A Thesis submitted for the degree of

Doctor of Philosophy

In
Life Sciences

‘Novel Approaches for Risk Management of Legionella bacteria in Domestic Water Systems’

By

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Declaration of Own Work

I declare that this research entitled **'Novel Approaches for Risk Management of Legionella bacteria in Domestic Water Systems'** is entirely my own work and that where material could be construed as the work of others, it is fully cited and referenced, and/or with appropriate acknowledgement given.

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Abstract

Legionella pneumophila, the causative agent of Legionnaires' disease, is a water born pathogenic bacteria commonly found in natural and manmade water systems such as rivers, lakes, wet soil, hot and cold water storage systems (being able to survive at temperatures between 6-63˚C, and proliferating between 20-45˚C), showerheads, cooling towers and spa pools. The main pathway of exposure to *Legionella* is by inhaling the aerosols containing the microorganism. Legionnaires' disease can be fatal if not diagnosed and treated at the right time. Practical *Legionella* control starts with a risk assessment of the water system and followed by the regular monitoring and water sampling. UK Health and Safety Executive (HSE) have implemented strict legislations to protect the public from Legionnaires' disease. This research highlights and addresses three major data gaps identified in *Legionella* control and management strategy employed in the UK and worldwide; namely, (i) the underestimation of microbiological threat in current cold water storage sampling strategy, (ii) the inability of current qPCR diagnostic methods to detect live *Legionella* in water samples, and (iii) the lack of predictive 'risk management system' for *Legionella* control in domestic water systems.

During my PhD, 15 relevant cold water storage tanks (selected from more than 6000 tanks surveyed at different sites located in different London Boroughs) were used to investigate the risk factors that contribute towards Legionella proliferation, and revealed serious shortcomings in the appropriateness of the water sample taken for regulatory testing. Secondly, molecular biology research was carried out to develop an accurate, reliable and rapid testing method for the detection and quantification of live Legionella using qPCR techniques. This was successfully achieved by extracting RNA from a *Legionella* lenticule, converting the RNA into cDNA and amplifying the cDNA using qPCR techniques. Finally, regular monitoring data from 120 London buildings (60 known to be *Legionella* positive and 60 known to be *Legionella* negative) was used to identify the possible risk factors contributing towards *Legionella* outbreaks. Data for these factors was then used to develop a predictive risk model for *Legionella* contamination using Principal Component Analysis (PCA). The model was validated with 66 new London buildings and 9 out of London buildings. The model showed 100% accuracy in predicting the risk of *Legionella* by distinguishing infected and non-infected sites in London as well as for the sites in out of London.

Acknowledgements

"I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand" (Isaiah 41:10)

Inspiration for this research is from my M.Sc. course and the practical experience I gained through my professional career and is a milestone in my professional development. I remember and thank all the people who have helped and advised me throughout my profession as a water treatment engineer.

Firstly, I thank Almighty God Jesus who is always with me to guide, protect and support to live a successful life and I commit my devotion to him until the end of my life.

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Abbreviations and Acronyms

ACOP L8	Approved Code of Practice, Legislation 8
Ag^{2+}	Silver ions
BCDC	British Communicable Disease Surveillance Centre
BCYE	Buffered Charcol-Yeast Extract
CAP	Community Acquired Pneumonia
CAPNETZ	Competence Network for Community Acquired Pneumonia
cDNA	complimentary DNA
cfu	colony forming units
CKD	Chronic Kidney Diseases
COG	Clusters of Orthologous Groups
COSHH	Control of Substance Hazardous to Health
CPVC	Chlorinated Poly Vinyl Chloride
C_T	Threshold Cycle
Cu^{2+}	Copper ions
DNA	Deoxyribonucleic Acid
Dot/Icm	Defective in organelle trafficking/Intracellular multiplication.
dsDNA	double-stranded DNA
ECDC	European Centre for Disease Prevention and Control
ELDSNet	Legionnaires' Disease Surveillance Network
EMA	Ethidium Monoazide
EPA	Environmental Protection Agency
EU	European Union
FRET	Fluorescence Resonance Energy Transfer
gDNA	genomic DNA

GRP	Glass Reinforced Plastic
GU	Genomic Unit
HDPE	High Density Poly Ethylene
HSE	Health and Safety Executive
HSG	Health and Safety Guideline
LCV	Legionella Containing Vacuole
LD	Legionnaire's Disease
LHS	Left Hand Side
LP	Legionella pneumophila
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry.
MIQE	Minimum Information required for the publication of qPCR Experiments
MRSA	Methicillin-Resistant Staphylococcus Aureus
PCA	Principal Component Analysis
PMA	Propidium Monoazide
PPB	Parts Per Billion
PPM	Parts Per Million
PVC	Poly Vinyl Chloride
qPCR	quantitative Polymerase Chain Reaction
RA	Risk Assessment
RHS	Right Hand Side
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulphate
ssDNA	single-stranded DNA
T4SS	Type IV Secretion System
TMV	Thermostatic Mixing Valve
UAT	Urinary Antigen Test

UK	United Kingdom
UKAS	United Kingdom Accreditation Service
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	Ultra Violet
VBNC	Viable But Non-Culturable
WC	Water Closet
WHO	World Health Organisation

List of Publications

Peter, A., Thompson, K. C., & Routledge, E. J. (2017). Barriers to effective Legionella control in a changing world: a practitioner's view. Environmental Technology Reviews, 6(1), 145-155.

Peter, A and Routledge, E (2018). Present-day monitoring underestimates the risk of exposure to pathogenic bacteria from cold water storage tanks. PloS one, 13(4), e0195635.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 This research project

This research project entitled 'Novel Approaches for Risk Management of *Legionella* bacteria in Domestic Water Systems' presents novel applied research taken from the perspective of a *Legionella* control practitioner.

1.2 Discovery of Legionnaires' disease (LD)

On 21st July 1976, about four thousand World War II legionnaires with their families and friends gathered together in Philadelphia, United States of America (USA) to participate in the 58th American Legion's Convention. About six hundred participants were staying in Bellevue Stratford Hotel, Philadelphia (Honigsbaum, 2016). The very next day of the opening day, some of the participants fell ill with flu like symptoms, fever, cough and breathing difficulties. On 27th July 1976, one of the participants had died with the above symptoms. Within a week, more than 234 participants were showed the same symptoms and 34 had died. Five months later, in January 1977, Dr. Joseph McDade and his team succeeded to identify the causative agent of illness, which was isolated from the lung tissues of the infected participants. This, previously unrecognised bacterium named as '*Legionella* pneumophila' and the illness named as Legionnaires' disease (LD) a potentially fatal form of pneumonia (Oliva et al., 2018); Dingman, 2017; Ferguson, 2016).

1.3 Legionella species

The genus *Legionella* is comprised of different *Legionella* species, including *Legionella* pneumophila. New species of *Legionella*, and serogroups, have been discovered since the original discovery in 1977. More than 60 *Legionella* species (Table 1.1) (ECDC, 2017b; Cunha et al., 2016), encompassing at least 70 serogroups (approximately half of

which have been isolated from, or detected in, clinical specimens), have been identified so far; many of which are found to be pathogenic (Benitez and Winchell, 2016; Gomez-Valero et al., 2014). The most commonly detected species in the United Kingdom (UK), European Union (EU) and United States of America (USA) associated with LD is L. pneumophila serogroup-1 (Eisenreich and Heuner, 2016; Essig et al., 2016; Gomez-Valero et al., 2014, Mercante and Winchell, 2015).

