18.1% of the samples were positive for free-living amoebae (FLA) using culture. All samples positive for *Legionella* spp. were also positive for FLA. Samples with a high heterotrophic plate count (HPC ≥ 5×10^3 CFU/L) were also significantly associated with high concentrations of *Legionella* spp. DNA, VBNC *Legionella* spp./*L. pneumophila* ($p < 0.01$) and *V. vermiformis* ($p < 0.05$). Temporary water stagnation arising through intermittent usage (< 2 hours of usage per month) significantly ($p < 0.01$) increased the amount of *Legionella* spp. DNA, VBNC *Legionella* spp./*L. pneumophila*, and *V. vermiformis;* however, it did not significantly impact the HPC load. In contrast to stagnation, no relationship was observed between the microbes and water temperature. In conclusion, *Legionella* spp. (DNA and VBNC) was associated with *V. vermiformis,* heterotrophic bacteria, and stagnation occurring through intermittent usage. This is the first study to monitor VBNC *Legionella* spp. within a hospital water system. The high percentage of false negative *Legionella* spp. results provided by the culture method supports the use of either qPCR or VFC+qPCR to monitor *Legionella* spp. contamination within hospital water systems.

9.2 Introduction

Legionella is an opportunistic premise plumbing pathogen and etiological agent of Legionnaires' disease (LD), a potentially fatal pneumonia like infection (Cunha et al., 2016). *Legionella* is ubiquitous in natural and engineered water systems and transmitted through aspiration or inhalation of *Legionella* contaminated water or aerosols (Schwake et al., 2021). Globally the incidence of LD has been increasing. In 2021, the US Centers for Disease Control

and Prevention (CDC) reported 8260 confirmed cases of LD in USA (Centers for Disease Control and Prevention, 2022). In Australia, 524 confirmed cases of legionellosis were reported in 2020 (Australian Government, 2021b). According to the European Centre for Disease Prevention and Control (ECDC) 11,298 confirmed cases of LD were documented across European countries in 2019. However, in 2020 the number decreased to 8,372; this reduction may be associated with COVID-19 pandemic lockdown restrictions or a decrease in focus on LD. In 2021, 10,723 confirmed cases of LD were documented of which 5.4% were nosocomial infections (The European Legionnaires' disease Surveillance Network, 2022). The actual number of legionellosis cases is understated, because in the majority of cases Pontiac fever remains unnoticed and the etiological agent of pneumonia remains unrecognized (Cassell et al., 2019). There are at least 60 distinct species of *Legionella,* with *L. pneumophila* sg.1 being the most common cause of outbreaks (Miyashita et al., 2020, Khodr et al., 2016). Initially, cooling towers were considered to be the main source of *Legionella* spp., but subsequent investigations have identified that engineered water systems are a major source of LD (Kanarek et al., 2022). Those at greatest risk of infection are the elderly and immunocompromised individuals, and as such nosocomial outbreaks associated with hospital engineered water systems are of significant concern (Bartram et al., 2007).

A range of factors influence the survival and persistence of *Legionella* spp. in hospital water systems including: biofilms, nutrients, disinfectants, protozoa hosts, water temperature, flow dynamics and stagnation (Nisar et al., 2020b, Whiley et al., 2017, Abdel-Nour et al., 2013). Naturally, *Legionella* spp. infects and survives within a wide range of polyphyletic protozoan hosts, with *Acanthamoeba* and *Vermamoeba* the most commonly identified hosts in potable water (Nisar et al., 2020a, Boamah et al., 2017, Best and Abu Kwaik, 2018). Intracytoplasmic *Legionella* spp. are protected from adverse environmental conditions (Best and Abu Kwaik, 2018), with *Legionella* spp. released from host protozoa more virulent and pathogenic in nature (Boamah et al., 2017, Fields et al., 2002). Additionally, *Legionella* spp. intrinsically tolerate water disinfection treatments by entering into a metabolically inactive but highly resistant and potentially pathogenic “viable but non-culturable” (VBNC) state (Kirschner, 2016). Under suitable environmental conditions, and in the presence of protozoa hosts, VBNC *Legionella* spp. can resuscitate back into a culturable state (Dietersdorfer et al., 2018). VBNC *Legionella* spp. are a significant challenge to water quality management as they cannot be detected using the standard culture-based method (International Organization for Standardization, 2017, Standards Australia, 2017). *Legionella* spp. specific quantitative PCR (qPCR) assay is an alternative method typically used to detect the genomic load of *Legionella* spp. (International Organization for Standardization, 2019); however, it cannot distinguish between culturable,

dead and VBNC *Legionella* spp. (Kirschner, 2016). As such, there are currently limited studies that investigate the survival of VBNC *Legionella* spp. in engineered water systems.

Water stagnation in engineered water systems is categorized into two different types; permanent, and temporary stagnation (Nisar et al., 2020b, Peter and Routledge, 2018). Permanent stagnation is complete stagnation of plumbing structures, such as dead-ends and dead-legs (Nisar et al., 2020b). However, in engineered water systems, water can also stagnate in storage tanks, plumbing piping network, and within components at the water outlets for a few hours to weeks and even months (Manuel et al., 2009, Bartram et al., 2007). This type of water stagnation is known as intermittent or temporary stagnation (Peter and Routledge, 2018, Manuel et al., 2009). The relationship between *Legionella* spp. and permanent stagnation is well characterized (Totaro et al., 2018, Nisar et al., 2020b). However, less is known about the relationship between *Legionella* spp. and temporary stagnation. Therefore, this study examined the role of temporary stagnation arising through intermittent water usage on the persistence of *Legionella* spp. and free-living amoebae (FLA) within a hospital water system.

This study was the first study to utilize a novel method to enumerate VBNC *Legionella* spp. and *L. pneumophila* from environmental water samples and investigate relationships with protozoan hosts. This study utilized the Enware Smart® Flow monitoring system to examine the relationships between water flow (arising through water outlet usage) and temperature with *Legionella* spp., *L. pneumophila* and amoeba hosts. The specific aims of this study were as follows, to: (1) determine the prevalence of *Legionella* spp./*L. pneumophila* and FLA in a hospital water system; (2) examine the relationship between *Legionella* spp. and potential protozoan hosts; and (3) monitor the effect of sampling phases (months), water temperature, flow dynamics and stagnation on persistence of *Legionella* spp. in the hospital water system. To our knowledge, this is the first comprehensive study that has quantified VBNC *Legionella* spp. and FLA from a hospital water system under dynamic flow and temperature conditions.

9.3 Materials and Methods

9.3.1 Sample collection and processing

From March 2021 to June 2022, water (n = 120) and biofilm (n = 46) samples were collected from the engineered water system of an Australian hospital located in New South Wales, Australia. The sampling was done in different phases, where the categorization was: March 2021 as phase 1, April 2021 as phase 2, November 2021 as phase 3 and June 2022 as phase 4. All water and biofilm samples were collected, transported and stored as recommended by standard guidelines (Centers for Disease Control and Prevention, 2019, International

Organization for Standardization, 2018). For the water samples, 1 L first flush hand basin or shower water was collected in sterile wide-mouth screw capped plastic bottles (2105-0032, Nalgene™). For the biofilm samples, visible biofilm was scraped from the inside of tap faucet or shower head using sterile polyurethane-tipped swabs (CleanFoam® TX751B, Texwipe®), then 5 to 10 mL of water was added and placed with the swab in a sterile screw capped tube. For both the water and biofilm samples, 0.5 mL 0.1 N sodium thiosulfate (124270010, ACROS Organics™) was added to neutralize pre-existing chlorine-based chemical disinfectants. All samples were transported and kept at 5 ± 2°C and processed within 72 hours. The samples were vacuum filtered through 47 mm diameter 0.22 µm polycarbonate membrane (GTTP04700, Isopore™). The filtered residues were resuspended in 3 mL sterile distilled water. This sample suspension was used for further microbiological and molecular testing.

9.3.2 Water flow and temperature data

Parameters related to water temperature and flow dynamics were monitored in the hospital water system using the Enware Smart® Flow monitoring system. Briefly, this monitoring system measures water system delivery temperatures using temperature probes located at the hot water inlet, cold water inlet, and outlet of the thermostatic mixing valves (TMV) and the hot water inlet and cold water inlet of hand basin faucets. Water flow was measured using flow switches located at the hot water inlet and cold water inlet of both the TMVs and hand basin faucets (Whiley et al., 2019). The temperature data of the hot water supply, cold water supply and outlet was collected for the entire duration of the sampling period. For analysis these measurements were separated into a period one week and one month prior to a water sampling event. In terms of flow regime, the total duration (hours) and number (counts) of flushing events for a period of one week and one month prior to sampling were recorded. The total duration (hours) of flushing events were divided into low and high flow regimes with categorization as: low flow regime; 0 to < 2 hours per month, and high flow regime; ≥ 2 to 40 hours per month.

9.3.3 Molecular analysis

Quantification of *Legionella* spp. (16 rDNA gene) and *L. pneumophila* (*mip* gene) was performed using ISO/TS12869:2019 quantitative polymerase chain reaction (qPCR) assays (International Organization for Standardization, 2019). The 18S rDNA gene was amplified for the quantification of *Acanthamoeba* and *Vermamoeba vermiformis* (Qvarnstrom et al., 2006, Scheikl et al., 2016). *Legionella* spp. (GenBank Acc CP021281), *L. pneumophila* (GenBank Acc KR902705), *Acanthamoeba castellanii* (GenBank Acc U07413) and *V. vermiformis* (GenBank Acc KT185625) gBlocks gene fragments (IDT™) were used as a positive control and for the preparation of a standard curve using ten-fold serial dilutions. Using the Aquadien™

kit (3578121, BIO-RAD Laboratories Ltd.), genomic DNA was extracted from each water and biofilm sample before being subjected to a qPCR assay. The qPCR reaction mixture consisted of microbe-specific primers (Bio-Rad Laboratories Ltd.), 1X PCR reaction buffer (2X SsoAdvanced™ universal probes supermix:172-5281, Bio-Rad Laboratories Ltd.), and DNA template. To detect the potential presence of environmental inhibitors of the qPCR assays, both the purified and a one in ten dilution of extracted DNA was used as template (Nisar et al., 2022, Hayes-Phillips et al., 2019). Using a Rotor-Gene Q thermal cycler (Qiagen Ltd.), each template DNA was subjected to the qPCR assay in triplicate (Nisar et al., 2022). All fluorescence labelled probes and primers used in this study are presented in **Table 15.1** (Appendix-4).

9.3.4 Microbiological analysis

Isolation of culturable *Legionella* spp. and *L. pneumophila* was performed in accordance with the standard guidelines (International Organization for Standardization, 2017, Standards Australia, 2017). Briefly, samples were heat treated (50 ± 1°C for 30 ± 2 minutes) and/or acid treated (HCl-KCl buffer treatment for 5 ± 0.5 minutes) to reduce the contamination of interfering microbes. An aliquot of treated sample was then spread on *Legionella* agar (CM1203, Oxoid Ltd.) supplemented with GVPC (glycine, vancomycin, polymyxin B and cycloheximide: SR0152, Oxoid Ltd.) and *Legionella* growth supplement (α-ketoglutarate, buffer/potassium hydroxide, ferric pyrophosphate, and $_{L}$-cysteine: SR0110C, Oxoid Ltd.). The inoculated plates were incubated at 37 ± 1°C for 7 days and examined every day. Suspected *Legionella*-like colonies were counted from each plate and evaluated by *Legionella* latex agglutination test kit (DR0800, Oxoid Ltd.). This kit identifies genus *Legionella* and further characterizes various species and serogroups with overall 99% sensitivity and 100% specificity. Furthermore, all *Legionella*-like colonies were confirmed through *Legionella* spp. specific qPCR assays. To determine the heterotrophic plate counts (HPC), an aliquot from each sample was inoculated on R_2A agar (CM0906, Oxoid Ltd.) and incubated at 35 ± 1°C. The colonies were counted after 2, 5 and 7 days of incubation. The results for *Legionella* spp. and heterotrophic bacteria were expressed in colony forming units (CFU)/L for water samples and CFU for the biofilm samples. Isolation of culturable FLA was performed by inoculating an aliquot of each sample on heat-inactivated (57°C for 45 minutes) *Escherichia coli* American Type Culture Collection 700891™ supplemented 1.5% non-nutrient agar (Eco-NNA: CM0003, Oxoid Ltd.) (Nisar et al., 2022). The plates were incubated at 25 ± 1°C for 14 days and amoebal growth was examined daily using an inverted light microscope (AMEFC4300, EVOS™ FL, Thermo Fisher Scientific). All monoxenic amoebae cultures were characterized by microscopic examination and sequence analysis of 18S rDNA gene.

9.3.5 Quantification of VBNC *Legionella* and *L. pneumophila*

VBNC *Legionella* spp. and *L. pneumophila* were detected and quantified by flow cytometry-cell sorting and qPCR (VFC+qPCR) assay (Nisar et al., 2023). Briefly, 300 µL sample suspension was resuspended in 200 µL of filter sterilized staining buffer (0.01% Tween-20 and 1 mM EDTA in 1X PBS, pH 7.4 ± 0.1), followed by addition of 48 µM propidium iodide (PI) and 420 nM thiazole orange (TO) dyes (cell viability kit Cat # 349480, Becton Dickinson, Franklin Lakes, USA). The mixture was incubated at 5°C for 15 minutes. Then, using a FACS Aria Fusion instrument, (Becton Dickinson, Franklin Lakes, USA) cells were analysed and segregated into dead (PI/TO), alive (potentially culturable: TO), and injured (potentially VBNC: PI/TO) cell populations. From each sample, the injured cell fraction was isolated and subjected to DNA extraction and quantification of *Legionella* spp. and *L. pneumophila* gene markers (Nisar et al., 2023).

9.3.6 Data analysis

The data are described in logarithmic form with base 10 (log_{10}). The percentage of both bacterial and amoebae isolates was determined based on phases or contamination levels and plotted in Microsoft® Excel®. Statistical calculations were made using R studio (version 4.2.2) and graphically presented by using “ggplot2 (version 3.3.6)” package (Wickham, 2016). Briefly, the Shapiro-Wilk test was used to assess normality of analysed quantitative parameters. For comparison of the means of the quantitative parameters (i.e., either GU or CFU of *Legionella* spp./*L. pneumophila*, HPC, *Acanthamoeba* and *V. vermiformis*), a non-parametric Kruskal-Wallis test was used. Finally, the non-parametric Spearman’s correlation (ρ) test was used to evaluate relationships among different variables (i.e., *Legionella* spp./*L. pneumophila*, HPC, *Acanthamoeba* and *V. vermiformis*). A statistically significant difference among the quantitative parameters was defined by *p* values of less than 0.05.

9.4 Results

9.4.1 Occurrence of *Legionella* and *L. pneumophila*

Table 9.1 presents an overview of the percentage of samples identified as positive for *Legionella* spp. and FLA using the different detection methods. All samples that were qPCR positive for *Legionella* spp. were also positive for FLA and VBNC *Legionella.* Specifically, 21.7% (n = 36/166) of total samples were positive for *Legionella* spp. DNA (16S rDNA gene) with a concentration range of 9×10^2 to 1.5×10^6 GU (**Tables 9.1** and **9.2**). *L. pneumophila* DNA (*mip* gene) was present in 21% samples (n = 35/166) with a concentration ranging from 3.5×10^2 to 9×10^4 GU (**Tables 9.1** and **9.2**). All *L. pneumophila* positive samples were also positive for *Legionella* spp. During phase 1, 58.06% (n = 18/31) of the samples tested positive for *Legionella* spp. DNA, whereas in the 2nd phase 7.31% (n = 3/41), and 4th phase 28.84% (n = 15/52) of the collected samples tested positive for *Legionella* spp. DNA (**Figure 9.1**). However, in phase 3 none of the samples were positive for either *Legionella* spp. or *L. pneumophila* DNA. Standard culturing demonstrated that only four samples (two in phase 1 and two in phase 4) were positive for culturable *Legionella* spp., which were identified as non-*pneumophila Legionella* using serology and qPCR. The VFC+qPCR assay demonstrated that all samples positive for either *Legionella* spp. or *L. pneumophila* DNA (according to the qPCR assay) also contained VBNC cells (**Figure 9.1** and **Table 9.1**). Therefore, of the 36 samples that were positive for VBNC *Legionella* spp.*,* the standard microbiological culturing assay returned a false negative result for 32 of them (88.9%). For analysis, the VBNC *Legionella* spp. and *L. pneumophil*a samples were categorized into three groups based on concentration i.e., low (< 10^3 GU/L), medium (≥ 10^3 to 10^4 GU/L) and high (> 10^4 GU/L) contamination. Based on this grouping it was found that in phase 1, 14.3% (n = 4/31) of the water samples were positive for high VBNC *Legionella* spp. contamination, whereas in the 4th phase 33.3% (n = 10/52) water samples were positive for high VBNC *Legionella* spp. contamination (Appendix-4, **Figure 15.1 A**). It was found that the lower level of VBNC *L. pneumophila* occurred more frequently in the samples collected during phase 1 (53.5%, n = 15/31) and 4 (23.3%, n = 7/52). Only 6.7% (phase 4: n = 2/52) water samples contained high levels of VBNC *L. pneumophil*a contamination (Appendix-4, **Figure 15.1 B**). Based on sampling sites it was found that in hand basin water, 13.4% (n = 9/67) samples were positive for high VBNC *Legionella* spp. contamination, whereas in shower water, 9.4% (n = 5/53) samples were positive for high VBNC *Legionella* spp. contamination (Appendix-4, **Figure 15.2 A**). However, the majority of hand basin (22.4%, n = 15/67) and shower (15.1%, n = 8/53) water samples contained low levels of VBNC *L. pneumophila* contamination (Appendix-4, **Figure 15.2 B**). Overall, both qPCR and VFC+qPCR assays clearly demonstrated that the standard culturing assay is frequently unable to detect *Legionella* spp./*L. pneumophila* present in the hospital water system.

9.4.2 Occurrence of heterotrophic bacteria and FLA

Both the hand basin and shower water samples contained HPC counts ranging from 10 to 1.5 × 10^5 CFU/L (**Table 9.2**). In the biofilm samples the HPC load ranged from 15 to 7.5 × 10^4 CFU/sample (**Table 9.2**). In case of FLA, the *V. vermiformis* gene marker was present in 40.9% (n = 68/166) of samples with concentrations ranging from 7.5 × 10^2 to 7.5 × 10^7 GU (**Tables 9.1** and **9.2**). The *Acanthamoeba* gene marker was detected only in hand basin water (3.01%, n = 5/166) and biofilm samples (1.2%, n = 2/166) with a range of 1 × 10^3 to 8 × 10^3 GU (**Tables 9.1** and **9.2**). Culturable amoebae were identified in 18.1% (n = 30/166) samples; however, due to fungal overgrowth 13 isolates were unable to develop monoxenic cultures, of these 13, five isolates showed acrasid amoebae-like morphology. Only 17 isolates developed monoxenic cultures which were further characterized on the basis of cellular morphology and sequence analysis of 18S rDNA gene. Light microscopy revealed that isolates harboured monotactic morphotype and developed spherical cysts consisting of distinct inner and outer walls. Based on 18S rDNA sequencing, all these monoxenic isolates were identified as *V. vermiformis*.

9.4.3 Relationship among *Legionella*, HPC, and FLA

All shower and hand basin water samples were classified into two groups based on the HPC levels i.e., low (10 to < 5 × 10^3 CFU/L) and high (≥ 5 × 10^3 CFU/L) contamination. Kruskal-Wallis analysis demonstrated that quantity of both *Legionella* spp. DNA and VBNC *Legionella* spp. were significantly ($p < 0.001$) higher in water samples with high levels of HPC load (**Table 9.3** and **Figure 9.2**). Similarly, water samples having greater levels of HPC load harboured significantly higher concentrations of *L. pneumophila* DNA (Kruskal-Wallis test, $p < 0.01$), VBNC *L. pneumophila* (Kruskal-Wallis test, $p < 0.01$), and *V. vermiformis* (Kruskal-Wallis test, $p < 0.05$) (**Tables 9.3** and **Figure 9.2**). Furthermore, all samples characterized as positive for *Legionella* spp./*L. pneumophila* (DNA, culturable, and VBNC cells) were also positive for either the *V. vermiformis* gene marker or culturable amoebae. Furthermore, Spearman's analysis demonstrated both *Legionella* spp./*L. pneumophila* DNA and *Legionella* spp./*L. pneumophila* VBNC cells were positively correlated ($p < 0.001$) with *V. vermiformis* (**Table 9.4**). Overall, these results suggested that in hospital water system, high levels of HPC load and *V. vermiformis* are positively associated with both *Legionella* spp./*L. pneumophila* DNA and VBNC cells.

Table 9.1: Prevalence of *Legionella* spp.*, Vermamoeba vermiformis*, *Acanthamoeba* and total free-living amoeba in a hospital water system using the different methods of detection.

Sample (n)	***Number of Legionella* positive samples (%)**					**Number of free-living amoeba positive samples (%)**		
	qPCR assay		**VFC+qPCR**		**Culture assay**	**qPCR assay**		**Culture assay**
	Legionella	***L. pneumophila***	***Legionella***	***L. pneumophila***		***Acanthamoeba***	***V. vermiformis***	
Sampling phase 1 (March 2021)								
Hand basin water (n = 16)	11	11	11	11	1	3	15	9
Shower water (n = 12)	4	4	4	4	1	0	9	6
Tap faucet biofilm (n = 3)	3	3	3	3	0	1	2	1
Total (n = 31)	**18 (58.06%)**	**18 (58.06%)**	**18 (58.06%)**	**18 (58.06%)**	**2 (6.45%)**	**4 (12.9%)**	**26 (83.87%)**	**16 (51.61%)**
Sampling phase 2 (April 2021)								
Hand basin water (n = 17)	2	2	2	2	0	0	11	8
Shower water (n = 13)	1	1	1	1	0	0	4	2
Tap faucet biofilm (n = 11)	0	0	0	0	0	0	5	0
Total (n = 41)	**3 (7.31%)**	**3 (7.31%)**	**3 (7.31%)**	**3 (7.31%)**	**0**	**0**	**20 (48.78%)**	**10 (24.39%)**
Sampling phase 3 (November 2021)								
Hand basin water (n = 18)	0	0	0	0	0	1	0	1
Shower water (n = 14)	0	0	0	0	0	0	0	0
Tap faucet biofilm (n = 10)	0	0	0	0	0	0	1	1
Total (n = 42)	**0**	**0**	**0**	**0**	**0**	**1 (2.38%)**	**1 (2.38%)**	**2 (4.76%)**
Sampling phase 4 (June 2022)								
Hand basin water (n = 16)	8	7	8	7	0	1	10	0
Shower water (n = 14)	5	5	5	5	0	0	7	0
Tap faucet biofilm (n = 22)	2	2	2	2	2	1	4	2
Total (n = 52)	**15 (28.84%)**	**14 (26.92%)**	**15 (28.84%)**	**14 (26.92%)**	**2 (3.84%)**	**2 (3.84%)**	**21 (48.38%)**	**2 (3.84%)**

Table 9.2: The minimum and maximum microbial concentrations present in the positive water and biofilm samples.

Microbes	Minimum concentration	Maximum concentration
Hand basin water (n = 67)		
Legionella DNA (GU/L)	1×10^3	1.5×10^6
VBNC *Legionella* (GU/L)	2.5×10^2	6.5×10^5
L. pneumophila DNA (GU/L)	3.5×10^2	9×10^4
VBNC *L. pneumophila* (GU/L)	6×10^2	8.5×10^4
Vermamoeba vermiformis (GU/L)	7.5×10^2	7.5×10^7
Acanthamoeba (GU/L)	1×10^3	5×10^3
Heterotrophic plate count (CFU/L)	10	1.5×10^5
Shower water (n = 53)		
Legionella DNA (GU/L)	9×10^2	7×10^4
VBNC *Legionella* (GU/L)	3.5×10^2	2.5×10^4
L. pneumophila DNA (GU/L)	3.5×10^2	9.5×10^3
VBNC *L. pneumophila* (GU/L)	70	4.5×10^3
Vermamoeba vermiformis (GU/L)	1×10^3	4×10^7
Acanthamoeba (GU/L)	0	0
Heterotrophic plate count (CFU/L)	10	1.5×10^5
Tap faucet biofilm (n = 46)		
Legionella DNA (GU)	1×10^4	3.5×10^5
VBNC *Legionella* (GU)	1.5×10^2	3×10^4
L. pneumophila DNA (GU)	7.5×10^2	1.5×10^4
VBNC *L. pneumophila* (GU)	1×10^2	1×10^4
Vermamoeba vermiformis (GU)	1×10^3	1×10^6
Acanthamoeba (GU)	4.5×10^3	8×10^3
Heterotrophic plate count (CFU)	15	7.5×10^4

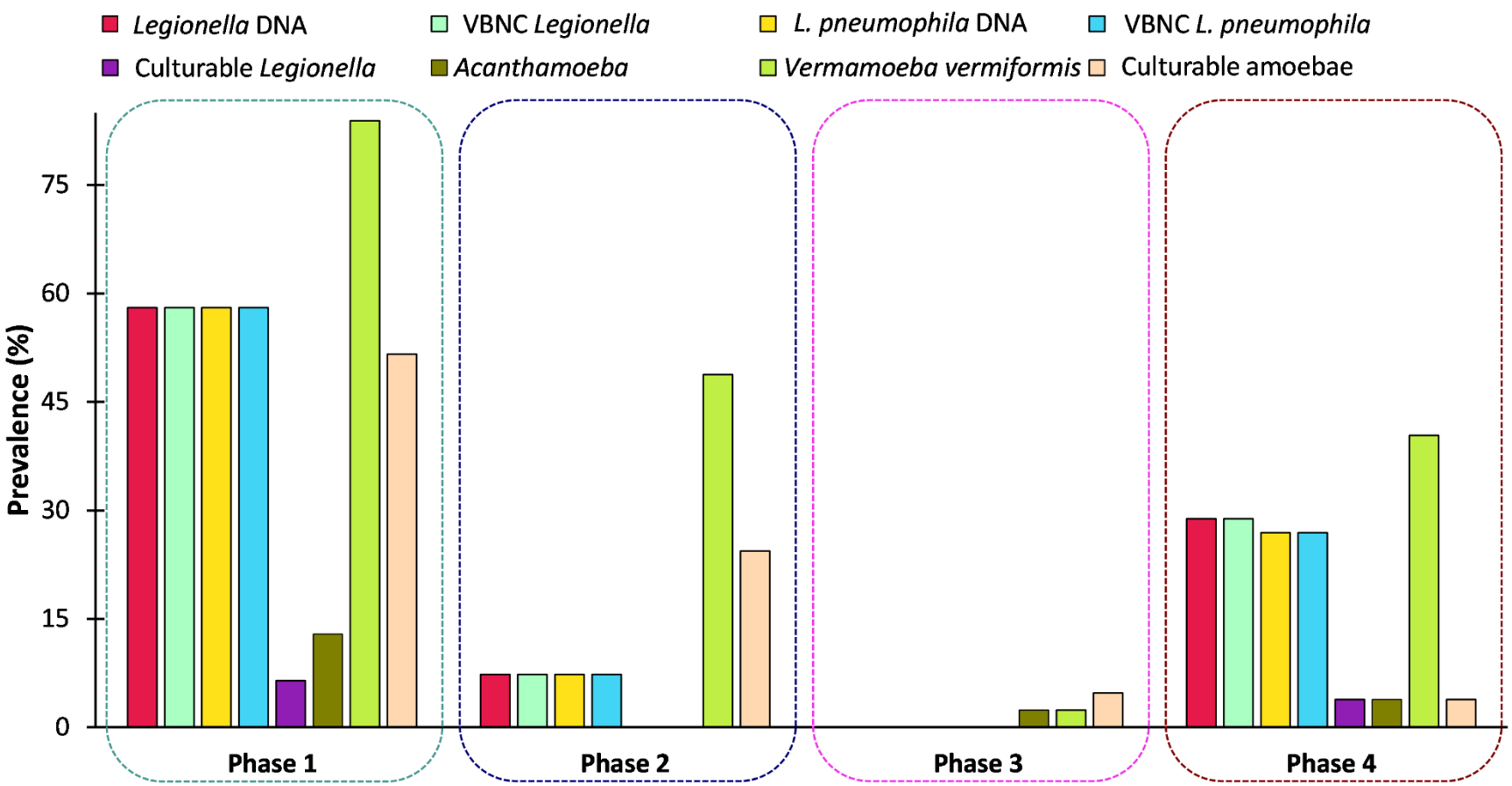

Figure 9.1: Prevalence (%) of *Legionella* spp. and free-living amoeba in a hospital water system. A total of 166 water (hand basin and shower) and biofilm (tap faucet) samples were collected in March 2021 (Phase 1), April 2021 (Phase 2), November 2021 (Phase 3), and June 2022 (Phase 4). Total amounts of *Legionella* spp./*L. pneumophila*, *Acanthamoeba*, and *Vermamoeba vermiformis* were detected and quantified by qPCR assays. Culturable *Legionella* spp. and amoebae were detected by standard microbiological culturing procedures. VBNC *Legionella* spp. and VBNC *L. pneumophila* were detected and quantified by flow cytometry-cell sorting and qPCR assay.

Table 9.3: Effect of flow regime (flushing duration one-month prior to sampling) and heterotrophic plate counts of hand basin and shower water microbes.

Microbes in hand basin and shower water	Flow regime (one-month prior sampling) *		Heterotrophic plate count **	
	Low (0 to < 2 hours/month)	High (≥ 2 to 40 hours/month)	Low (10 to < 5×10^3 CFU/L)	High (≥ 5×10^3 to 1.5×10^5 CFU/L)
Total *Legionella* (GU/L)	1.783 ± 2.043	0.637 ± 1.559	0.532 ± 1.443	1.604 ± 2.015
VBNC *Legionella* (GU/L)	1.560 ± 1.771	0.551 ± 1.366	0.413 ± 1.100	1.455 ± 1.839
Total *L. pneumophila* (GU/L)	1.472 ± 1.671	0.495 ± 1.284	0.429 ± 1.148	1.326 ± 1.698
VBNC *L. pneumophila* (GU/L)	1.206 ± 1.372	0.416 ± 1.107	0.344 ± 0.921	1.106 ± 1.442
Vermamoeba vermiformis (GU/L)	3.578 ± 2.835	1.755 ± 2.508	1.983 ± 2.763	2.952 ± 2.755
Heterotrophic plate count (CFU/L)	3.742 ± 1.010	3.656 ± 0.855	-	-

Data is log transformed and shown as mean ± standard deviation.

* Microbial loads (except heterotrophic plate count) in low flow regime are significantly higher than high flow regimes (Kruskal-Wallis analysis, $p < 0.05$)

** Microbial loads in high heterotrophic plate counts are significantly higher than low heterotrophic plate counts (Kruskal-Wallis analysis, $p < 0.05$)

Table 9.4: Correlation between *Legionella* spp. and *Vermamoeba vermiformis* in hand basin and shower water samples.

	Spearman's rank correlation			
	***Legionella* DNA**	**VBNC *Legionella***	***L. pneumophila* DNA**	**VBNC *L. pneumophila***
Vermamoeba vermiformis	ρ = 0.5819 $p < 0.001$	ρ = 0.5833 $p < 0.001$	ρ = 0.5955 $p < 0.001$	ρ = 0.5826 $p < 0.001$

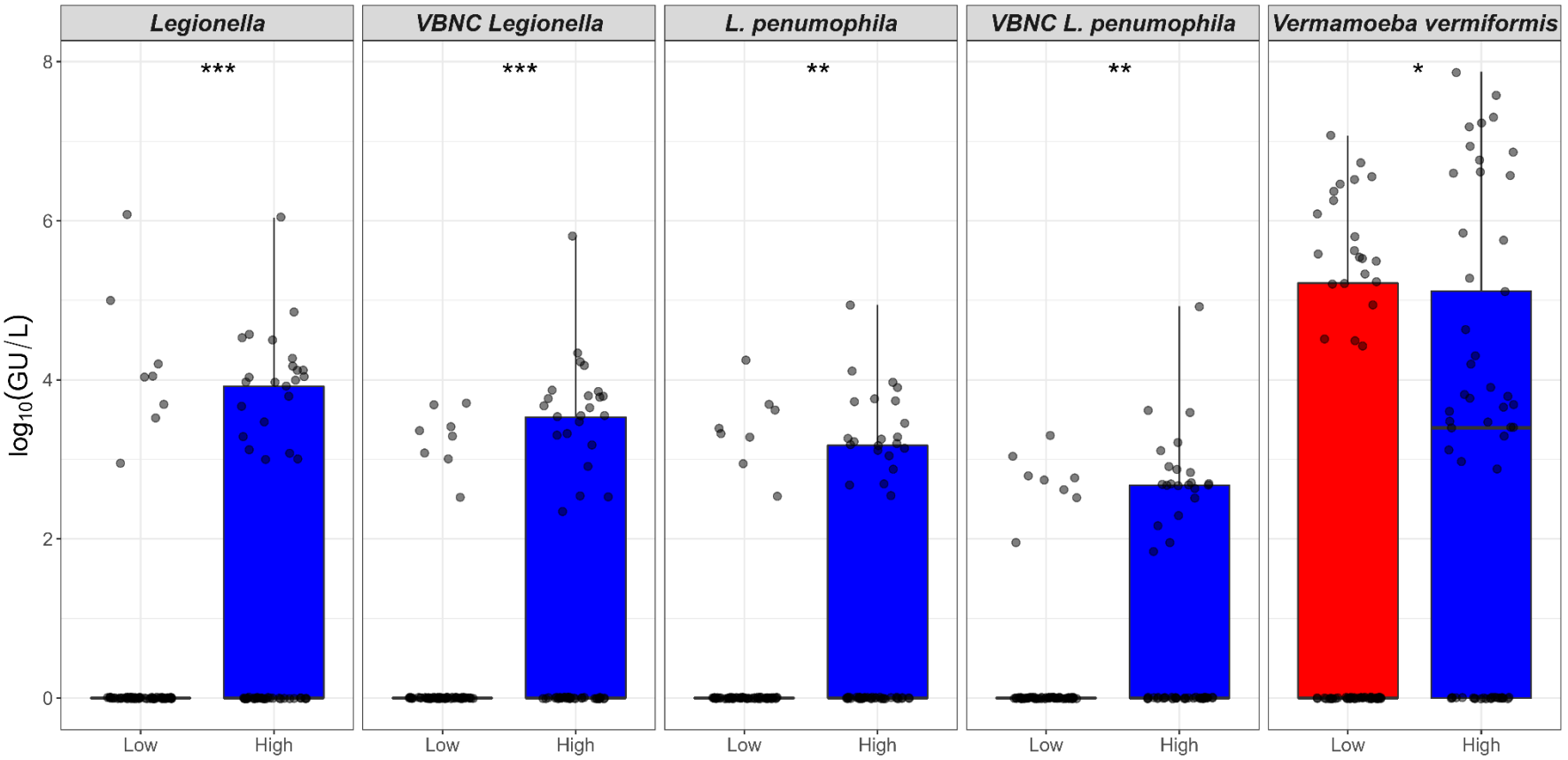

Figure 9.2: Relationship between the heterotrophic plate count and *Legionella* spp./*Vermamoeba vermiformis*. X-axis represents HPC level that is categorized into low (10 to < 5×10^3 CFU/L) and high (≥ 5×10^3 to 1.5×10^5 CFU/L) contamination. Y-axis represents $\log_{10}$(GU/L) of *Legionella* spp., VBNC *Legionella* spp., *L. pneumophila*, VBNC *L. pneumophila*, and *V. vermiformis*.

9.4.4 Influence of flow regimes and on *Legionella*, HPC, and free-living amoebae

The total duration (hours) of flushing events for one month prior to sampling, was categorized into: low (0 to < 2 hours/month) and high (≥ 2 to 40 hours/month) flow regimes. The Kruskal-Wallis analysis indicated that the concentrations of *Legionella* spp. DNA ($p < 0.01$), *L. pneumophila* DNA ($p < 0.01$), VBNC *Legionella* spp. ($p < 0.001$), VBNC *L. pneumophila* ($p < 0.001$) and *V. vermiformis* DNA ($p < 0.05$), were all higher in low flow regimes compared with high flow regimes (**Table 9.3** and **Figure 9.3**). When the total duration (hours) of flushing events for only one week prior to sampling was examined, no association was observed with any of the microbial concentrations measured. The HPC load did not show any measurable difference in the low vs high flow regimes either one month or one week prior to sampling (**Table 9.3**). In contrast with the total flow duration, the total number of flow counts (number of flushing events) for either one week or one month prior to sampling was not associated with any significant change in any of the microbial concentrations measured. In conclusion, a month of reduced usage (< 2 hours water flushing per month) supports the proliferation of *Legionella* spp./*L. pneumophila* and *V. vermiformis* in hospital water system.

9.4.5 Influence of water temperature and on *Legionella*, HPC, and FLA

The water outlets (hand basins and showers) of the hospital water system received water from both the cold water supply and hot water supply (Appendix-4, **Figures 15.3** and **S5**). The temperature data for each sample location were averaged over one week and one month prior to sample collection (Appendix-4, **Figures 15.3** and **15.5**). The average temperatures (mean ± SD) measured from the cold water supply were (21.78 ± 1.98°C per week and 22.01 ± 1.69°C per month), hot water supply (23.64 ± 3.15°C per week and 23.69 ± 3.08°C per month) and outlet water (23.74 ± 2.43°C per week and 23.76 ± 1.95°C per month). No relationships between microbial concentration and water temperatures were observed. This is likely due to the average water temperature being similar for both hot and cold water supplies, with increases in hot water temperature occurring through hot water usage having a limited effect on the overall average temperature due to the periods of stagnation and inactivation occurring in between usages (Appendix-4, **Figures 15.4** and **15.6**).