Table 1. 1 Currently known *Legionella* species

1. L. anisa,	2. L. birminghamensis,
3. L. bozemanii,	4. L. cardiaca,
5. L. cincinnatiensis,	6. L. clemsonensis,
7. L. dumoffii,	8. L. erythra,
9. L. feeleii,	10. L. gormanii,
11. L. hackeliae,	12. L. jamestowniensis,
13. L. jordansis,	14. L. lansingensis,
15. L. londiniensis,	16. L. longbeachae,
17. L. lytica,	18. L. maceachernii,
19. L. micdadei,	20. L. nagasakiensis,
21. L. oakridgensis,	22. L. parisiensis,
23. L. pneumophila,	24. L. sainthelensi,
25. L. steelei,	26. L. tucsonensis,
27. L. wadsworthii,	28. L. waltersii
29. L. adelaidensis,	30. L. beliardensis,
31. L. brunensis,	32. L. busanensis,
33. L. cherrii,	34. L. drancourtii,
35. L. dresdenensis,	36. L. drozanskii,
37. L. fairfieldensis,	38. L. fallonii,
39. L. geestiana,	40. L. gratiana,
41. L. gresilensis,	42. L. impletisoli,
43. L. israelensis,	44. L. massiliensis,
45. L. moravica,	46. L. nautarum,
47. L. norrlandica,	48. L. quateirensis,
49. L. quinlivanii,	50. L. rowbothamii,
51. L. rubrilucens,	52. L. santicrucis,
53. L. saoudiensis,	54. L. shakespearei,
55. L. spiritensis,	56. L. steigerwaltii,
57. L. taurinensis,	58. L. thermalis,
59. L. tunisiensis,	60. L. worsleiensis,
61. L. yabuuchiae	

Source:https://ecdc.europa.eu/sites/portal/files/documents/ELDSNET_2017-revised_guidelines_2017-web.pdf.

1.4 Legionella ecology

Legionella pneumophila (LP) are gram-negative, motile, rod-shaped bacteria (Figure. 1.1) found naturally in fresh water habitats (Hellinga et al., 2015; Dietrich et al., 2001) and can occur in compost samples, natural soil and potting soil (van Heijnsbergen et al., 2016; Travis et al., 2012), and manmade water storage systems (Vaccaro et al., 2016; HSE, 2015). The Legionella cell envelope is composed of branched-chain fatty acids and distinctive ubiquinone (protein electron carriers used in cellular respiration), whose structural differences are used to classify different *Legionella* species (Morens et al., 2004) –see Section 1.3 (*Legionella* species). In nutrient rich environments *Legionella* pneumophila is approximately 2-20μm in length and 0.3-0.9μm in width. In contrast, in nutrient-deficient environments, the bacteria become long and filamentous (Mekkour et al., 2013).

Despite their association with water, *Legionella* are not considered to be a free-living waterborne bacterium. Instead, they parasitize and reside within free-living protozoans (amoeba) found in freshwater and wet soil (Boamah et al., 2017). The fact that *Legionella* bacteria can multiply intracellularly in a number of different protozoan species is an integral aspect of their ecology. Amoebae also protect *Legionella* from adverse environmental conditions, resulting in increased resistance to acids, antibiotics, biocides and osmotic and thermal stresses (Berjeaud et al., 2016; Greub and Raoult, 2004). Some amoebae species expel biocide-resistant vesicles containing large numbers of *Legionella* bacteria, which subsequently act as airborne agents for the transmission of *Legionella* bacteria (Newton et al., 2010) – see Section 1.6.

The specificity of conditions and nutrients needed for *Legionella* to proliferate and culture in the lab has resulted in them being described as 'fastidious bacteria'. L-cysteine is required as the primary growth factor and ferric iron (often associated with metal rust in domestic water systems) is also essential for optimal growth of this organism. Unlike other organisms, energy is derived from amino acids in *Legionella* species, rather than carbohydrates (Percival and Williams, 2014). *Legionella* can remain dormant in cool water, but will multiply when the water temperature rises to a suitable level (typically between 20 and 45°C). Unlike other bacteria, *Legionella* can survive and multiply in hot water (up to 50°C) (HSE, 2016; Temmerman et al., 2006). Like any other living organisms, *Legionella* also requires nutrients to multiply (Refer Chapter 3, 3.4.5 to 3.4.7). There are a number of nutrient sources including the

organisms which commonly encountered within the water systems itself, for example, amoebae, algae and other bacteria. Amoebae are one of the predominant members of protozoan family which is also found in many biofilm communities, play an important role in the lifecycle of *Legionella* pneumophila by providing habitat, protection and nutrients (Oliva and Buchrieser, 2018; Declerck, 2010). Some studies have reported that the presence and concentration of Legionella pneumophila in biofilms is directly proportional to the biomass of protozoa (Abu Khweek and Amer, 2018). Amoebae can provide not only habitat for Legionella pneumophila but also the debris from dead amoebae can act as nutrient to encourage the proliferation as well as replication (Temmerman et al., 2006).

Importantly, *Legionella* is increasingly detected at high levels in the manmade environment, with contamination of domestic hot and cold water storage systems, showerheads, cooling towers, spa pools, humidifiers, water closet (WC) cisterns, water fountains, water features, air conditioning systems, spas and irrigation systems being reported (Cunha et al., 2016; Garrison et al., 2016; Khodr et al., 2016). Within the built environment, *Legionella* proliferation is also encouraged by water stagnation, and the presence of sludge, slime, scale, corrosion products, biofilms and a pH range of 6-8 (CDC, 2018a; Garrison et al., 2016; ECDC, 2011). *Legionella* is capable of surviving at temperatures ranging from <0 to 60°C (there is some evidence for survival at even higher temperatures – see Rhoads et al., 2016), and can reproduce at temperatures between 20-45°C, with maximum virulence at ~ 37°C (Sharaby et al., 2017). Factors positively associated with *Legionella* proliferation in manmade systems include water stagnation, water pH (between 6.0 to 8.0), the existence of sludge, scale and corrosion products (possibly acting as nutrients), and the presence of biofilms including protozoa (within which it can persist during extreme conditions) (Eisenreich and Heuner, 2016; Oliva et al., 2018).

1.5 Pathogenicity of Legionella bacteria

The ability of *Legionella* to replicate within environmental protozoa also provides it with the capability to replicate in alveolar macrophages in humans, resulting in a form of lobar pneumonia (Newton et al., 2010). Indeed, the term 'pneumophila' means lung-loving (Tsai et al., 1979), and due to the abundance of nutrients and favorable

temperatures, lungs are an ideal environment for *Legionella* to grow and breed. Once *Legionella* enters the lungs, it penetrates deeply into the gas exchange (alveolar) region (Tsai et al., 1979). Within the human respiratory tract, airborne particles deposition is mainly governed by the size of the particles. Normally, the upper region of the respiratory tract expels most of the particles >2 microns in size; however, this expulsion is muted in smokers and alcoholics, and is the reason why smokers and alcoholics are more susceptible to *Legionella* infection (Sopena et al., 1998).

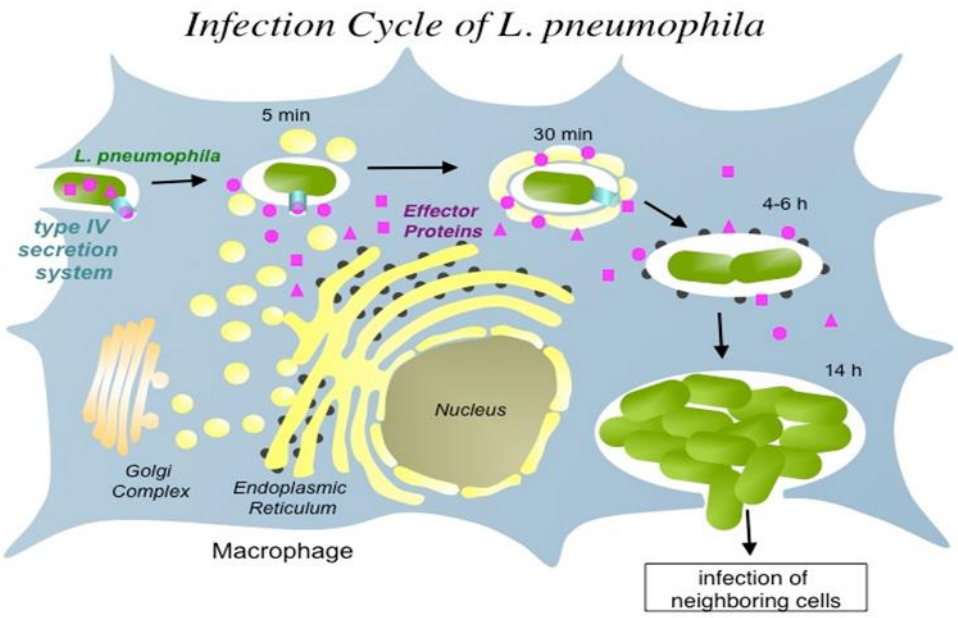


Figure 1.1 Infection cycle of *Legionella* pneumophila within the alveolar macrophage: The *Legionella* Dot/Icm T4SS system produces multiple effector proteins (pink circles and triangles) that initially form a vacuole around the bacteria enabling it to hide from the macrophage lysosome fusion. The vacuole interacts with membranes of the endoplasmic reticulum of the macrophage and becomes similar to the rough ER in appearance and studded with ribosomes. Within the *Legionella* containing vesicle (LCV) the bacteria multiply, and eventually lyse the cell to release more bacteria into the lung.

(Source:
http://www.nature.com/nrmicro/journal/v7/n1/fig_tab/nrmicro1967_F1.html)

Pathogenic bacteria have developed a number of strategies to infect host cells to cause various diseases. Most of the gram negative bacteria make use of type IV

secretion system (T4SS) to distribute their toxins into human host cells where they create a favourable environment for their survival and replication. The first cellular level of the human lungs defence system is the alveolar macrophages and phagocytes which try to ingest and destroy any invader. In most cases, this attempt is successful, but macrophages (or phagocytes) fail to digest the *Legionella* bacteria. Instead, *Legionella* consumes the phagocyte as a nutrient and begins to replicate within the host cell (Figure 1.1) (Bouyer et al., 2007). Intracellular replication of *Legionella* within macrophages requires a special type IV secretion system (T4SS), known as the 'defect in organelle trafficking/intracellular multiplication (Dot/Icm)' system. This Dot/Icm system acts as a carrier for multiple effector proteins, orchestrating a variety of protein-protein interactions within the host cell to form a unique vacuole that escapes lysosome fusion and interacts with membranes and vesicles of the secretory pathway. The formation of this special *Legionella* containing vacuole (LCV) is necessary for the replication of the pathogen and its spread to new cells (Shin and Roy, 2008).

The pathogenicity of *Legionella* pneumophila is therefore a consequence of its ability to penetrate and grow within alveolar macrophages (Shin and Roy, 2008). In most cases *Legionella* bacteria resist the hosts' immune system by cleavage of immunoglobulin and by using its polar flagellum to avoid ciliary clearance (Kabus, 2017; Molofsky and Swanson, 2004). *Legionella* bacteria replication happens exponentially every two hours and the lungs become overloaded with *Legionella* in just a few days, making breathing difficult and leading to death in untreated.

Human health hazards associated with exposure to *Legionella* pneumophila are severe, and appropriate precautions are necessary to minimise the risk of *Legionella* exposure within society. Despite these precautions, hundreds of cases of *Legionella* outbreaks occur every year in the UK (HSE, 2013), and thousands of cases occur worldwide (Borges et al., 2016; Chamberlain et al., 2017). Domestic hot and cold water systems are one of the main sources of Legionella pneumophila exposure in the built environment, and is responsible the majority of the reported cases of Community Acquired Pneumonia (CAP) in the UK and worldwide (Murdoch et al., 2018; Cunha et al., 2016).

1.6 Exposure to Legionella bacteria

Primary mode of transmission of *Legionella* is by inhaling or aspirating aerosols or soil (Figure 1.2) containing the bacteria (Craun, 2018; Soda et al., 2017; Travis et al., 2012). There are some instances where LD may have been contracted by inhaling bacteria during the ingestion of contaminated water (Tateda et al., 2003). Recent studies have also identified exposure to potting compost, soil, and gardening activities to be risk factors (Kenagy et al., 2017; Van Heijnsbergen et al., 2016), as 12% of garden soil samples and up to 69.3% of composts samples have been found to be contaminated with *Legionella* species including *Legionella* pneumophila and *Legionella* longbeachae (Van Heijnsbergen et al., 2016; Conza et al., 2013a). Once the bacteria enters the lungs, the normal incubation period is 2-16 days (occasionally this may take even longer) before the subject starts to show symptoms of the disease (CDC, 2018b; NHS, 2018).

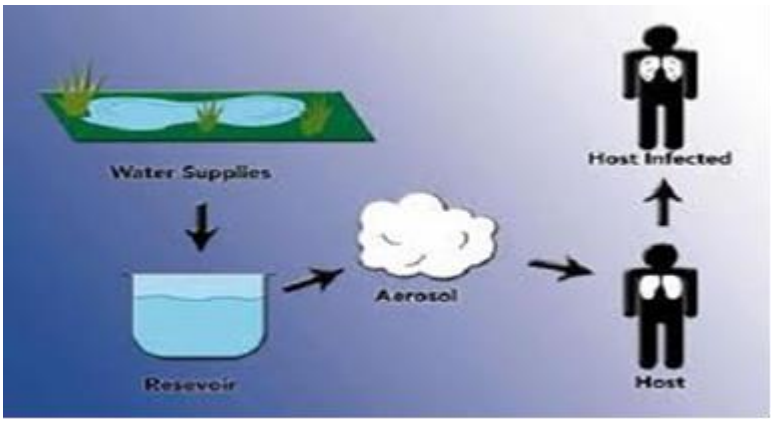


Figure 1.2 Root of infection from water supply, water storage, aerosols generation and contraction with human host. (Source: http://blogs.bournemouth.ac.uk/research/2012/05/17/bu-student-identifies-*Legionella*-pneumophila-in-windscreen-washer-fluid/).

Until recently, it was also believed that LD could not be transmitted from one person to another (Correia et al., 2016). In this example, a forty eight year old cooling tower engineer in Portugal passed the disease on to his 74 year old healthy mother who was caring for him. Both patients died within 1 week of each other. Analysis of urine specimens from both patients were positive for *Legionella* antigens, and culture

analysis of respiratory secretions obtained from both the patients confirmed a severe infection with *Legionella* pneumophila serogroup 1. Separate *Legionella* whole genome sequencing carried out using on isolates at the National Institute of Health in Lisbon revealed that both genomes matched one another. Further investigation revealed that the second patient was in close contact with the first patient during the time of taking care of the first patient in a poorly ventilated room and the timeline of second infection was highly coherent; 6-7 days for the typical incubation of *Legionella* pneumophila. This study concluded by reporting this to be an example of person to person transmission of LD (Correia et al., 2016; Borges et al., 2016).

1.7 Seasonal patterns

Generally, most of the cases of LD especially LD outbreaks are closely related to seasonal patterns, with the highest number of reported outbreaks occurring in the late summer to autumn (EPA, 2015a). Globally there is an increasing trend of Legionnaires disease in summer season (Simmering et al., 2017; Cunha et al., 2016) and latest studies have suggested that the recent increases in cases of CAP could be due to increases in precipitation, warm and humid weather associated with climate change (Raeven et al., 2016; Beauté et al., 2016; Sakamoto, 2015). Fisman and team have reported that wet humid weather often occurs six to 10 days prior to the occurrence of LD (Fisman et al., 2005). In addition, a study by the US Nationwide Inpatient Sample and US weather data found that LD risk significantly increased when weather was warm and humid, with a dose-response relationship between relative humidity and the likelihood for Legionellosis. When the mean temperature was 15–26°C with humidity >80.0%, the diagnosed cases of LD among CAP were 3.1 times higher than that with humidity level of <50.0%. In other words, the likelihood of LD within CAP cases increases when weather is warm and humid (Simmering et al., 2017). Increased survival of *Legionella* pneumophila at high relative humidity is reported, although aerosol viability data from laboratory studies might not represent the true environmental situation (Phin et al., 2014)

A study in the Netherlands to investigate the short-term effects of the weather on Legionellosis reported peak weekly incidences of LD to occur when mean weekly temperatures reached 17.5^0C, with high relative humidity. A slight increase in average weekly sunshine (1 hour) resulted in an additional increase of 1. 8% (95% CI 1. 2–2. 4). A mean weekly cloud cover of 7-8 oktas was found to cause the relative humidity to rise

to very high levels. Indeed, a 1% increase in relative humidity was associated with a 4 - 6% (95% CI 4. 7–8. 2) increase in the cases of LD. When the average weekly precipitation intensity was 3 mm/h (P=0. 004), the reported cases of LD was maximal, therefore, the report stated that the LD cases are highest when average weekly precipitation falls in the range of 40 - 60 mm in summer season. Thus, this study also established a correlation between LD with humidity, temperature and precipitation as during humid and showery summer season, there was significant increase in the number of reported cases of LD. This investigative study concluded that the result obtained from this study can be used for the future prediction of LD cases in The Netherlands during showery summer season (Karagiannis et al., 2009).