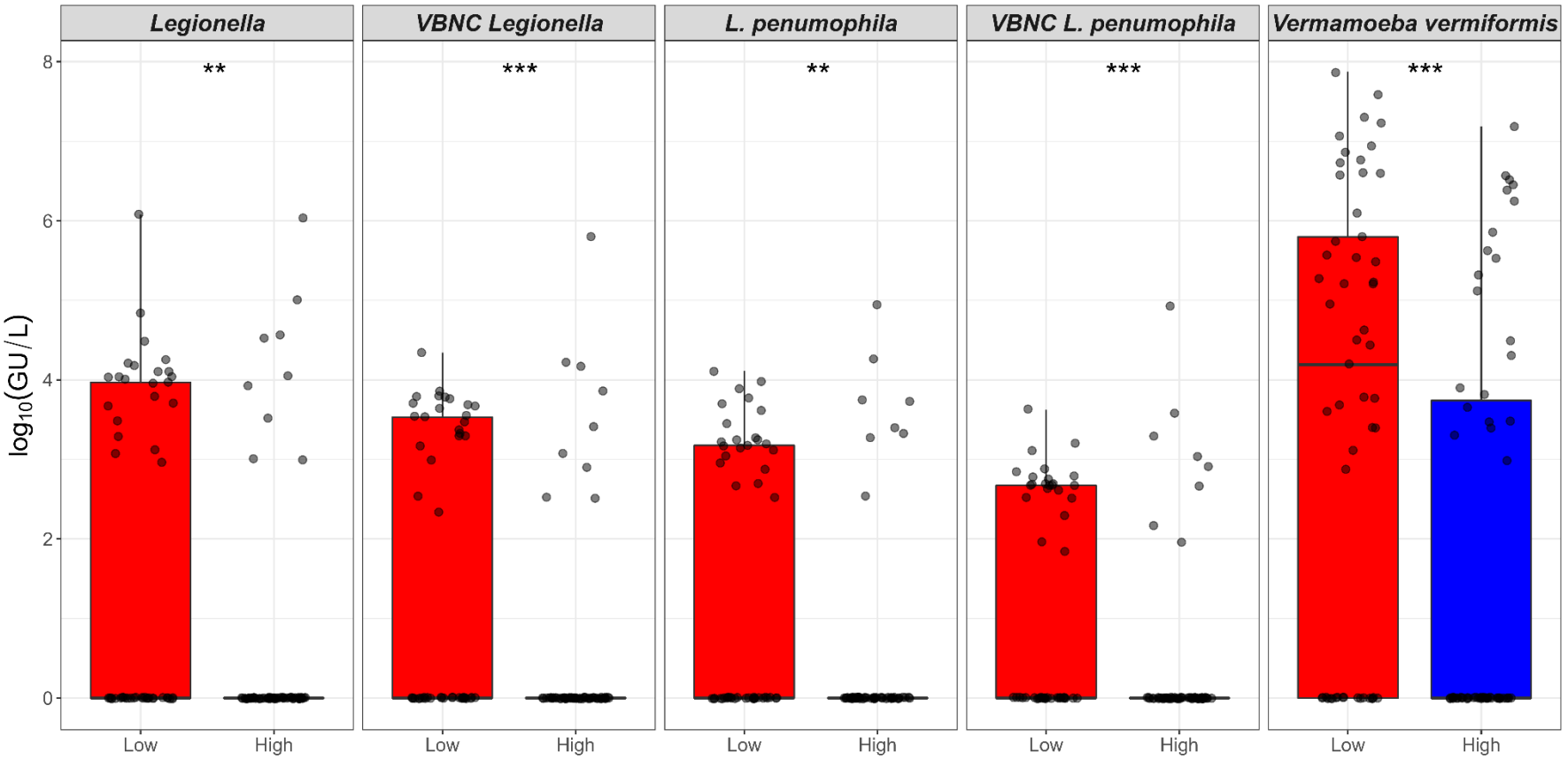

Figure 9.3: Relationship between intermittent water usage and the presence of *Legionella* spp./*Vermamoeba vermiformis*. X-axis represents total duration (hours) of flushing events recorded for one-month prior to sampling. Flushing was categorized into low; 0 to < 2 hours, and high flow regime; ≥ 2 to 40 hours. Y-axis represents $\log_{10}$(GU/L) of *Legionella* spp., VBNC *Legionella* spp., *L. pneumophila*, VBNC *L. pneumophila*, and *V. vermiformis*.

9.5 Discussion

In this study, it was identified that 31.3% (n = 21/67) hand basin water, 18.9% (n = 10/53) shower water and 10.9% (n = 5/46) biofilm samples were positive for either *Legionella* spp. or *L. pneumophila* gene marker (**Table 9.1**). According to the literature, the majority of engineered water systems of hospital and healthcare facilities are contaminated with *Legionella* spp. or *L. pneumophila*. In Poland, 74.7% of water samples from hospitals and other large building structures tested positive for *Legionella* spp., and *L. pneumophila* sg2-14 was the most prevalent serogroup (Sikora et al., 2015). A similar study conducted in Hungary that examined water samples from healthcare facilities and other buildings showed that 60% samples were positive for *Legionella* spp. (predominantly *L. pneumophila* sg2-14) (Barna et al., 2016). A study conducted in 20 different hospitals in Spain reported that 37.2% of water samples were colonized with *L. pneumophila* sg1 and *L. pneumophila* sg2-14 (Sabrià et al., 2004). In Taiwan, 63% of samples collected from hospital water systems tested positive for *Legionella* spp. and *L. pneumophila* sg1 (Yu et al., 2008). Comprehensive national surveillance studies conducted in 13 different states of the USA reported that 70% of hospital water systems were contaminated with *Legionella* spp. (Stout et al., 2007). A recent study conducted in Australia detected 41% samples of water and biofilms from hospital and residential buildings were colonized with *Legionella* spp. (Nisar et al., 2022). The lower *Legionella* spp. prevalence in this study could be due to the fact this was a case study of a single hospital that has been proactive in their water quality risk management compared with other hospitals.

All previous studies on *Legionella* spp. in engineered water systems have either used standard culturing or a qPCR assay to detect *Legionella* spp., and none have screened for the presence of VBNC *Legionella* spp. In the present study, VFC+qPCR assay showed that all water and biofilm samples positive for *Legionella* spp./*L. pneumophila* gene marker also contained VBNC cells. The quantity of total *Legionella* spp. detected by qPCR assay was greater than VBNC cells, which clearly highlights that the hospital water system harboured both dead and VBNC *Legionella* spp. (**Figure 9.1**). Our findings suggest that the standard *Legionella* spp./*L. pneumophila* guidelines should include quantification of VBNC cells.

Currently, there is still much debate around the exact infective dose for *Legionella* spp. (Bartram et al., 2007). An analysis by Sikora et al. (2015) estimated that legionellosis outbreaks may occur sporadically when water is contaminated with 10^3 to 10^5 CFU/L and when *Legionella* spp. counts exceed 10^5 CFU/L an outbreak of legionellosis can occur (Sikora et al., 2015). However, these estimates are based on the number of culturable cells (CFU/L), so it is challenging to determine the relative risk associated with the concentrations of VBNC cells (GU/L). In contrast with culturable *Legionella* spp., VBNC cells infect with lower pathogenicity

and take a longer time to infect amoebae (Nisar et al., 2023). Therefore, future research is needed to determine the infectious dose of VBNC *Legionella* spp. to understand the role of VBNC *Legionella* spp. in nosocomial infections and the public health risk posed by this concentration of VBNC *Legionella* spp. in engineered water systems.

In hospital water systems, *Legionella* infects and survives within protozoan hosts, including *Acanthamoeba* and *V. vermiformis* (Nisar et al., 2020a). In this study, it was identified that *V. vermiformis* (gene marker and culturable) was the most commonly identified amoebae associated with *Legionella* spp. prevalent in the water and biofilm samples (**Table 9.4**). This is supported by previous studies that have demonstrated *V. vermiformis* to be widely present in potable water (Kuiper et al., 2006, Nisar et al., 2020a). Similarly, microbiome analysis of potable water also showed *V. vermiformis* as the most prevalent protozoa (Delafont et al., 2016, Delafont et al., 2013). Water samples of dental units of Italian hospitals were found to be highly contaminated with *V. vermiformis* (60%) (Spagnolo et al., 2019). Similarly, a study conducted in hospitals of South Africa identified that 69% of samples were positive for *V. vermiformis* and 30.6% for *Acanthamoeba* (Muchesa et al., 2018). A recent study conducted in Australia examined water and biofilm samples from hospital and residential buildings showed the presence of FLA in 69% of the samples. It was also found that in all tested samples, *V. vermiformis* (55%) was the more frequently detected FLA (Nisar et al., 2022). In comparison with *Acanthamoeba*, *V. vermiformis* is more sensitive to disinfection treatments (Nisar et al., 2020a). Therefore, high levels of *V. vermiformis* could be attributed to decay and lower levels of residual chemical disinfectants in the hospital water system.

To our knowledge HPC loads have not been linked to any known legionellosis outbreak and the relationship between HPC levels and opportunistic premise plumbing pathogens is still unclear (Bartram et al., 2003). The SA Health guidelines (2013) use HPC load as an indicator of water quality and recommend that if HPC load is ≥ 10^2 CFU/mL in warm water systems then the disinfection procedures for engineered water system should be considered. In this study it was found that the water samples with high HPC loads (≥ 5 × 10^3 CFU/L) contained high quantities of both *Legionella* spp. and *L. pneumophila* (**Figure 9.2** and **Table 9.3**). These results are in accordance with a previous study conducted on engineered water systems of residential buildings, which also showed a positive relationship between HPC levels and concentrations of *Legionella* spp. (Ley et al., 2020). Similarly, it was also found that samples with high HPC loads harbored high levels of *V. vermiformis* (**Figure 9.2** and **Table 9.3**). The relationship between bacteria and FLA consists of three major types of interactions i.e., mutualism, parasitism, and predation (Shi et al., 2021). Generally, FLA are considered natural predators of bacteria, which could account for the high levels of *V. vermiformis* observed in the

presence of high levels of HPC (Rodriguez-Zaragoza et al., 2005). It was also identified that all samples positive for *Legionella* spp./*L. pneumophila* were also positive for FLA. Furthermore, Spearman's analysis demonstrated a strong positive correlation between *Legionella* spp./*L. pneumophila* and *V. vermiformis*. In engineered water systems, FLA exist in both trophozoite (metabolically active) and cyst (dormant) states (Zhang and Lu, 2021). The trophozoites support intracellular proliferation of *Legionella* spp. and transformation of VBNC *Legionella* spp. into a culturable state (Shadrach et al., 2005, Solomon et al., 2000, Watanabe et al., 2016, Berk et al., 2008, Boamah et al., 2017). Amoebae cysts protect intracellular *Legionella* spp. from prolonged chemical and physical disinfection treatments (Boamah et al., 2017, Dobrowsky et al., 2016). The significant role amoebae play in *Legionella* spp. survival in potable water systems suggests that guidelines for the control of *Legionella* spp. must consider acceptable limits of amoeba within these systems as a measure to control *Legionella* spp. concentrations.

Water stagnation within building distribution systems promotes the accumulation of biomass, decay of chemical disinfectants, and alters the water quality (Bedard et al., 2018). Therefore, this study investigated the effect of temporary stagnation induced by intermittent flushing and water usage on *Legionella* spp.*,* with a special focus on VBNC *Legionella* spp. It was found that an increase in temporary stagnation once a month prior to sampling significantly ($p < 0.01$) increased the quantity of total *Legionella* spp./*L. pneumophila* and VBNC *Legionella* spp./*L. pneumophila* population; however, increased stagnation one week prior to sampling was not associated with increased risk (**Figure 9.3** and **Table 9.3**). This supports guidelines that recommend routine flushing of outlets to manage *Legionella* spp. within engineered water systems (enHealth, 2015). To our knowledge, this is first study in which the effect of temporary stagnation, HPC load, and *V. vermiformis* on VBNC *Legionella* spp./*L. pneumophila* in hospital water systems has been investigated. This study averaged water temperatures across one week or one month prior to sampling for both the hot and cold water pipelines/outlets. As a result, water temperatures were more similar to each other than anticipated. This is likely to explain the lack of a statistically significant difference in *Legionella* concentrations associated with different temperatures. Future research with a larger dataset is needed to explore the temperature relationship further.

9.6 Conclusion

In building plumbing systems, temporary stagnation arising through intermittent usage causes water quality to deteriorate. This study identified that temporary stagnation for over a month (< 2 hours of usage per month) promotes the persistence of VBNC *Legionella* spp./*L. pneumophila*. Similarly, FLA and heterotrophic bacteria present in this temporary stagnant

environment positively interact with *Legionella* spp./*L. pneumophila*. Therefore, temporary stagnation, FLA and heterotrophic bacteria must be managed for the proper control and prevention of LD. This study also showed that the standard microbiological culture method used to detection *Legionella* spp. returned a false negative result for 88% of the VBNC *Legionella* spp. positive samples. As all samples positive for VBNC *Legionella* spp. were also qPCR positive, this suggests that qPCR may be a more appropriate detection method for routine surveillance. However, future research is needed to investigate the concentrations of VBNC *Legionella* spp. that pose a risk to public health to enable interpretation of these results to inform improved *Legionella* spp. guidelines.

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Authors contributions

MAN and HW conceived and designed research. MAN performed the experiments. GB assisted in the flow cytometry. KER, MHB, HW and RB provided technical assistance. JX and JH assisted in sampling and data collection. MAN and HW drafted and edited the manuscript, HW, KER, MHB, RB, GB, JX, and JH corrected and contributed to the manuscript. All authors approved of the final manuscript.

Conflicts of interest

Author Jason Hinds and James Xi are employed by Enware Pty Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Hospital water microbiome and flow dynamics

Microbes present in engineered water systems play a key role in the survival and persistence of opportunistic premise plumbing pathogens, including *Legionella*. This chapter addresses objective 9 and consists of an unpublished manuscript. In this chapter the role of water temperature, seasoning, flow dynamics and water stagnation on the planktonic prokaryotic microbial communities of hospital water system was investigated.

10 The composition of planktonic prokaryotic communities in a hospital building water system depends on both incoming water and flow dynamics

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This article is an unpublished manuscript.

Keywords: Engineered water system, potable water, flow dynamics, water stagnation, opportunistic premise plumbing pathogens

10.1 Abstract

In recent years, the frequency of nosocomial infections has increased. Hospital water systems support the growth of microbes, especially opportunistic premise plumbing pathogens. In this study, planktonic prokaryotic communities present in water samples taken from hospital showers and hand basins, collected over three different sampling phases, were characterized by 16S rRNA gene amplicon sequencing. Significant differences in the abundance of various prokaryotic taxa were found through univariate and multivariate analysis. Overall, the prokaryotic communities of hospital water were taxonomically diverse and dominated by biofilm forming, corrosion causing, and potentially pathogenic bacteria. The phyla Proteobacteria, Actinobacteriota, Bacteroidota, Planctomycetota, Firmicutes, and Cyanobacteria made up 96% of the relative abundance. The α-diversity measurements of prokaryotic communities showed no difference in taxa evenness and richness based on sampling sites (shower or hand basins), sampling phases (months), and presence or absence of *Vermamoeba vermiformis*. However, β-diversity measurements showed significant clustering of prokaryotic communities based on sampling phases, with the greatest difference observed between the samples collected in phase 1 vs phase 2/3. Importantly, significant difference was observed in prokaryotic communities based on flow dynamics of the incoming water. The Pielou's evenness diversity index revealed a significant difference (Kruskal Wallis, $p < 0.05$) and showed higher species richness in low flow regime (< 13 minutes water flushing per week and ≤ 765 flushing events per six months). Similarly, Bray-Curtis dissimilarity index found significant differences (PERMANOVA, $p < 0.05$) in the prokaryotic communities of low vs medium/high flow regimes. Furthermore, linear discriminant analysis effect size showed that several biofilm forming (e.g., Pseudomonadales), corrosion causing (e.g., Desulfobacterales), extremely environmental stress resistant (e.g., Deinococcales), and potentially pathogenic (e.g., *Pseudomonas*) bacterial taxa were in higher amounts under low flow regime conditions. This study demonstrated that a hospital building water system consists of a complex microbiome that is shaped by incoming water quality and the building flow dynamics arising through usage.

10.2 Introduction

Hospital water systems are a significant but often overlooked source of nosocomial infections (Anaissie et al., 2002, Hayward et al., 2020). Potable water supplied to healthcare facilities typically has undergone chemical or physical disinfection. However, despite these disinfection procedures, building plumbing systems are colonized by diverse prokaryotic and eukaryotic communities (Falkinham III et al., 2015b, Ji et al., 2015). Plumbing pipes, shower heads, tap faucets, and aerators are reservoirs of opportunistic premise plumbing pathogens (OPPPs),

especially *Acinetobacter spp.*, *Legionella spp.*, Non-Tuberculous *Mycobacterium*, and *Pseudomonas spp.* (Proctor et al., 2018, Proctor et al., 2016). Generally, planktonic microbes enter in the hospital water system from the municipal water system (Liu et al., 2016, Whiley et al., 2014) and can form or become part of pre-existing biofilms. These biofilms protect microbes from disinfection treatments and other environmental stresses (Hayward et al., 2022). Premise plumbing consists of pipes with a high surface-to-volume ratio, in which decay of chemical disinfectant is higher, an important factor in stimulating growth of microbes (Falkinham III et al., 2015b). Many abiotic factors such as water temperature, disinfection treatment, plumbing material, age of plumbing system, and flow dynamics, impact growth and survival of microbes growing in these biofilms (Chan et al., 2019, De Sotto et al., 2020, Lee et al., 2021). The detachment of biofilms plays a key role in contaminating circulating water and further spreading, colonizing and transmission of OPPPs (Vartoukian et al., 2010, Zhang et al., 2018). Standard microbiological culturing methods are unable to fully identify and characterize prokaryotic communities present in water samples (Wang et al., 2017). Specifically, these methods cannot detect fastidious and viable but nonculturable (VBNC) bacteria (Ramamurthy et al., 2014, Vartoukian et al., 2010). Therefore, in this study, amplicon sequencing of the16S rDNA gene, a culture-independent method, was used for the characterization of the prokaryotic communities of a hospital water system. Several studies have examined microbial communities found within plumbing biofilms; however, less is known about the planktonic prokaryotic communities present in the building water distribution systems (Chan et al., 2019, De Sotto et al., 2020, Lee et al., 2021) and their link to plumbing biofilms. Furthermore, hospital water distribution systems are extremely dynamic with fluctuating and complex environmental variables arising through complex design. These variables include different types of outlets, intermitted use, changes in incoming water quality, temperature fluctuations, and different disinfection strategies. Little is known about the role of these environmental variables in driving the diversity of these microbial communities.

The aims of this study were: (1) to characterize planktonic prokaryotic communities of hospital water systems; and (2) to develop a better understanding of the biotic (amoebae host) and abiotic (sampling site, phases, water dynamics and temperature) factors that drive the alterations in the structure and composition of prokaryotic communities. To achieve these aims, the composition of prokaryotic communities of hospital water collected from showers and hand basins was characterized by 16S rRNA gene amplicon sequencing, followed by diversity and statistical analyses to investigate relationships among prokaryotic communities and various physical and biological parameters.

10.3 Materials & Methods

10.3.1 Sample collection and processing

A total of 70 water samples were collected from hand basins and showers from an Australian hospital situated in New South Wales. Samples were collected during three sampling rounds in March, April, and November 2021. The samples were collected and transported in accordance with standard guidelines (Centers for Disease Control and Prevention, 2019, International Organization for Standardization, 2018). Briefly, 1 L of first flush water samples from shower and hand basins were collected in sterile screw capped wide-mouth plastic bottles (2105-0032, Nalgene™). To quench pre-existing chlorine-based disinfectants, 0.5 mL 0.1 N $Na_2S_2O_3$ (124270010, ACROS Organics™) was added. All samples were transported and stored at 5 ± 2°C and processed within three days. Collected samples were concentrated by vacuum filtration onto a 47 mm diameter 0.22 µm polycarbonate membrane (GTTP04700, Isopore™) and filtered residues were resuspended in 3 mL sterile distilled water. This sample suspension was used for microbiological and molecular analyses.

10.3.2 Water flow and temperature data

Water flow and temperature was measured at the basins and thermostatic mixing values (TMVs, for showers) using Enware's Smart Flow® TMV monitoring system (Whiley et al., 2019). For flow dynamics, both the number (counts) and duration (minutes) of flushing events for a period of one week and six months prior to sampling, were studied. Both parameters were divided into three different flow regimes, namely low, medium, and high. The duration (minutes) of flushing events for one week prior to sampling was categorised as: low flow regime, 0 to < 13 minutes/week; medium flow regime, ≥ 13 to < 30 minutes/week; and high flow regime, ≥ 30 to 85 minutes/week. For the number (counts) of flushing events for the six months prior to sampling the categorisation was: low flow regime, 0 to 765 counts/six months; medium flow regime, 800 to 1,185 counts/six months; and high flow regime, 1,200 to 2,500 counts/six months. The temperature of water at hot and cold-water supply outlets for one week and six months prior to sampling was recorded.

10.3.3 Isolation and characterization of amoebae

For the isolation of amoebae, 0.1 mL of filtrate was cultured on heat-inactivated (57°C for 45 minutes) *Escherichia coli* American Type Culture Collection (ATCC®) 700891™ supplemented 1.5% non-nutrient agar (CM0003, Oxoid Ltd) plates. The plates were incubated under aerobic conditions at 25°C for two weeks. Monoxenic amoebae cultures were characterized by microscopy and sequencing of 18S rDNA gene (Nisar et al., 2022).

10.3.4 DNA extraction and 16S rRNA gene sequencing

DNA extraction was performed using the Aquadien™ kit (3578121, Bio-Rad Laboratories Ltd.) following the manufacturer's instructions with the addition of a pre-treatment of 10 µL of 25 mg/mL lysozyme (L6876, Sigma-Aldrich®) that was added to the sample suspension and incubated for 15 minutes at 37°C. The extracted DNA was subjected to polymerase chain reaction (PCR) with a target amplicon size of 391 base pairs (bp). The bacteria and archaea specific forward oligo 515F (5´-GTGYCAGCMGCCGCGGTAA-3´) and reverse oligo 806R (5´-GGACTACNVGGGTWTCTAAT-3´) targeting the V4 region of 16S rRNA gene was used (Apprill et al., 2015, Parada et al., 2016, Earth Microbiome Project). Nextera adapter sequences (specific to the sequencing platform) were also contained on the primer pairs. The PCR consisted of; 26.5 µL Milli-Q® water, 10 µL 5X Q5® reaction buffer (B9027S, New England Biolabs®), 1 µL 10 mM dNTP mix (U1518, Promega®), 0.5 µL Q5® hot start high-fidelity DNA polymerase (M0493L, New England Biolabs®), 1 µL of each oligo and 10 µL of 10 ng template DNA. Thermal cycler conditions used for amplification were: one cycle of initial melting at 98°C for 60 s; followed by 30 cycles of melting at 98°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s; then one cycle of final extension at 72°C for 10 minutes. Finally, 10 µL of each amplified product was run on a GelRed® (41003, Biotium) stained 2% agarose gel and the molecular mass was estimated using 100 bp DNA ladders (G210A, Promega®). The amplicons were dispatched to Australian Genome Research Facility (AGRF Ltd, Australia) for barcode indexing and 2 x 250 paired end sequencing using the Illumina MiSeq™ System.

10.3.5 Bioinformatic analysis

The data were analysed using Mothur (version 1.48.0) according to the standard protocol (Kozich et al., 2013). Briefly, the raw sequencing (FastQ format) files were quality filtered and, after trimming, a maximum 275 bp region was selected. The sequences with homopolymers longer than eight base pairs and greater than two mismatches were removed. The selected trimmed regions (V4 region of bacterial 16S rRNA gene) were aligned with SILVA database (version 138.1) to generate the count tables (Quast et al., 2013). The VSearch (version 2.21.1) was used for the removal of any chimeric sequences (Rognes et al., 2016). Finally, using OptiClust algorithm, the sequences were grouped into operational taxonomic units (OTU) based on 0.03 distance limit (equivalent to 97% similarity) (Westcott and Schloss, 2017).

10.3.6 Ecological and statistical analysis

The prokaryote abundance table was Log(x+1) scale transformed then α-diversity and β-diversity indices were analysed using the PRIMER v7 with PERMANOVA+ software (Plymouth Routines in Multivariate Ecology Research, United Kingdom) (Clarke and Gorley, 2015, Gorley et al., 2015). The core microbiome, linear discriminant analysis effect size, and interactive pie

charts of comparative abundance profiles were generated using MicrobiomeAnalyst, an online tool based on multiple R packages including matR, phyloseq, VAMPS and vegan (Dhariwal et al., 2017, Chong et al., 2020). For univariate statistical analysis, the data were transformed into total sum scaling (TSS) format. The normality of data was tested by the Shapiro-Wilk test. The *p* values less than 0.05 were considered statistically significant and represented as follows: *** $p < 0.001$; ** $p < 0.01$; $*$ $p < 0.05$; and ns non-significant. The parametric and non-parametric statistical analyses were performed using the R program (version 4.2.2), and graphically presented by using the "ggplot2 (version 3.3.6)" package (Wickham, 2016).

10.3.6.1 Alpha (α) diversity analysis

The α-diversity (intra-sample variances) was measured as richness (number of microbial taxa) and evenness (abundance of those microbial taxa) of each sample. The Pielou's evenness, Simpson and Shannon diversity indices were estimated for the calculation of α-diversity in the selected environmental parameters, i.e., sampling sites (hand basins and showers), phases (seasons), presence or absence of amoebae, number and duration of water flushing events, and water temperature. A non-parametric method, the Kruskal-Wallis test, was used to determine if the estimated diversity index was significantly different in the selected environmental parameters.

10.3.6.2 Beta (β) diversity analysis

The β-diversity (inter-sample variances) was measured as variations in prokaryotic community composition (identity of microbial taxa) among samples within an ecological habitat. The purpose of considering differences among selected environmental parameters was to identify any predictable difference(s) in composition of prokaryotic community. The environmental parameters considered for this analysis were sampling sites (hand basins and showers), phases (seasons), presence or absence of amoebae, number and duration of water flushing events and water temperature. To determine the differences in the composition of the prokaryotic communities, the Bray-Curtis dissimilarity index (resemblance measure) was used. The obtained non-Euclidean matrices were visualized using Principal Coordinates Analyses (PCoA) and Canonical Analysis of Principal coordinates (CAP). The statistical significance of differences in composition of prokaryotic community was determined by a non-parametric method Permutational Multivariate Analysis of Variance (PERMANOVA). The similarity percentage (SIMPER) analysis (cut off for low contributions 70%) was used to examine contribution of individual taxa to the variations in the composition of prokaryotic communities for each environmental parameter. The results retrieved from SIMPER analysis were used to demonstrate the dissimilarities in the composition of prokaryotic communities on either PCoA or CAP plots.

10.3.6.3 Univariate statistical analysis

The abundance table data was transformed into TSS format followed by univariate statistical analysis. To test a significant difference between means of selected parameters, a non-parametric Kruskal-Wallis test was applied. For graphical presentation the values are presented in logarithmic (log_{10}) scale.

10.3.6.4 Co-occurrence correlation analysis

Co-occurrence of different bacterial genera (especially potentially pathogenic and corrosive) was identified by performing non-parametric Spearman's correlation analysis (ρ) to a significance threshold of $p < 0.05$. The correlation matrices were plotted by using "ggcorplot (version 0.1.4)" package (Kassambara, 2022).

10.4 Results

10.4.1 Taxonomic composition of prokaryotic communities

Of the 70 water samples, 46 (22 from hand basins and 24 shower water samples) were able to be successfully 16S rRNA amplified and sequenced. After bioinformatic processing, a total of 20,743 archaeal and bacterial OTUs were identified to genus level. All samples were dominated with bacterial taxa (666 archaeal and 20,077 bacterial OTU). The hospital water system consisted of very complex prokaryotic communities comprising of up to 57 bacterial (99.89%) and eight archaeal (0.11%) phyla. The Proteobacteria (79.16%) was the most prevalent phylum in all samples and mainly comprised of two classes α-Proteobacteria (41.06%) and γ-Proteobacteria (38.09%). The phylum Actinobacteriota (6.88%) was the second largest prokaryotic group in the samples and mainly consisted of Actinobacteria (4.53%) and Rubrobacteria (1.97%) classes. The other phyla that accounted for more than 1% relative abundance of the total population were: Bacteroidota (4.23%), Planctomycetota (2.86%), Firmicutes (2.03%) and Cyanobacteria (1.42%) (Appendix-5, **Figure 16.1**). At the family level, the dominant bacterial families across all samples were: Comamonadaceae (22.18%), Sphingomonadaceae (12.63%), Beijerinckiaceae (12.49%), Cycloclasticaceae (4.98%) and Xanthobacteraceae (4.22%) (**Figure 10.1**). Data analysis at genus level demonstrated that many sequences were not classified at that level and the lowest taxonomic classification was attained at family level or in some cases even at order level. Importantly, the most abundant genera identified in all samples belonged to the unclassified genus of family Comamonadaceae. Overall, the most abundant genera present in the samples were: Comamonadaceae genus (20.3%), *Methylobacterium-Methylorubrum* genus (9.5%), *Cycloclasticus* (4.98%), *Bradyrhizobium* (3.83%), and *Reyranella* (3.59%) (Appendix-5, **Figure 16.2**). The genera considered to be the essential (core) part of hospital water were

Bradyrhizobium, Comamonadaceae genus, *Methylobacterium-Methylorubrum*, *Reyranella*. *Sphingomonas*, Sphingomonadaceae geuns, Rhodobacteraceae genus, and *Cycloclasticus*.

10.4.2 Potentially pathogenic bacteria

According to the available literature, more than 25 different potentially pathogenic bacterial genera have been found in potable water (Pereira et al., 2017). Analysis found the presence of potentially pathogenic bacterial genera namely, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, *Bosea*, *Campylobacter*, *Coxiella*, *Helicobacter*, *Legionella*, *Methylobacterium-Methylorubrum*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, *Vibrio* and *Yersinia*. The relative abundance of each genus was very diverse, ranging from 0.001% for *Campylobacter* to 50% for *Methylobacterium-Methylorubrum* (Appendix-5, **Figure 16.3**). Co-occurrence of these potentially pathogenic bacterial genera was studied using Spearman's correlation analysis at $p < 0.05$. *Legionella* showed a positive correlation with *Coxiella*, *Staphylococcus*, *Streptococcus*, *Vibrio*, and *Yersinia* ($\rho = 0.581, 0.505, 0.656, 0.575$ and 0.542, respectively). *Pseudomonas* showed a positive correlation with *Acinetobacter*, *Coxiella*, *Staphylococcus*, *Streptococcus* and *Yersinia* ($\rho = 0.530, 0.506, 0.501, 0.584$ and 0.511 respectively). *Streptococcus* showed a positive correlation with *Acinetobacter*, *Coxiella*, *Staphylococcus*, *Vibrio* and *Yersinia* ($\rho = 0.539, 0.884, 0.694, 0.876$ and 0.671, respectively). The positive correlation shows that these pathogenic bacteria co-occur in hospital water systems, and environmental conditions that support the presence of one pathogen may support multiple pathogens (Appendix-5, **Figure 16.4**).

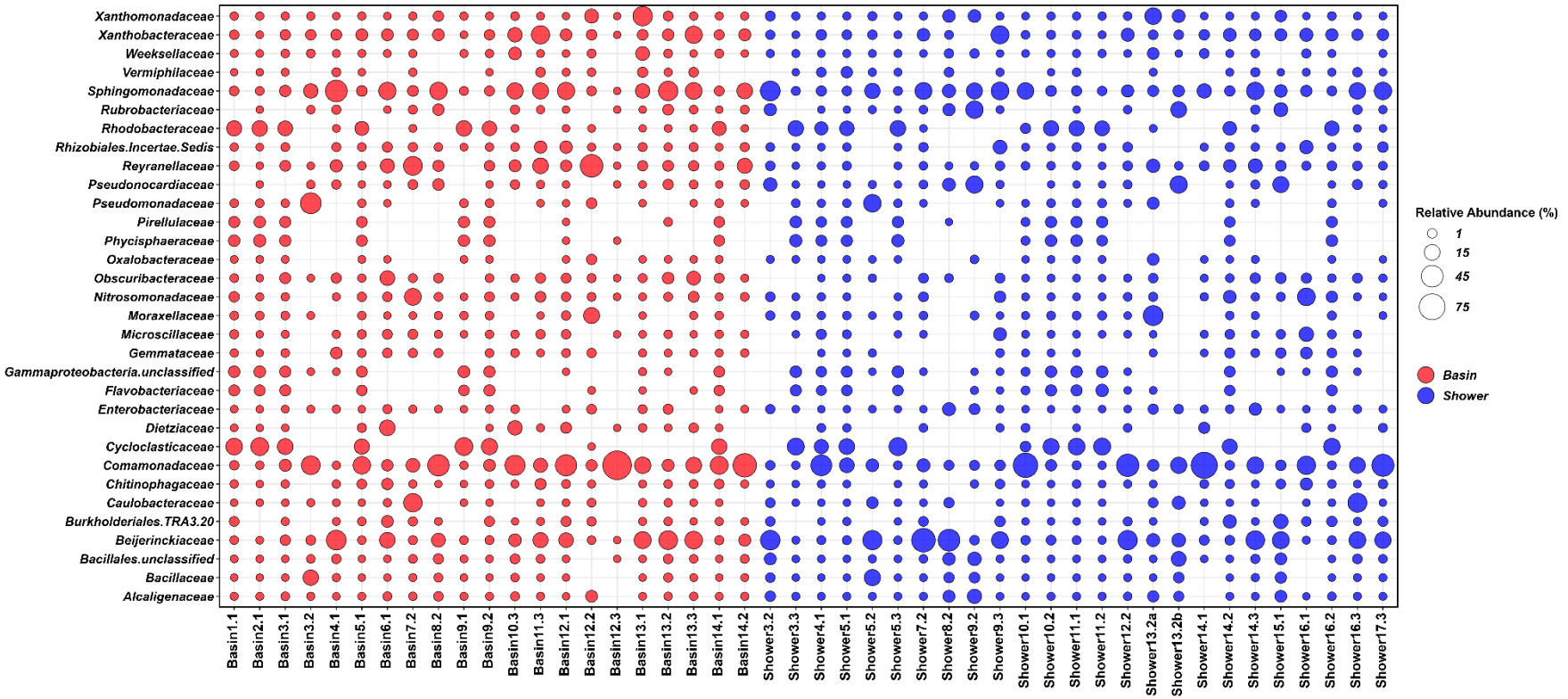

Figure 10.1: Abundant bacterial families (n = 32) with relative abundance ≥ 3% present in hospital hand basins (red coloured) and shower (blue coloured) water samples. The spot size represents % relative abundance of bacterial families (Y-axis) in the samples (X-axis).

10.4.3 Potentially corrosive bacteria

Fourteen potentially corrosive bacterial genera, each with abundance ranging from 0.001% for *Desulfomicrobium* to 0.085% for *Sulfurovum*, were identified in the samples (Appendix-5, **Figure 16.5**). The corrosive bacteria were identified as sulfate-reducing bacteria (*Desulfovibrio*), sulfur-reducing bacteria (*Desulfobacter*, *Desulfobacterium*, *Desulfobulbus*, *Desulfomicrobium*, *Desulfomonile*, *Desulfovermiculus*, *Desulfovibrio*, *Desulfuromonas*, *Desulfuromusa*, *Sulfurimonas*, and *Sulfurovum*), sulphur-oxidizing bacteria (*Alicyclobacillus*), and iron-oxidizing bacteria (*Mariprofundus* and *Pedomicrobium*) (Loto, 2017, Sun et al., 2014a). Spearman's analysis ($p<0.05$) showed that abundance of *Sulfurovum* was positively correlated with *Desulfovermiculus*, *Sulfurimonas*, *Desulfobacter*, *Desulfobacterium*, *Desulfovibrio*, *Desulfuromusa* and *Desulfomicrobium* (ρ = 0.983, 0.973, 0.713, 0.674, 0.519 and 0.511, respectively). Similarly, *Sulfurimonas* positively correlated ($p < 0.05$) with *Desulfovermiculus*, *Desulfobacter*, *Desulfovibrio*, *Desulfobacterium*, *Desulfomicrobium* and *Desulfuromusa* (ρ = 0.980, 0.688, 0.616, 0.589, 0.510 and 0.508, respectively). Likewise, *Desulfovermiculus* demonstrated positive correlation with *Desulfobacter* (ρ = 0.671) and *Desulfobacterium* (ρ = 0.620). Similarly, *Desulfovibrio* revealed positive correlation with *Desulfobacter* (ρ = 0.549) and *Desulfovermiculus* (ρ = 0.600). *Desulfuromusa* showed positive correlation with *Desulfovermiculus* (ρ = 0.533). These results showed that various genera of sulfur-reducing bacteria co-occur in the hospital water system (Appendix-5, **Figure 16.6**). The presence of these bacteria result in corrosion, damage of plumbing systems and support the formation of biofilms and deterioration of water quality.

10.4.4 Presence of environmental amoeba

Of the 46 samples, 21 were positive for amoeba using culture detection. Microscopic examination found the presence of locomotive forms with a monotactic morphotype (hand basins = 11 and shower = 10). Sequence analysis of 18S rRNA gene characterized all isolates as of *Vermamoeba vermiformis*.

10.4.5 V. vermiformis and prokaryotic communities

Examining the relationship between *V. vermiformis* and the prokaryotic communities showed that there was no difference in Pielou's evenness diversity index between *V. vermiformis* positive and negative water samples. Similarly, β-diversity did not differ between the *V. vermiformis* positive and negative samples. In the available literature, six different bacterial phyla are considered as part of the amoebae microbiomes (Sallinger et al., 2021). Data analysis found that out of these six phyla, five were present in both hand basins and shower water samples, namely Proteobacteria (79.16%), Actinobacteriota (6.88%), Bacteroidetes (4.23%), Firmicutes (2.03%) and Acidobacteria (0.18%). Bacterial genera notably

Acinetobacter, *Aeromonas*, *Bacillus*, *Campylobacter*, *Coxiella*, *Delftia*, *Francisella*, *Helicobacter*, *Legionella*, *Methylobacterium-Methylorubrum*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, *Vibrio*, and *Yersinia* which interact with amoebae host in natural environment (Thomas et al., 2010, Sallinger et al., 2021), were present in both hand basins and shower water samples. Importantly, genera like *Aeromonas*, *Legionella*, *Mycobacterium*, and *Stenotrophomonas* which replicate intracellularly in *V. vermiformis* were present in the samples (Pagnier et al., 2015, Cabello-Vilchez et al., 2014, Nisar et al., 2022, Cateau et al., 2014, Delafont et al., 2019, Nisar et al., 2020a). Furthermore, univariate analysis demonstrated that abundance of *Hyphomicrobium*, *Sphingobium*, Acidobacteriae, Beijerinckiaceae-28-YEA-48, Comamonadaceae, and Enterobacterales genera was significantly higher in *V. vermiformis* positive samples (Appendix-5, **Figure 16.7**).