Another study conducted in Taiwan using long term daily data from 1995-2011 reported changing weather to be a significant risk factor in LD cases. In this study, Chen and team investigated the impacts of temperature, precipitation and relative humidity on LD. Daily precipitation levels of 61–80 mm (95% CIs 1.106–5.978) in warm season significantly increased the number of cases of LD compared to 21–40 mm (95% CIs=1.074–2.513) precipitation levels of the same season in previous years. In contrast, this study did not identify any linear correlation between relative humidity alone and increased number of cases of LD and concluded that the increased daily precipitation in humid warm region is a critical risk factor for maximising the occurrence of Legionnaires' disease (Chen et al., 2014).

Investigation reports from the UK also confirm that warm and wet weather conditions can significantly increase the number of cases of community acquired Legionnaires' disease due to the increased rate of Legionella proliferation (Halsby et al., 2014). Predicted global warming could become one of the most risky contributing factors of increased community acquired LD in EU and in the UK. Possible temperature increases within domestic cold water systems could encourage Legionella proliferation and replication. Moreover, increased humidity may prolong Legionella pneumophila survival in aerosols thereby increasing the likelihood of exposure to Legionella via inhalation of contaminated aerosols (Simmering et al., 2017; Prussin II et al., 2017).

Another source of evidence for extensive *Legionella* proliferation during wet warm weather conditions is the PHE data of reported cases of LD from 2007 to 2016. The

MET office reported August 2007to be the wettest summer month since records began in 1914 (MET, 2008; ncdc, 2008). Later the MET office reported 2007 to be the year of heaviest rainfall ever recorded (MET, 2008). In light of these weather reports, it is interesting to note that the maximum number of LD cases reported in the UK also occurred in 2007 since *Legionella* monitoring began in the UK in 1988 (Figure 1.3) (PHE, 2016a).

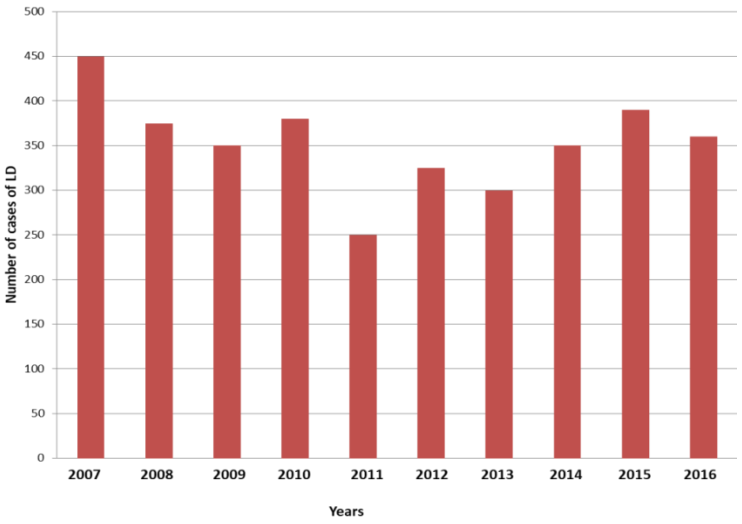


Figure 1.3 Graphical representation of the number of reported cases of LD in England and Wales on annual basis from 2007 to 2016; maximum number of reported cases of LD was in 2007. Source: (PHE, 2016a).

1.7.1 *Future perspectives - Climate change*

A study carried out by the European Legionnaires' Disease Surveillance Network (ELDSNet) found that 10582 out of 11836 suspected cases of LD were confirmed between 2009 and 2010 in the European Union. Out of the 10582 confirmed cases, 71% (n=7397) were community acquired. Healthcare related cases were only 8% (n=893) and the remaining 20% (n=2187) were travel-related. The UK Health Technical Memorandum for Safe water in healthcare premises – Part B also confirms that "the incidence of healthcare associated waterborne illness, including Legionnaires' disease, is relatively low" (HTM, 2016). 43% of total reported cases (n=5100) were found in individuals aged 65 and above (Beaute et al., 2013). This study also confirmed a significant increase of reported cases in 2010 (n=775) possibly as a result of the exceptionally warm summer that year (Barriopedro et al., 2011). Long-term climate

predictions indicate that EU and UK temperatures and precipitation are likely to increase in the near future as a part of global warming (Vautard et al., 2014; Hajat et al., 2014; Chou et al., 2012) and environmental conditions are likely to become increasingly favourable to the proliferation of Legionella bacteria in water systems (Sakamoto, 2015; Hicks et al., 2007).

1.8 Legionellosis

Legionella bacteria are known to cause three types of clinical infections that differ in their severity, termed Legionnaires' disease (LD) Pontiac fever and Lochgoilhead fever (CDC, 2018c; Goldberg et al., 1989). Legionellosis is the general term used to describe all the diseases caused by *Legionella* bacteria (HSE, 2013). Out of these three illnesses, Legionnaires' disease is the most serious one (being a potentially fatal pneumonic form of the disease) and other two are considered as less serious and non-fatal.

In 2015, 96.1% of culture-confirmed cases of LD in Europe were attributed to *Legionella* pneumophila (HPS, 2017). However, recent reports from different parts of the world, including Australasia, indicate that LD can also be caused by another species, known as *Legionella* longbeachae (Isenman et al., 2016; Cameron et al., 2016), especially in the immunosuppressed population as well as people with underlying diseases (Kenagy et al., 2017; Edelstein and Christian, 2015). *Legionella* pneumophila is responsible for up to 17.5% of cases of diagnosed community acquired pneumonia (CAP)(Para et al., 2018). In Europe itself, the reported cases of CAP ranged from 1.6 to 9 cases per 1000 in adult population every year (Chen et al., 2018). A review of 41 studies of community acquired pneumonia (CAP) in Europe estimated *Legionella* species to be responsible for 1.9% of outpatients, 4.9% of hospitalised patients and 7.9% of ICU patients (Simmering et al., 2017). *Legionella* pneumophila is responsible for 80–85% of *Legionella* infections worldwide, and together serogroups 1 and 6 are responsible for two-thirds of all Legionnaires Disease cases (Victor et al., 2002). Although *Legionella* pneumophila serogroup 1 is the most frequently identified cause of LD, recent studies suggest that about 20% of LD cases are caused by *Legionella* pneumophila serogroup 3, *Legionella* pneumophila serogroup 9, *Legionella* pneumophila serogroup 6 and *Legionella* longbeachae with non-specific symptoms, such as cough and fever (Ito et al., 2017).

LD has no particular clinical features to distinguish this disease from other types of pneumonia. Majority of the cases start with a headache, dry cough, fever and sometimes diarrhoea, but laboratory investigation is necessary to confirm the diagnosis. The normal incubation period of *Legionella* species is thought to be 2–10 days; however, recent studies have reported longer incubation periods up to 19 days. Males are more at risk than females in terms of Legionnaires disease and is estimated that of all the reported cases of LD, 60–70% are male (WHO, 2018; ECDC, 2017; Beauté, 2017). Generally, fatality rate of LD is 8–12%, but there are evidences that in the cases of elderly people, patients with pre-existing medical conditions, smokers, and the cases of delayed diagnosis and treatment shows higher mortality rate up to 34% (Phin et al., 2014).

Lochgoilhead fever is caused by a species called *Legionella* micdadei and its incubation period is up to nine days and this illness is named after an outbreak in Lochgoilhead, Scotland (Gobin et al., 2009). Lochgoilhead fever is associated with only milder flu-like symptoms (Benin et al., 2002) and there have been no recorded deaths associated with this form of Legionellosis (www.cdc.gov/ncidod). Pontiac fever is caused by another species of *Legionella*; the normal incubation period is 2-3 days and is an acute self-limiting flu-like illness with symptoms that mimic influenza such as nausea, malaise, sore throat, a non-productive cough vomiting, and abdominal pain, without pneumonia (Diederen, 2008).The diagnosis of Pontiac fever is made on the basis of clinical, epidemiological and environmental microbiology findings. It is reported that the infection rate is more than 90% among exposed population and approximate recovery rate is one week (Tossa et al., 2006).

1.9 Factors affecting the virulence of Legionella

Whatever their specific cause of LDS, approximately 10-15% of the reported cases are fatal. Although most of the population is susceptible to these infections, susceptibility to disease can be higher in certain groups (Percival and Williams, 2014; EPA, 2016; Borges et al., 2016; Edelstein and Christian, 2015). Major contributing factors affecting susceptibility to *Legionella* bacteria are a weakened immune system, being over 50 years of age, and suffering from existing illness such as respiratory problems, kidney disease, diabetes and cancer (HSE, 2000; Qin, 2012). Currently, one in six people (18%) living in the UK are reported to be aged 65 and above, and is

estimated that this figure will be one in four (25%) by 2050 (ONS, 2017). Data from Eurostat on population structure and aging clearly shows an increasing trend for an aging population within European Union (EU) countries (Table 1.2). By 2080, the total proportion of people over 65 years of age residing in the EU is expected to reach 28.7% compared to 18.9% in 2015 (Marois et al., 2018; PSA, 2016; Eurostat, 2017).

Table 1.2 Aging population trend in Europe during the period 2005 – 2015 taken from Eurostat

	0–14 years old		15–64 years old		65 years old or over	
	2005	2015	2005	2015	2005	2015
EU-28 (1)	16.3	*15.6*	67.2	*65.6*	16.6	*18.9*
Belgium (1)	17.2	17.0	65.6	64.9	17.2	18.0
Bulgaria	13.7	13.9	68.9	66.2	17.4	20.0
Czech Republic	14.9	15.2	71.1	67.0	14.1	17.8
Denmark	18.8	17.0	66.1	64.4	15.0	18.6
Germany (1)	14.5	13.2	66.9	65.8	18.6	21.0
Estonia (2)	15.4	16.0	68.0	65.2	16.6	18.8
Ireland	20.7	*22.1*	68.2	*64.9*	11.1	*13.0*
Greece	15.1	14.5	66.7	64.5	18.3	20.9
Spain	14.5	15.2	69.0	66.3	16.6	18.5
France (1)	18.7	*18.6*	65.1	*63.0*	16.3	*18.4*
Croatia (2)	15.9	14.7	66.7	66.5	17.3	18.8
Italy	14.1	13.8	66.4	64.5	19.5	21.7
Cyprus	19.9	16.4	68.0	69.0	12.1	14.6
Latvia	15.0	15.0	68.4	65.6	16.6	19.4
Lithuania	17.1	14.6	67.1	66.6	15.8	18.7
Luxembourg (1)	18.6	16.7	67.3	69.2	14.1	14.2
Hungary (1)	15.6	14.5	68.8	67.6	15.6	17.9
Malta	17.6	14.3	69.0	67.2	13.3	18.5
Netherlands	18.5	16.7	67.5	65.4	14.0	17.8
Austria	16.1	14.3	67.9	67.2	15.9	18.5
Poland (1)	16.7	15.0	70.2	69.5	13.1	15.4
Portugal	16.0	*14.4*	66.8	*65.4*	17.2	*20.3*
Romania	17.5	*15.5*	68.4	*67.5*	14.2	*17.0*
Slovenia (1)	14.4	14.8	70.2	67.3	15.3	17.9
Slovakia	17.1	15.3	71.3	70.7	11.7	14.0
Finland	17.5	16.4	66.6	63.7	15.9	19.9
Sweden	17.6	17.3	65.2	63.1	17.2	19.6
United Kingdom	18.1	*17.7*	65.9	*64.6*	15.9	*17.7*
Iceland	22.3	20.4	65.9	66.1	11.8	13.5
Liechtenstein	17.6	15.1	71.3	68.9	11.1	16.0
Norway	19.7	18.0	65.6	65.8	14.7	16.1
Switzerland (1)	16.3	14.9	67.9	67.3	15.8	17.8
Montenegro (2)	20.8	18.5	66.7	67.8	12.5	13.7
FYR of Macedonia (2)	20.0	16.8	69.1	70.5	10.9	12.7
Albania	26.5	18.6	65.1	69.0	8.3	12.5
Serbia (1)	15.8	14.4	67.0	67.2	17.1	18.5
Turkey	27.5	24.3	65.9	67.8	6.7	8.0

(1) Break in time series in various years between 2005 and 2015.
(2) The population of unknown age is redistributed for calculating the age structure.
Source: Eurostat (online data code: demo_pjanind)

In 2009, 23% of the total population of Japan was over 65 years of age, and this is expected to rise above 33% by 2030 (Muramatsu and Akiyama, 2011). Also, in the United State of America (USA), people aged 65 and above is expected to reach 24% of the total population by 2060 compared to 15% in 2016, with further increases by 2075 (PRB, 2016). In a recent report by the WHO it states that "as people get older, they become less active and the overall evidence for adults aged 65 years and above showed that they become more susceptible to disease compared to active individuals" (WHO, 2017). However, once men reach 45 years they are known to be at higher risk of contracting LD (Farnham et al., 2014), and this risk increases with age up to 65 years of age and above. All these projections indicate the necessity of precautionary measures

to be implemented to protect the health of this potential vulnerable population in terms of CAP.

Among the over 65 year olds, diabetes and Chronic Kidney Diseases (CKD) are the most common risk factors for LD (Russo et al., 2018; Cai and Chen, 2016), and about 40% of individuals aged 65 and above living in the UK suffer from long standing illnesses (LLUK, 2015). Currently, 12 million people in the United Kingdom were reported to be over 65 years old, and forecasts suggest this figure will rise to approximately 19 million by 2050 (Parliament, 2015). As revealed by the 2011 Census Analysis, only 3% of over 65 year olds reside in care homes across England & Wales, and 2.5% in London (ONS 2014). A recent estimate suggests that there are 5,153 nursing homes and 12,525 residential homes in the UK and approximately 405,000 people aged 65 and above live in these homes (LLUK, 2015). By inference, approximately 10.7 million over 65s in the UK therefore reside within their own flats and apartments of residential estates sharing communal hot and cold water facilities and these elderly population also fall in the same risk category i.e. 'potential high risk' as the population in healthcare premises in terms of LD.

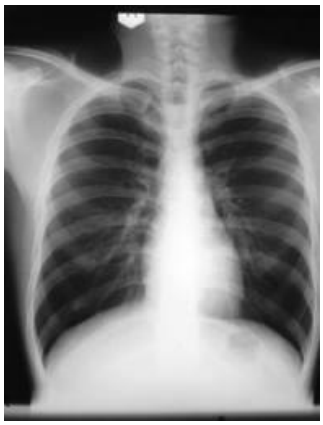
1.10 Clinical diagnosis of LD

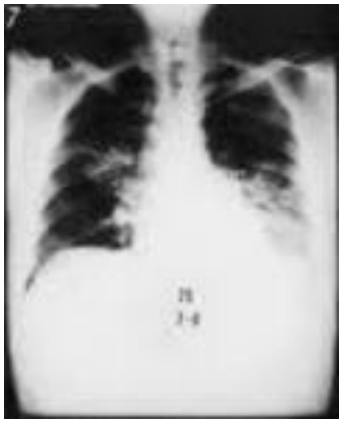
Initial symptoms of Legionnaires' disease (LD) are a high fever, headache, chills and muscular pain. Generally, LD patients become lethargic and develop a non-productive cough at the beginning, but eventually it becomes productive and some patients (about a third) may develop watery diarrhea and vomiting, and half the cases report symptoms of confusion, and being delirious (Cunha et al., 2016). Not everyone who is exposed will develop the symptoms of LD and in some cases human hosts may only have flu-like symptoms (Kuroda and Takeuchi, 2011). In contrast, some of the infected patients develop blood-streaked or pus-forming sputum with severe pneumonia symptoms, and this can be fatal if not diagnosed and treated at the right time (EWGLI, 2011).

The clinical diagnosis of Legionnaires' disease was done by culture (isolation of any *Legionella* species from lung tissue, respiratory secretions, pleural fluid etc.) and serological investigation. But these methods are time consuming with low sensitivity (Phin et al., 2014). Since early-1990s, clinical diagnosis was done by analysing

Legionella pneumophila serogroup 1 antigen in the urine of infected patients (Garrison et al., 2016; Musher et al., 2014). However, *Legionella* urinary antigen test was not recognised as a diagnostic method until 1996; but, later this method was identified as reasonably reliable diagnostic procedure and it was commercialised as routine test kits (Garrison et al., 2016; Helbig et al., 2003). Currently, urinary antigen detection method is used for clinical diagnosis of in 70-80% of LD cases in Europe and USA. However, there are remarkable limitations in this method as it detects only *Legionella* pneumophila species but very poor sensitivity with non-*Legionella* pneumophila strains (Reller et al., 2003). Currently PCR diagnostic method is considered far more sensitive than rapid antigen tests (Musher et al., 2014). Unfortunately, commonly prescribed antibiotics such as 'Erythromycin' or 'clarithromycin' are ineffective against *Legionella,* but can slow or stop the intracellular multiplication of *Legionella* thereby giving the immune system a chance to regain control (Haranaga et al., 2007). Once the immune system gains the upper hand, recovery can start and may eventually clear the infection, although permanent lung damage can occur (Figure 1.4) (Tateda et al., 2003). *Legionnaires' disease* can be fatal if not diagnosed on time and treated appropriately (Cunha et al., 2016; LDOIT 2014).