10.4.6 Sampling sites (outlets) and prokaryotic communities

There were no statically significant differences in the α-diversity indices calculated between shower and hand basins water. Similarly, Bray-Curtis dissimilarity index based PCoA plots revealed no statically significant predictable clustering on the basis of sampling site (hand basins or shower). Despite both diversity indices demonstrating that the composition of the prokaryotic communites present in hand basins and shower water were similar, some variations were found when comparative abundance profiles were examined at the genus level. The interactive pie charts demonstrated that eight genera, i.e., *Blastomonas*, *Bradyrhizobium*, Comamonadaceae genus, *Dietzia*, *Novosphingobium*, Obscuribacteraceae genus, *Reyranella*, *Sphingomonadaceae* genus and *Sphingomonas*, accounted for 46.45% in hand basins water, but only accounted for 24.34% of shower water. Eleven different genera, i.e., Alcaligenaceae, Bacillales, *Bosea*, Burkholderiales-TRA3-20, *Cycloclasticus*, Gammaproteobacteria, *Phreatobacter*, Phycisphaeraceae-CL500-3, *Prauserella*, Rhodobacteraceae, and *Rubrobacter* accounted for 26.17% in shower water, but only accounted for 9.83% in hand basin water (**Figure 10.2**). Furthermore, univariate analysis showed that relative abundance of Comamonadaceae genus, *Mycobacterium*, *Novosphingobium*, *Reyranella*, *Sphingomonas*, and Xanthobacteraceae genus was significantly higher in hand basins water compared to shower water (Appendix-5, **Figure 16.8**). Together, these results suggest subtle differences in structure of prokaryotic communities, primarily in the relative abundance of certain genera, present in hand basins water compared with shower water.

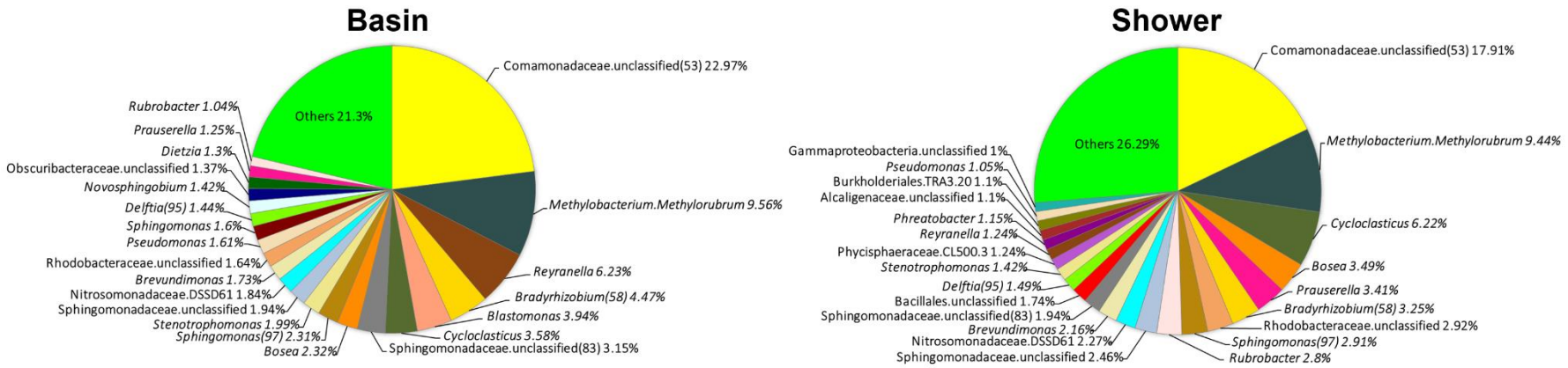

Figure 10.2: Interactive pie charts depicting the relative abundance (%) of bacterial genera based on two different sampling sites.

10.4.7 Sampling phases and prokaryotic communities

The water samples were collected at three sampling times, March 2021 (phase 1), April 2021 (phase 2) and November 2021 (phase 3). The α-diversity indices based on three different sampling phases did not show any significant difference in the prokaryotic communities. However, the results of Bray-Curtis dissimilarity index based PCoA and CAP plots generated predictable clustering based on the sampling phases (PERMANOVA: $p < 0.07$, $R^2 = 0.07$). At genus and family levels, the prokaryotic communities of phase 1 and phase 2 were dissimilar and clustered in distinct clusters (PERMANOVA: $p < 0.05$). Similarly, prokaryotic communities of phase 1 and phase 3 seem dissimilar, although the dissimilarity was less pronounced (PERMANOVA: at genus level $p = 0.07$ and at family level $p = 0.09$). However, phase 2 and phase 3 shared an average similarity of 30.22% and 40.36% at genus and family levels, respectively. SIMPER analysis was used for ranking of each taxon according to their contribution to intragroup differences in the composition of prokaryotic communities. Comparisons between the phase 1 with both phase 2 and 3 showed 11 different genera including Bacillales genus, Burkholderiales-TRA3-20 genus and Comamonadaceae genus cumulatively accounted for ≈ 7% of the variance (Appendix-5, **Figure 16.9**). Similarly, at family level comparisons between the phase 1 with both phase 2 and 3 showed 12 different families cumulatively accounted for ≈ 10% of the dissimilarity (**Figure 10.3**). Furthermore, the interactive pie plots demonstrated that abundance of Alcaligenaceae genus, Bacillales genus, *Blastomonas*, *Bosea*, *Bradyrhizobium*, *Brevundimonas*, Burkholderiales-TRA3-20 genus, Comamonadaceae genus, *Cycloclasticus*, *Delftia*, *Dietzia*, Gammaproteobacteria genus, *Methylobacterium-Methylorubrum*, Nitrosomonadaceae-DSSD61 genus, *Novosphingobium*, Obscuribacteraceae genus, *Phreatobacter*, Phycisphaeraceae-CL500-3 genus, *Prauserella*, *Pseudomonas*, *Reyranella*, Rhodobacteraceae genus, *Rubrobacter*, Sphingomonadaceae genus, *Sphingomonas*, and *Stenotrophomonas* genera differed based on the sampling phases (**Figure 10.4**). In phase 1, *Blastomonas*, Burkholderiales-TRA3-20, *Cycloclasticus*, Gammaproteobacteria, Phycisphaeraceae-CL500-3, and Rhodobacteraceae genera accounted for 17.7% relative abundance, compared with only 5.79% in phase 2 and 6.14% in phase 3. Whereas in phase 1 *Brevundimonas*, *Methylobacterium-Methylorubrum*, and *Sphingomonas* genera accounted for only 7.26%, compared to 18.3% for phase 2 and 16.55% for phase 3. On the family level, the phase 1 samples were enriched with Burkholderiales-TRA3-20, Chitinophagaceae, Cycloclasticaceae, Gammaproteobacteria, Microscillaceae, Phycisphaeraceae, Pirellulaceae, and Rhodobacteraceae families and accounted for 18.73%, compared with only 6.57% of phase 2 and 6.9% of phase 3. In the phase 2 and 3 samples, Beijerinckiaceae, Reyranellaceae, and Caulobacteraceae families exceeded 20% relative abundance compared with only 10% of phase 1 (Appendix-5, **Figure 16.10**). Moreover, univariate analysis identified that abundance of 33 different genus varied significantly based

on sampling phases (Appendix-5, **Figure 16.11**). All these results demonstrated difference in composition of prokaryotic communities based on sampling phases. The average climatic temperature for the sampling periods was provided by the Bureau of Meteorology: phase 1, T_{max} mean = 22.9°C, T_{min} mean = 17.3°C; phase 2, T_{max} mean = 22.7°C, T_{min} mean = 15.0°C; and phase 3, T_{max} mean = 20.6°C, T_{min} mean = 15.4°C. Whereas the rainfall (monthly total): phase 1 = 118.6 mm; phase 2 = 12.6 mm; and phase 3 = 112 mm. Changes observed between phases are likely due to changes in incoming water usage or to disinfection protocols, climatic conditions did not seem to have any significant impact on these results.

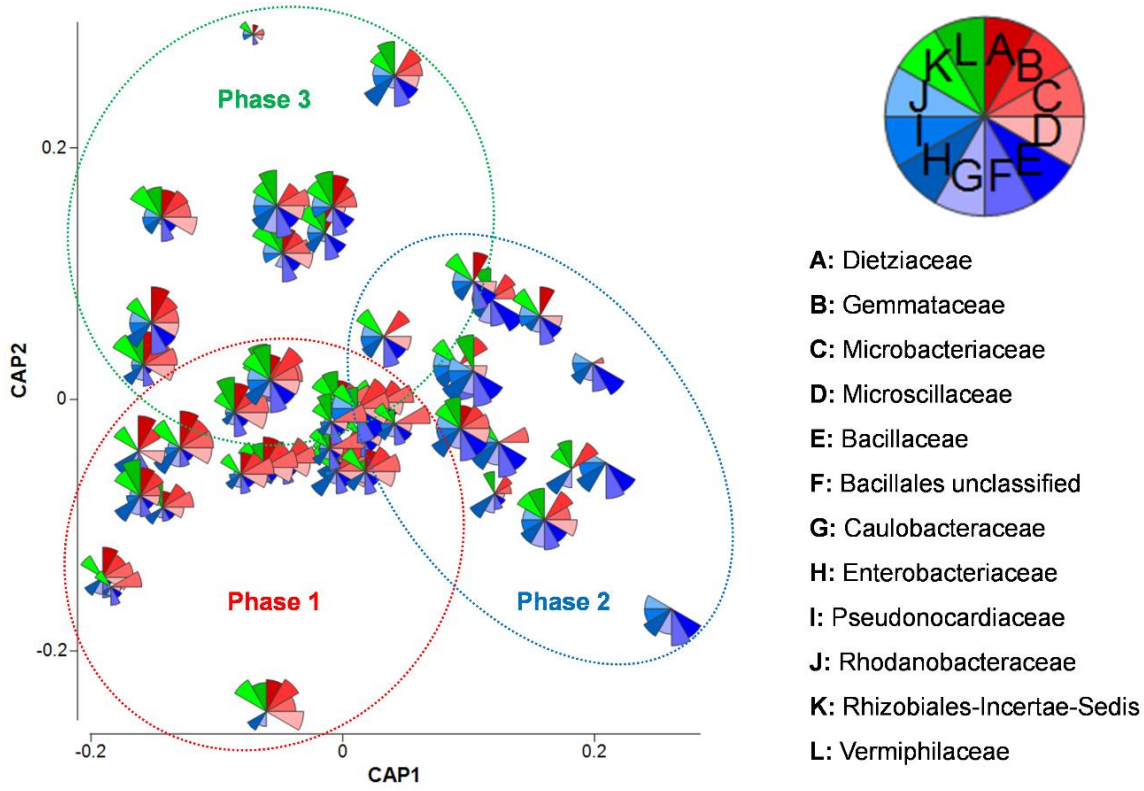

Figure 10.3: Canonical analysis of principal coordinates (CAP) representing Bray-Curtis dissimilarity index displaying the variation in prokaryotic communities on the basis of sampling phases (months). Each point representing microbiome measurement for a water sample. The overlayed similarity percentage (SIMPER) analysis showing the 12 bacterial families responsible for ≈ 10% of variance.

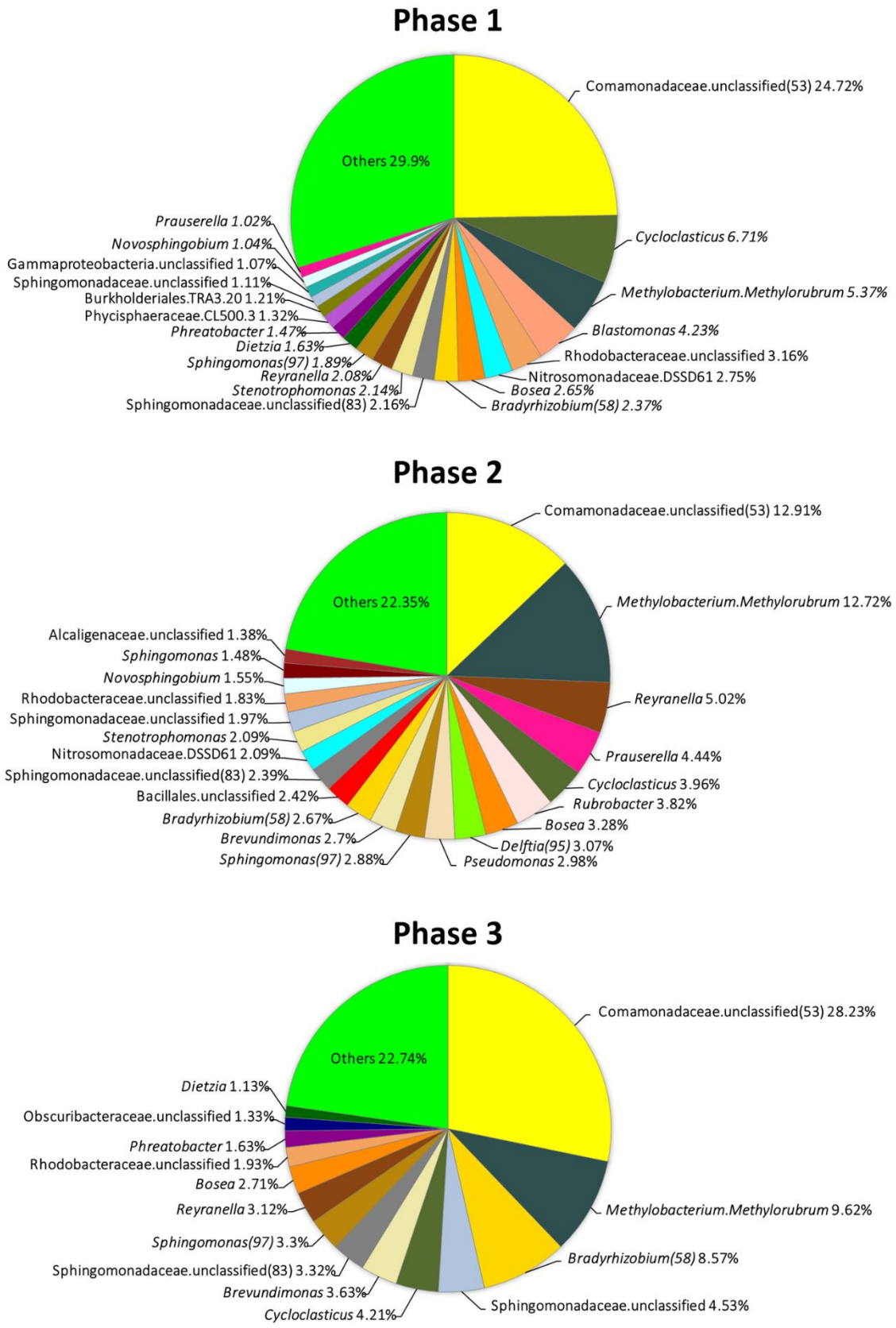

Figure 10.4: Interactive pie charts depicting the relative abundance (%) of bacterial genera based on three different sampling phases (months).

10.4.8 Influence of flow regimes on prokaryotic communities

To understand the effect of water dynamics on prokaryotic communities, both the number (counts) and duration (minutes) of flushing events for a period of one week and six months prior to sampling, were studied (Appendix-5, **Table 16.1**). Comprehensive data analysis revealed that behaviour of both parameters i.e., duration of flushing events (minutes) for one week and number of flushing events (counts) for six months prior to sampling were similar.

10.4.8.1 One week prior to sampling (total duration of flow events)

Total duration (minutes) of flushing events for one week prior to sampling, was categorised into: low (0 to < 13 minutes/week); medium (≥ 13 to < 30 minutes/week); and high (≥ 30 to 85 minutes/week) flow regimes. The Pielou's evenness diversity index revealed a significant difference (Kruskal Wallis, $p < 0.05$) and showed comparatively higher species evenness in a low flow regime compared to a medium flow regime (Appendix-5, **Figure 16.12**). Generally, greater species evenness represents greater stability and robustness. These results showed that a low flow regime (more stagnation) promoted growth and propagation of prokaryotic communities. In order to evaluate the relationship between flow dynamics and prokaryotic communities, ordination analysis based on Bray-Curtis dissimilarity index was performed and illustrated in a PCoA plot (**Figure 10.5**). This analysis showed a predictable clustering based on the flow regime (PERMANOVA: $p = 0.02$, $R^2 = 0.088$). PERMANOVA comparing the flow regimes indicated that variance was attributed to the low vs medium ($p = 0.02$), whereas the medium flow regime and high flow regime shared an average similarity of 30.53%. According to SIMPER analysis, 10 different genera were identified, which cumulatively accounted for ≈ 7% of variance between the low flow regime with both medium and high flow regimes (**Figure 10.5**). According to the interactive pie plots, three genera i.e., *Blastomonas*, *Cycloclasticus*, and Phycisphaeraceae-CL500-3 genus had higher relative abundance in the low flow regime and accounted for 14.23% compared with 2.27% of the medium flow regime and 3.27% of the high flow regime. However, *Bosea*, *Bradyrhizobium*, *Delftia*, *Methylobacterium-Methylorubrum*, Nitrosomonadaceae-DSSD61 genus, *Phreatobacter*, Sphingomonadaceae genus and *Sphingomonas* genera had higher relative abundance in the medium flow (33.6%) and high flow regimes (38.21%), compared to the low flow regime (13.09%) (**Figure 10.6**). At the family level, in the the low flow regime Cycloclasticaceae, Flavobacteriaceae, Gammaproteobacteria family, Phycisphaeraceae, Pirellulaceae, Puniceicoccaceae, and Rhodobacteraceae families accounted for 21.72% relative abundance, compared with 3.67% of the medium flow regime and 5.42% of the high flow regime. However, in the low flow regime Bacillales, Beijerinckiaceae, Nitrosomonadaceae, Obscuribacteraceae, Rhizobiales-Incertae-Sedis, and Xanthobacteraceae families accounted for 9.79% relative abundance compared with 28.33% of the medium flow regime and 28.98% of the high flow regime (Appendix-5,

Figure 16.13). Linear discriminant analysis (LDA) effect size (LEfSe), a widely used bioinformatic tool to find potential biomarkers of a specific environment, was employed to identify potential bacterial taxa associated with flow dynamics. According to the LEfSe analysis, the low flow regime samples were enriched with many bacterial orders including; Campylobacterales, Chlamydiales, Clostridiales, Desulfobacterales, Desulfovibrionales, Methylococcales, Pseudomonadales, Rhodobacterales, Rubrobacterales, and Sphingobacteriales, and families; Cyanobiaceae, Cyclobacteriaceae, Cycloclasticaceae, Flavobacteriaceae, Gammaproteobacteria, Nostocaceae, Pseudomonadales, Puniceicoccaceae, Rhodobacteraceae, Sphingobacteriaceae, Vibrionaceae, Woeseiaceae, and Xanthobacteraceae ($p < 0.05$, LDA score > 2.0). Similarly, the low flow regime samples were enriched with many bacterial genera including *Blastococcus*, *Coxiella*, *Cycloclasticus*, Flavobacteriaceae genus, Pseudomonadales genus, Rhodobacteraceae genus, *Rubrobacter*, *Sphingobacterium*, unclassified Sphingomonadaceae genus, *Sulfurimonas*, and *Sulfurovum* ($p < 0.05$, LDA score > 2.0). Whereas the medium flow regime was enriched with some important families i.e., Mycobacteriaceae, Nocardiopsaceae, Pseudonocardiaceae, Reyranellaceae, and Rubrobacteriaceae ($p < 0.05$, LDA score > 2.0). LEfSe analysis at genus level demonstrated that medium flow regime was enriched with many important genera including *Bosea*, *Mycobacterium*, Obscuribacteraceae genus, *Prauserella*, *Reyranella*, unclassified Sphingomonadaceae genus, and *Sphingomonas* ($p < 0.05$, LDA score > 2.0).

10.4.8.2 Six months prior to sampling (total number of flow events)

Total number (counts) of flushing events for six months prior to sampling, was categorised into: low (0 to 765 counts/six months); medium (800 to 1185 counts/six months); and high (1,200 to 2,500 counts/six months) flow regimes. The Pielou's evenness diversity index demonstrated a significant difference and revealed comparatively higher species evenness in the low flow regime compared to the medium flow regime (Appendix-5, **Figure 16.14**). The results of the genus level Bray-Curtis dissimilarity index based PCoA plots generated predictable clustering based on the flow regime (PERMANOVA: $p = 0.017$, $R^2 = 0.097$). PERMANOVA comparing the flow regimes indicated that variance was attributed to the low vs medium ($p = 0.012$), whereas the medium and high flow regimes shared 31.27% of average similarity. SIMPER analysis comparisons between the low flow regime with both the medium and high flow regimes showed 11 different genera cumulatively accounted for ≈ 7% of variance. Moreover, it was found that the variations were associated with comparative abundance profiles at both genus and family levels. The interactive pie plots suggested that abundance of three genera, i.e., *Cycloclasticus*, Gammaproteobacteria, and Phycisphaeraceae-CL500-3, which accounted for 12.68% of the low flow regime, in contrast with 1.75% of the medium flow regime and 3.5% of the high flow regime. However, *Bradyrhizobium*, *Methylobacterium-Methylorubrum*,

Phreatobacter, *Reyranella*, and *Sphingomonas* genera had a higher relative abundance in the medium flow (28.24%) and high flow regimes (21.63%), compared to the low flow regime (11.84%) (**Figure 10.7**). At the family level, in the low flow regime Cycloclasticaceae, Flavobacteriaceae, Gammaproteobacteria, Phycisphaeraceae, Pirellulaceae, Puniceicoccaceae, and Rhodobacteraceae families accounted for 23.02% of relative abundance, compared with 3.15% for the medium and 5.76% for high flow regimes. However, Obscuribacteraceae, Reyranellaceae, Rhizobiales-Incertae-Sedis, Xanthobacteraceae, and Xanthomonadaceae families had a higher relative abundance in the medium flow (15.58%) and high flow regimes (13.7%), compared to the low flow regime (4.7%) (Appendix-5, **Figure 16.15**). According to the LEfSe analysis, the low flow regime samples were enriched with many prokaryotic orders including; Bacillales, Campylobacterales, Chlamydiales, Clostridiales, Deinococcales, Desulfobacterales, Desulfobacterales, Desulfobulbales, Desulfovibrionales, Desulfuromonadales, Methylococcales, Pseudomonadales, Rhodobacterales, and Sphingobacteriales, and families including; Aeromonadaceae, Cyanobiaceae, Cyclobacteriaceae, Hyphomicrobiaceae, Nostocaceae, Pseudomonadales family, Puniceicoccaceae, Rhodobacteraceae, Sulfurimonadaceae, Trueperaceae, and Woeseiaceae (p < 0.05, LDA score > 2.0). Similarly, the low flow regime samples were enriched with many bacterial genera including *Bacillales*, *Bosea*, *Cycloclasticus*, *Pseudomonas*, *Puniceicoccus*, Rhodobacteraceae, unclassified Sphingomonadaceae, and *Truepera* (p < 0.05, LDA score > 2.0). Whereas the medium flow regime was enriched with some important families i.e., Beijerinckiaceae, Obscuribacteraceae and Sphingomonadaceae and, genera i.e., *Methylobacterium-Methylorubrum*, *Mycobacterium*, and *Sphingomonas* (p<0.05, LDA score > 2.0). Overall, these results demonstrated that flow dynamics and stagnation modulate the composition of prokaryotic communities.

10.4.9 Influence of temperature on prokaryotic communities

The temperatures of all water samples, i.e., outlet water, hot water, and cold-water supplies, measured both one week and six months prior to sampling, ranged from 15 to 80°C (Appendix-5, **Table 16.1, Figures 16.16** and **16.17**). The influence of water temperature was also examined but was not found to have a significant influence on the structure and composition of the prokaryotic communities. Either these parameters were not responsible for changing the structure and composition of prokaryotic communities in this hospital water system or the number of samples were insufficient to detect the influence of these parameters.

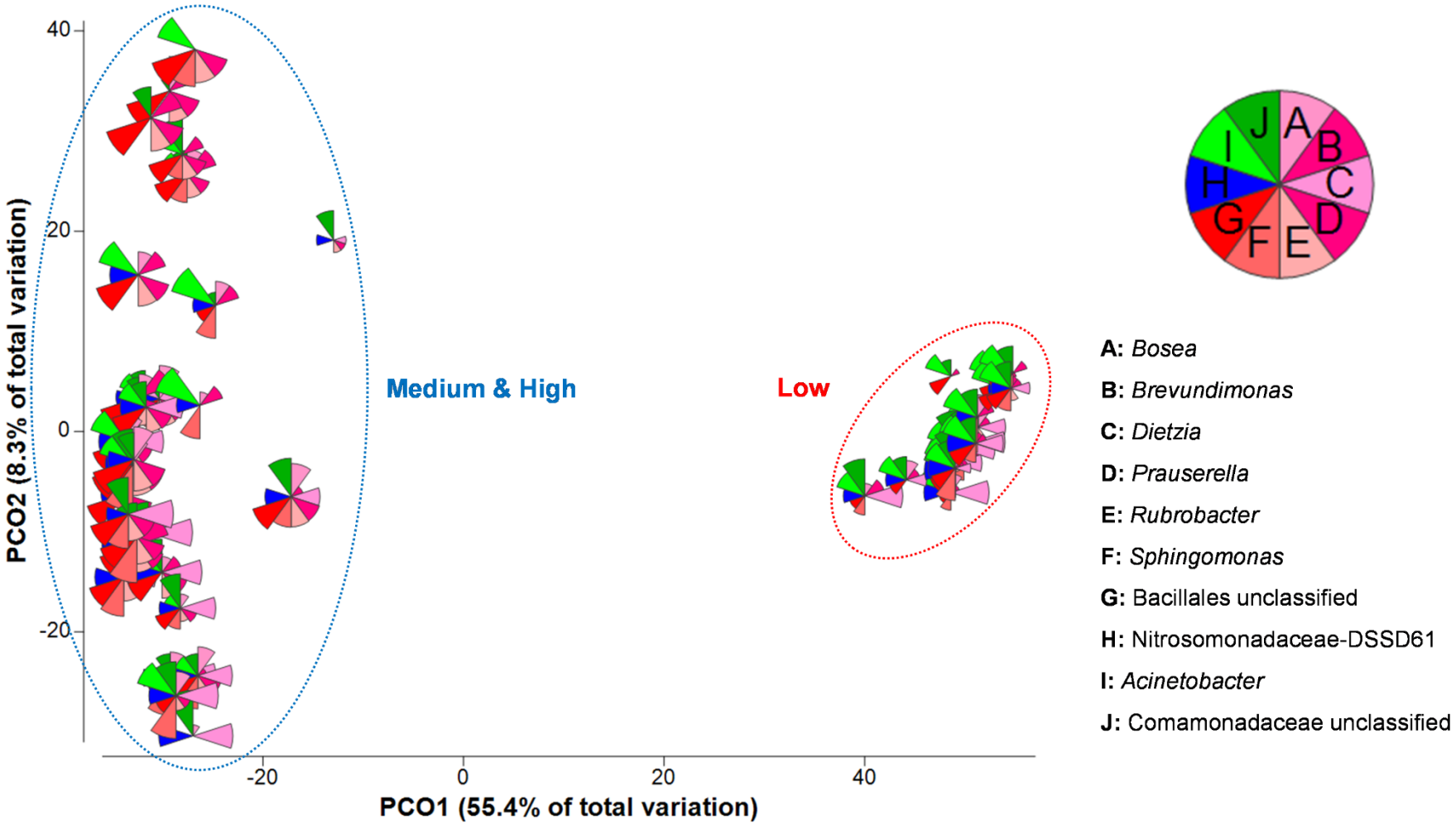

Figure 10.5: Principal coordinates analyses (PCoA) representing Bray-Curtis dissimilarity index displaying the variation in prokaryotic communities on the basis of flow regimes (one week prior to sampling). Each point representing microbiome measurement for a water sample. The overlayed similarity percentage (SIMPER) analysis showing the 10 bacterial genera responsible for ≈ 7% of variance.

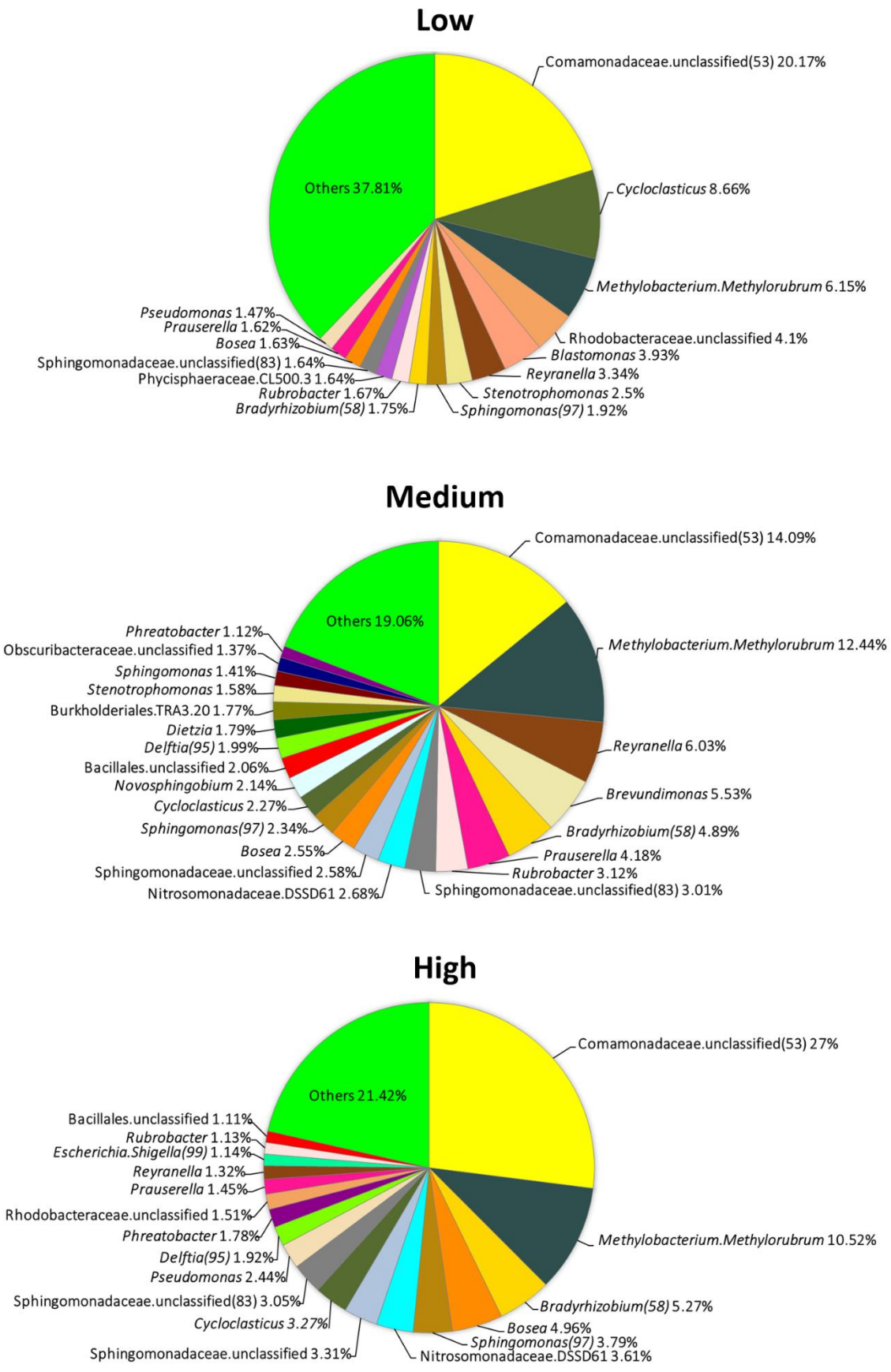

Figure 10.6: Interactive pie charts depicting the relative abundance (%) of bacterial genera affected by three different flow regimes (one week prior to sampling).

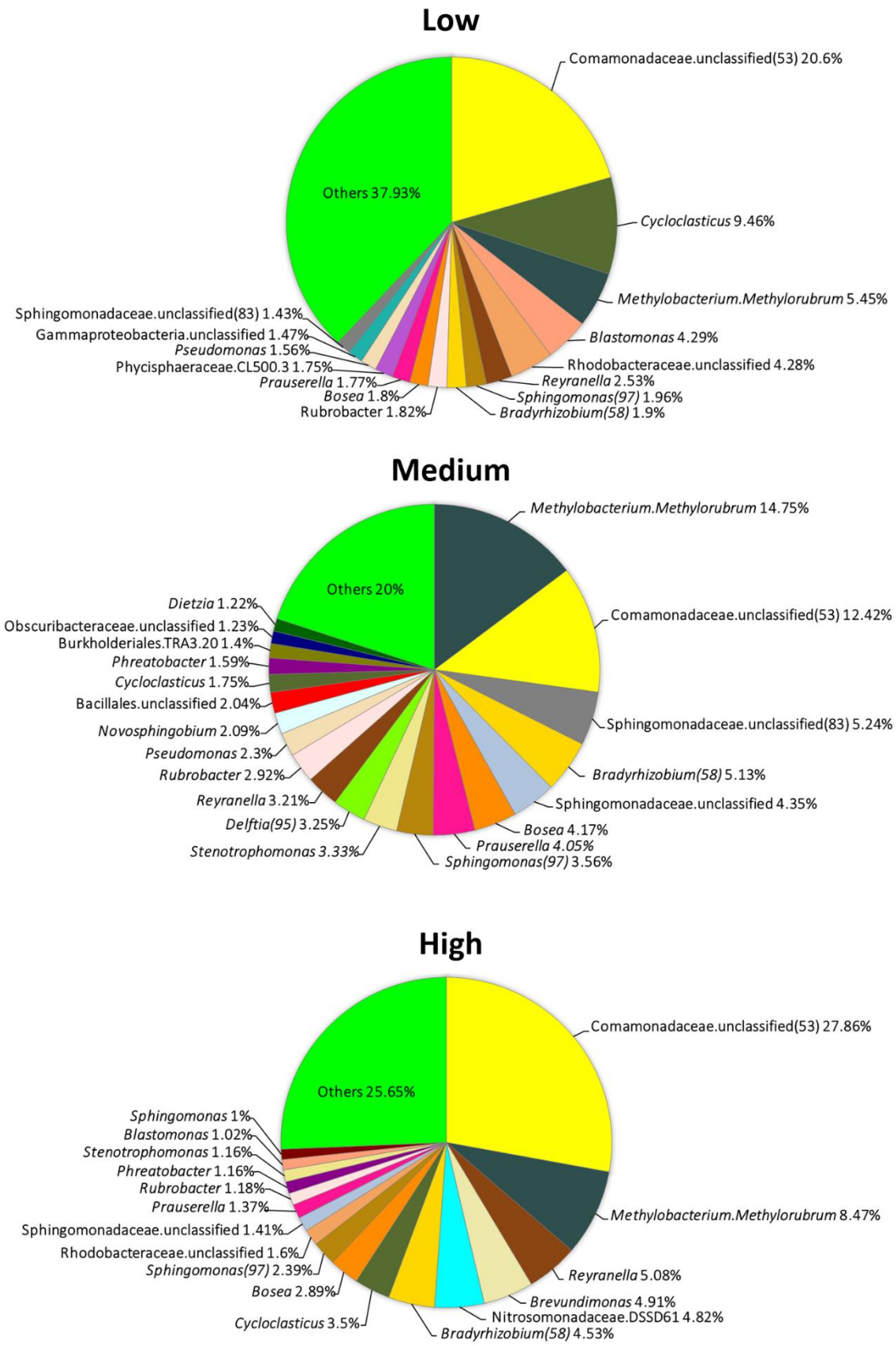

Figure 10.7: Interactive pie charts depicting the relative abundance (%) of bacterial genera affected by three different flow regimes (six months prior to sampling).

10.5 Discussion

Microbiome diversity is defined as abundance, composition, evenness, and richness of taxa as well as interactions between different taxa (Gibbons and Gilbert, 2015, Vitorino and Bessa, 2018). These parameters play a vital role in processes responsible for ecosystem functioning and stability (Escalas et al., 2019). Analysis of 16S rRNA gene amplicons of water samples of the hospital water system led to the identification of approximately 3,000 genera, suggesting a high level of prokaryotic richness and diversity. Generally, it is considered that in contrast to other environments, water has a low prokaryotic diversity (Vierheilig et al., 2015). In this study, the high abundance of genera comprising unclassified and uncultured prokaryotes demonstrated the complexity and uniqueness of the hospital water system microbiome. Furthermore, presence of biofilm forming, denitrifying, sulfate-reducing, sulfur-oxidizing, and sulfur-reducing bacteria, suggest a functional complexity and stability of hospital water system microbiome. Bacteria mediated oxidation of metals can lead to corrosion of engineered water system infrastructure (Edwards, 2004). These corrosion reactions damage the surfaces of plumbing pipes, connections, and values, which results in accumulation of corrosion reaction products on the metal surfaces (Sun et al., 2014b). These complex reactions and their by-products provide a habitat for OPPPs (Wang et al., 2012b). Both the corrosion reaction by-products and OPPPs deteriorate water quality (Edwards, 2004).