LD is usually diagnosed by the culture of samples of sputum, lung tissues, blood, urine or by chest radiograph (Valster et al., 2011; McDade et al., 1977). One of the most complicated issues with LD is the difficulty in distinguishing clinically between patients with LD and other types of pneumonias caused by Chlamydophila, Streptococcus and Mycoplasma; all of which have identical radiological and clinical findings (Sharma et al., 2017; Sopena et al., 1998). Legionnaires' disease has a wide spectrum of severity and frequency of extra pulmonary manifestations; and there are many reported cases of continued progression of pneumonia despite of appropriate treatment, with the possibility of respiratory system failure and breathing arrest. In cases of severely LD infections, clinical improvement can be very slow, and there may be no signs of improvement for more than a week even after appropriate antibiotic therapy has been administered (Kao et al., 2017; Roig and Rello, 2003).





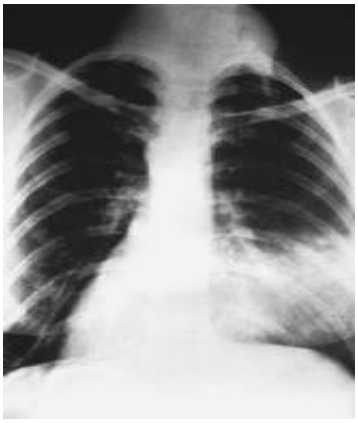


Figure 1.4a. **Figure.1.4b** **Figure.1.4c**

Figure 1.4a represents a normal and healthy chest X-Ray with full lung capacity (dark shaded area). Figure 1.4b. Chest X-Ray after *Legionella* infection; dark shaded area has been diminished due to the lung damage and reduced lung capacity. Figure 1.4c. Chest X-Ray years later of infection, dark shaded area is still remaining almost same as that of after the infection indicates that the permanent lung damage can occur during *Legionella* infection.

(Source: http://www.mevis-research.de/~hhj/Lunge/xSammlungInf2Fr.html)

1.11 The epidemiology of Legionnaires disease

Epidemiology is the scientific study of diseases; in terms of incidence, distribution, and possible control of diseases and other factors relating to various health problems (WHO, 2018). An epidemiologic investigation determines the nature and source of *Legionella* infection; therefore, epidemiological investigation is extremely important in any outbreak of Legionnaires' disease.

Until recent years, epidemiology data of Legionnaires' disease was limited to countries such as USA, Europe, Canada, Australia, New Zealand Japan and Singapore where LD is considered as a notifiable and fatal infectious disease. In Europe, LD surveillance schemes are in place since 1995 and many epidemiological studies and researches are in progress in terms of Legionnaires' disease. However, LD is likely to be underestimated or unrecognised in many countries; thus the global quantification of LD is difficult (Phin et al., 2014). According to the available global data, the age and sex distribution of LD cases are almost similar between countries and most cases occur in older people, with very rare in children (Greenberg et al., 2006). In 2011, the average rate of infection in EU countries was 9.2 per one million people, but the range was 0-21.4 per million among EU countries. In Europe, the highest number of cases was

reported from Spain, France and Italy; however, globally highest numbers were reported from USA with an increase of 11.5 per million in 2009 compared to 3.9 per million in 2000 (Beauté et al., 2013; Jacquinet et al., 2015).

LD is broadly classified into three categories; community acquired, nosocomial and travel related. Community acquired pneumonia (CAP) is defined as the LD cases without any history of overnight stays in hospital or healthcare facilities or holiday or travel abroad or hotel accommodation prior to showing symptoms of the disease. Nosocomial can be defined as the infection received from hospital or nursing home or any other healthcare facility for at least 10 days prior to showing symptoms of Legionnaires' disease. This can be a hospitalised patient, an outpatient, a hospital staff or a healthcare worker. Travel related cases are when an individual is clinically and microbiologically diagnosed with Legionnaires' disease, and who has spent at least one night in a holiday place, business accommodation or in a hotel anywhere in the world prior to showing symptoms of the disease. This overnight stay can be in camp sites, rented holiday apartments, tourist facilities, ships and any other accommodation other than that mentioned in CAP and nosocomial related cases (LDCD, 2016). Many studies and investigations have identified that the long-time un-occupancy of hotel rooms with large number of outlets cause water stagnation within the pipeworks and outlets encourage the proliferation of *Legionella* bacteria, unless proper control measures and monitoring systems are in place (Phin et al., 2014; Ricketts et al., 2010; WHO, 2018a). Recent studies have reported that the same scenario is applicable in the case of cruise ship in terms of Legionnaires' disease (Guyard and Low, 2011; Garrison et al., 2016). Travel related cases of LD varies from countries to countries as 1.68 cases per one million nights were reported from Greece in 2009, but in the same year only 0.55 cases per one million nights were reported from the UK (ECDC, 2017).

1.12 Epidemiological investigations

Two common 'terms' widely used in terms of Legionnaires' disease are 'outbreaks' and 'cluster'. An 'outbreak' can be defined as two or more cases of LD closely linked in time in terms of weeks (less than a month) and where there is epidemiological evidence that the source of infection is common but no microbiological evidence is collected (LDCD, 2016). In the UK, Europe and USA, an incident control team should be appointed to investigate any confirmed outbreaks. A cluster is defined as more than two cases that appear to be linked by work place, residence, community settings,

hospital or other healthcare premises and which have close proximity in dates of infection (LDCD, 2016). Knowledge of the potential source of LD is an essential step in preventing further spread of the disease (Greig et al., 2004).

Once a diagnosis is confirmed, all infected patients and their close contacts need to be interviewed by using a standardised questionnaire to collect information on current disease, place of residence, potential risk factors and movements two weeks prior the illness occurred. In most of the cases, these interviews were conducted in the hospital where the patient is admitted and further interviews with close contacts are conducted with the use of their personal diaries to clarify the details of their movements prior to the outbreak. This investigation can be extended to the routes taken to and between home, shops, workplaces, leisure centres and any other locations including any trips out of the local area (PHE, 2018). The travel details are extremely important in epidemiological investigation as this is the key factor to establish the outbreak as travel related or local. If there is no evidence of travel related outbreak, the detailed history of local movements down to road and postcode level is conducted wherever possible. Any identification of new confirmed cases, the case histories need to be compared to find out the possible association of infection. In some cases, new information can emerge from investigating the confirmed cases of new diagnosis, and in such cases repeated interviews with infected patients and their close contacts can help to accelerate the progress of epidemiological investigation by linking information from various sources to reach a potential common source (HPS, 2014; White et al., 2013; Kirrage et al., 2006). A Legionnaires 'disease outbreak followed by an epidemiological investigation in Genesee County, Michigan during 2014-2015 is a typical example of this kind. Complete investigation report was obtained during 2016-2017 which includes review of completed medical records of infected patients, death certificate information, environmental testing results, laboratory testing summaries, reports of interview with patients, water system details, residents details etc. (Michigan, 2018).

1.13 LD in the UK

According to the reports from PHE published in December 2017, the number of potential cases of Legionellosis reported during the year 2016 was 510. Out of these, 357 cases were confirmed of Legionellosis. The total number of cases of legionellosis in 2016 was lower than in 2015 (389 confirmed cases). In contrast, the total number of

cases in 2016 remain higher than the annual confirmed case numbers between 2011 and 2014 and is above the 349 mean annual number of cases observed since 2006 (PHE, 2016). In England and Wales, average fatality rate for confirmed cases of Legionnaires' disease during 2014 – 2016 was 7.2% with community acquired 9.8%, nosocomial 22.2% and travel related (abroad and UK) 3% (PHE,2016). Another report from NHS indicated that during the period of 2011 to 2013, there were 84 deaths confirmed from LD in England and Wales (NHS, 2015). In addition, there are other reports indicating that the actual number of deaths and infected cases are much more than officially estimated. These findings are supported by another report from the British Lung Foundation, which showed that 0.5-1.1% of adults in the UK get pneumonia each year (BLF, 2018); however, in most of these cases are treated only on the basis on their primary symptoms without diagnosing the actual cause of infection. Therefore, it is likely that the number of infected cases of LD and the number of deaths caused by LD reported by NHS are not a reflection of the true extent of Legionnaires Disease in the UK.