According to the available literature, prokaryotic communities of engineered water systems are often dominated by members of the phylum Proteobacteria (≥ 45%), which is supported by the present study (Sun et al., 2014a, Rahmatika et al., 2022, Withey et al., 2021, El-Chakhtoura et al., 2015, Zhou et al., 2023). In potable water, genera of both α-Proteobacteria and γ-Proteobacteria classes are the most dominant bacteria (El-Chakhtoura et al., 2015, Lin et al., 2014, Sun et al., 2014a). Members of α-Proteobacteria are primary colonizers within biofilms, and often considered as the pioneers of biofilm formation (Douterelo et al., 2014, Kalmbach et al., 1997). In contrast with previous studies, it was identified that phylum Actinobacteriota (Appendix-5, **Figure 16.1**) was the second most dominant bacterial group (Lin et al., 2014, El-Chakhtoura et al., 2015, Sun et al., 2014a). The phylum Cyanobacteria (1.42%) was present in majority of the water samples (Appendix-5, **Figure 16.1**). Cyanobacteria are ubiquitous in surface water and have been detected in potable water (Revetta et al., 2011, Codony et al., 2003, Dugan and Williams, 2006) but can also grow and proliferate in dark conditions (Richardson and Castenholz, 1987). At the order level, the most abundant taxa were the Burkholderiales (25.97%), Rhizobiales (17.92%) and Sphingomonadales (12.63%), which are well known to colonize biofilms (Huang et al., 2021, Belgini et al., 2014). Further analysis showed that Comamonadaceae, Sphingomonadaceae, and Beijerinckiaceae were the three most dominant families in all samples and represented cumulatively 47.3% of the relative

abundance. Members of the Comamonadaceae family are commonly found in different environments including lake and potable water (Lautenschlager et al., 2013), as well as in cooling tower and water systems (Pereira et al., 2017, Wang et al., 2013). Importantly, bacteria of Comamonadaceae family can consume a wide range of substrates for survival, and therefore out-compete other bacterial genera (Lautenschlager et al., 2013, Vuono et al., 2015). Likewise, members of the Sphingomonadaceae family can produce biofilms in potable water and are capable of tolerating chlorine disinfection procedures (Sun et al., 2013, Gulati and Ghosh, 2017). Data analyses demonstrated that *Blastomonas* (1.89%), *Novosphingobium* (1.43%), and *Sphingomonas* (3.58%) were present in majority of the water samples (Appendix-5, **Figure 16.2**). In particular, these genera play an important role in biofilm formation and have the ability to colonize different surfaces and co-aggregate with other microbes (Soto-Giron et al., 2016). In addition, these genera also have the ability to resist and tolerate disinfection procedures (Narciso-da-Rocha et al., 2014). These results indicate that the prokaryotic communities existing in hospital water (hand basins and shower) are potentially influenced by the biofilm growing on the surfaces of the building plumbing systems.

Generally, the physico-chemical conditions at sampling sites (outlets) are considered an important parameter responsible for structuring the composition of prokaryotic communities (Withey et al., 2021, Proctor et al., 2018). However, this study demonstrated that both hand basins and shower water samples had similar evenness. Supporting this, Bray-Curtis dissimilarity analysis suggested that the sampling site (outlets) did not influence the prokaryotic composition, significantly, which is not surprising as all samples were collected from a single hospital. Instead, it was identified that sampling phases (months) played a significant role in variations in prokaryotic communities (**Figure 10.3**). Routine flushing events replace pre-existing microbes and introduce new microbes coming from the municipal water system. It is well-established that the microbial communities in plumbing systems are dispersed from the inflow source to the downstream networks (Whiley et al., 2014). Furthermore, environmental factors such as weather and temperature can also play an important role in structuring the prokaryotic communities (Calero Preciado et al., 2021). According to the weather data, sampling phase 2 and 3 had a similar mean minimum temperature (T_{min} mean = 15.0°C and 15.4°C, respectively). Additionally, mean maximum temperature of sampling phase 1 and 3 were similar (T_{max} mean = 22.9°C and 22.7°C, respectively). In the case of rainfall, both sampling phases 1 and 3 had quite similar rainfall values (phase 1 = 118.6 mm, and phase 3 = 112 mm). These results suggested that both climatic parameters (temperature and rainfall) were not key plays in changing the structure and diversity of planktonic prokaryotic communities in hospital water system. Most likely, these changes were attributed to the incoming municipal water supplies and the water disinfection protocols.

Focusing on the potentially pathogenic bacteria, it was apparent that at least 20 genera were present in majority of the samples (Appendix-5, **Figure 16.3**). Survival and persistence of these OPPPs in such a nutrient deficient environment is attributable to their ability to infect free-living amoebae, with the associated resistance against chemical and physical disinfection procedures (Falkinham III et al., 2015b, Nisar et al., 2020a). Importantly, members of five different bacterial phyla i.e., Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Actinobacteriota, considered essential part of amoebae microbiome, were abundantly present in all shower and hand basins water samples (Appendix-5, **Figure 16.1**) (Sallinger et al., 2021). These results point towards the importance of amoebae, especially *V. vermiformis,* that play a key role in survival and persistence of pathogenic bacteria in hospital water system (Nisar et al., 2020a).

In building plumbing systems, due to complexity of plumbing design, structural components (tap faucets, aerators, and thermostatic mixing valves) and fluctuations in water temperature, insufficient water flow or low water usage is unable to introduce fresh water into the building plumbing system (Rhoads et al., 2014). Therefore, prolonged periods of stagnation or low water usage deteriorate water quality. Four key reactions are attributed to stagnation: (1) decay of chemical disinfectants and failure of disinfection treatments; (2) formation of chemical disinfectant by products; (3) corrosion of plumbing materials and; (4) growth of microbes (Proctor et al., 2020). Increased microbial growth always accelerates the rate of the first three reactions. According to the enHealth guidelines, weekly flushing of unused water outlets (taps, shower, etc.) is highly recommended as part of water safety plans to control for the clinically important OPPP *Legionella* (enHealth, 2015). Furthermore, previous studies have identified that OPPPs, e.g., *Flavobacterium*, *Legionella*, *Methylobacterium*, *Mycobacterium*, *Pseudomonas*, *Sphingobium*, and *Sphingomonas*, proliferate during stagnation (Zhang et al., 2021b, Zhang et al., 2021a, Rahmatika et al., 2022, Nisar et al., 2020b). Similarly, in our data, the Pielou's evenness diversity index demonstrated that prokaryotic communities exhibited greater evenness during low flow regime (both in case of duration of flushing events for one week and number of flushing events for six months prior to sampling) (Appendix-5, **Figures 16.12** and **16.14**). This implies that during stagnation (low flow regime), the microbes present in the plumbing system propagated and increased their population numbers. This could allow for the selection of microbes that survive and grow better in low nutrient environments (which increased evenness). Similarly, Bray-Curtis dissimilarity analysis generated statistically significant clustering based on flow regimes (both in case of duration of flushing events for one week and number of flushing events for six months prior to sampling) (**Figures 10.5**). According to this analysis, low flow regime prokaryotic communities were significantly different, whereas the medium and high flow regimes shared considerable similarities. It is important to

notice that the low flow regime resulted in high abundance of biofilm forming (*Pseudomonas*, Rhodobacteraceae genus, and Sphingomonadaceae genus), spore-forming (Bacillales genus), highly resistant to physical and chemical disinfection treatments (Deinococcales order), and corrosion related (Desulfobacterales, Desulfobulbales, Desulfovibrionales, and Desulfuromonadales orders) bacterial taxa (Albuquerque et al., 2005, Soto-Giron et al., 2016, Sun et al., 2014a, McKenney et al., 2013). In conclusion, these results demonstrated that flow dynamics and stagnation play key role in structuring the composition of prokaryotic communities in hospital water system.

10.6 Conclusions

Limited studies have focused on prokaryotic communities in water from hospital plumbing systems. In the present study, culture-independent 16S rRNA gene amplicon sequencing demonstrated that just like plumbing system biofilms, the water circulating in plumbing system is comprised of diverse and unique prokaryotic communities. Collectively, our results demonstrated that the sampling time, flow dynamics and water stagnation alter the structure and composition of prokaryotic communities growing in engineered water system. Furthermore, the stagnation and restricted water circulation supports the growth and proliferation of opportunistic premise plumbing pathogens, biofilm forming and corrosion causing bacteria.

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Author contribution statement

MAN and HW conceived and designed research. MAN performed the experiments. JH and JX assisted in sampling and data collection. KER, MHB, HW, RB, TJ and SCL provided technical assistance. MAN and HW drafted and edited the manuscript. KER, MHB, RB, TJ, SCL, JH and JX corrected and contributed to the manuscript. All authors approved of the final manuscript.

Conflict of interests

The authors declare no conflict of interest.

11 Discussion

Legionella is an opportunistic waterborne intracellular human pathogen (Berjeaud et al., 2016, Fields et al., 2002). Despite advancements in disease management and surveillance systems, the incidence and outbreak rate of legionelloses remains high. As such, there is a need for improved water management strategies to control *Legionella* in engineered water systems. To inform future guidelines, this research investigated the relationships between *Legionella,* protozoan hosts, and intermittent stagnation in building potable water distribution systems. A novel method to quantify VBNC *Legionella* from environmental samples was developed and used in this study, which has provided some unique insights into the ecology of this pathogen. This study was conducted in four phases:

1. In first phase of the study a viability based flow cytometry-cell sorting and qPCR (VFC+qPCR) assay was designed to detect and quantify VBNC *Legionella* and *L. pneumophila* from environmental samples (**Section 4**). It was identified that:
    a. Standard selective decontamination steps (thermal and acid treatment) recommended by ISO11731:2017-05 culturing method generates VBNC *Legionella*.
    b. The VFC+qPCR assay is an effective and rapid method for detection and quantification of VBNC *Legionella*/*L. pneumophila* from environmental samples.
2. The second phase of the study consisted of two parts. In the first part, a comprehensive literature review was conducted to identify potential protozoan hosts that support the persistence of *Legionella* in engineered water system (**Section 5**). In the second part, water and biofilm samples collected from hospital and domestic water systems were screened for *Legionella* and free-living amoebae (**Section 6**). This phase identified that:
    a. In hospital and domestic water systems, *Acanthamoeba* and *Vermamoeba vermiformis* are natural hosts of *L. pneumophila* (systematic literature review).
    b. Hospital and domestic water systems of Australia are highly contaminated with *Legionella* (41%) and free-living amoebae (69%).
    c. In engineered water systems, *Legionella* always coexists with free-living amoebae.
    d. In engineered water systems, *V. vermiformis* (55%), *Acanthamoeba* (11%), *Stenamoeba* (≈ 1.5%), and *Allovahlkampfia* (≈ 1%) are the most abundant free-living amoebae.
    e. In engineered water systems, *Legionella* exists in the cytoplasm of *V. vermiformis* and *Allovahlkampfia*.

3. The third phase of this study consisted of three parts. The first part was a literature review to investigate the role of stagnation and flow dynamics on the persistence of *Legionella* in engineered water systems (**Section 7**). In the second part, a laboratory scale plumbing system was used to determine the role of intermittent stagnation on *Legionella* under controlled conditions (**Section 8**). The third part was a collaboration with Enware Pty Ltd. in which the *Legionella* presence in a hospital water distribution system was compared with the temperature and flow dynamics data measured by Enware Smart® monitoring system located at the thermostatic mixing values and faucet outlets (**Section 9**). This phase identified that:
    a. Permanent and temporary stagnation deteriorate water quality and support the growth of *Legionella* and *L. pneumophila* (systematic literature review).
    b. In the laboratory scale plumbing system, once-a-day water flushing significantly ($p < 0.001$) reduced the quantity of biofilm-associated culturable *Legionella*, compared to once-a-week water flushing (temporary stagnation).
    c. In the hospital water system, intermittent water usage (temporary stagnation < 2 hours water flushing per month) increases the concentration of *Legionella*/*L. pneumophila* and *V. vermiformis*.
    d. The hospital water system was highly contaminated with VBNC *Legionella*/*L. pneumophila* (22%).
    e. The concentration of *Legionella*/*L. pneumophila* positively correlates with the concentration of heterotrophic bacteria and *V. vermiformis*.
4. In the fourth phase of this study, the effect of stagnation, flow dynamics, water temperature, sampling season, and free-living amoebae on the structure and composition of planktonic prokaryotic communities of hospital water system was investigated (**Section 10**). This phase identified that:
    a. The hospital water system consists of diverse planktonic prokaryotic communities, which includes OPPPs, biofilm former, intrinsically disinfectant resistant, and corrosion associated bacteria.
    b. Water stagnation, flow dynamics and seasonal (sampling phases) changes the composition of hospital water planktonic microbiome.

11.1 Standard guidelines and legionellosis risk assessment

The survival and persistence of *Legionella* in engineered water systems is influenced by many factors, including flow dynamics and failure of disinfection treatments. Furthermore, *Legionella* growth in engineered water systems is associated with prokaryotic (specifically bacteria) and eukaryotic (specifically free-living amoebae) communities. Therefore, it important to consider

water temperature, flow dynamics, water stagnation, structure and chemical nature plumbing system, type of disinfection treatment, dose and time of disinfection treatment, heterotrophic bacterial load, free-living amoebae, intrinsic resistance against disinfection treatments, and VBNC state while designing any strategy to manage and control *Legionella* (Falkinham III et al., 2015a, Bartram et al., 2007, Whiley et al., 2017, Kirschner, 2016, Proctor and Hammes, 2015, Cullom et al., 2020). Worldwide, current guidelines and regulations for the management of *Legionella* in engineered water systems recommend the regular surveillance and elimination of factors promoting growth of *Legionella* (enHealth, 2015, European Study Group for *Legionella* Infections, 2020, European Study Group for *Legionella* Infections, 2017, Bartram et al., 2007). Based on this study, it is suggested that the standard guidelines to manage *Legionella* in engineered water systems need to address the following five points more precisely:

1. *Legionella* detection and quantification;
2. *Legionella* infectious dose;
3. *Legionella* and water stagnation;
4. *Legionella* and free-living amoebae;
5. *Legionella* and prokaryotic communities.

11.1.1 *Legionella* detection and quantification in engineered water systems

In Australia, the USA, and European countries legionellosis is considered one of the most important waterborne illnesses (Australian Government, 2021a, Centers for Disease Control and Prevention, 2022, The European Legionnaires' disease Surveillance Network, 2022, enHealth, 2015). Therefore, routine surveillance of *Legionella* in engineered water systems is standard practice. Standard microbiological culturing and qPCR assay recommended by the International Organization for Standardization (ISO) and World Health Organization (WHO) are extensively used for the routine surveillance and outbreak investigation (International Organization for Standardization, 2017, International Organization for Standardization, 2019, Standards Australia, 2017, Bartram et al., 2007). In this study (**Section 6**), domestic and hospital water and biofilm samples were screened using standard culturing and qPCR assays. The direct qPCR assay identified that that 41% samples were positive for *Legionella*, 33% for *L. pneumophila*, whereas standard culturing detected only 7% culturable *Legionella* spp. Similarly, characterization of water and biofilm samples from hospital water system (**Section 9**), demonstrated that 22% samples were qPCR positive for *Legionella* spp. but none of the samples were positive for culturable cells. However, a culture-negative result does not represent absence of *Legionella* and a qPCR assay positive result does not represent presence of viable and infectious *Legionella* in an engineered water system (Kirschner, 2016).

Both assays are unable to either detect or characterize VBNC *Legionella*. Therefore, in this study flow cytometry-cell sorting and qPCR (VFC+qPCR) assays (**Section 4**) was designed to detect and quantify the VBNC *Legionella* spp. from environmental samples. Using the VFC+qPCR assay, it was identified that 22% hospital water and biofilm samples were positive for VBNC *Legionella* (**Section 9**). It is well established that the VBNC state is a survival strategy of certain Gram-negative bacteria in response to environmental stresses and starvation (Li et al., 2014). VBNC *Legionella* are potentially infectious in nature and transform into more pathogenic culturable cells with help of host protozoa (Boamah et al., 2017, Fields et al., 2002, Dietersdorfer et al., 2018). Despite, the potential importance of VBNC *Legionella* from a public health perspective, standard detection protocols cannot identify it. The standard guidelines recommend culture-based method (ISO11731:2017-05 and AS5132:2017) for the detection and quantification of *Legionella* from engineered water systems (European Study Group for *Legionella* Infections, 2017, enHealth, 2015). Similarly, the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) (2018) emphasises the microbiological testing of *Legionella* (ANSI/ASHRAE Standard 188-2018; section 7.3.5.1). The European Study Group for *Legionella* Infections (ESGLI) guidelines (2017) point out two main concerns with the qPCR method (ISO/TS12869:2019) namely., it provides results in genomic units (GU) instead of colony forming units (CFU) (ESGLI 2017, paragraph 1.41) and detects/quantify both dead and VBNC cells (ESGLI 2017, paragraph 2.28). Similarly, concerns are raised in the *Legionella* Control Guidelines of Australian standard guidelines (enHealth 2015, section 3.2.1, table 3). In comparison with the standard qPCR method, the VFC+qPCR assay specifically quantifies VBNC *Legionella*. This study suggested instead of standard culturing, qPCR and VFC+qPCR assays should be used in testing of *Legionella* high risk areas such as hospitals and aged care buildings.

11.1.2 *Legionella* infectious dose in engineered water systems

Legionella is ubiquitously present in engineered and natural water systems from very low to very high concentrations (Parthuisot et al., 2010, Casini et al., 2018, Dai et al., 2019, Hayes-Phillips et al., 2019). However, the exact number of *Legionella* in engineered water systems required to potentially infect a human has not been universally determined (Bartram et al., 2007). The infectious dose is multifactorial and can depend on the virulence of pathogen and susceptibility of potential host (Leggett et al., 2012). Different regions use different guideline values for *Legionella* concentrations in engineered and warm water systems (Bartram et al., 2007, Kirschner, 2016). In the case of *Legionella*, the guidelines used by different countries are based on the standard microbiological culturing assay (Bartram et al., 2007, Kirschner, 2016). A study conducted on guinea pigs using an aerosol infection model identified that the median infectious dose of *L. pneumophila* is < 129 culturable cells, whereas the median lethal

dose (LD_{50}) is ≈ 10^5 culturable cells (Berendt et al., 1980). Sikora et al. (2015) identified that if a water system is contaminated with 10^3 to 10^5 CFU/L *Legionella*, then legionellosis may occur sporadically. However, when the *Legionella* count exceeds 10^5 CFU/L, an outbreak of legionellosis may happen (Sikora et al., 2015). Whereas, the European Study Group for *Legionella* Infections (ESGLI) categorized culturable *Legionella* concentration in engineered water system into three groups, specifically; group I: < 10^2 to 10^3 CFU/L, group II: > 10^3 to < 10^4 CFU/L, and group III: ≥ 10^4 CFU/L (European Study Group for *Legionella* Infections, 2017). These guidelines totally ignore VBNC *Legionella* which are also infectious in nature. In this study using the VFC+qPCR assay, VBNC *Legionella* was identified in 22% of hospital water and biofilm samples (**Section 9**). The concentration of VBNC *Legionella* ranged from 2.5 × 10^2 to 6.5 × 10^5 GU/L and 3.5 × 10^1 to 6.5 × 10^3 GU/mL in water and biofilm samples, respectively. At this time, standard guidelines do not provide information about the infectious dose of VBNC *Legionella*; therefore, it is very difficult to predict whether these water samples can be a cause of any future legionellosis outbreaks. A previous study demonstrated that in contrast to culturable *Legionella*, the VBNC *Legionella* are less pathogenic and infectious in nature (Cervero-Arago et al., 2019). Similarly, in **Section 4** *Legionella*-amoebae coculture assays demonstrated that VBNC *Legionella* are less infectious and take longer time to infect *Acanthamoeba polyphaga*, compared to culturable cells. Therefore, for proper management of *Legionella*, the infectious dose for both culturable and VBNC cells in engineered water systems should be determined.

11.1.3 *Legionella* and water stagnation in engineered water systems

Stagnation is important factor responsible for failure of disinfection treatment, loss of temperature control, and persistence of *Legionella* (Bartram et al., 2007). Manufactured water systems experience permanent and temporary stagnation. The permanent stagnation is primarily associated with plumbing structures such as dead-ends and dead-legs and has been responsible for several legionellosis outbreaks (Patterson et al., 1994, Tercelj-Zorman et al., 2004, Bartley et al., 2016). In hospital water systems, removal of permanent stagnation sites significantly reduced *L. pneumophila* contamination (Totaro et al., 2018, Totaro et al., 2020). According to the WHO (2007) *Legionella* control guidelines, permanent stagnation points should be avoided in the design of building plumbing systems. If it is difficult to remove dead-ends or dead-legs regular water flushing should be adopted. Similarly, New South Wales (NSW) Health guidelines (2018) for *Legionella* control in cooling water systems recommend installation of a flushing point in vicinity of dead-legs, from which water should be flushed from once a week. Both guidelines only address permanent water stagnation. Both guidelines only address permanent water stagnation. Conversely, the standard guidelines of *Legionella* control suggested by the Government of South Australia recommend water flushing on a weekly basis

for maintenance of warm water systems (SA Health, 2013). The enHealth (2015) guidelines recommend once-a-week flushing for the management of *Legionella* in manufactured water of hospitals and age care facilities. Similarly, ESGLI recommends once-a-week flushing in little used water outlets of building cold and hot water systems (European Study Group for *Legionella* Infections, 2017).

It is well established that elimination of water stagnation hot spots can significantly reduce *Legionella* in engineered water systems (Totaro et al., 2018, Totaro et al., 2020). However, in buildings, water also stagnates in water storage tanks, piping network, and components of water outlets (i.e., shower heads, tap faucets, etc.) for a few hours to weeks (Manuel et al., 2009, Bartram et al., 2007). This type of water stagnation is known as temporary or intermittent stagnation. Deliberate obstruction of water flow in the net-zero energy or green buildings also promotes temporary stagnation (Rhoads et al., 2016). The lockdown strategy for the management of COVID-19 pandemic was another example of a temporary stagnation event (Rhoads and Hammes, 2021). In comparison to permanent stagnation, there are fewer studies that comprehensively examine the effect of temporary or intermittent stagnation on survival and persistence of *Legionella* and associated microbes. The standard guidelines provide information for prolonged stagnation but do not provide comprehensive information about temporary stagnation and deliberated water obstruction in green buildings. In this piece of work, the effect of temporary stagnation on *Legionella* and associated microbes was specifically studied using a laboratory scale plumbing model and a real-world hospital water system (**Section 8**).

In **Section 8**, the results of laboratory scale plumbing model suggested that once-a-day water flushing for a period of four weeks significantly ($p < 0.001$) reduced the population of biofilm-associated alive and culturable *L. pneumophila* (1.5 $\log_{10}$) compared with weekly flushing. Similarly, Spearman correlation analysis also suggested that population of biofilm-associated alive and culturable are negatively correlated with once-a-day water flushing ($\rho = -0.9$, $p < 0.001$). In **Section 9**, the results of the hospital water system also suggested that hand basins and showers experiencing intermittent flushing (< 2 hours per month) were more likely to be colonized by *L. pneumophila*. It was also identified that *L. pneumophila* survived and persisted in a VBNC state in these hospital showers and hand basins. The results of both laboratory scale and real-world studies clearly suggested that without management of temporary or intermittent stagnation control of *Legionella*/*L. pneumophila* is not possible. In conclusion, the current guidelines of WHO, SA Health, NWS Health and enHealth which recommends once-a-day water flushing to control *Legionella*/*L. pneumophila* in engineered water systems (Bartram et al., 2007, SA Health, 2013, NSW Health, 2018, enHealth, 2015).

11.1.4 *Legionella* and free-living amoebae in engineered water system

In engineered water systems *Legionella* coexists with protozoan hosts. Among protozoan hosts, the free-living amoebae are widely distributed in engineered water systems. The relationship between *Legionella* and free-living amoebae is very complex and requires more attention. The trophozoites of free-living amoebae support intracellular replication of *Legionella* (Boamah et al., 2017). A single *A. polyphaga* trophozoite can produce more than 1000 culturable *Legionella* (Buse and Ashbolt, 2012). Furthermore, trophozoites are responsible for resuscitation of VBNC *Legionella* into more pathogenic and virulent culturable *Legionella* (Boamah et al., 2017, Fields et al., 2002). In engineered water systems, free-living amoebae, especially *Acanthamoeba* and *V. vermiformis,* play an important role in the survival of *Legionella* and provide resistance against some disinfection treatments. The trophozoites and cysts of free-living amoebae are intrinsically resistant against the chemical and physical disinfection treatments (Thomas et al., 2004, Critchley and Bentham, 2009, Garcia et al., 2007, Dupuy et al., 2014). The cysts of free-living amoebae protect intracellular *Legionella* from chlorine, chlorine dioxide monochloramine, and ozone (Loret et al., 2005). It has also been observed that trophozoites of *Acanthamoeba* infected with *Legionella* are more resistant against chemical disinfection treatments (Garcia et al., 2007). Therefore, the relationship between *Legionella* and free-living amoebae is not a classical host-parasite relationship.

Previous studies showed that *V. vermiformis* is present in natural freshwater bodies i.e., rivers and lakes, at concentrations ranging from 5 to 75 cells/L (Kuiper et al., 2006), whereas *Acanthamoeba* is more prevalent in recreational hot water springs and water treatment plants (Ji et al., 2014). In USA, it was reported that the majority of water of domestic buildings are contaminated with *Acanthamoeba*, *Vermamoeba*, and *Vahlkampfia* (Stockman et al., 2011). In this study (**Section 6**), microbiological and molecular screening of hospital and domestic water samples demonstrated that 69% of samples were positive for free-living amoebae. Molecular characterization of free-living amoebae isolates identified *Acanthamoeba*, *Allovahlkampfia*, *Stenamoeba*, or *V. vermiformis* as potential hosts of *Legionella*. Importantly, *Allovahlkampfia* and *Stenamoeba* were identified as new hosts of *Legionella* in engineered potable water systems. This demonstrates that in engineered water systems, it is necessary to characterize all free-living amoebae in order to identify all potential *Legionella* hosts. This study also found that *V. vermiformis* was the predominant host in the sampled Australian engineered water systems. Similarly, the second study (**Section 9**), which was an investigation into a single hospital water system, found *V. vermiformis* to be the predominant *Legionella* host. Both laboratory scale (**Section 8**) and screening of hospital and domestic water systems (**Sections 6** and **9**) demonstrated strong positive associations between *Legionella* and *V. vermiformis* (Spearman's correlation analysis $p < 0.05$). These studies clearly demonstrated

that for proper management and control of *Legionella* in engineered water systems, free-living amoebae must be considered. The standard water treatment guidelines are specifically designed for highly pathogenic free-living amoebae such as *Naegleria fowleri* and *Acanthamoeba* and ignore potential hosts of *Legionella* (National Health and Medical Research Council and National Resource Management Ministerial Council, 2022). An *in vitro* study identified that 1 mg/L chlorine is sufficient to inhibit the growth of *Acanthamoeba* and *Vermamoeba* trophozoites, whereas 5 mg/L chlorine inactivates the cysts of these free-living amoebae (Critchley and Bentham, 2009, Nisar et al., 2020a). However, *Legionella*-infected, biofilm associated, or intrinsically-resistant *Acanthamoeba*/*Vermamoeba* trophozoites and cysts are more resistant against chemical and physical disinfection procedures (Storey et al., 2004, Dupuy et al., 2014, Dupuy et al., 2011, Rhoads et al., 2015, Dobrowsky et al., 2016) (**Section 5**). Therefore, future guidelines must consider these three parameters while designing any appropriate water disinfection procedure.

The laboratory scale biofilm model (**Section 8**) demonstrated that once-a-day water flushing significantly increased the amount biofilm-associated *V. vermiformis* (Spearman's correlation: $\rho = 0.706$, $p < 0.001$) whereas flushing decreased the concentration of biofilm-associated *Acanthamoeba* (Spearman's correlation: $\rho = -0.949$, $p < 0.001$). Furthermore, this increase in biofilm-associated *V. vermiformis* was positively associated with an increase in VBNC *Legionella* (Spearman's correlation: $\rho = 0.848$, $p < 0.001$). In the case study, investigation of the hospital water system (**Section 9**) identified that the temporary water stagnation (< 2 hours flushing/month) significantly (Kruskal-Wallis analysis, $p < 0.05$) increased the concentration of planktonic *V. vermiformis*. Importantly, this increase in was also positively associated with an increase in VBNC *Legionella* (Spearman's correlation: $\rho = 0.581$ and 0.595, respectively; $p < 0.001$). Both laboratory scale and real world studies clearly demonstrated importance of *V. vermiformis* in survival and persistence of *Legionella* in engineered water system. To our knowledge, the effect of permanent and temporary water stagnation on growth and proliferation of free-living amoebae, especially *V. vermiformis,* has not yet been investigated. Therefore, further studies are required for proper understanding how these free-living amoebae behave in building plumbing systems under different hydraulic regimes.

11.1.5 *Legionella* and prokaryotic communities in engineered water system

Bacterial colonization within building plumbing systems is a very complex process. Generally, microbes including OPPPs come from municipal water supplies and colonize in plumbing structures (Whiley et al., 2014). The chemical nature and structure of plumbing materials play a significant role in bacterial colonization and persistence. Metal plumbing materials directly react and neutralize chemical disinfectants, whereas synthetic organic materials leach organic

compounds which potentially support microbial growth and indirectly neutralize the chemical disinfectants (Colbourne and Ashworth, 1986, Cullom et al., 2020). It is recommended that assimilable organic carbon in untreated water must be 10 to 32 µg/L, and in disinfected water 100 µg/L, to reduce the risk of microbial growth and persistence (Hammes et al., 2010, Kooij, 1992, Volk and LeChevallier, 2000). These organic and inorganic compounds accumulated in engineered water systems provide ideal conditions for colonization of *Legionella* (Sciuto et al., 2021).

In engineered water systems *Legionella* coexists with bacterial biofilms. Generally, the bacterial load in engineered water system is estimated using HPC (Bartram et al., 2003). However, it is difficult to interpret the public health significance of heterotrophic bacteria (HPC load) growing in engineered water systems because the correlation with legionellosis outbreak is debated (Bartram et al., 2003). In this study the laboratory scale biofilm model experiment (**Section 8**) identified a strong positive association of culturable *Legionella* with both biofilm-associated (Spearman's analysis: $\rho = 0.929$, $p < 0.001$) and planktonic (Spearman's analysis: $\rho = 0.802$, $p < 0.001$) heterotrophic bacteria. Furthermore, it was also noted that HPC load is negative associated with water flushing frequency (Spearman's analysis: biofilm-associated $\rho = -0.942$, $p < 0.001$ and planktonic $\rho = -0.683$, $p < 0.001$) i.e., intermittent water usage increases HPC load. The analysis of hospital water samples (**Section 9**) demonstrated that the samples with high levels of HPC load (ranging from $\geq 5 \times 10^3$ to 1.5×10^5 CFU/L) contained VBNC *Legionella*/*L. pneumophila* in high numbers. Both laboratory-scale and hospital water studies demonstrated a strong association between *Legionella* and HPC load.

The EWGLI guidelines provide information about relationship between *Legionella* and aerobic plate count for cooling towers. To minimize the risk of legionellosis the cooling water samples are categorized into four group i.e., group I: $\leq 10^4$ CFU/L aerobic count and no culturable *Legionella*, group II: $\leq 10^4$ CFU/L aerobic count and $\leq 10^3$ CFU/L *Legionella*, group III: $\geq 10^4$ to $\leq 10^5$ CFU/L aerobic count and $\geq 10^3$ to $\leq 10^4$ CFU/L *Legionella*, and group III: $> 10^5$ CFU/L aerobic count and $> 10^4$ CFU/L *Legionella*. Only group I conditions are acceptable for cooling towers, but other conditions require either further testing or disinfection treatments (European Study Group for *Legionella* Infections, 2017). The SA Health guidelines (2013) use HPC load as an indicator of water quality and recommend that if HPC load is $\geq 10^5$ CFU/mL in cooling water system and $\geq 10^2$ CFU/mL in warm water system then plumbing system should be considered for disinfection treatments. However, all these standard guidelines do not comprehensively discuss relation between *Legionella* and heterotrophic bacteria in building cold and hot water systems. Moreover, these guidelines are specifically designed for culturable *Legionella*, and VBNC cells are not considered.

It is well established that the microbes growing in engineered water systems are associated with failure of disinfection treatments (Sciuto et al., 2021). Microbes, especially nitrifying bacteria, present in engineered water systems decay chemical disinfectants such as monochloramine and support biofilm formation (Lipponen et al., 2004, Sciuto et al., 2021). In this study (**Section 10**), molecular analysis of the hospital water system demonstrated that the planktonic prokaryotic communities of both shower and hand basin water were taxonomically diverse and dominated by potentially pathogenic, intrinsically resistant against disinfectant, biofilm forming, and corrosion causing bacteria. It was also identified that stagnation is the key factor responsible for stability and persistence of these prokaryotic communities in hospital water system. In summary, prokaryotic communities present in engineered water system support growth of *Legionella*. Furthermore, temporary, and permanent stagnation are important factors responsible for persistence of these microbial communities in engineered water systems. The standard guidelines do not discuss how to manage temporary stagnation for management of microbial communities.

11.2 Recommendations for management of legionelloses

This research work investigated *Legionella* and explored the role of various biotic and abiotic factors influencing *Legionella* presence in engineered water systems from the public health point of view. For proper management and control of any possible *Legionella* outbreak following recommendations must be considered:

1. For proper surveillance of *Legionella* in engineered water systems of healthcare buildings, VBNC cells must be detected and quantified. Alternatively, standard qPCR detection assays could be used but acknowledgement must be made that this may be an over estimation due to amplification of DNA from dead cells.
2. To control colonization of *Legionella* in engineered water systems, free-living amoebae, especially *V. vermiformis* and *Acanthamoebae*, should be included in regular surveillance and considered when designing future disinfection strategies.
3. Although high HPC counts have not been directly linked to any known legionellosis outbreaks, HPC was found to be significantly associated with the quantity of alive *Legionella* in engineered water systems. This supports the current guidelines that use HPC as an indicator of building water quality. Current guidelines (such as the EWGLI) that do not currently include recommendations around HPC and building water quality should be updated.
4. Stagnation is a key risk factor associated with persistence and growth of *Legionella*. Current guidelines need to place a greater emphasis on the need for regular flushing of outlets to prevent temporary stagnation and increased risk of *Legionella.*

11.3 Future research

The data presented within this research work call for the following further research:

1. *Legionella* is intrinsically resistant against disinfection treatments and transforms into VBNC sate during environmental stresses. Therefore, further molecular studies are required to investigate the mechanisms of intrinsic resistance and survival strategies of VBNC state.
2. In environmental samples the concentration of *Legionella* varies. Molecular methods are highly sensitive and can quantify very low concentrations of *Legionella*. However, the infectious dose of *Legionella* in engineered water systems is not known. Understanding the infectious dose is required for proper management of *Legionella* and to identify the potential sites of any possible disease outbreaks.
3. Free-living amoebae are the primary hosts and reservoirs of *Legionella* in engineered water systems. Therefore, further studies are required to investigate the role of different biotic and abiotic factors such as microbial communities, flow dynamics, and disinfectants in persistence and survival of free-living amoebae in engineered water systems.

11.4 Conclusions

Legionella is an important bacterial pathogen of public health significance. In engineered water systems it is associated with free-living amoebae, biofilms, stagnation, and flow dynamics. As a protection strategy under unfavourable conditions, it transforms into the highly infectious viable but nonculturable (VBNC) state. Current surveillance methods are relying on classical culturing and molecular detection assays that are unable to detect VBNC *Legionella*. Therefore, surveillance of VBNC *Legionella* in engineered water systems should be included in standard guidelines. Similarly, the current recommendations for *Legionella* risk management in engineered water systems provide limited advice about temporary stagnation and free-living amoebae. These factors are interlinked and support growth and recolonization of *Legionella*. Therefore, multidimensional approaches are required for the control and management of *Legionella* in engineered water systems. This should include the prevention of water stagnation, in combination with additional disinfection strategies. Future water management protocols should incorporate treatment strategies to control free-living amoebae and microbial communities to reduce the risk to end users.

12 Appendix-1

Supplementary data of manuscript entitled “Detection and quantification of viable but non-culturable (VBNC) *Legionella pneumophila* from water samples using flow cytometry-cell sorting and quantitative PCR” (**Section 4**).

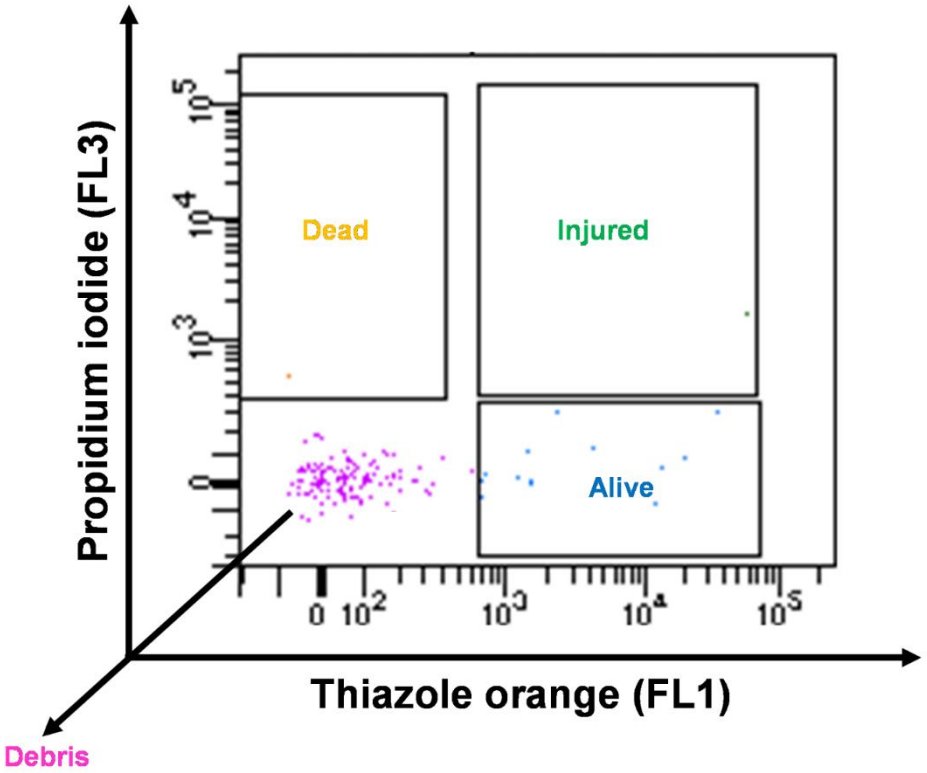

Figure 12.1: Cytogram represent “staining buffer” stained with “thiazole orange” and “propidium iodide” dyes.

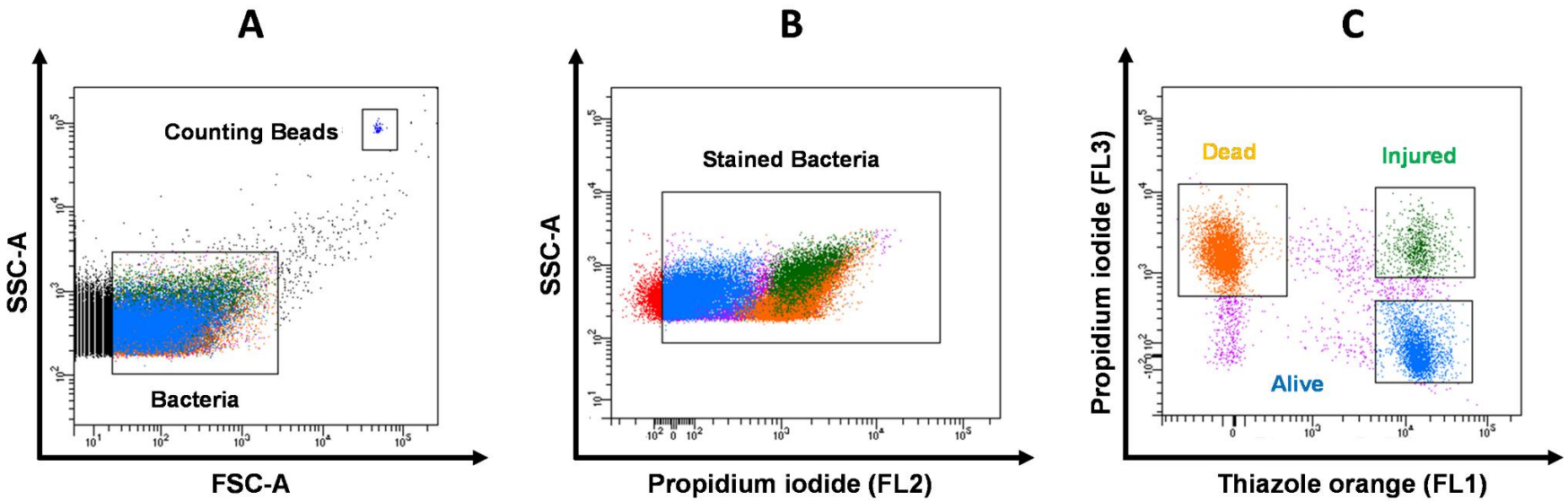

Figure 12.2: Flow cytometry of *L. pneumophila* grown on BCYE-GVPC agar at 37°C. A: Discrimination of bacterial population based on forward (FSC) and side scatter (SSC) parameters. B and C: Cytograms represent three different bacterial populations: thiazole orange-stained alive (blue), thiazole orange-stained injured (green) and propidium iodide-stained dead (orange) cells.

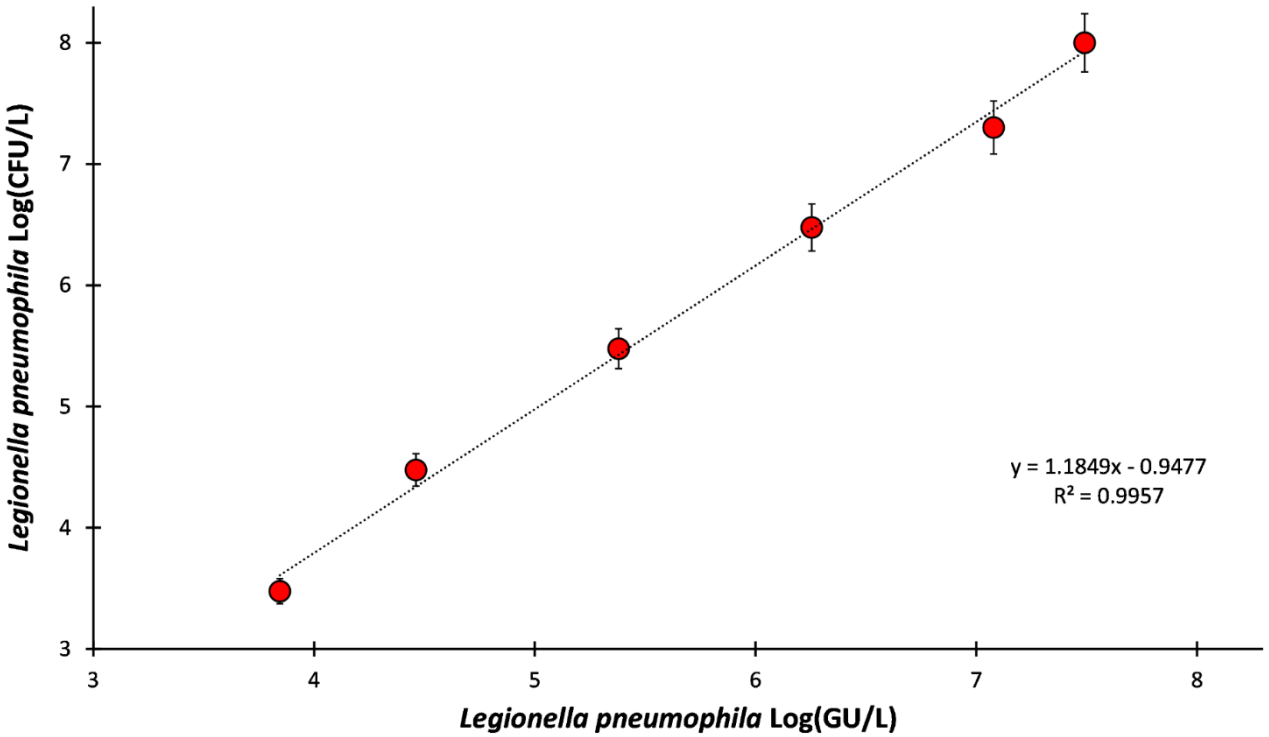

Figure 12.3: Relationship between *L. pneumophila* genomic unit (GU/L) enumerated using qPCR and colony forming units (CFU/L) enumerated using culture. Six different dilutions of pure *L. pneumophila* culture (concentrations ranging from 10^3 to 10^8 CFU/L) were processed according to ISO/TS12869:2019 and ISO11731:2017-05.

Table 12.1: Comparison of Legiolert kit, standard *Legionella* culturing and qPCR assays, amoebae co-culture assay and "viability based flow cytometry-cell sorting and qPCR" assay on two environmental samples.

Sample	Standard Culturing	Legiolert kit	Amoebae coculture assay	Standard qPCR assay	Viability based flow cytometry-cell sorting and qPCR assay
Shower water 1	Negative	Negative	Positive	3.9×10^4 GU/L	3.2×10^3 GU/L
Shower water 2	Negative	Negative	Positive	3.5×10^4 GU/L	1×10^3 GU/L

13 Appendix-2

Supplementary data of manuscript entitled “Molecular screening and characterization of *Legionella pneumophila* associated free-living amoebae from domestic and hospital water systems” (**Section 6**).

Table 13.1: Sequences of oligos and fluorogenic probes.

Name	Sequence and fluorogenic signal (5´-3´)	Assay conditions
***Legionella* 16S rDNA gene specific qPCR primers and probe (International Organization for Standardization, 2019)**		
Forward Primer	GGAGGGTTGATAGGTTAAGAGCT	Step 1: one cycle of 95°C/3 min Step 2: 43 cycles of 95°C/20 s and 60°C/60 s
Reverse Primer	CCAACAGCTAGTTGACATCGTTT	
Probe	6 FAM–AGTGGCGAAGGCGGCTACCT–Iowa Black FQ	
***L. pneumophila mip* gene specific qPCR primers and probe (International Organization for Standardization, 2019)**		
Forward Primer	CCGATGCCACATCATTAGC	Step 1: one cycle of 95°C/3 min Step 2: 43 cycles of 95°C/20 s and 60°C/60 s
Reverse Primer	CCAATTGAGCGCCACTCATAG	
Probe	6 FAM–TGCCTTTAGCCATTGCTTCCG–Iowa Black FQ	
***Acanthamoeba* 18S rDNA gene specific qPCR primers and probe (Qvarnstrom et al., 2006)**		
Forward Primer	CCCAGATCGTTTACCGTGAA	Step 1: one cycle of 95°C/3 min Step 2: 40 cycles of 95°C/20 s and 63°C/60 s
Reverse Primer	TAAATATTAATGCCCCCAACTATCC	
Probe	6 FAM–CTGCCACCGAATACATTAGCATGG–Iowa Black FQ	
***V. vermiformis* 18S rDNA gene specific qPCR primers and probe (Scheikl et al., 2016)**		
Forward Primer	TAACGATTGGAGGGCAAGTC	Step 1: one cycle of 95°C/5 min Step 2: 45 cycles of 95°C/20 s and 60°C/60 s
Reverse Primer	ACGCCTGCTTTGAACACTCT	
Probe	6 FAM–TGGGGAATCAACCGCTAGGA–Iowa Black FQ	
Eukaryotic 18S rRNA universal primers (Moreno et al., 2018)		
Forward Primer	GCCGCGGTAATTCCAGCTC	Step 1: one cycle of 95°C/5 min Step 2: 28 cycles of 98°C/30 s, 57°C/30 s and 72°C/30 s Step 3: one cycle of 72°C/5 min
Reverse Primer	CYTTCGYYCTTGATTRA	
***Legionella* 16S rDNA gene specific fluorescence *in situ* hybridisation probe (Manz et al., 1995)**		
LEG705	Alexa Fluor 488-CTGGTGTTCCTTCCGATC	Hybridization: 55 ± 1°C/100 min
Eukaryotic 18S rDNA gene specific fluorescence *in situ* hybridisation probe (Lim et al., 1993)		
EUK1209	Alexa Fluor 647-GGGCATCACAGACCTG	Hybridization: 55 ± 1°C/100 min

Table 13.2: Physical, environmental, and climatic data for samples.

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
1	Domestic	Shower	Water	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
2	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	5 to 9	2 to 10/day
3	Domestic	Shower	Water	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
4	Domestic	Shower	Water	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
5	Domestic	Shower	Water	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
6	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	5 to 9	2 to 10/day
7	Domestic	Shower	Water	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
8	Domestic	Shower	Water	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
9	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	5 to 9	2 to 10/day
10	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	5 to 9	2 to 10/day
11	Domestic	Shower	Water	October 2020	-	-	Electric	No	-	Less than 5	2 to 10/day
12	Domestic	Shower	Water	March 2020	10.5	20.6	-	-	-	-	-
13	Domestic	Shower	Water	March 2020	10.5	20.6	-	-	-	-	-
14	Domestic	Shower	Water	October 2020	12	21.9	Gas hot water	No	More than 20	More than 20	2/day
15	Domestic	Shower	Water	October 2020	12	21.9	Gas hot water	No	More than 20	More than 20	1/day
16	Domestic	Shower	Water	October 2020	-	-	-	-	-	-	-
17	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	Less than 5	2/day
18	Domestic	Shower	Water	April 2020	11.3	22.3	Gas hot water	No	More than 20	-	2 to 10/day
19	Domestic	Shower	Water	November 2020	10.2	29.8	Gas hot water	No	Less than 5	Less than 5	2/day

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
20	Domestic	Shower	Water	November 2020	10.2	29.8	Gas hot water	No	Less than 5	Less than 5	Less than 1/month
21	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	Less than 5	2 to 10/day
22	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	Less than 5	2 to 10/day
23	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	Less than 5	2 to 10/day
24	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	Less than 5	2 to 10/day
25	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	Less than 5	2 to 10/day
26	Domestic	Shower	Water	July 2020	6.8	15.1	Gas hot water	No	More than 20	Less than 5	2 to 10/day
27	Domestic	Shower	Water	September 2020	11	20.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
28	Domestic	Shower	Water	September 2020	11	20.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
29	Domestic	Shower	Water	September 2020	11	20.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
30	Domestic	Shower	Water	September 2020	11	20.5	Gas hot water	No	More than 20	5 to 9	2 to 10/day
31	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	More than 20	Less than 5	2/day
32	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	More than 20	Less than 5	1/week
33	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	Less than 5	Less than 5	Less than 1/month
34	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	Less than 5	Less than 5	Less than 1/month
35	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	Less than 5	Less than 5	2/day
36	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	More than 20	5 to 9	1/week
37	Domestic	Shower	Water	October 2020	12	21.2	Gas hot water	No	More than 20	5 to 9	2/day
38	Domestic	Shower	Water	March 2020	10.5	20.6	Electric	Yes	More than 20	10 to 14	1/week
39	Domestic	Shower	Water	March 2020	10.5	20.6	Electric	Yes	More than 20	10 to 14	1/day

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
40	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	Less than 5	1/day
41	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	Less than 5	1/day
42	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	Less than 5	1/day
43	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	Less than 5	Less than 1/month
44	Domestic	Shower	Water	April 2020	-	-	Solar hot water	Yes	More than 20	Less than 5	2/day
45	Domestic	Shower	Water	April 2020	12.7	21.1	Solar hot water	Yes	More than 20	Less than 5	2/day
46	Domestic	Shower	Water	October 2020	-	-	-	-	More than 20	More than 20	2 to 10/day
47	Domestic	Shower	Water	April 2020	-	-	Electric	Yes	-	-	1/day
48	Domestic	Shower	Water	April 2020	12.8	21.4	Gas hot water	No	More than 20	More than 20	1/day
49	Domestic	Shower	Water	April 2020	9.2	18.6	Electric	Yes	More than 20	Less than 5	1/day
50	Domestic	Shower	Water	April 2020	9.2	18.6	Electric	Yes	More than 20	Less than 5	Less than 1/month
51	Domestic	Shower	Water	April 2020	9.2	18.6	Electric	Yes	More than 20	Less than 5	1/day
52	Domestic	Shower	Water	March 2020	12.7	24.9	Gas hot water	No	5 to 9	5 to 9	2 to 10/day
53	Domestic	Shower	Water	March 2020	12.7	24.9	Gas hot water	No	5 to 9	5 to 9	Less than 1/month
54	Domestic	Shower	Water	October 2020	9.3	18.3	Electric	Yes	5 to 9	5 to 9	1/day
55	Domestic	Shower	Water	October 2020	9.3	18.3	Electric	Yes	5 to 9	5 to 9	1/day
56	Domestic	Shower	Water	October 2020	9.3	18.3	Electric	Yes	5 to 9	5 to 9	1/day
57	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	10 to 14	2 to 10/day
58	Domestic	Shower	Water	October 2020	-	-	Gas hot water	No	More than 20	10 to 14	1/day

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
59	Domestic	Shower	Water	March 2020	-	-	Gas hot water	-	-	Less than 5	2 to 10/day
60	Domestic	Tap faucet	Biofilm	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	More than 10/day
61	Domestic	Tap faucet	Biofilm	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	More than 10/day
62	Domestic	Tap faucet	Biofilm	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	More than 10/day
63	Domestic	Tap faucet	Biofilm	February 2020	16.3	25.5	Gas hot water	No	More than 20	5 to 9	More than 10/day
64	Domestic	Tap faucet	Biofilm	October 2020	11.8	21.4	Gas hot water	No	More than 20	10 to 14	-
65	Domestic	Tap faucet	Biofilm	October 2020	11.8	21.4	Gas hot water	No	More than 20	10 to 14	-
66	Domestic	Tap faucet	Biofilm	October 2020	11.8	21.4	Gas hot water	No	More than 20	10 to 14	-
67	Domestic	Tap faucet	Biofilm	October 2020	11.8	21.4	Gas hot water	No	More than 20	10 to 14	-
68	Domestic	Tap faucet	Biofilm	February 2020	16.3	25.5	Gas hot water	No	More than 20	10 to 14	More than 10/day
69	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
70	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	2 to 10/day
71	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
72	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
73	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	1/day
74	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
75	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
76	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
77	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
78	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	2 to 10/day
79	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
80	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
81	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
82	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
83	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	More than 10/day
84	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
85	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
86	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
87	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	2 to 10/day
88	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
89	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
90	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
91	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
92	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	1/day
93	Hospital	Basin	Water	March 2021	17.3	22.9	Electric	-	-	-	-
94	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	More than 10/day

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
95	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
96	Hospital	Shower	Water	March 2021	17.3	22.9	Electric	-	-	-	-
97	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
98	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
99	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	-
100	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
101	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
102	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
103	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	-
104	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
105	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	1/day
106	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
107	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
108	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
109	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
110	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	More than 10/day
111	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	More than 10/day
112	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	More than 10/day
113	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
114	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
115	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
116	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
117	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
118	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
119	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
120	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	1/day
121	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	-
122	Hospital	Basin	Water	April 2021	15	22.7	Electric	-	-	-	-
123	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
124	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	More than 10/day
125	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	-
126	Hospital	Shower	Water	April 2021	15	22.7	Electric	-	-	-	2 to 10/day
127	Hospital	Tap faucet	Biofilm	March 2021	17.3	22.9	Electric	-	-	-	-
128	Hospital	Tap faucet	Biofilm	March 2021	17.3	22.9	Electric	-	-	-	-
129	Hospital	Tap faucet	Biofilm	March 2021	17.3	22.9	Electric	-	-	-	-
130	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
131	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
132	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
133	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
134	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
135	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
136	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
137	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
138	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-

Sample ID	Sampling Details				Temperature*		Features of Plumbing System				
	Building type	Shower or basin	Sample type	Collection month	Mini (°C)	Max (°C)	Water heating system	Hot water storage	Age of building (Years)	Age of water heating system (Years)	Outlet usage
139	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-
140	Hospital	Tap faucet	Biofilm	April 2021	15	22.7	Electric	-	-	-	-

14 Appendix-3

Supplementary data of manuscript entitled “Once-a-day flushing of a model plumbing system significantly reduced colonisation of *Legionella*” (**Section 8**).

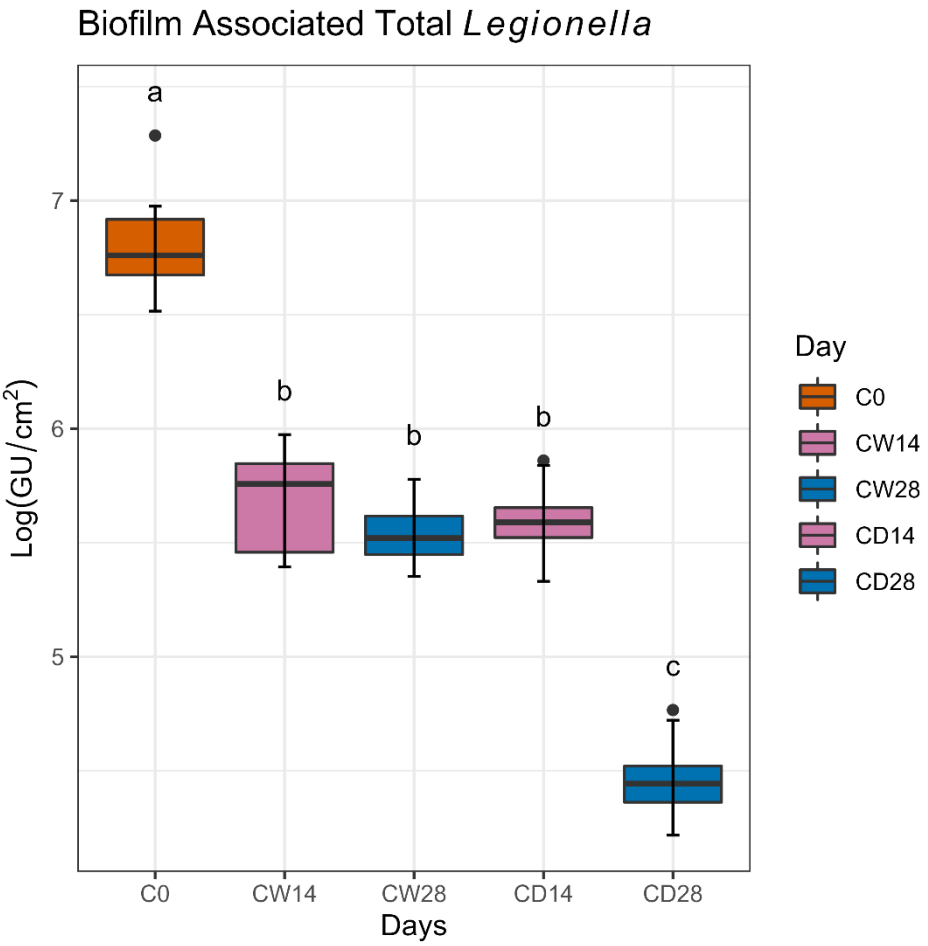

Figure 14.1: Impact of the flushing frequency on biofilm-associated total *Legionella* quantified by the qPCR assay. The log transformed data are presented as mean ± standard deviation of nine to eighteen replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing- day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

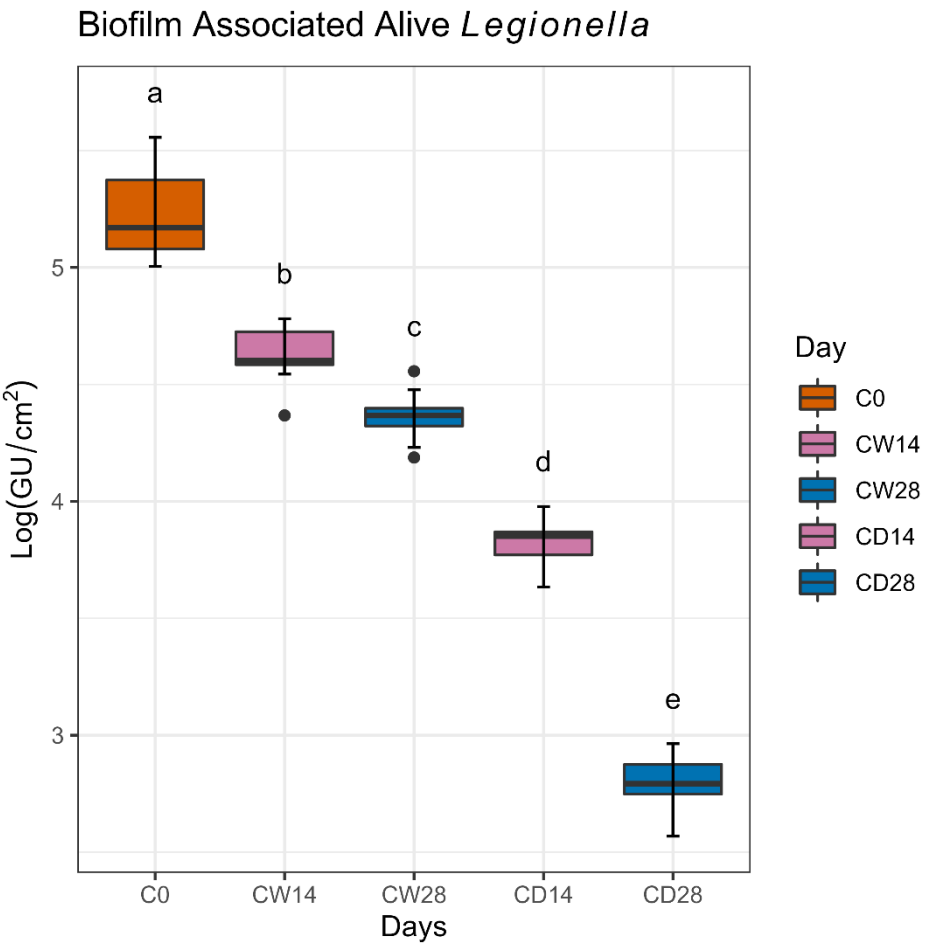

Figure 14.2: Impact of the flushing frequency on biofilm-associated alive *Legionella* (potentially culturable) quantified by a flow cytometry-cell sorting and subsequent qPCR assay. The log transformed data is presented as mean ± standard deviation of nine to eighteen replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing- day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

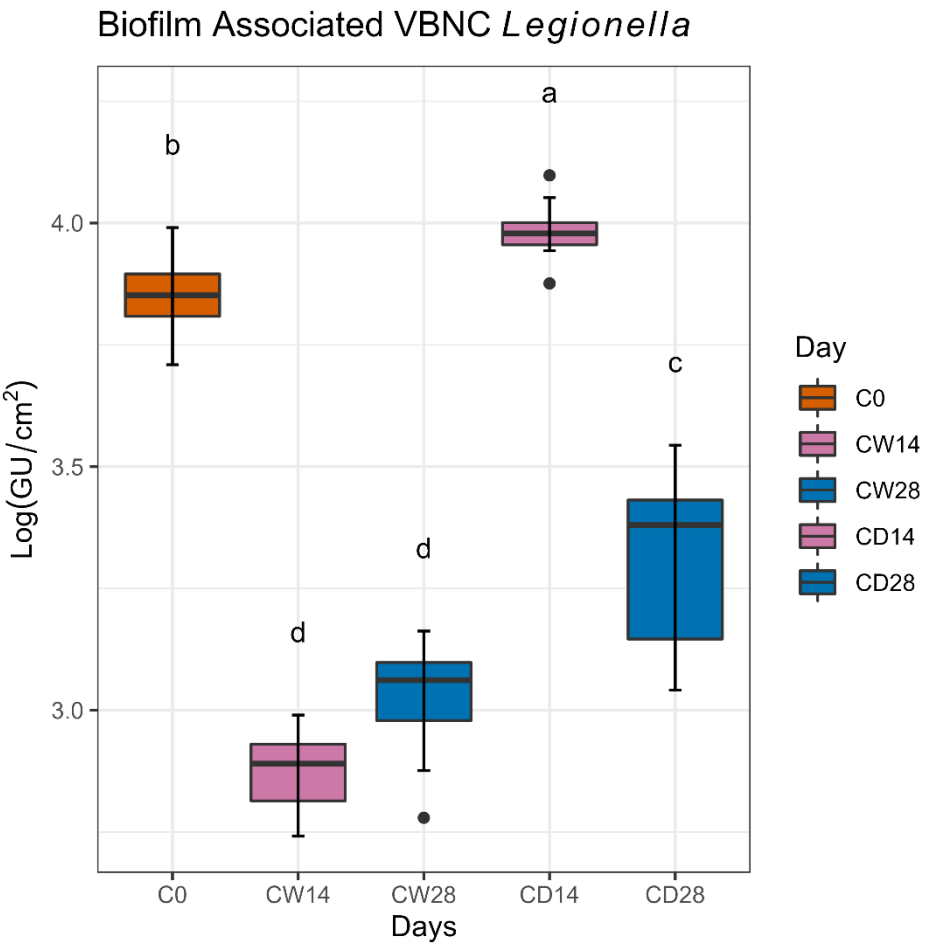

Figure 14.3: Impact of the flushing frequency on biofilm-associated VBNC *Legionella* quantified by "flow cytometry-cell sorting and qPCR assay". The log transformed data is presented as mean ± standard deviation of nine to eighteen replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing-day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

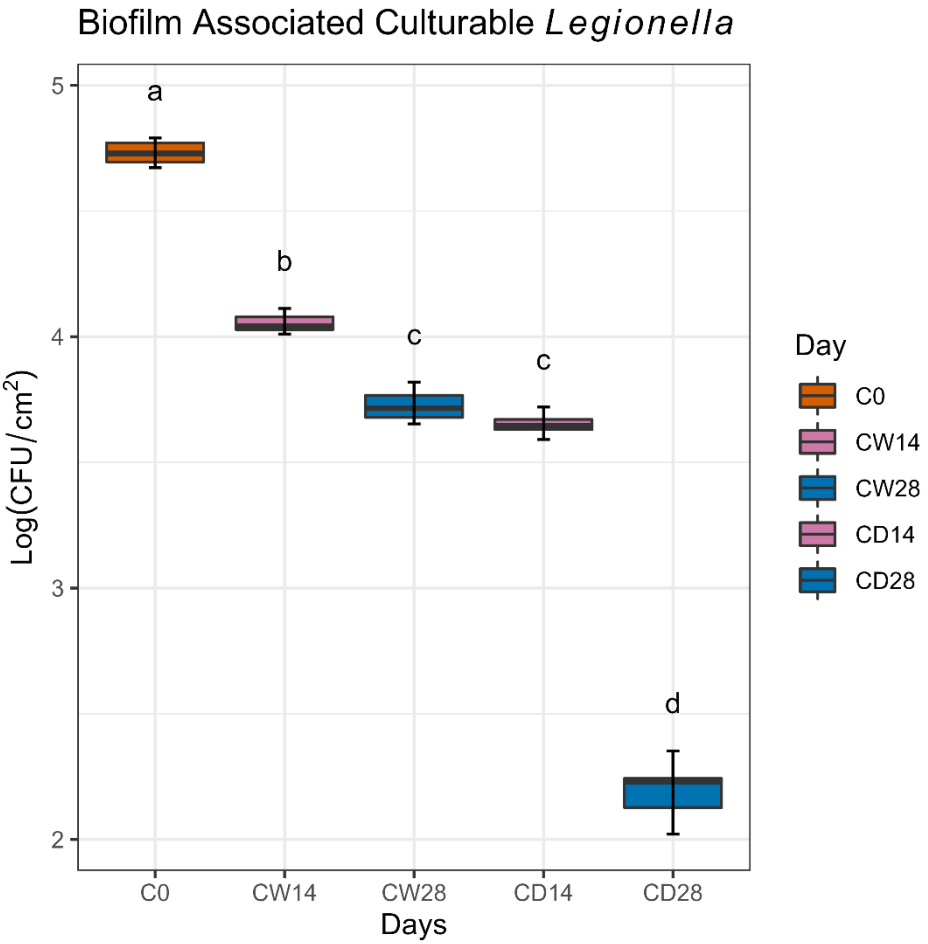

Figure 14.4: Impact of the flushing frequency on biofilm-associated culturable *Legionella* quantified by "standard culturing assay". The log transformed data is presented as mean ± standard deviation of six to twelve replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing- day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

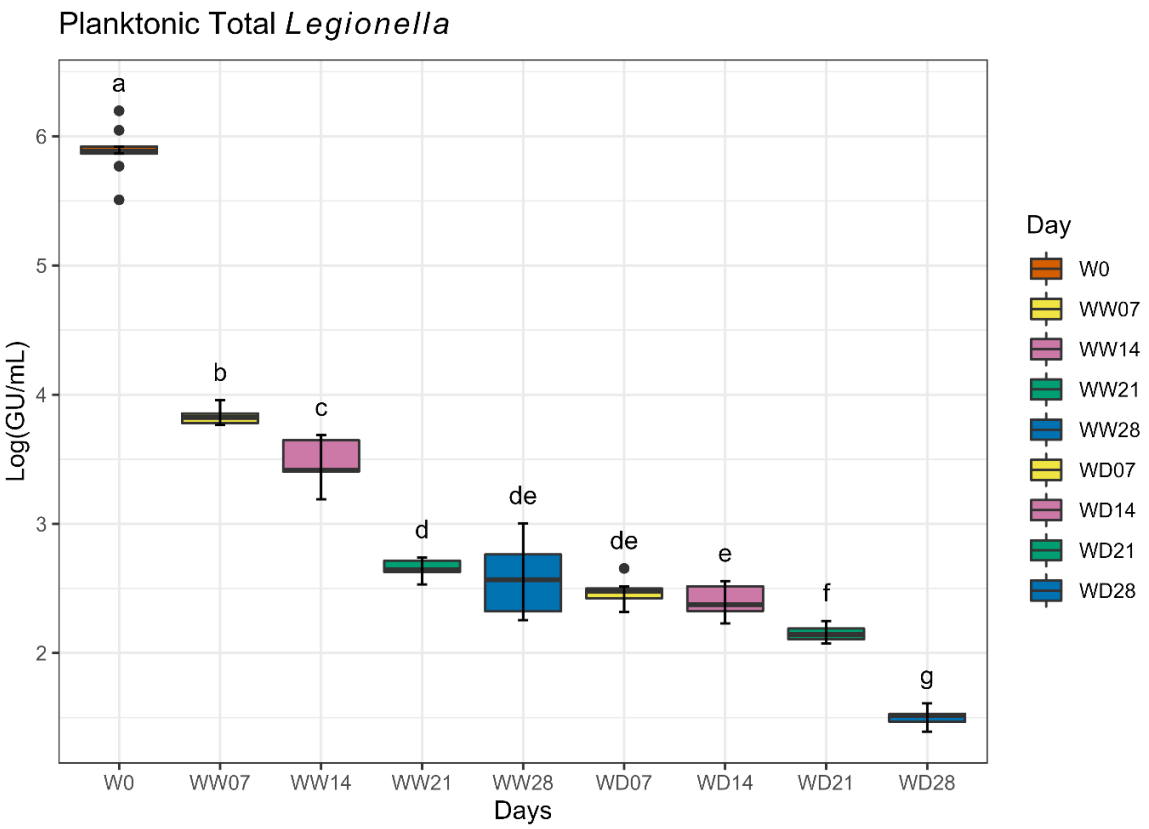

Figure 14.5: Impact of the flushing frequency on planktonic total *Legionella* quantified by "qPCR assay". The log transformed data is presented as mean ± standard deviation of nine replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing- day 07 sampling, WD14: once-a-day flushing-day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

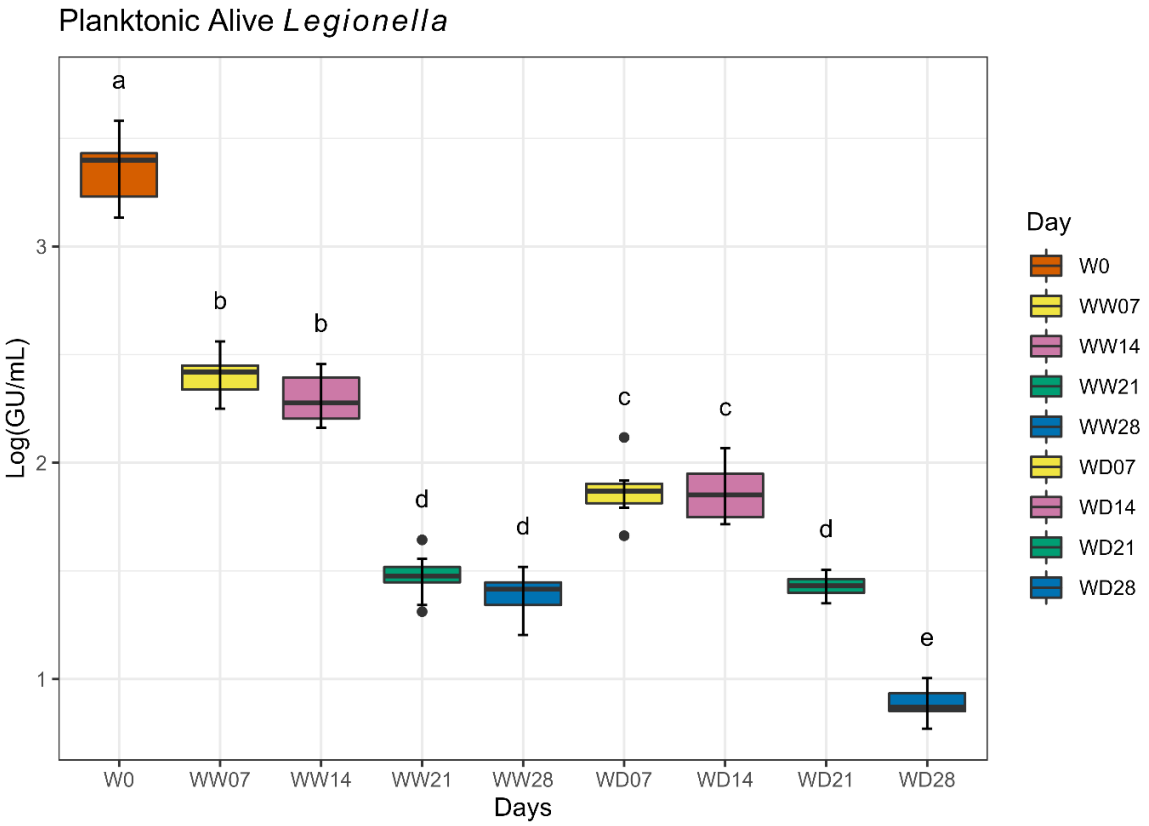

Figure 14.6: Impact of the flushing frequency on planktonic alive *Legionella* (potentially culturable) quantified by "flow cytometry-cell sorting and qPCR assay". The log transformed data is presented as mean ± standard deviation of nine replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing-day 07 sampling, WD14: once-a-day flushing- day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

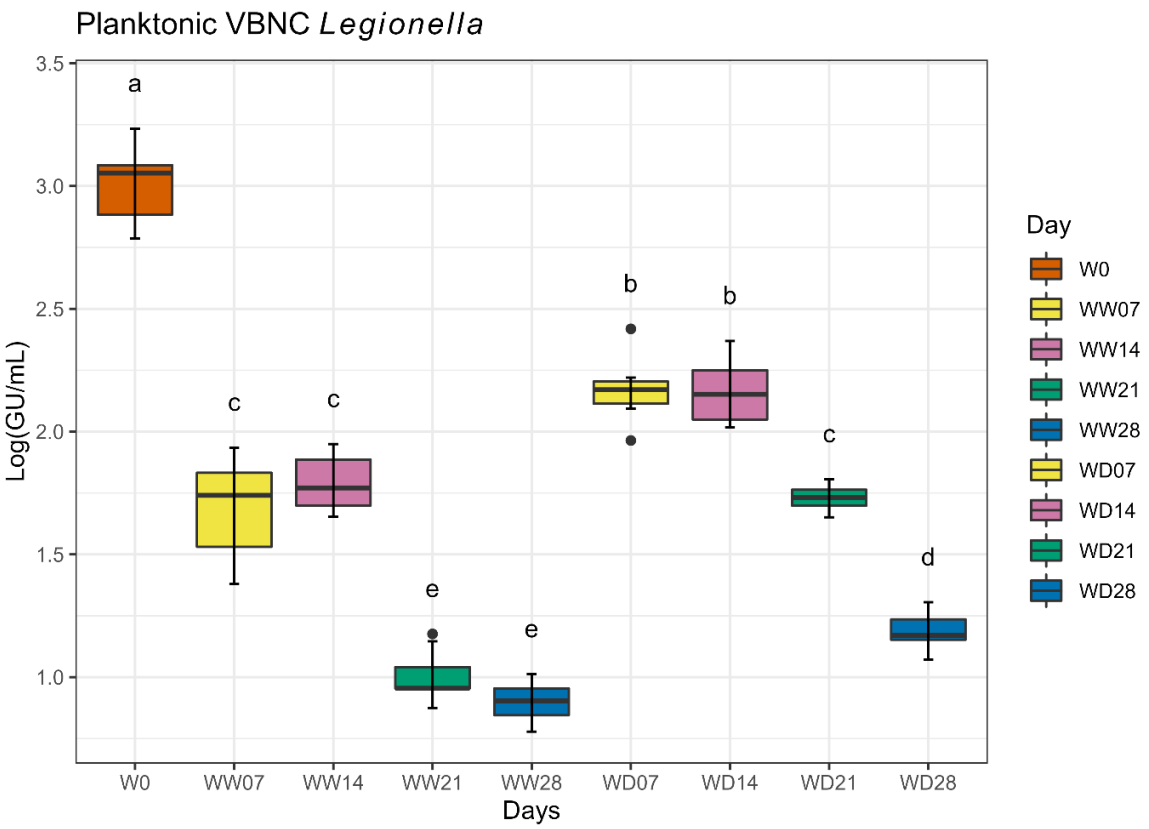

Figure 14.7: Impact of the flushing frequency on planktonic VBNC *Legionella* quantified by "flow cytometry-cell sorting and qPCR assay". The log transformed data is presented as mean ± standard deviation of nine replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing- day 07 sampling, WD14: once-a-day flushing- day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