Many studies have reported that *Legionella* species are the predominant causative agent for community-acquired pneumonia (CAP) (Hashmi et al., 2017; Viasus et al., 2013). WHO reported that *Legionella* species are responsible for 2–15% of hospital admissions for community-acquired pneumonia (Sakamoto, 2015). However, some of the studies have reported that *Legionella* is not the main causative agent for CAP (Ryu et al., 2017; Dissanayake et al., 2016). This contradictory report indicates the fact that analysing for *Legionella* species is not recommended for all patients contracted with pneumonia, but is recommended only for particular patient subgroups. This can result in the overall underestimation of the role of *Legionella* species in CAP (Decker et al., 2016).

Another study conducted in 1992-1993 estimated the annual cost of managing 261,000 reported cases of community-acquired pneumonia in the UK to be in the order of £440.7 million at 1993 prices, and the 83,153 cases of CAP that were treated in hospital accounted for 96% of the annual cost (Guest and Morris, 1997). This study concluded with a warning that new strategies should be developed and implemented to prevent the rising number of CAP and related hospitalisation; otherwise, this cost could be significantly high in the future (Guest and Morris, 1997).

An investigation in 2003 related to a community acquired Legionnaires' disease outbreak in the UK, reported 28 cases of LD with a fatality rate of 7% (2 deaths), and all

infected cases were epidemiologically linked to one location (Kirrage et al., 2006). Another investigation on Nosocomial outbreaks of Legionnaires' disease in England and Wales during the period of 1980 to 1992 reported that 62% of the total outbreaks of LD were associated with hospitals or other healthcare premises, with the remaining being individual or single isolated cases (Joseph et al., 1994). Recent evidence suggests that LD cases in the UK are steadily rising every year; a recent report from the Chartered Institute of Plumbing & Heating Engineering (CIPHE) also supporting this concern of increasing number of LD (CIPHE, 2017). However, the actual number of outbreaks and infected cases is likely to be much higher than reported figures as majority of the cases with pneumonia symptoms are treated without a clinical diagnosis of the actual cause of the infection (Decker et al., 2016).

According to the PHE (PHE, 2016), the majority of LD cases in the UK are related to travel abroad. However, the use of domestic water facilities, especially showers, upon return from holiday may pose a very high risk in terms of LD exposure and infection. *Legionella* bacteria may proliferate in domestic water systems due to the water stagnation that is typical of periods of non-usage, for example in the holiday period. In addition, the cold water storage tank located in the loft space or roof may warm to a favourable temperature for *Legionella* bacteria proliferation in the summer if not replenished often with fresh water. Another report of PHE states that the maximum fatality rate is reported from nosocomial LD cases (Table 1.3), and the second largest death rate is from community acquired LD cases (PHE, 2016).

Table 1.3 Case fatality rates for confirmed cases of Legionnaires' disease between 2014 – 2016 in England and Wales.

	Cases	Deaths	Case Fatality Rate (%) (95% CI)
Community	580	57	9.8 (7.5 - 12.5)
Nosocomial	27	6	22.2 (8.6 - 42.3)
Travel Abroad	463	14	3.0 (1.7 - 5.0)
Total	1070	77	7.2 (5.7 - 8.9)

Source:https://www.gov.uk/government/statistics/legionnaires-disease-in-england-and-wales-2016

1.14 LD in the EU

Legionella species (especially *Legionella* pneumophila), is a leading cause of community-acquired pneumonia in many European countries. Many studies have reported that in recent years the number of cases of Legionnaires' disease detected in Europe has increased significantly and is mainly community acquired (Beauté, 2017). A report from Germany showed that the number of undiagnosed cases of LD is increasing in recent years because the urine antigen test does not recognise species of *Legionella* other than *Legionella* pneumophila following clinical presentation. Furthermore, the clinical presentation and outcome of community acquired *Legionella* pneumophila infection differs significantly from hospitalised patients and outpatients of nosocomial *Legionella* infections (Murdoch et al., 2018; von Baum et al., 2008).

European Surveillance Scheme for Travel Associated Legionnaires' Disease (EWGLINET) have a formal reporting procedure to monitor and provide epidemiological and microbiological information on the total number of reported cases of LD, travel-related and non-travel related detected in each EU countries. Since April 2010, EWGLINET is coordinated by ECDC and the name of the scheme changed to European Legionnaires' Disease Surveillance Network (ELDSNet). According to the reports from 30 EU countries in 2015, total reported cases of LD was 7034; 6573 (93.4%) of which were classified as confirmed and the remaining 461 (6.6%) cases were probable (ECDC, 2017). There are serious concerns about the current lack of coordinated testing procedures for LD which severely hampers outbreak investigations because fewer clinical isolates are available to match against environmental isolates (ECDC, 2015; Hartemann and Hautemaniere, 2011). For example, a travel related outbreak of LD occurred in Spain during the summer of 1993; five cases of LD were reported with one death related to a tour group from the UK. This tour group stayed at four different hotels in Spain with other French tourists. Genotypic and phenotypic comparison of *Legionella* pneumophila isolates obtained from one of the UK cases and the French case showed that they were indistinguishable from each other, and from environmental isolates obtained from the water facility of the hotel at which all five cases had stayed. A cohort study of the UK tour group was carried out to determine the extent of this outbreak and the international participation in this investigation has proved the importance of a European surveillance scheme for Legionnaires' disease

and the benefit of microbiological collaboration between *Legionella* testing laboratories in Europe (Joseph et al., 1996).

The world's largest outbreak of Legionnaires' disease reported to date occurred in July 2001 in Murcia (Spain). 449 cases of LD were confirmed among more than 800 suspected cases reported, although the mortality rate was only 1%. During epidemiological investigation, 600 completed questionnaires indicated that the point of exposure was an outdoor point in the Northern region of the city. An environmental isolate from the point of exposure and clinical isolates from the infected patients were identical and subsequently identified and supported the epidemiologic investigation and conclusion (García-Fulgueiras et al., 2003). These examples illustrate the importance of collaborative efforts to link disease outbreaks to sources of exposure on an international scale, given increasing mobility within society.

In general, LD raises serious concern in all 30 European Union and European Economic Area (EU/EEA) countries as recent data showed the number of reported cases of LD is rising every year (Table 1.4). Between 2005 and 2010, 5,500-6500 LD cases were reported in Europe annually making one case per 100,000 people (Beauté et al., 2013). But in 2015 alone, the reported cases of LD in EU were 7034 of which 6573 were confirmed cases (ECDC, 2017).

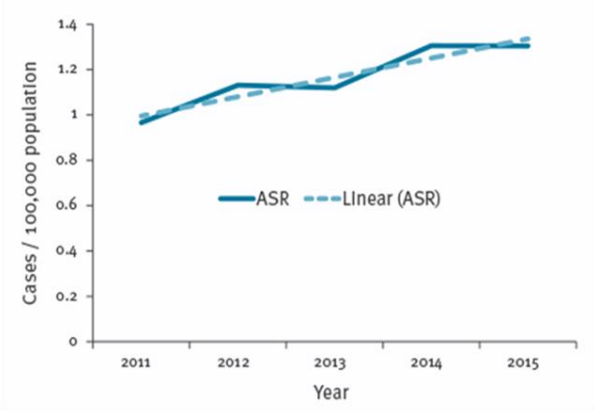
Between 2011–15, 29 countries had reported 30,532 LD cases to ECDC; 92.3% (28,188) were confirmed cases and 7.7% (2,344) were probable cases. There was a significant increase in confirmed cases of LD from 2011(90.6%) to 2015(93.3%). For example: Lativia, Poland and Romania had only a few confirmed cases in 2011(38%, 44%, and 0%, respectively) compared to 2015 when more than 70% of cases were confirmed in each country. In 2011, the total number of reported cases in these countries was 4,915, but in 2015, this figure has gone up to 6,986 due to better monitoring. Furthermore, the age-standardised rate ASR) also increased (Figure 1.5 A) from 0.97 LD cases per 100,000 population in 2011 to 1.30 LD cases per 100,000 population in 2015 which is equal to an annual average increase of 0.09 LD cases per 100,000 population (95% CI, 0.02–0.14; p = 0.02)(Beauté, 2017).