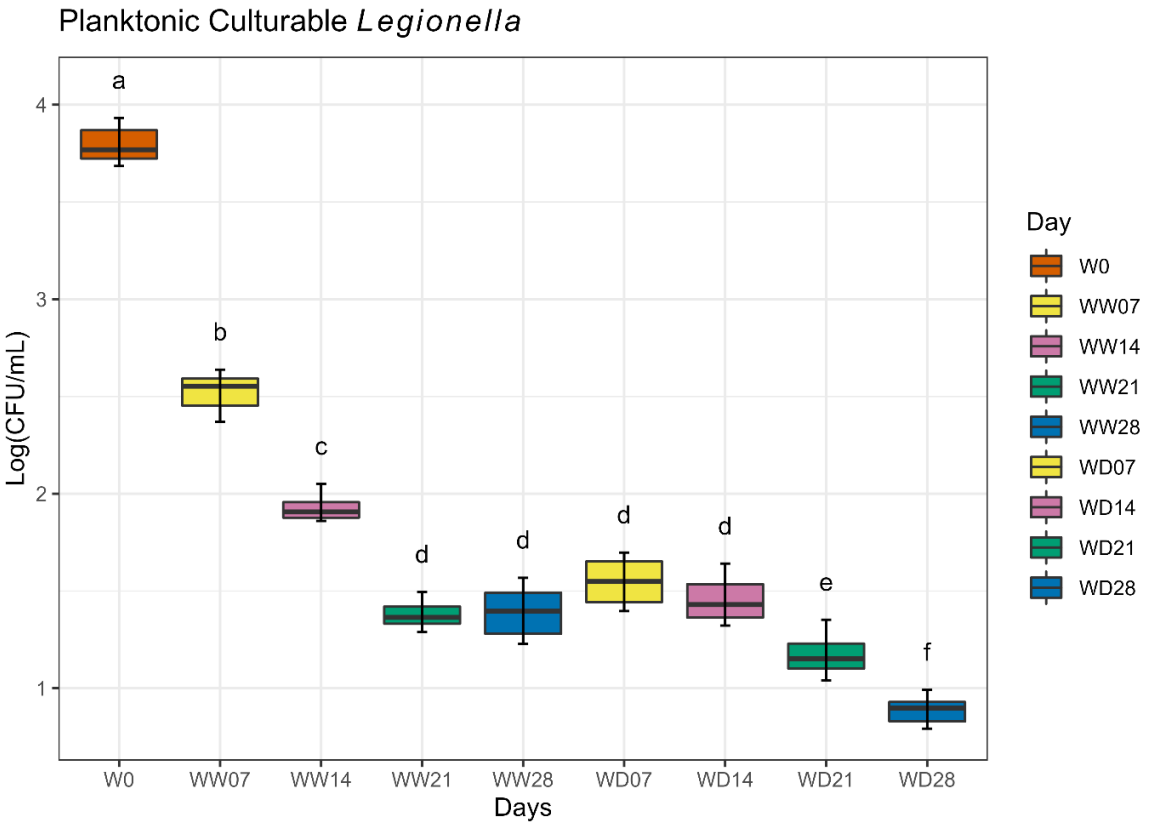

Figure 14.8: Impact of the flushing frequency on planktonic culturable *Legionella* quantified by "standard culturing assay". The log transformed data is presented as mean ± standard deviation of six replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing- day 07 sampling, WD14: once-a-day flushing- day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

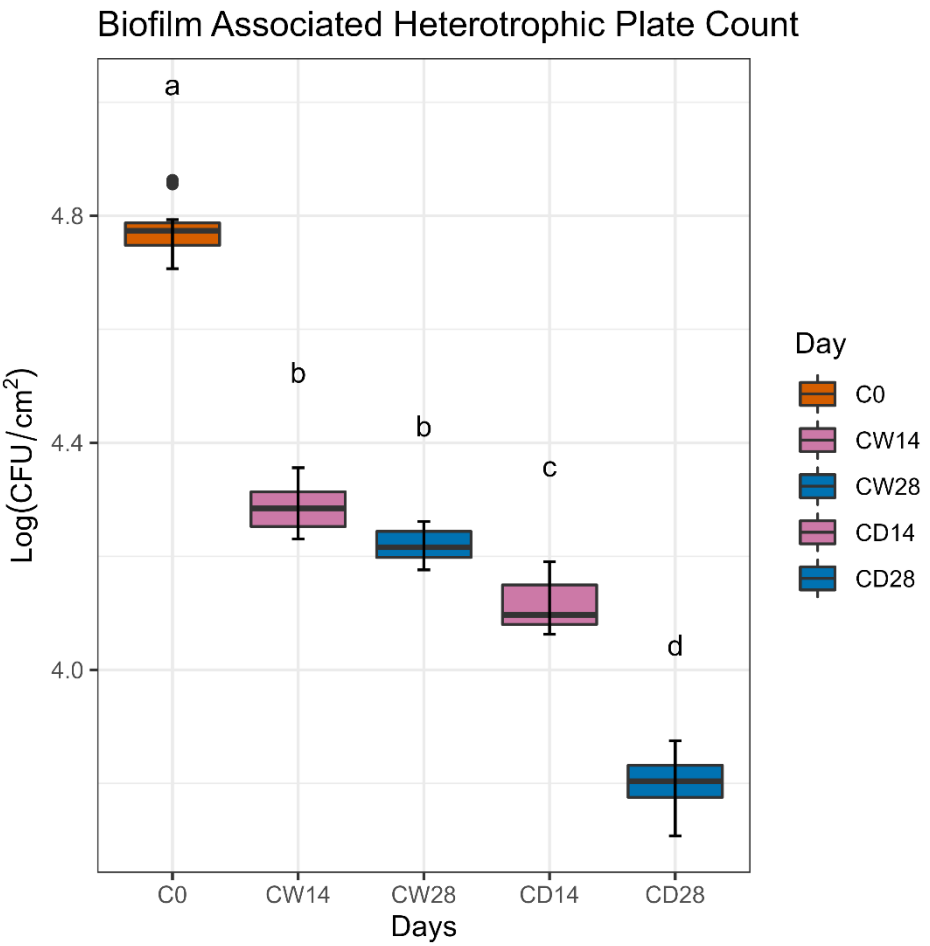

Figure 14.9: Impact of the flushing frequency on biofilm-associated culturable heterotrophic bacteria quantified by “culturing assay”. The log transformed data is presented as mean ± standard deviation of six to twelve replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing- day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

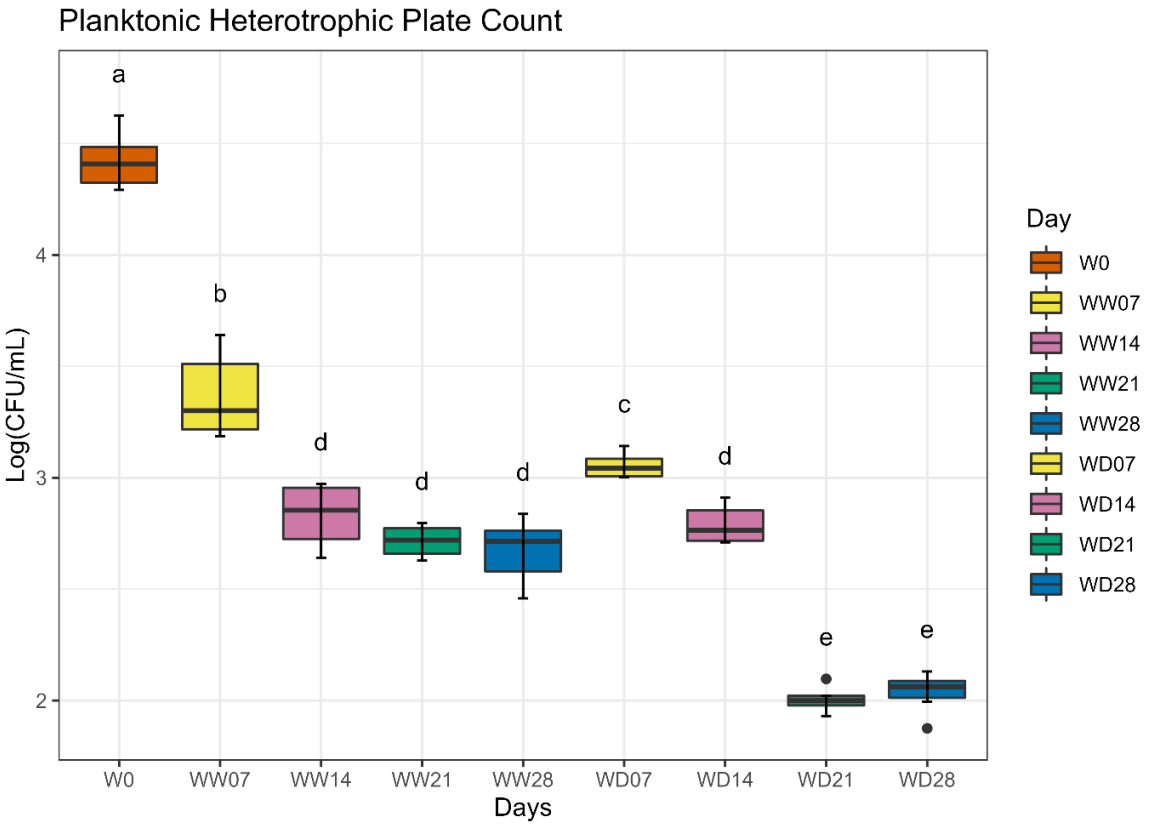

Figure 14.10: Impact of the flushing frequency on planktonic culturable heterotrophic bacteria by "culturing assay". The log transformed data is presented as mean ± standard deviation of six replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing- day 07 sampling, WD14: once-a-day flushing- day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

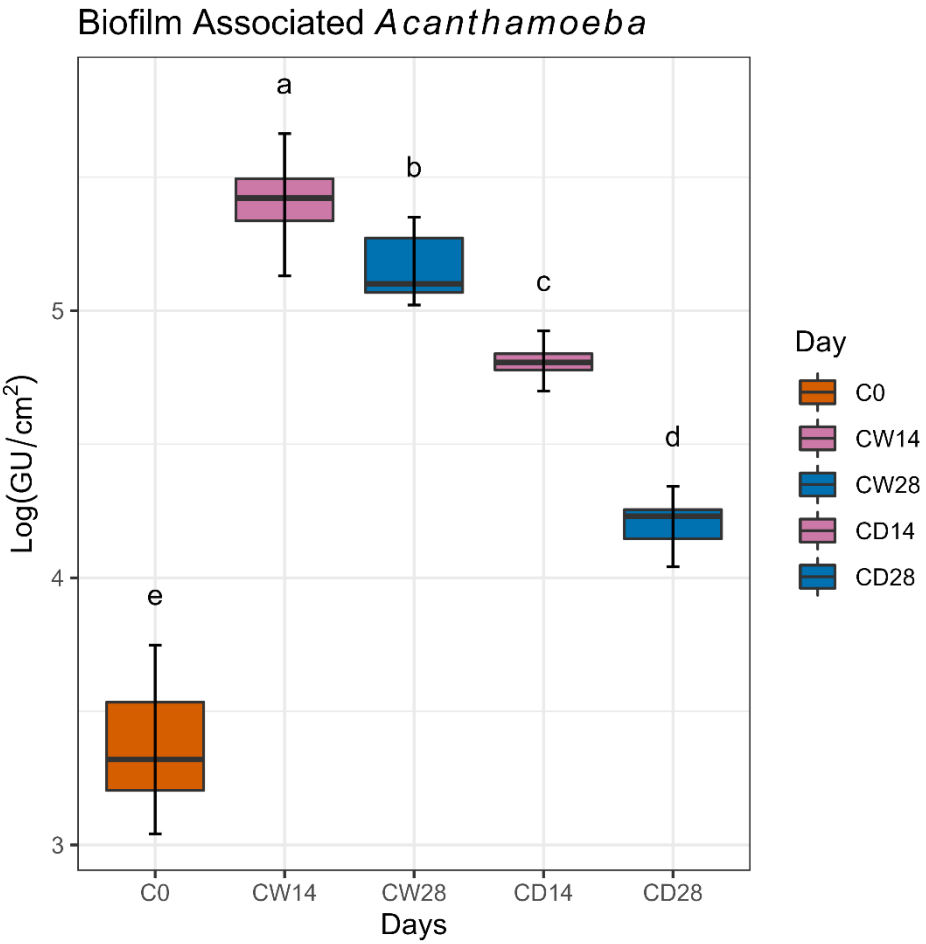

Figure 14.11: Impact of the flushing frequency on biofilm-associated *Acanthamoeba* quantified by "qPCR assay". The log transformed data is presented as mean ± standard deviation of nine to eighteen replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing- day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

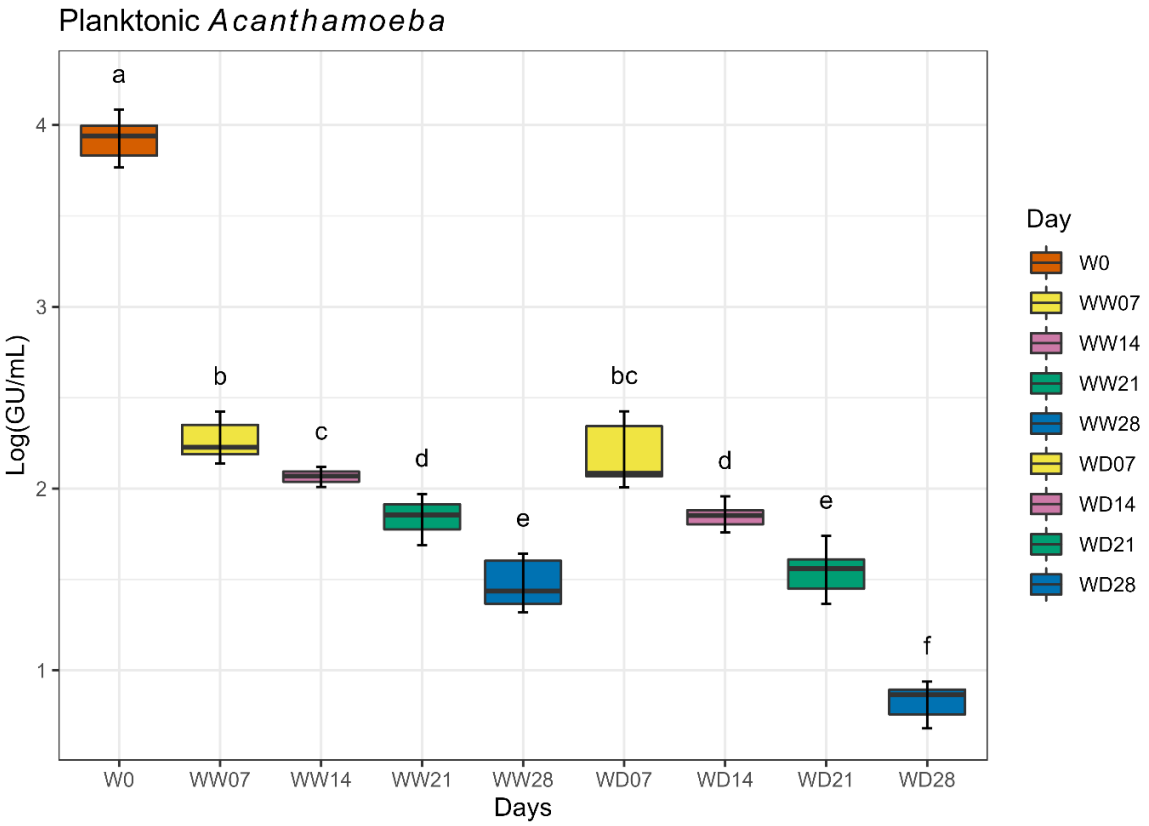

Figure 14.12: Impact of the flushing frequency on planktonic *Acanthamoeba* quantified by "qPCR assay". The log transformed data is presented as mean ± standard deviation of nine replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing- day 07 sampling, WD14: once-a-day flushing-day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

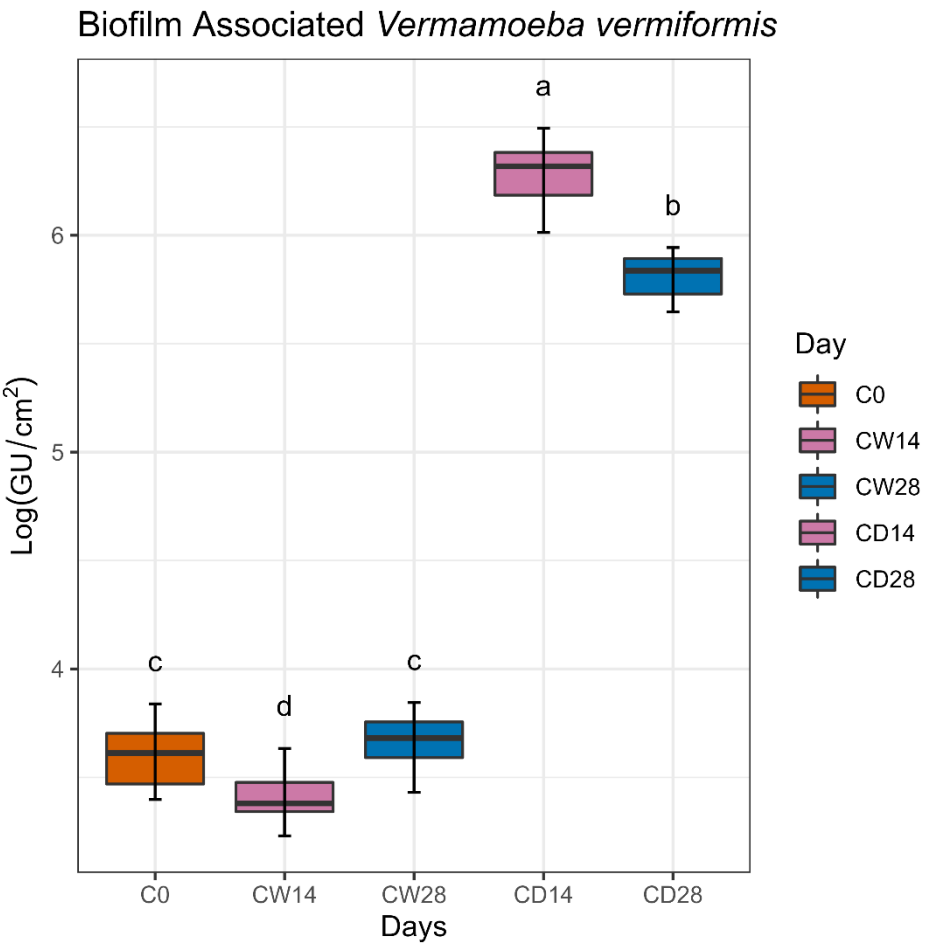

Figure 14.13: Impact of the flushing frequency on biofilm-associated *Vermamoeba vermiformis* quantified by “qPCR assay”. The log transformed data is presented as mean ± standard deviation of nine to eighteen replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. C0: colonization phase, CW14: once-a-week flushing-day 14 sampling, CW28: once-a-week flushing-day 28 sampling, CD14: once-a-day flushing- day 14 sampling, and CD28: once-a-day flushing- day 28 sampling.

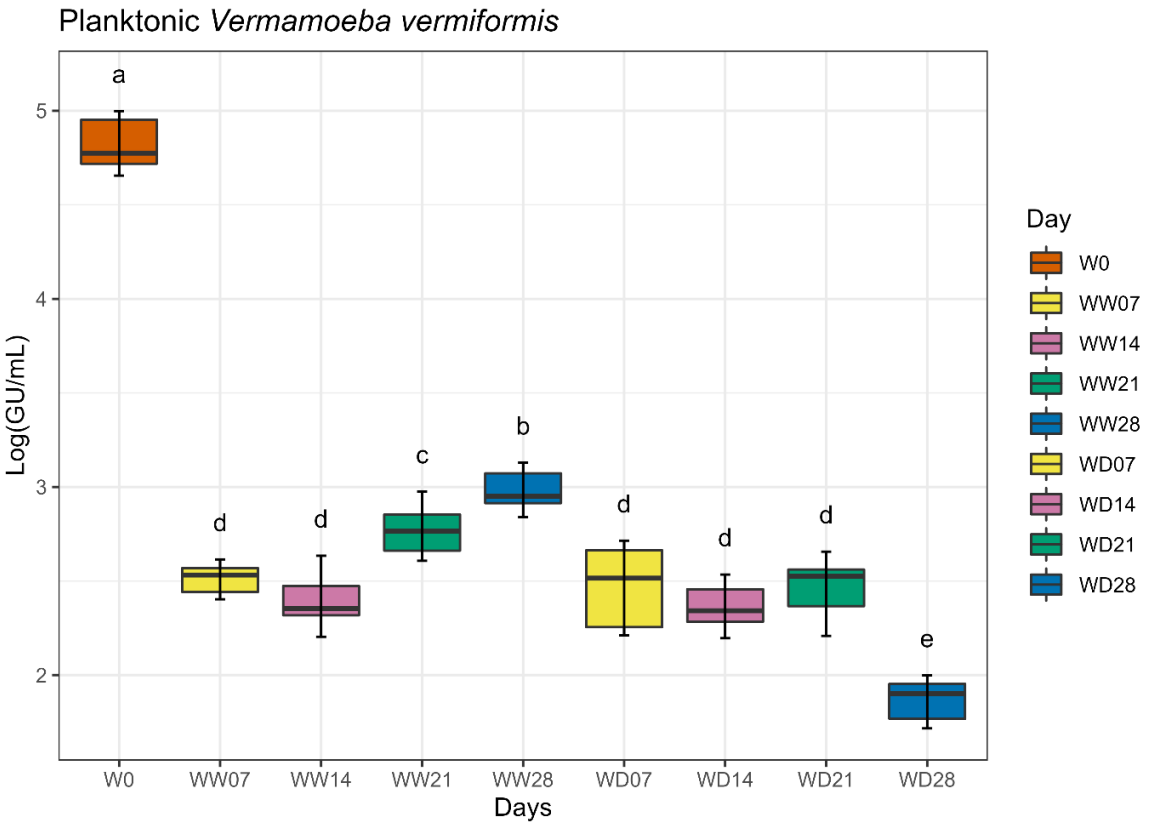

Figure 14.14: Impact of the flushing frequency on planktonic *Vermamoeba vermiformis* quantified by "qPCR assay". The log transformed data is presented as mean ± standard deviation of nine replicates. The same alphabetic letter represents statistical similarities at $p < 0.001$ according to Tukey's HSD test. W0: colonization phase, WW07: once-a-week flushing-day 07 sampling, WW14: once-a-week flushing-day 14 sampling, WW21: once-a-week flushing-day 21 sampling, WW28: once-a-week flushing-day 28 sampling, WD07: once-a-day flushing- day 07 sampling, WD14: once-a-day flushing-day 14 sampling, WD21: once-a-day flushing- day 21 sampling, and WD28: once-a-day flushing- day 28 sampling.

Table 14.1: Sequences of oligos and fluorogenic probes used in this study.

Name	Sequence and fluorogenic signal (5´ to 3´)	Assay conditions
***Legionella* 16S rDNA gene specific qPCR primers and probe (International Organization for Standardization, 2019)**		
Forward Primer	GGAGGGTTGATAGGTTAAGAGCT	Stage I: one cycle of 95°C for 3 min Stage II: 43 cycles of 95°C for 20 s and 60°C for 60 s
Reverse Primer	CCAACAGCTAGTTGACATCGTTT	
Probe	6 FAM–AGTGGCGAAGGCGGCTACCT–Iowa Black FQ	
***L. pneumophila mip* gene specific qPCR primers and probe (International Organization for Standardization, 2019)**		
Forward Primer	CCGATGCCACATCATTAGC	Stage I: one cycle of 95°C for 3 min Stage II: 43 cycles of 95°C for 20 s and 60°C for 60 s
Reverse Primer	CCAATTGAGCGCCACTCATAG	
Probe	6 FAM–TGCCTTTAGCCATTGCTTCCG–Iowa Black FQ	
***Acanthamoeba* 18S rDNA gene specific qPCR primers and probe (Qvarnstrom et al., 2006)**		
Forward Primer	CCCAGATCGTTTACCGTGAA	Stage I: one cycle of 95°C for 3 min Stage II: 40 cycles of 95°C for 20 s and 63°C for 60 s
Reverse Primer	TAAATATTAATGCCCCCAACTATCC	
Probe	6 FAM–CTGCCACCGAATACATTAGCATGG–Iowa Black FQ	
***V. vermiformis* 18S rDNA gene specific qPCR primers and probe (Scheikl et al., 2016)**		
Forward Primer	TAACGATTGGAGGGCAAGTC	Stage I: one cycle of 95°C for 5 min Stage II: 45 cycles of 95°C for 20 s and 60°C for 60 s
Reverse Primer	ACGCCTGCTTTGAACACTCT	
Probe	6 FAM–TGGGGAATCAACCGCTAGGA–Iowa Black FQ	
***Legionella* 16S rDNA gene specific fluorescence *in situ* hybridisation probe (Manz et al., 1995)**		
LEG705	Alexa Fluor 488-CTGGTGTTCCTTCCGATC	Hybridization: 55 ± 1°C for 100 min
Eubacterial 16S rDNA gene specific fluorescence *in situ* hybridisation probe (Amann et al., 1990)		
EUB338	Alexa Fluor 546-GCTGCCTCCCGTAGGAGT	Hybridization: 55 ± 1°C for 100 min
Eukaryotic 18S rDNA gene specific fluorescence *in situ* hybridisation probe (Lim et al., 1993)		
EUK1209	Alexa Fluor 647-GGGCATCACAGACCTG	Hybridization: 55 ± 1°C for 100 min

15 Appendix-4

Supplementary data of manuscript entitled “Water stagnation arising through intermitted usage associated with increased viable but non culturable *Legionella* and amoeba hosts in a hospital water distribution system” (**Section 9**).

Table 15.1: Sequences of oligos and fluorogenic probes used for qPCR assays.

Oligo name	Sequence and fluorogenic signal (5´→3´)	qPCR assay conditions
***Legionella* 16S rDNA gene specific qPCR primers and probe (International Organization for Standardization, 2019)**		
Forward oligo	GGAGGGTTGATAGGTTAAGAGCT	Step-I: one cycle of 95°C/3 min Step-II: 43 cycles of 95°C/20 s and 60°C/60 s
Reverse oligo	CCAACAGCTAGTTGACATCGTTT	
Probe	FAM–AGTGGCGAAGGCGGCTACCT–Q	
***L. pneumophila mip* gene specific qPCR primers and probe (International Organization for Standardization, 2019)**		
Forward oligo	CCGATGCCACATCATTAGC	Step-I: one cycle of 95°C/3 min Step-II: 43 cycles of 95°C/20 s and 60°C/60 s
Reverse oligo	CCAATTGAGCGCCACTCATAG	
Probe	FAM–TGCCTTTAGCCATTGCTTCCG–Q	
***Acanthamoeba* 18S rDNA gene specific qPCR primers and probe (Qvarnstrom et al., 2006)**		
Forward oligo	CCCAGATCGTTTACCGTGAA	Step-I: one cycle of 95°C/3 min Step-II: 40 cycles of 95°C/20 s and 63°C/60 s
Reverse oligo	TAAATATTAATGCCCCCAACTATCC	
Probe	FAM–CTGCCACCGAATACATTAGCATGG–Q	
***Vermamoeba vermiformis* 18S rDNA gene specific qPCR primers and probe (Scheikl et al., 2016)**		
Forward oligo	TAACGATTGGAGGGCAAGTC	Step-I: one cycle of 95°C/5 min Step-II: 45 cycles of 95°C/20 s and 60°C/60 s
Reverse oligo	ACGCCTGCTTTGAACACTCT	
Probe	FAM–TGGGGAATCAACCGCTAGGA–Q	

FAM: 6-carboxyfluorescein $\lambda_{(excitation)}/\lambda_{(emission)}$ 495/520 nm, channel for qPCR: $\lambda_{(source)}$ 470 nm and $\lambda_{(detector)}$ 510 nm; Q: Iowa Black® FQ quencher with absorbance spectrum range λ 420 nm to 620 nm with $\lambda_{(max)}$ 531 nm; s: seconds; min: minutes

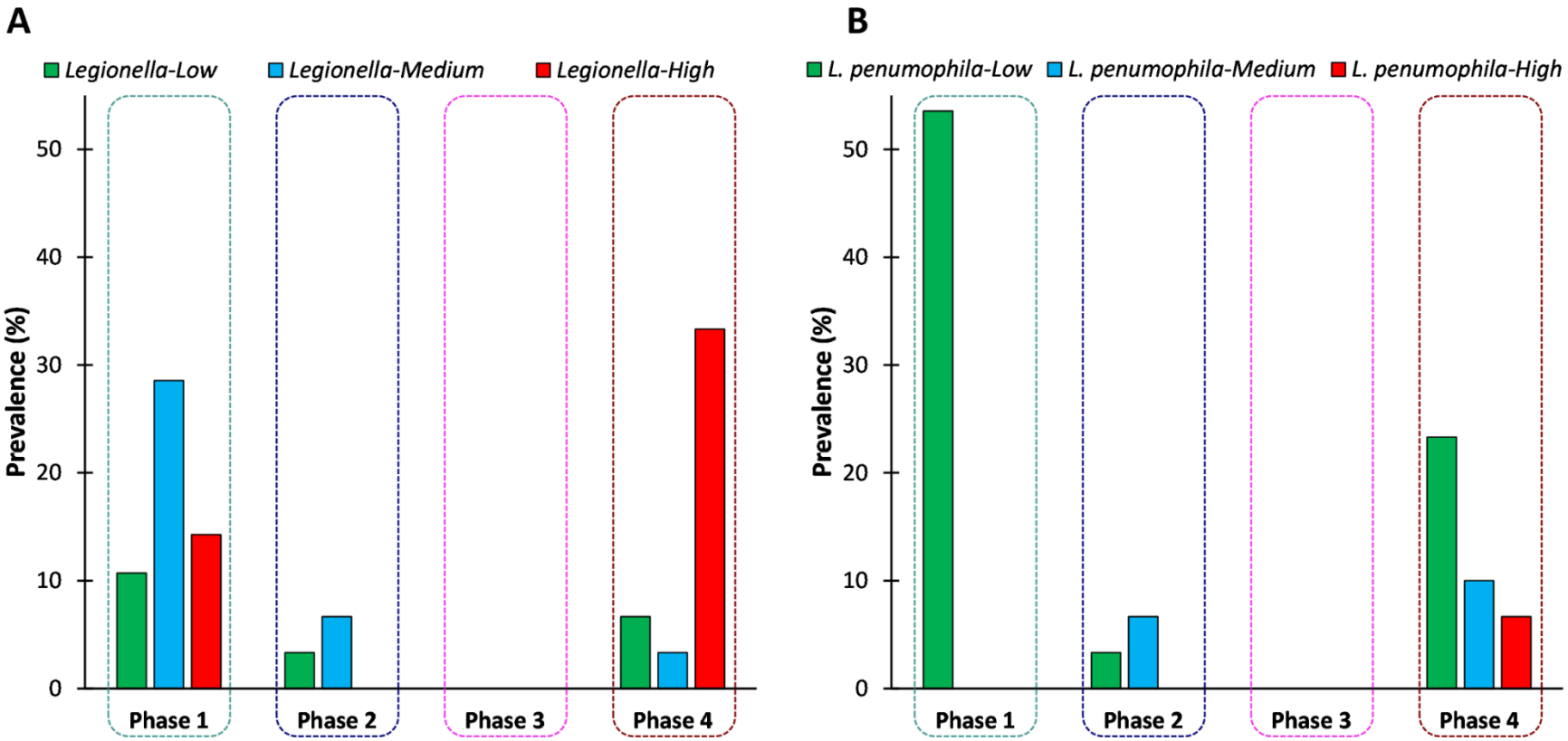

Figure 15.1: The percentage of water samples tested for VBNC *Legionella* (A) and VBNC *L. pneumophila* (B) with three levels of contaminations. The levels of VBNC cells were divided into three levels: low (< 10^3 GU/L, in green), medium (10^3 to 10^4 GU/L, in blue) and high (> 10^4 GU/L, in red) contamination. Total 120 water samples (hand basin and shower) were collected in March 2021 (phase 1), April 2021 (phase 2), November 2021 (phase 3), and June 2022 (phase 4). Y-axis represents % positive samples and X-axis represents sampling phases.

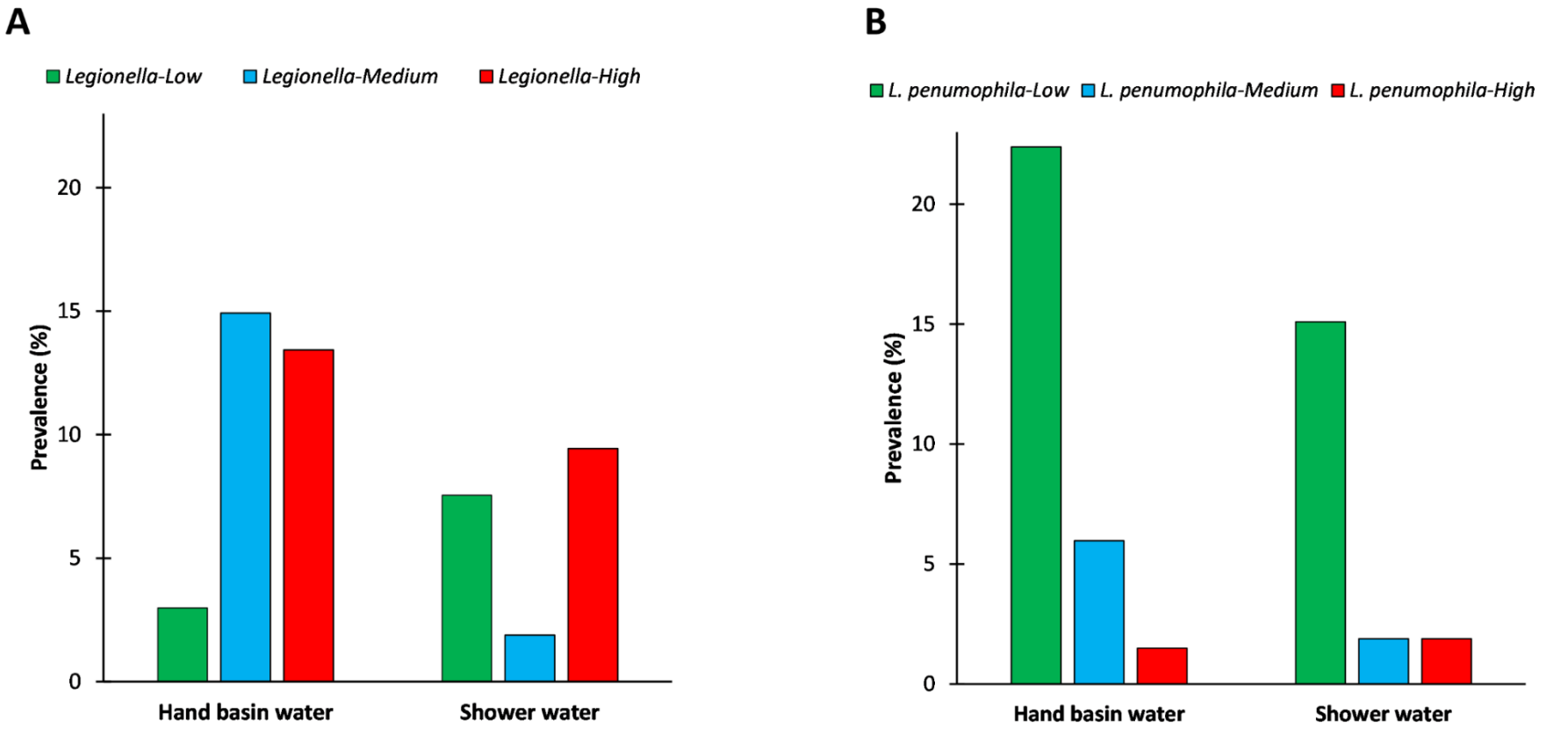

Figure 15.2: The percentage of water samples tested for VBNC *Legionella* (A) and VBNC *L. pneumophila* (B) with three levels of contaminations in hand basin (n = 67) and shower water (n = 53) samples. The levels of VBNC cells were divided into three levels: low (< 10^3 GU/L, in green), medium (10^3 to 10^4 GU/L, in blue) and high (> 10^4 GU/L, in red) contamination. Y-axis represents % positive samples and X-axis represents sample type.

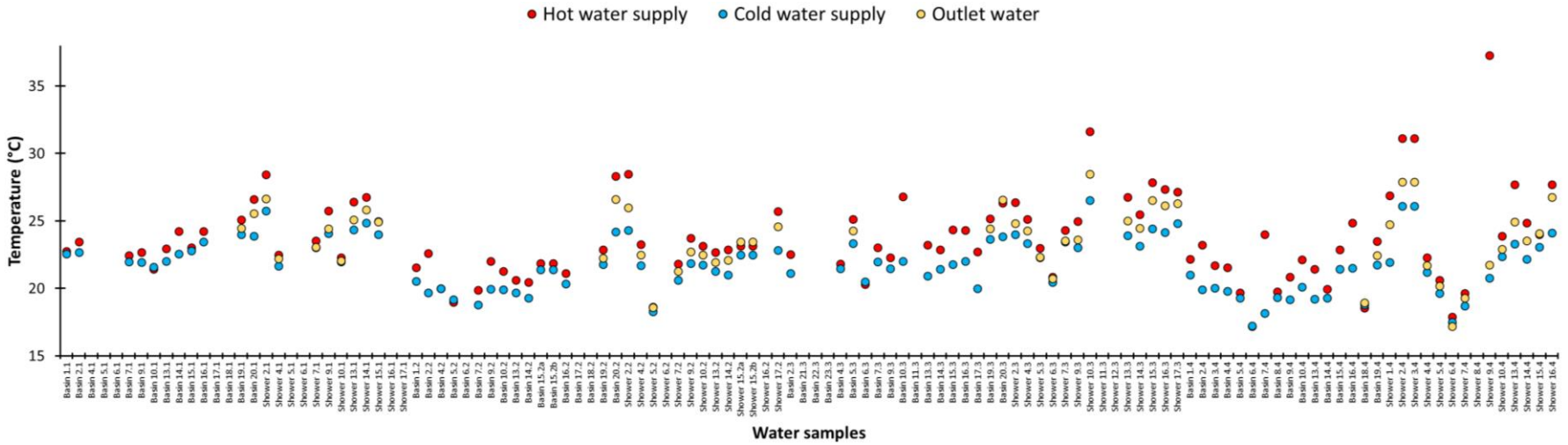

Figure 15.3: Average temperature of hot water supply, cold water supply and outlet water (hand basin and shower) recorded for one-week prior sampling. X-axis represents the tested water samples and y-axis represents temperature (°C). Y-axis represents temperature of water one-week prior sampling (cold water supply, hot water supply and outlet water) and X-axis represents the samples.

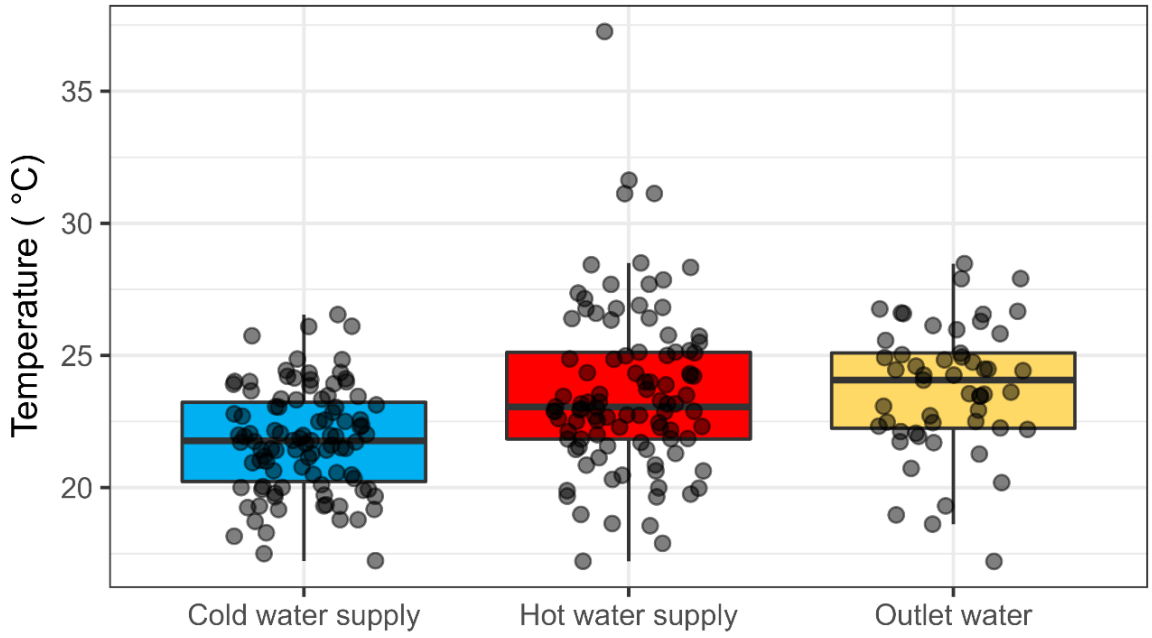

Figure 15.4: Average temperature of hot water supply, cold water supply and outlet water (hand basin and shower) recorded for one-week prior sampling. Y-axis represents temperature of water one-week prior sampling (cold water supply, hot water supply and outlet water) and X-axis represents the samples.

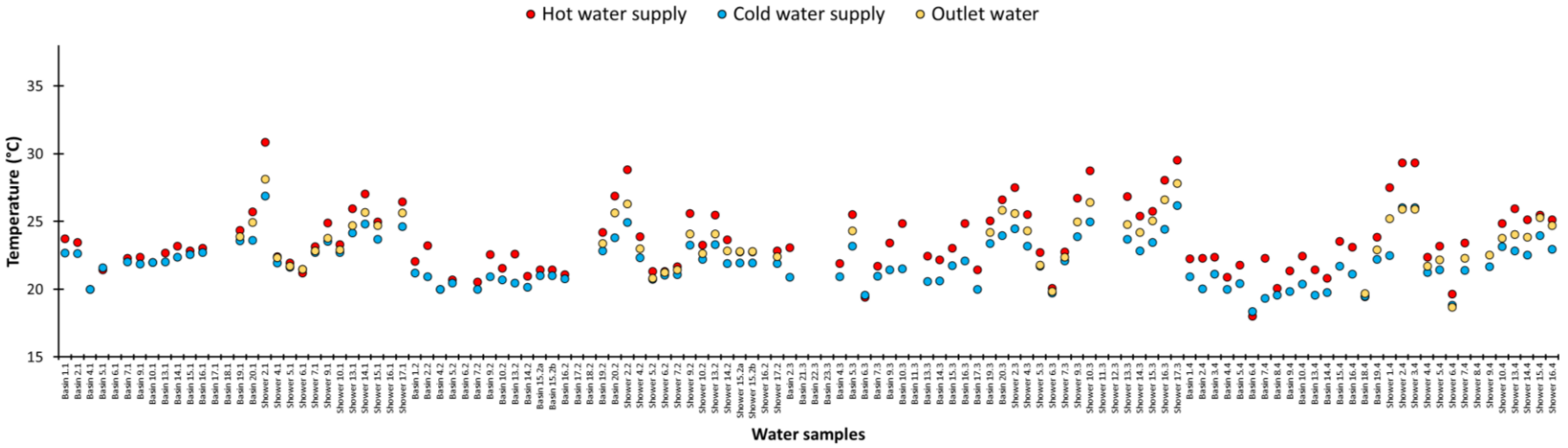

Figure 15.5: Average temperature of hot water supply, cold water supply and outlet water (hand basin and shower) recorded for one-month prior sampling. X-axis represents the tested water samples and y-axis represents temperature (°C). Y-axis represents temperature of water one-month prior sampling (cold water supply, hot water supply and outlet water) and X-axis represents the samples.

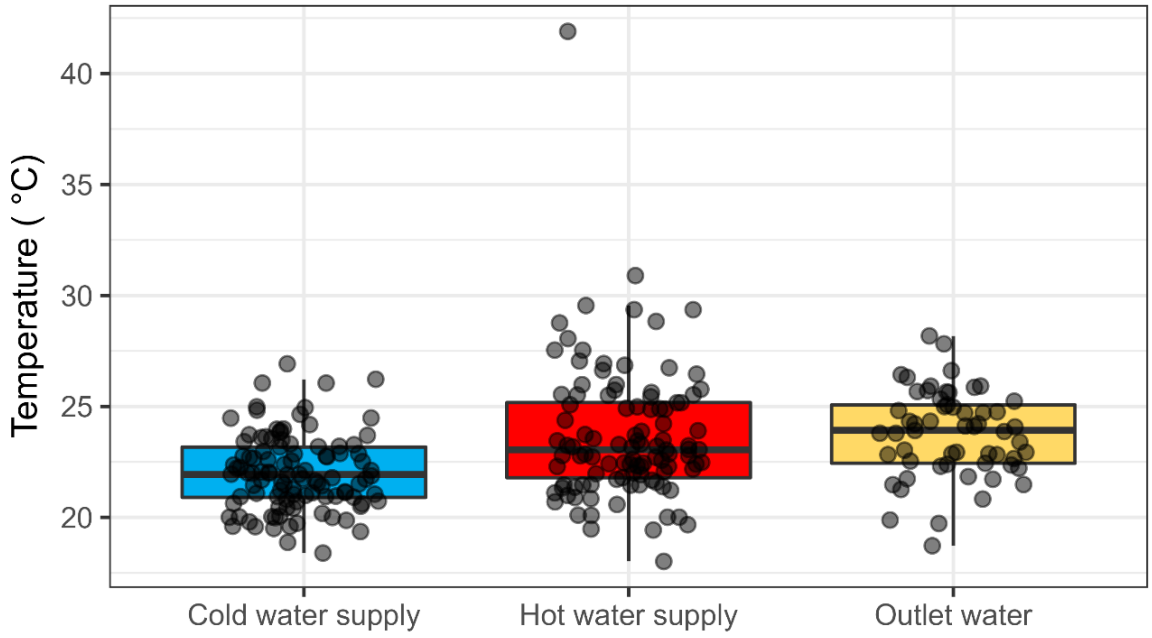

Figure 15.6: Average temperature of hot water supply, cold water supply and outlet water (hand basin and shower) recorded for one-month prior sampling. Y-axis represents temperature of water one-month prior sampling (cold water supply, hot water supply and outlet water) and X-axis represents the samples.

16 Appendix-5

Supplementary data of manuscript entitled “Composition of planktonic prokaryotic communities in hospital water system depends on water dynamics and stagnation” (**Section 10**).

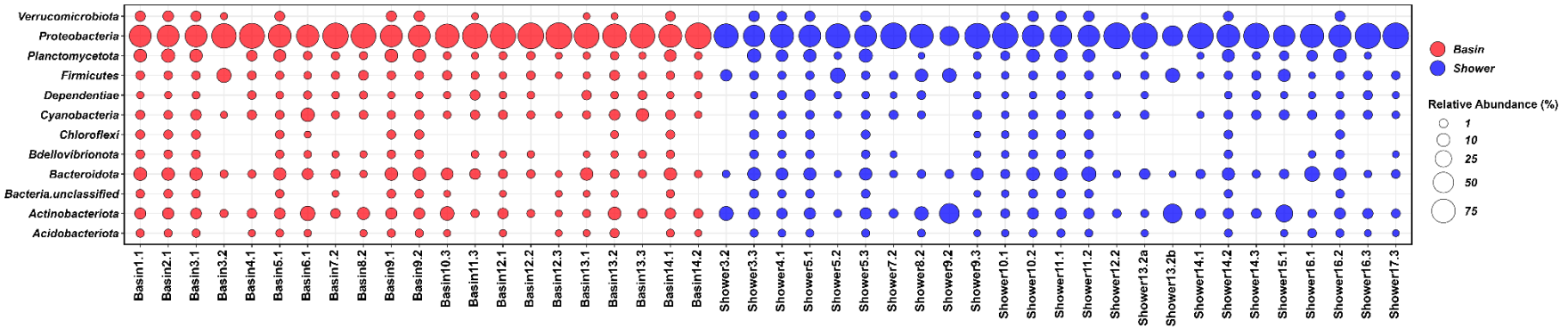

Figure 16.1: Abundant bacterial phyla (n = 12) with relative abundance ≥ 1% present in hospital basin (red coloured) and shower (blue coloured) water samples. The spot size represents % relative abundance of bacterial phyla (Y-axis) in the samples (X-axis).

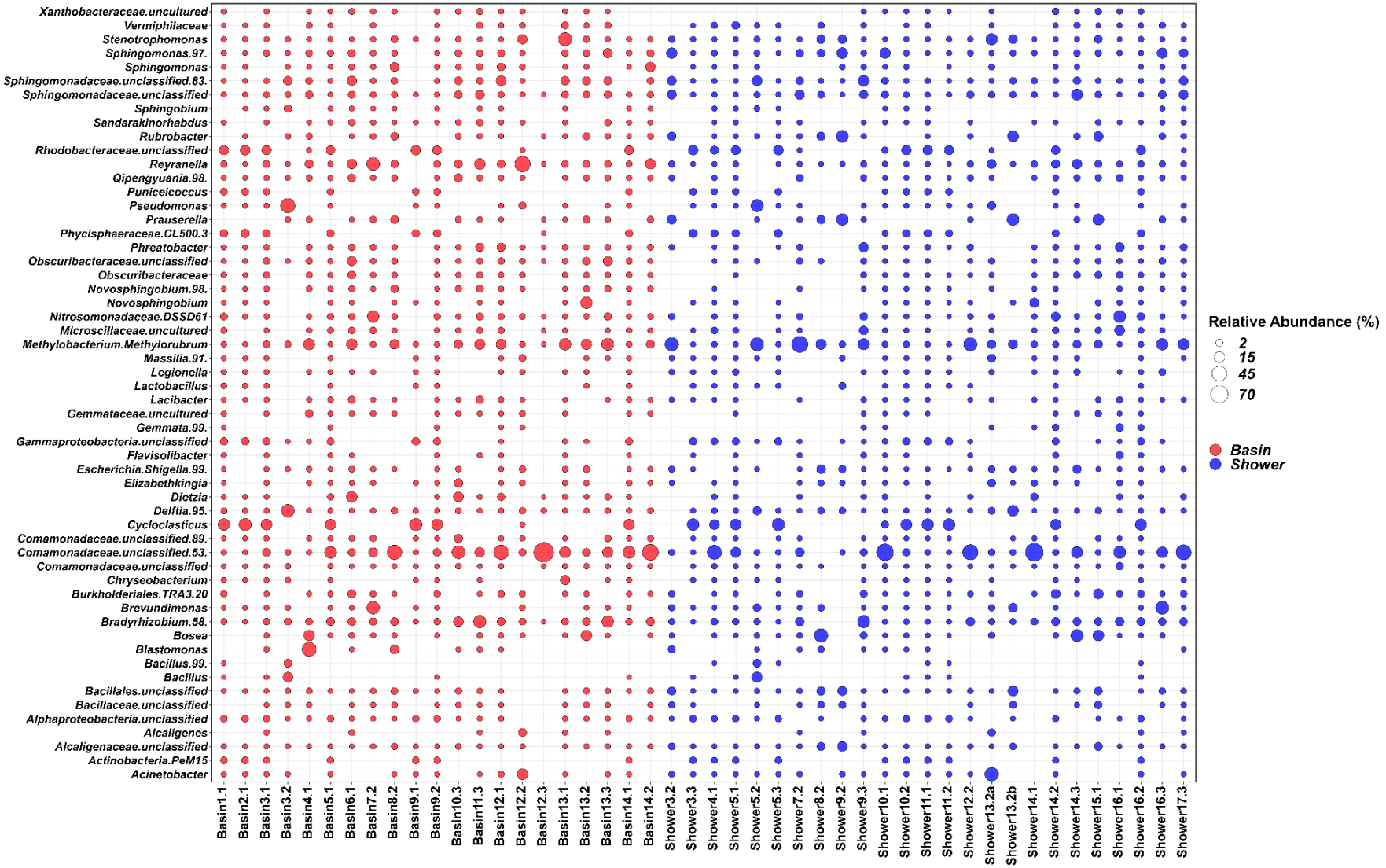

Figure 16.2: Abundant bacterial genera (n = 56) with relative abundance ≥ 1.5% present in hospital basin (red coloured) and shower (blue coloured) water samples. The spot size represents % relative abundance of bacterial genera (Y-axis) in the samples (X-axis).

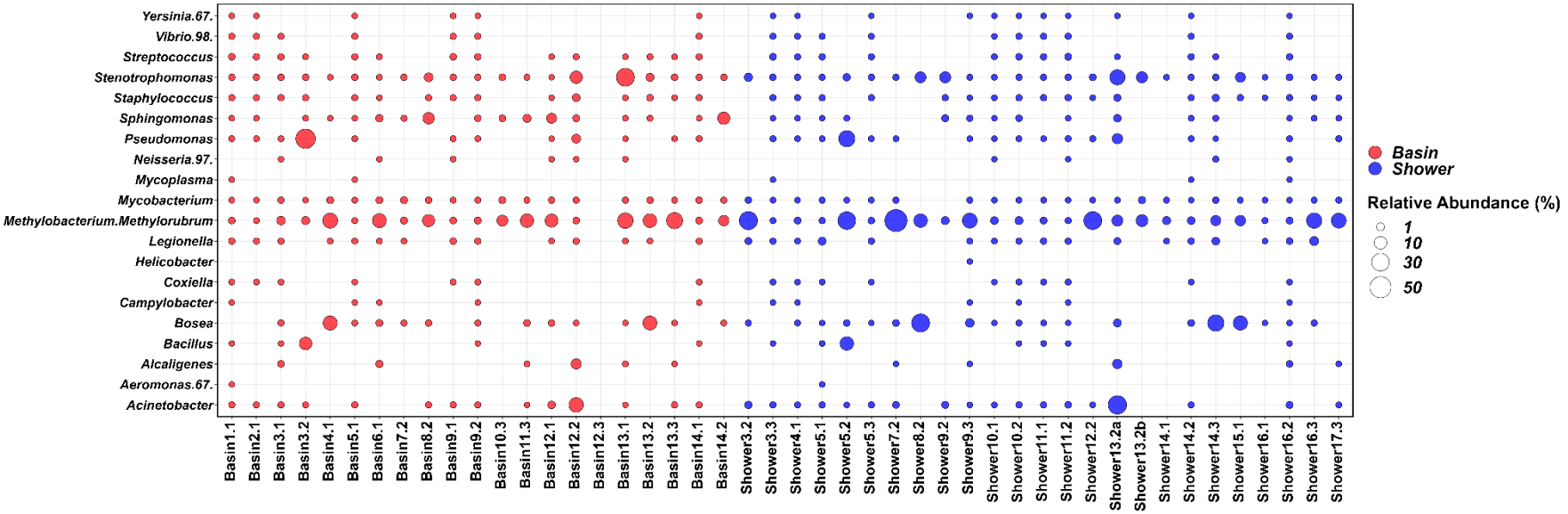

Figure 16.3: Potentially pathogenic bacteria (n = 20) present in hospital basin (red coloured) and shower (blue coloured) water samples. The spot size represents % relative abundance of potentially pathogenic bacterial genera (Y-axis) in the samples (X-axis).

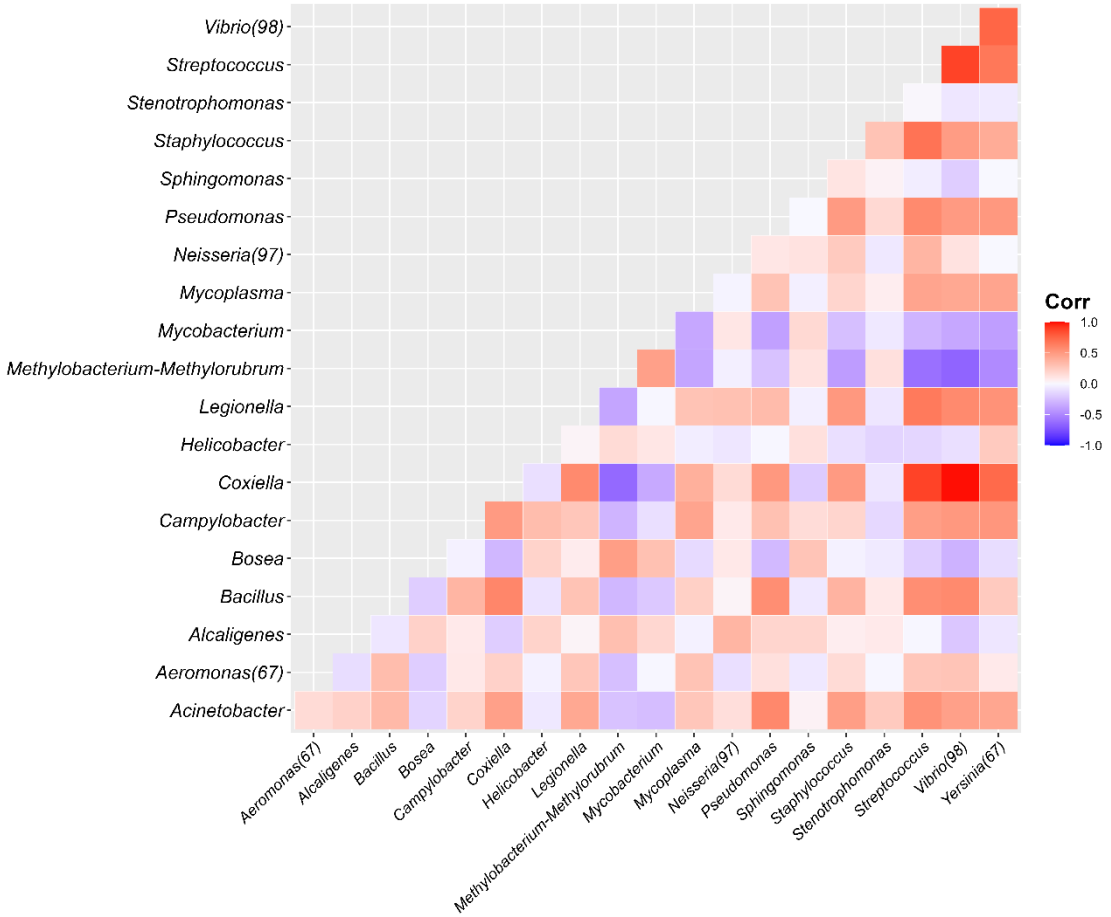

Figure 16.4: Spearman's correlation analysis of the potentially pathogenic bacterial genera (n = 20) of hospital shower and basin water samples. The heat map value shows the Spearman's correlation coefficient ρ to a significance threshold of $p < 0.05$, ranging from –1.0 (blue colour) to 1.0 (red colour). A minus value (in blue) demonstrates negative association, whereas a plus value demonstrates positive association (in red).

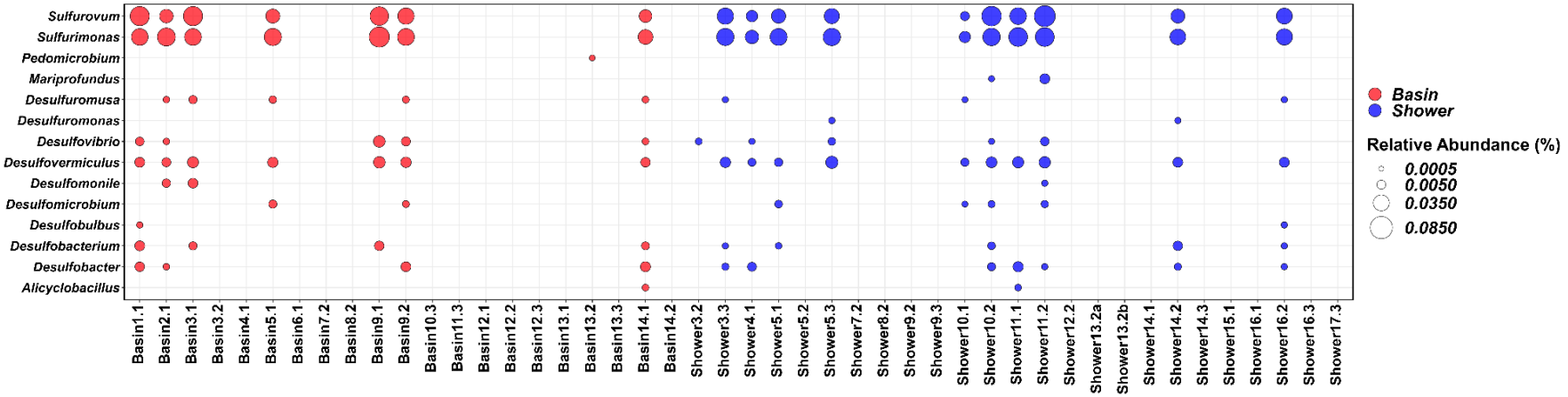

Figure 16.5: Potentially corrosion associated bacteria (n = 14) present in hospital basin (red coloured) and shower (blue coloured) water samples. The spot size represents % relative abundance of potentially corrosion associated bacterial genera (Y-axis) in the samples (X-axis).

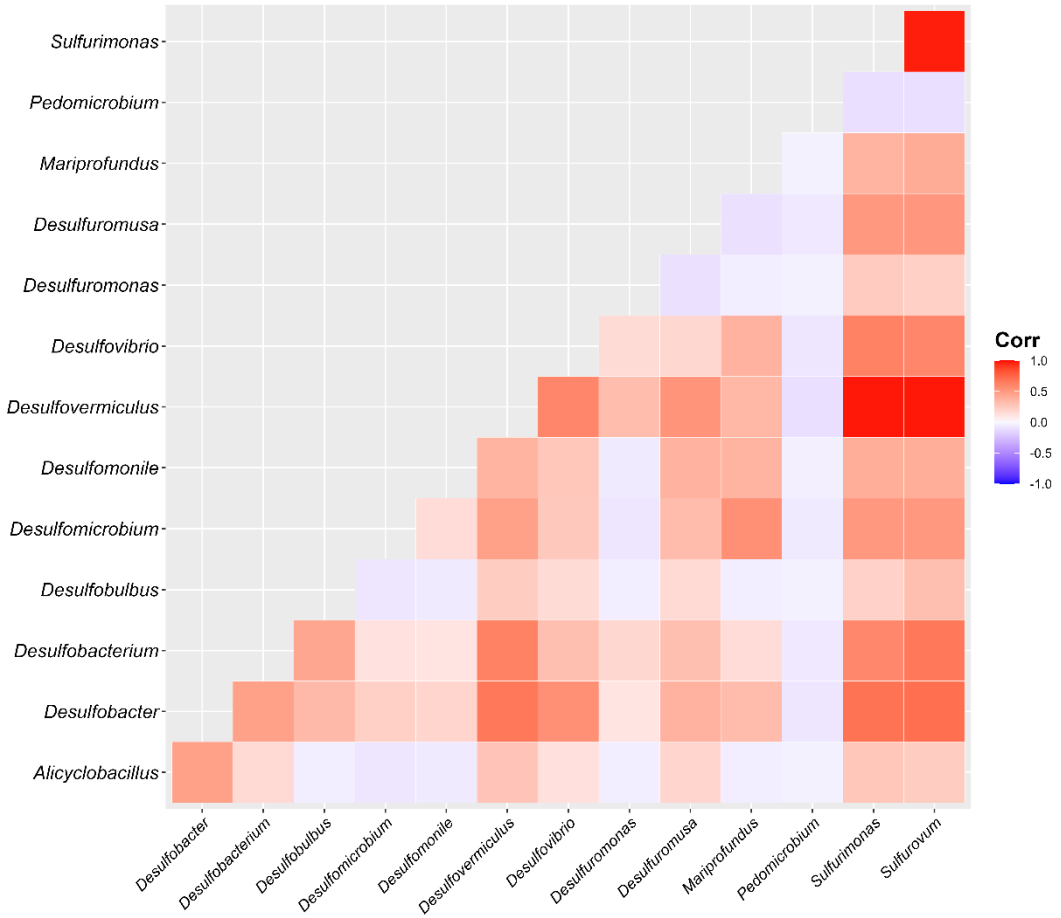

Figure 16.6: Spearman's correlation analysis of the potentially corrosion related bacterial genera (n = 14) of hospital shower and basin water samples. The heat map value shows the Spearman's correlation coefficient ρ to a significance threshold of $p < 0.05$, ranging from –1.0 (blue colour) to 1.0 (red colour). A minus value (in blue) demonstrates negative association, whereas a plus value demonstrates positive association (in red).

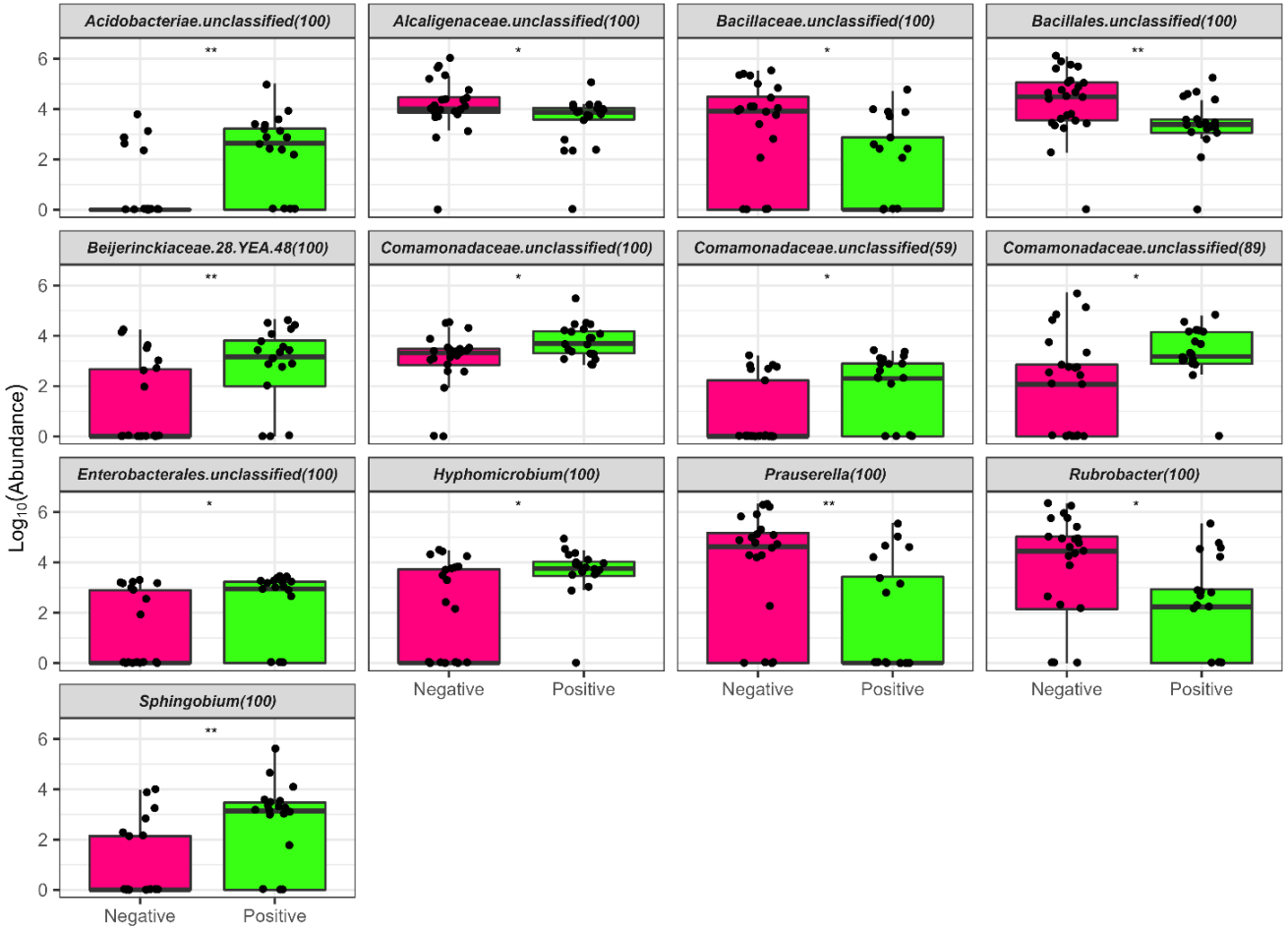

Figure 16.7: Relative abundance (Log_{10} transformed) of significant bacterial genera (n = 13) in presence (green coloured, positive) or absence (pink coloured, negative) of *Vermamoeba vermiformis*. Y-axis represents relative abundance (Log_{10} transformed), whereas X-axis represents presences (positive) and absence (negative) of *Vermamoeba vermiformis*. The *p* values are represented as follows: ** $p < 0.01$ and $*$ $p < 0.05$.

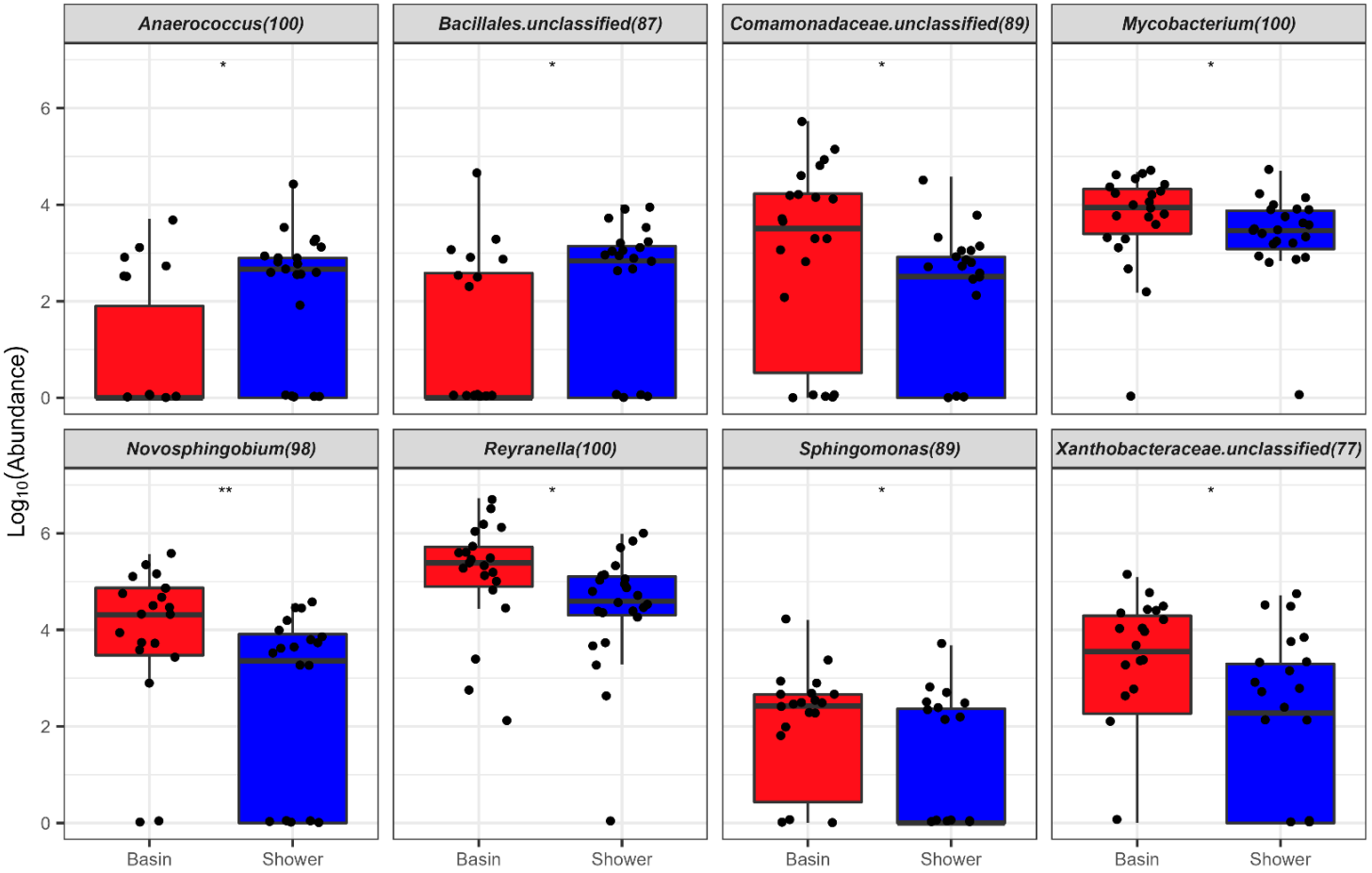

Figure 16.8: Relative abundance (Log_{10} transformed) of significant bacterial genera (n = 8) present in basin (red coloured) and shower (blue coloured) water samples. Y-axis represents relative abundance (Log_{10} transformed), whereas X-axis represents sampling site (hand basins and showers). The *p* values are represented as follows: ** $p < 0.01$ and $* \; p < 0.05$.

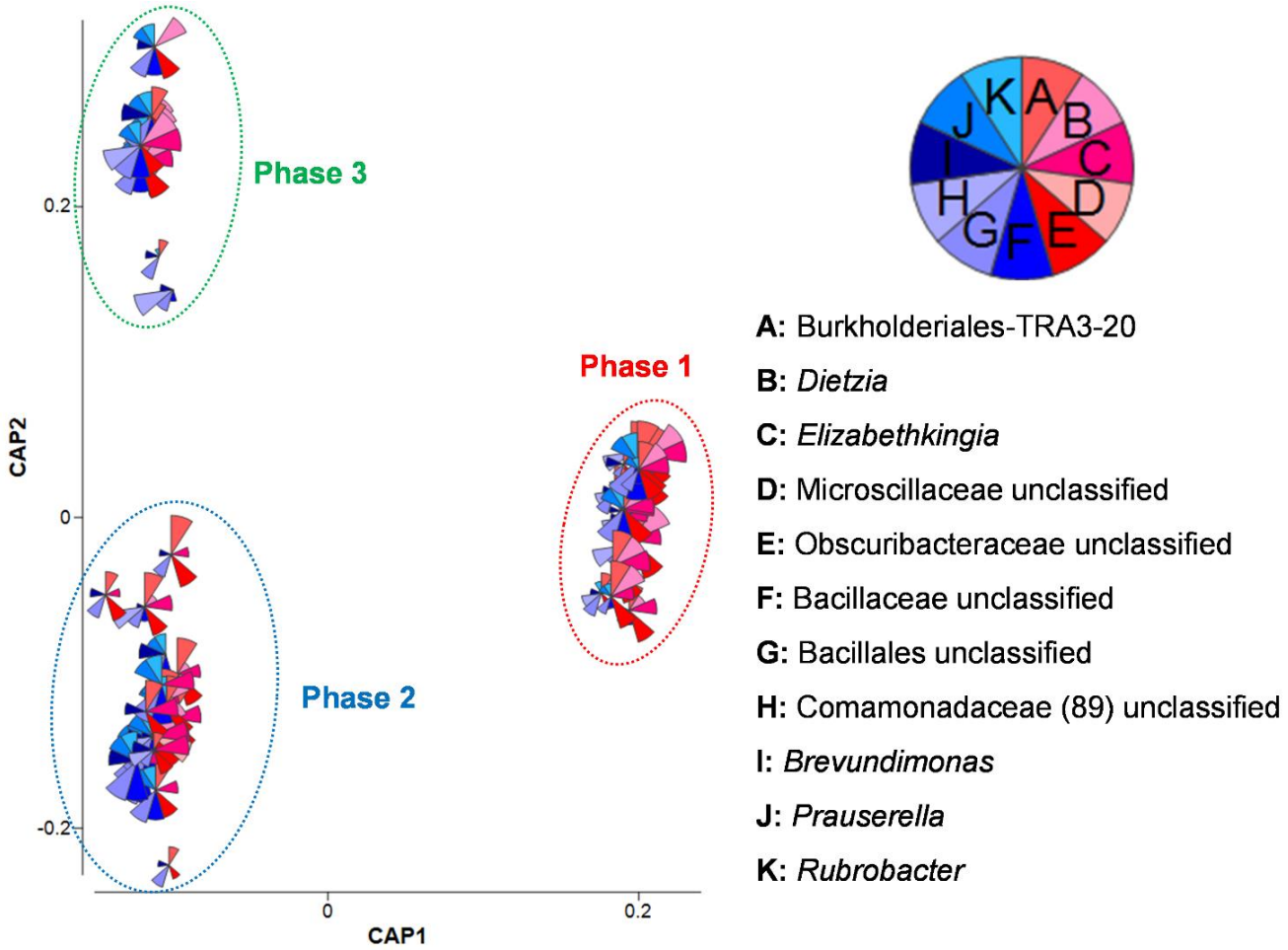

Figure 16.9: Canonical analysis of principal coordinates (CAP) based on Bray-Curtis dissimilarity index displaying the variation in prokaryotic communities on the basis of sampling phases (phase 1: March 2021, phase 2: April 2021, and phase 3: November 2021). The overlayed similarity percentage (SIMPER) analysis showing the 11 bacterial genera responsible for ≈ 7% of variance.

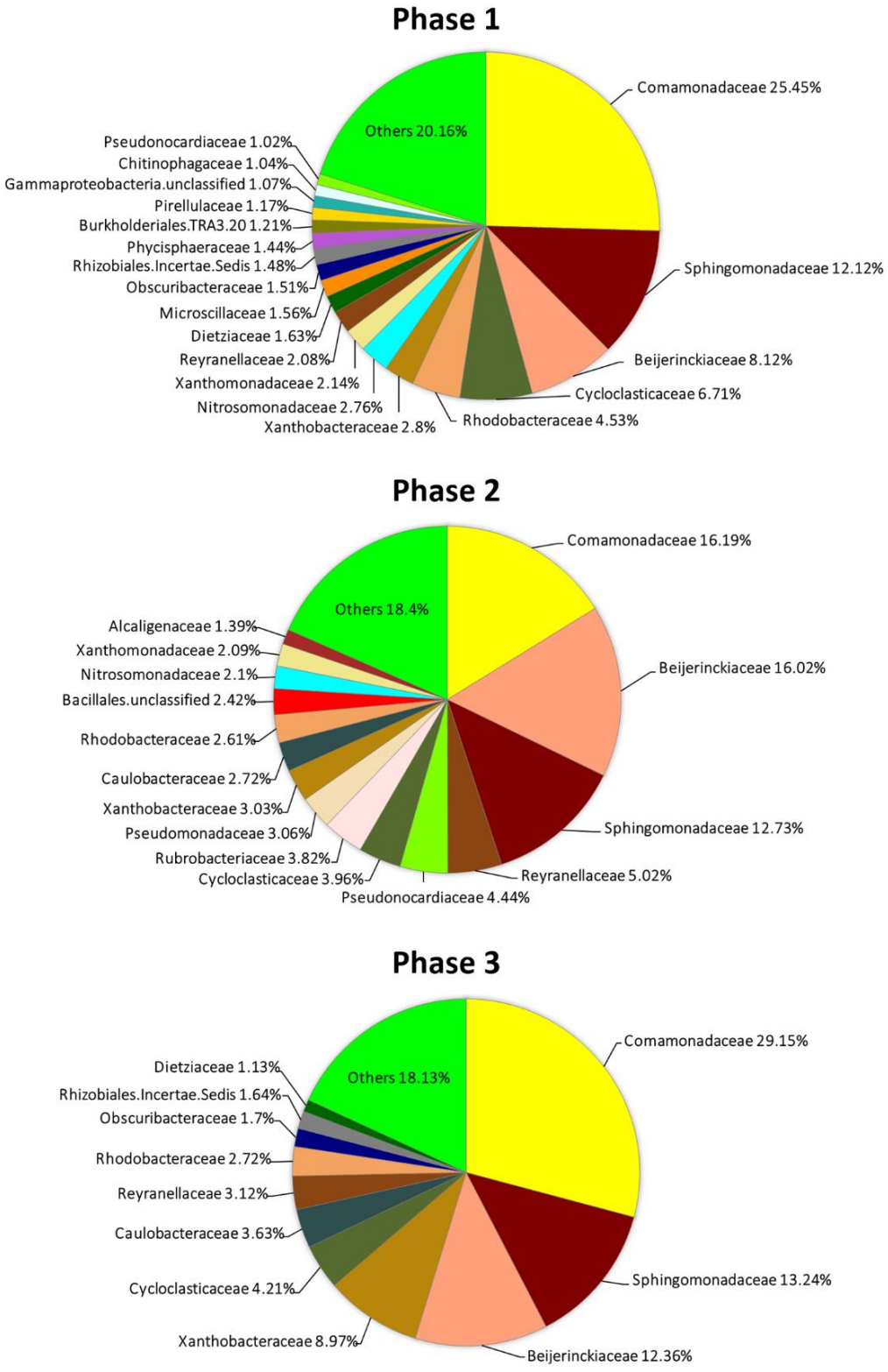

Figure 16.10: Interactive pie charts depicting the relative abundance (%) of bacterial families based on three different sampling phases (phase 1: March 2021, phase 2: April 2021, and phase 3: November 2021).

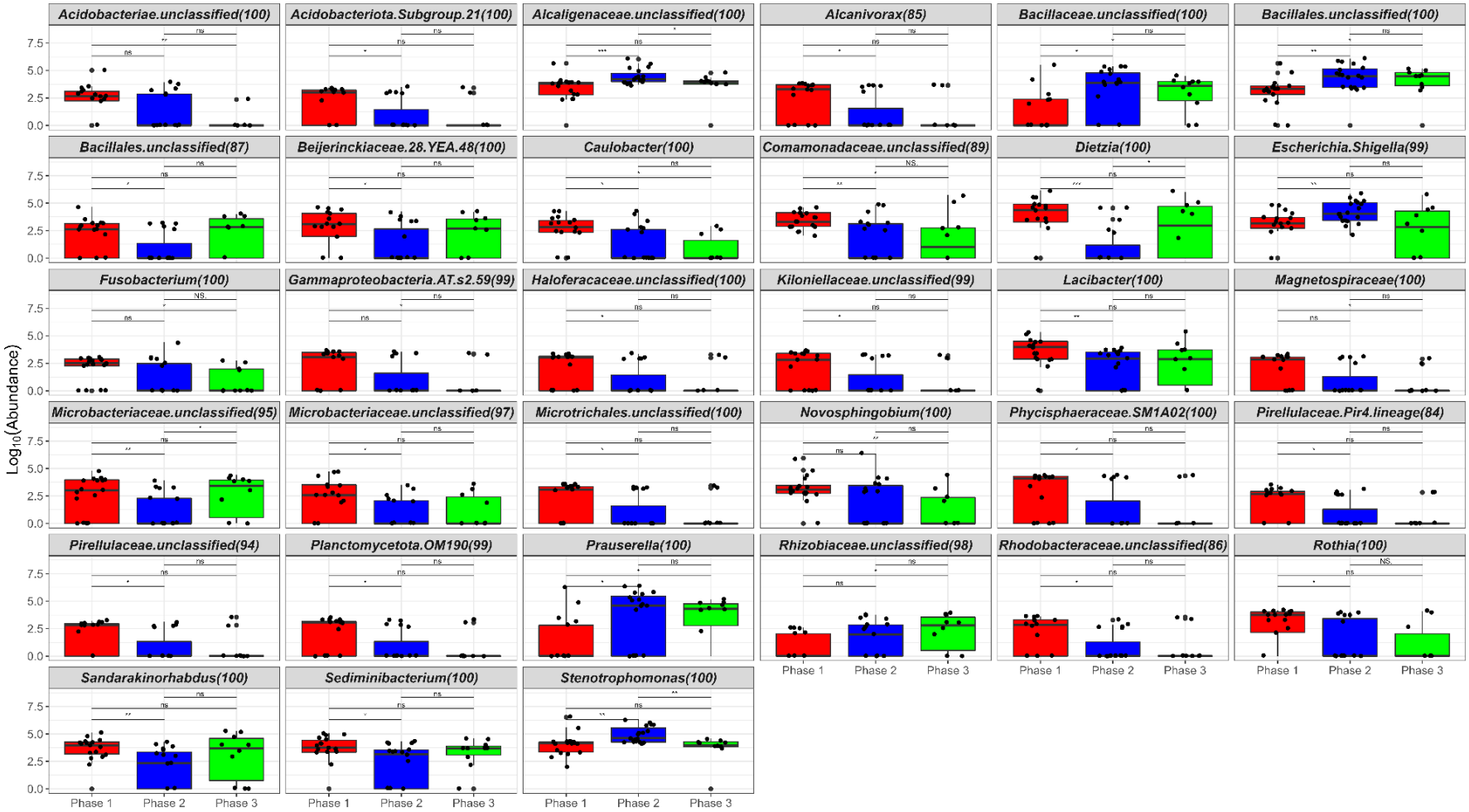

Figure 16.11: Relative abundance (Log_{10} transformed) of significant bacterial genera (n = 33) present in sampling phase 1 (red coloured), sampling phase 2 (blue coloured), and sampling phase 3 (green coloured). Y-axis represents relative abundance (Log_{10} transformed), whereas X-axis represents sampling phases (phase 1: March 2021, phase 2: April 2021, and phase 3: November 2021). The *p* values are represented as follows: *** $p < 0.001$, ** $p < 0.01$, $*$ $p < 0.05$, and ns non-significant.

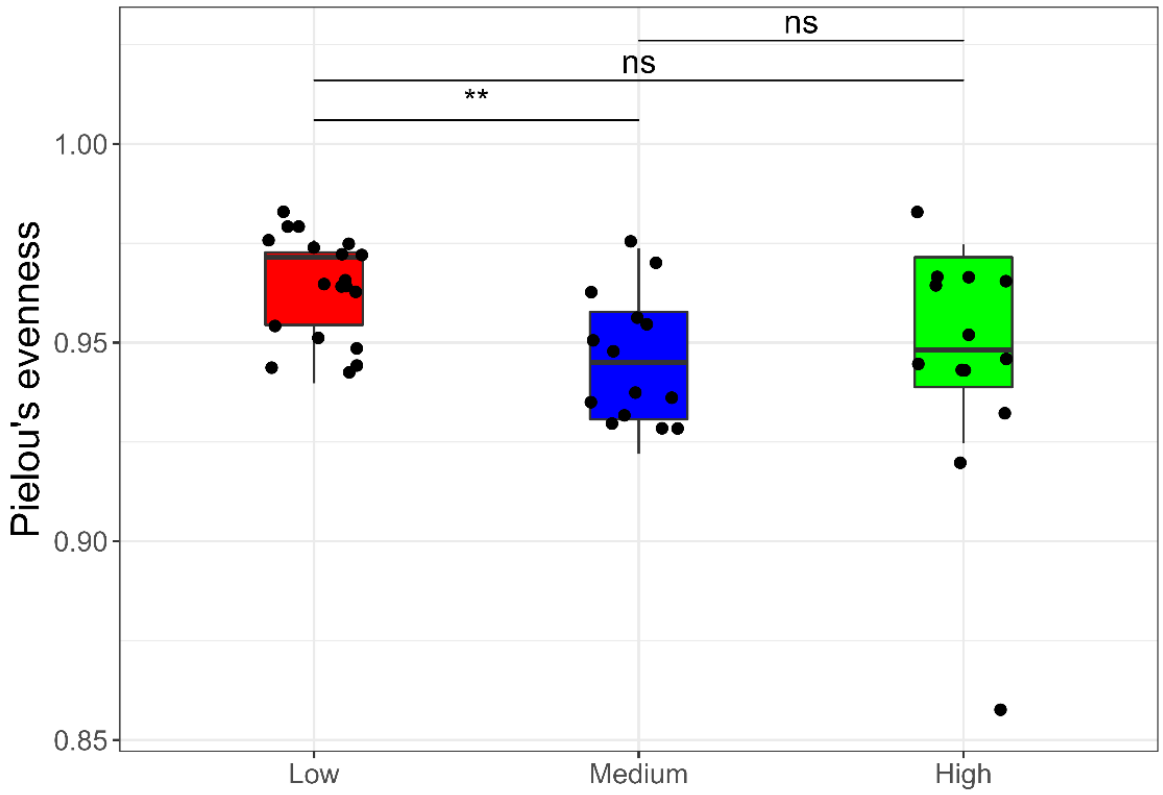

Figure 16.12: Pielou's evenness diversity index of prokaryotic communities at genus level affected by three different flow regimes (one week prior to sampling). Y-axis represents Pielou's evenness, whereas X-axis three different flow regimes one week prior to sampling (low flow regime: 0 to < 13 minutes/week, medium flow regime: ≥ 13 to < 30 minutes/week, and high flow regime: ≥ 30 to 85 minutes/week). The *p* values are represented as follows: ** $p < 0.01$ and ns non-significant.

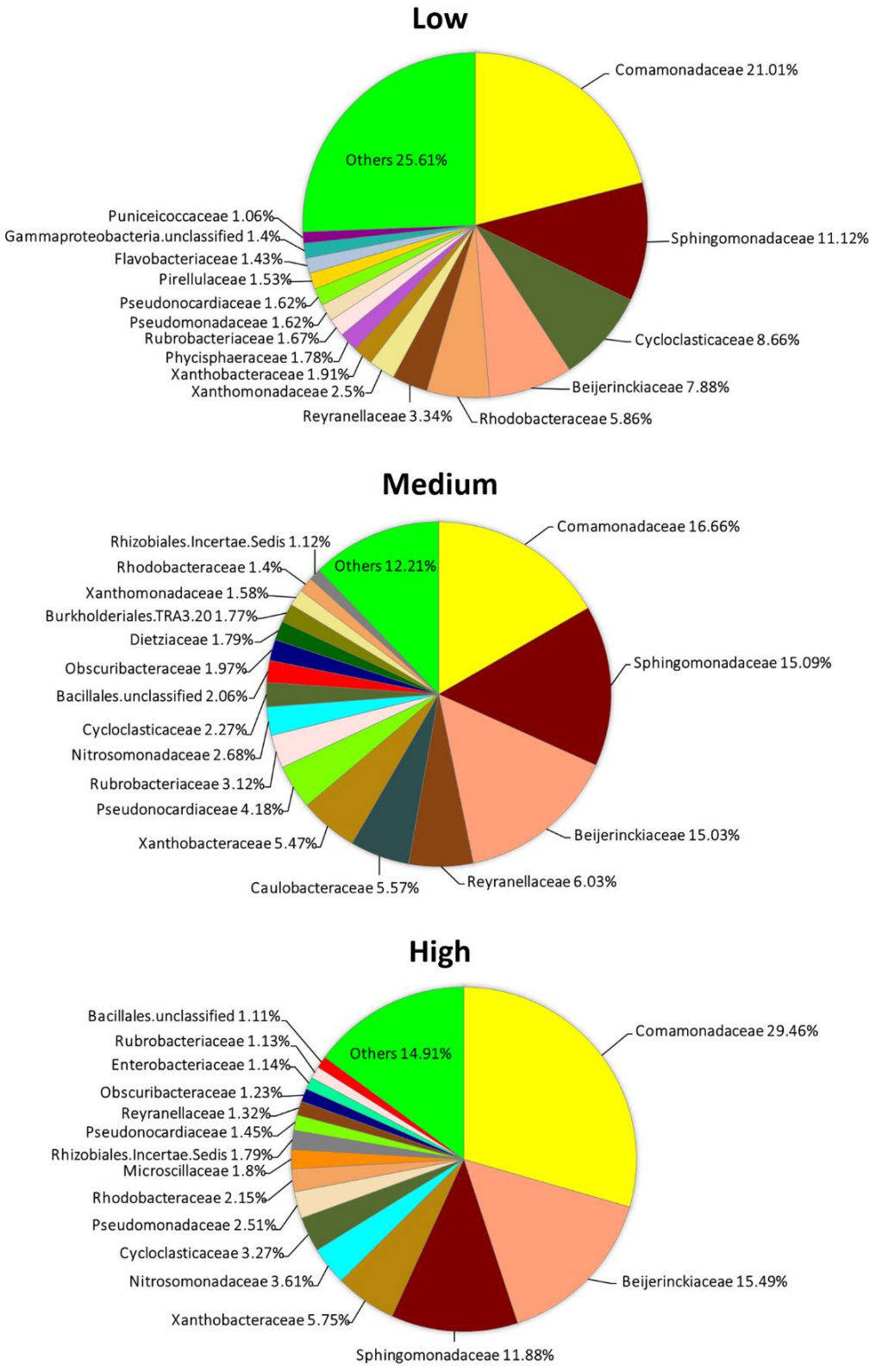

Figure 16.13: Interactive pie charts depicting the relative abundance (%) of bacterial families based on the three different flow regimes (one week prior to sampling). Low flow regime: 0 to < 13 minutes/week, medium flow regime: ≥ 13 to < 30 minutes/week, and high flow regime: ≥ 30 to 85 minutes/week.

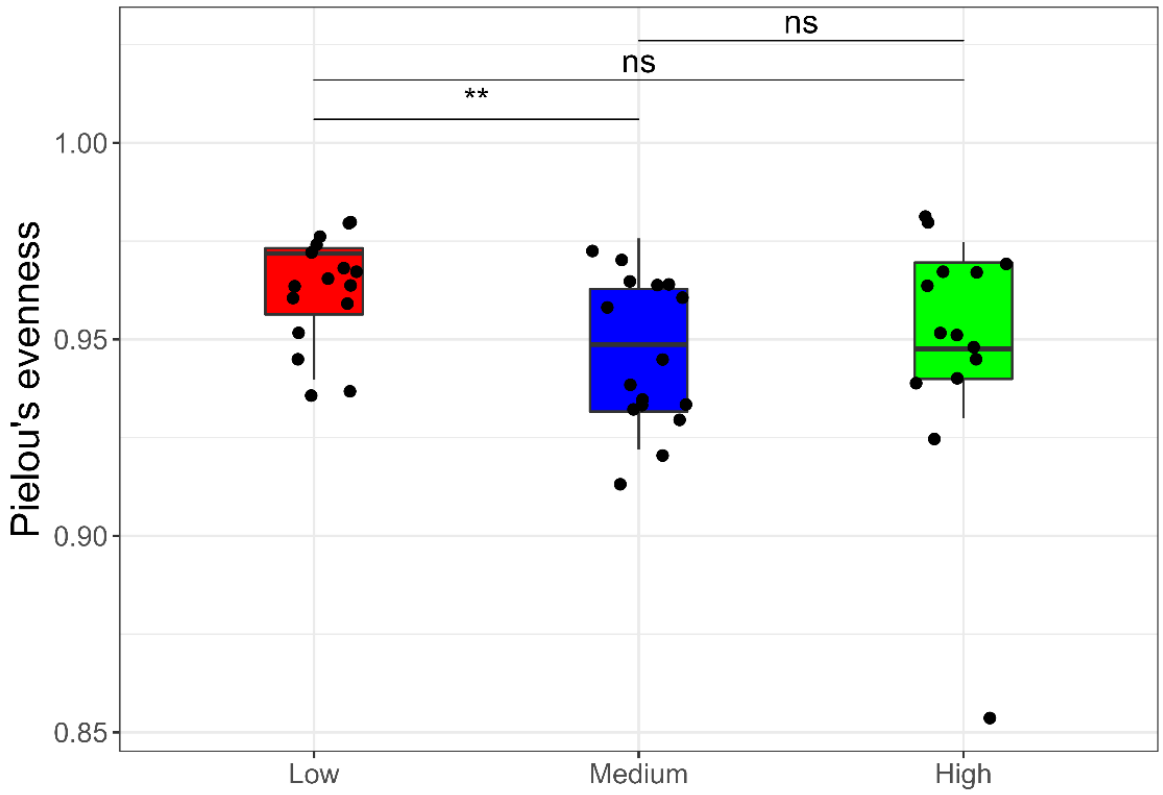

Figure 16.14: Pielou's evenness diversity index of prokaryotic communities at genus level affected by three different flow regimes (six months prior to sampling). Y-axis represents Pielou's evenness, whereas X-axis three different flow regimes six months prior to sampling (low flow regime: 0 to 765 counts/six months, medium flow regime: 800 to 1,185 counts/six months, and high flow regime: 1,200 to 2,500 counts/six months). The *p* values are represented as follows: ** $p < 0.01$ and ns non-significant.

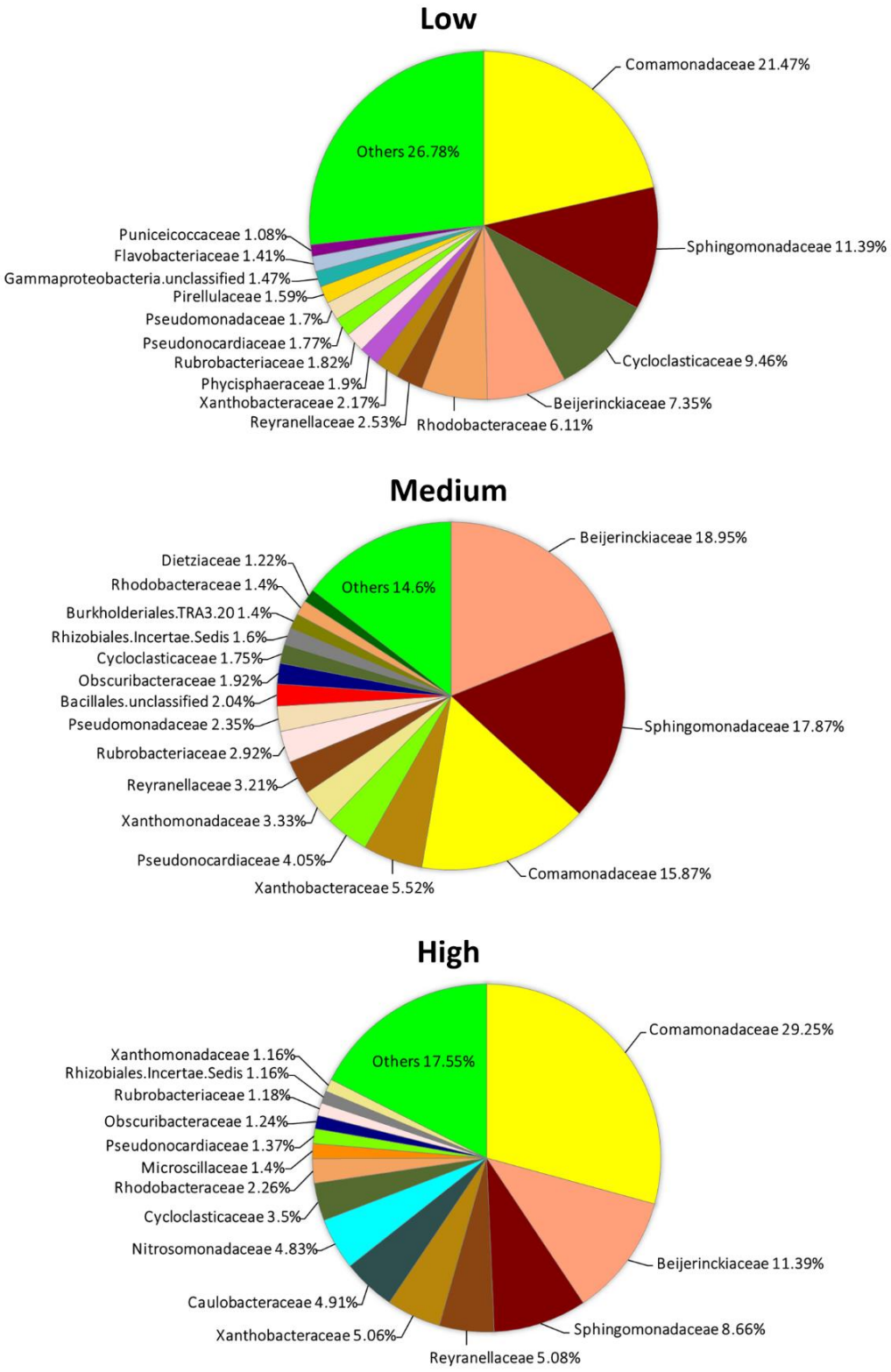

Figure 16.15: Interactive pie charts depicting the relative abundance (%) of bacterial families based on three different flow regimes (six months prior to sampling). Low flow regime: 0 to 765 counts/six months, medium flow regime: 800 to 1,185 counts/six months, and high flow regime: 1,200 to 2,500 counts/six months.

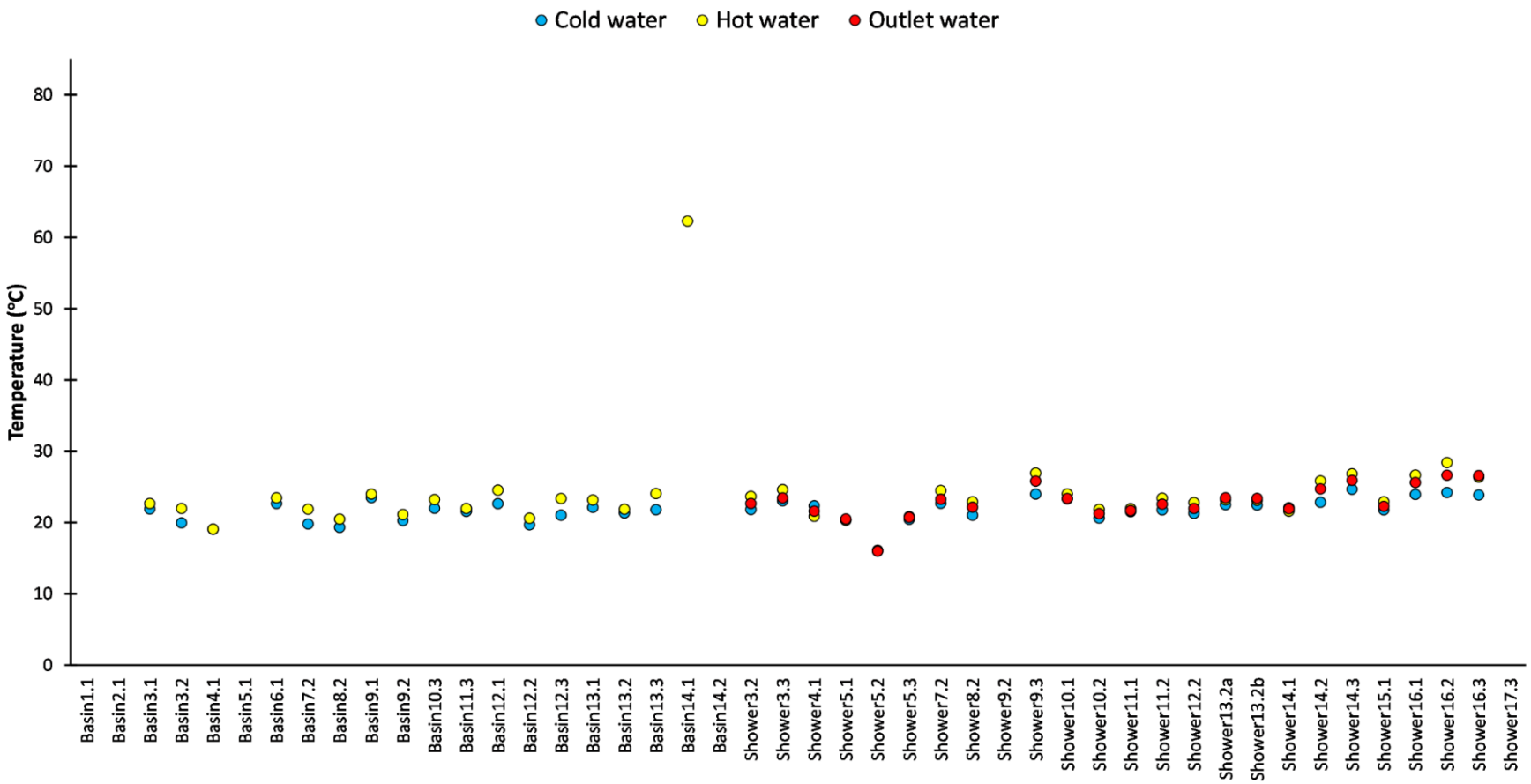

Figure 16.16: Average temperature of hot, cold and outlet water for hospital water samples, one week prior to sampling. Y-axis represents temperature of water one week prior to sampling (cold water supply, hot water supply and outlet water) and X-axis represents the samples.

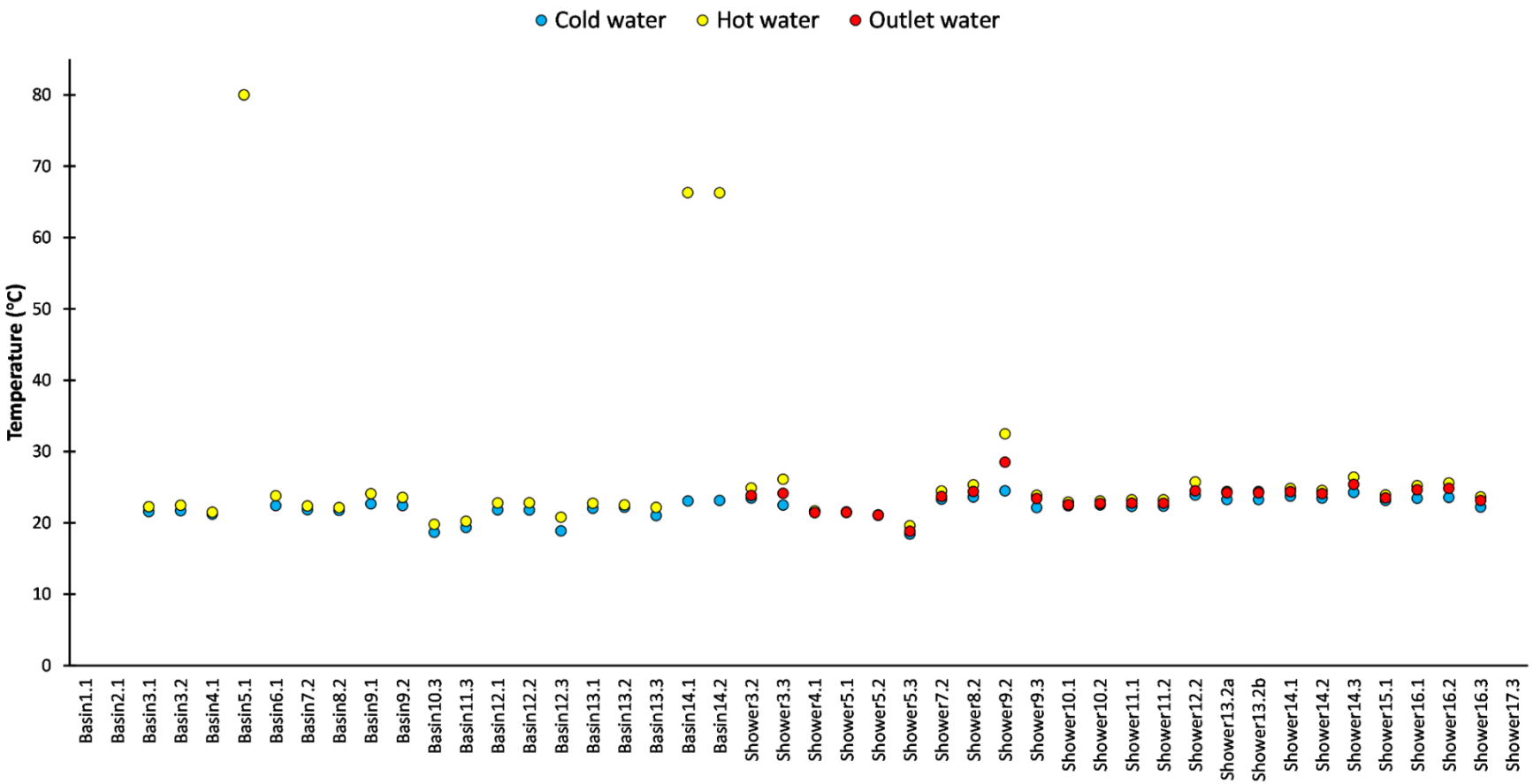

Figure 16.17: Average temperature of hot, cold and outlet water for hospital water samples, six months prior to sampling. Y-axis represents temperature of water six months prior to sampling (cold water supply, hot water supply and outlet water) and X-axis represents the samples.

Table 16.1: Biotic and abiotic parameters for the hospital water samples.

Samples	Sampling site	Sampling phase	*Vermamoeba vermiformis*	One week prior to sampling				Six months prior to sampling			
				Flow duration (minutes)	Water average temperature (°C)			Number of flow events (counts)	Water average temperature (°C)		
					Cold	Hot	Outlet		Cold	Hot	Outlet
Basin1.1	Basin	Phase 1	Negative	Low				Low			
Basin2.1	Basin	Phase 1	Negative	Low				Low			
Basin3.1	Basin	Phase 1	Positive	Low	21.91	22.68		Medium	21.57	22.28	
Basin3.2	Basin	Phase 2	Positive	High	19.95	21.98		Medium	21.7	22.49	
Basin4.1	Basin	Phase 1	Negative	Low	19.05	19.09		Low	21.22	21.5	
Basin5.1	Basin	Phase 1	Positive	Low				Low	80	80	
Basin6.1	Basin	Phase 1	Positive	Medium	22.68	23.47		Medium	22.42	23.81	
Basin7.2	Basin	Phase 2	Negative	Medium	19.8	21.88		High	21.86	22.39	
Basin8.2	Basin	Phase 2	Positive	Medium	19.33	20.47		High	21.76	22.15	
Basin9.1	Basin	Phase 1	Negative	High	23.5	23.99		High	22.69	24.09	
Basin9.2	Basin	Phase 2	Positive	Low	20.27	21.12		High	22.42	23.57	
Basin10.3	Basin	Phase 3	Negative	Medium	22	23.24		High	18.68	19.8	
Basin11.3	Basin	Phase 3	Negative	Medium	21.59	21.97		Medium	19.37	20.23	
Basin12.1	Basin	Phase 1	Positive	Medium	22.66	24.56		Medium	21.83	22.8	
Basin12.2	Basin	Phase 2	Positive	Low	19.68	20.6		High	21.8	22.83	
Basin12.3	Basin	Phase 3	Negative	High	21.04	23.37		High	18.89	20.8	
Basin13.1	Basin	Phase 1	Positive	Low	22.14	23.17		Medium	22.06	22.76	
Basin13.2	Basin	Phase 2	Negative	Medium	21.36	21.88		Medium	22.21	22.54	
Basin13.3	Basin	Phase 3	Negative	High	21.8	24.08		High	21.02	22.18	
Basin14.1	Basin	Phase 1	Positive	Low		62.29		Low	23.08	66.28	
Basin14.2	Basin	Phase 2	Positive	Low				Low	23.16	66.28	
Shower3.2	Basin	Phase 2	Negative	High	21.82	23.67	22.69	Medium	23.47	24.91	23.86
Shower3.3	Shower	Phase 3	Negative	High	23.05	24.63	23.46	High	22.51	26.13	24.14
Shower4.1	Shower	Phase 1	Positive	Low	22.35	20.88	21.6	Low	21.66	21.68	21.44
Shower5.1	Shower	Phase 1	Positive	Low	20.32	20.33	20.51	Low	21.45	21.55	21.48

Samples	Sampling site	Sampling phase	*Vermamoeba vermiformis*	One week prior to sampling				Six months prior to sampling			
				Flow duration (minutes)	Water average temperature (°C)			Number of flow events (counts)	Water average temperature (°C)		
					Cold	Hot	Outlet		Cold	Hot	Outlet
Shower5.2	Shower	Phase 2	Positive	Low	16.04	16.12	15.98	Low	21.06	21.14	21.1
Shower5.3	Shower	Phase 3	Negative	Medium	20.46	20.82	20.73	Low	18.44	19.61	18.84
Shower7.2	Shower	Phase 2	Positive	Medium	22.7	24.51	23.29	Medium	23.32	24.47	23.7
Shower8.2	Shower	Phase 2	Negative	High	21.03	22.92	22.15	High	23.62	25.34	24.4
Shower9.2	Shower	Phase 2	Negative	Low				Low	24.49	32.5	28.51
Shower9.3	Shower	Phase 3	Negative	High	23.99	26.96	25.82	Medium	22.16	23.88	23.43
Shower10.1	Shower	Phase 1	Negative	High	23.33	24.03	23.37	Medium	22.4	22.93	22.56
Shower10.2	Shower	Phase 2	Positive	Low	20.63	21.86	21.25	Medium	22.53	23.07	22.72
Shower11.1	Shower	Phase 1	Positive	Low	21.54	21.95	21.69	Low	22.32	23.25	22.77
Shower11.2	Shower	Phase 2	Negative	Low	21.78	23.42	22.58	Low	22.35	23.26	22.76
Shower12.2	Shower	Phase 2	Negative	High	21.3	22.8	21.99	High	23.89	25.76	24.49
Shower13.2a	Shower	Phase 2	Positive	Medium	22.5	23.2	23.47	Medium	23.28	24.39	24.23
Shower13.2b	Shower	Phase 2	Negative	Medium	22.45	23.13	23.4	Medium	23.28	24.39	24.23
Shower14.1	Shower	Phase 1	Positive	Low	22.08	21.61	21.94	Low	23.76	24.83	24.37
Shower14.2	Shower	Phase 2	Negative	Medium	22.85	25.84	24.73	Low	23.48	24.54	24.11
Shower14.3	Shower	Phase 3	Negative	High	24.67	26.84	25.93	Medium	24.27	26.43	25.41
Shower15.1	Shower	Phase 1	Negative	Medium	21.79	22.94	22.29	Medium	23.16	23.93	23.51
Shower16.1	Shower	Phase 1	Positive	High	23.94	26.67	25.62	High	23.44	25.22	24.64
Shower16.2	Shower	Phase 2	Positive	High	24.22	28.42	26.65	High	23.58	25.6	24.85
Shower16.3	Shower	Phase 3	Negative	Medium	23.87	26.37	26.6	High	22.24	23.64	23.15
Shower17.3	Shower	Phase 3	Negative	Low				Low			

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