Table 1.4 Number of reported cases of LD and age-standardised rates (ASR) per 100,000 populations, by reporting country and year, European Union/European Economic Area, 2011–2015.

Country	2011		2012		2013		2014		2015	
	Number	ASR	Number	ASR	Number	ASR	Number	ASR	Number	ASR
Austria	96	1.12	104	1.22	100	1.13	133	1.48	160	1.79
Belgium	79	0.71	84	0.45	155	1.37	200	1.72	196	1.44
Bulgaria	0	0.00	0	0.00	1	0.01	1	0.01	1	0.01
Cyprus	1	0.13	7	1.01	6	0.76	6	0.80	2	0.25
Czech Republic	57	0.52	56	0.53	67	0.63	110	1.03	120	1.10
Denmark	123	2.20	127	2.29	113	2.02	158	2.76	185	3.24
Estonia	7	0.51	3	0.24	10	0.76	8	0.59	6	0.43
Finland	9	0.16	10	0.18	15	0.24	10	0.17	17	0.27
France	1,170	1.84	1,298	2.01	1,262	1.92	1,348	2.04	1,389	2.07
Germany	635	0.73	628	0.72	810	0.90	832	0.92	865	0.95
Greece	18	0.16	29	0.25	38	0.33	27	0.24	29	0.25
Hungary	37	0.36	33	0.32	29	0.29	32	0.32	58	0.56
Iceland	3	1.35	2	0.71	0	0.00	4	1.30	1	0.36
Ireland	6	0.18	15	0.44	14	0.39	8	0.20	11	0.30
Italy	1,021	1.56	1,346	2.04	1,363	2.04	1,510	2.21	1,556	2.23
Latvia	49	2.32	48	2.33	34	1.63	38	1.86	22	1.09
Lithuania	2	0.07	9	0.31	1	0.04	8	0.28	7	0.25
Luxembourg	6	1.20	5	1.04	7	1.27	5	0.91	5	0.91
Malta	9	2.07	4	1.04	2	0.35	9	1.79	6	1.38
The Netherlands	311	1.88	304	1.83	308	1.83	348	2.04	419	2.39
Norway	33	0.72	25	0.52	40	0.83	51	1.06	60	1.22
Poland	18	0.05	8	0.02	11	0.03	12	0.03	23	0.06
Portugal	89	0.82	140	1.28	94	0.85	588	5.33	145	1.30
Romania	1	0.00	3	0.02	1	0.00	1	0.00	5	0.03
Slovakia	7	0.13	4	0.08	6	0.12	14	0.26	14	0.27
Slovenia	44	2.14	81	3.84	77	3.62	59	2.76	106	4.98
Spain	706	1.52	972	2.07	815	1.72	925	1.78	1,024	2.12
Sweden	127	1.32	102	1.04	122	1.25	136	1.38	142	1.42
United Kingdom	251	0.41	401	0.66	331	0.54	370	0.59	412	0.65
EU/EEA	4,915	0.97	5,848	1.13	5,832	1.12	6,951	1.31	6,986	1.30

Source: Beauté J(2017). On behalf of the European Legionnaires' Disease Surveillance Network. Legionnaires' disease in Europe, 2011 to 2015. Euro Surveill. 2017;22:pii=30566.

A



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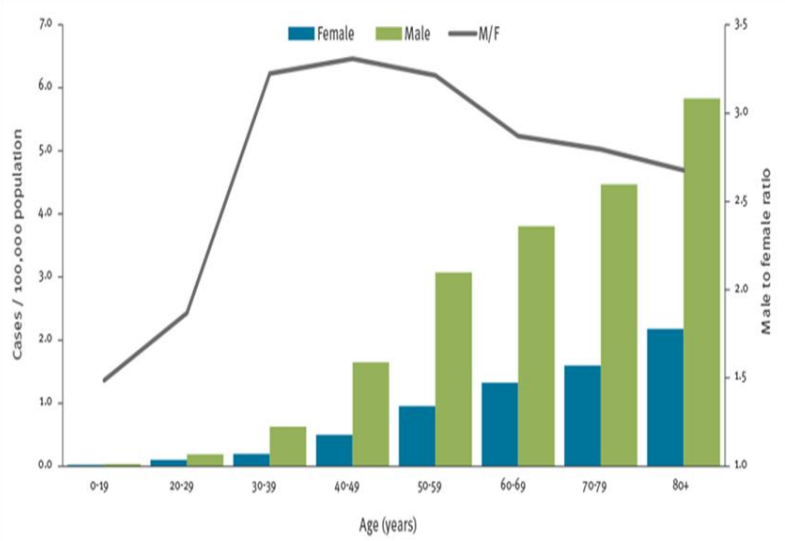


Figure 1.5 A - Age-standardised rate of Legionnaires' disease per 100,000 populations, European Union/European Economic Area, 2011–2015; B-Notification rates of Legionnaires' disease per 100,000 populations by sex and age group and male-to-female rate ratio by age group, European Union/European Economic Area, 2011–2015
Source:http://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2017.22.27.30566

Like the global ratio, LD across the EU is more common in males with an overall male-to-female rate ratio of 2.6:1. This ratio was almost unchanged during the period of 2011–15, but was 1.5:1 in people below 20 years, and 3.3:1 in the age group 40-49 (Figure 1.5B). In older age group (such as people over 65), this ratio varied significantly in each country (i.e. 1.1:1 in Slovakia cf. 5:1in Cyprus). During this study period, sources of infection were identified for 26,900 cases, of which 70.7% (19,019) were community acquired, 19.9% (5,357)were travel related, 7.3% (1,973) were nosocomial and 2.0% (551) were associated with other settings (Table 1.5)(ECDC, 2017).

Table 1.5 Main characteristics of reported Legionnaires' disease cases, European Union/European Economic Area, 2011–2015

Characteristics	Cases		Notification rate/100,000 population
	Number	Percentage (%)	
All cases	30,532	100	1.21
Age group (years)			
< 20	159	0.5	0.03
20–29	473	1.6	0.15
30–39	1,440	4.7	0.41
40–49	4,037	13.3	1.08
50–59	6,917	22.7	2.00
60–69	7,120	23.4	2.52
70–79	5,882	19.3	2.88
≥ 80	4,434	14.6	3.47
Unknown	70	NA	NA
Sex			
Male	21,618	71.1	1.75
Female	8,789	28.9	0.68
Unknown	125	NA	NA
Probable setting of infection			
Community	19,019	70.7	0.75
Travel abroad	3,098	11.5	NA
Domestic travel	2,259	8.4	NA
Nosocomial	1,322	4.9	NA
Other healthcare	651	2.4	NA
Other	551	2.0	NA
Unknown	3,632	NA	NA
Cluster status			
Sporadic cases	19,559	90.1	NA
Clustered cases	2,158	9.9	NA
Unknown	8,815	NA	NA
Outcome			
Alive	21,003	90.7	NA
Dead	2,161	9.3	0.09
Unknown	7,368	NA	NA

This study excluded Croatia from the analysis, because it only started reporting LD cases in 2013. Source: https://ecdc.europa.eu/en/publications-data/legionnaires-disease-europe-2015

Of all EU countries, 16 countries had identified the source of infections for more than 80% of their reported cases of LD. The proportion of sources varied from country to country. For example, Norway reported only 38.8% of LD cases to be community acquired whereas Slovenia had 96.1%. In contrast, Norway had 61.2% travel associated cases but in Spain, Latvia, Bulgaria and Italy had only less than 2% (Beauté, 2017).

1.15 LD worldwide

Legionnaires' disease is a global issue as it can affect anybody who inhales an infectious dose of *Legionella* bacteria (Skerrett, 2018). The number of cases of LD that occur globally is unknown as most of the countries (with the exceptions of the USA, EU/EEA and UK) do not have accurate data and reporting systems for LD. However, on the basis of individual studies and available limited data, it is estimated that two to nine percent of CAP globally is actually LD (Cunha et al., 2016). Recent reports from USA showed that the numbers of LD outbreaks have increased 450% over the past fifteen years; more than 6100 cases of LD were reported in the United States during 2016, and this number is likely to be underestimated as most cases of CAP are treated without proper diagnosis (CDC, 2018d; Granseth et al., 2017). The number of cases of LD has increased significantly since 2000 (Figure 1.6) possibly due to the increased testing for Legionnaires' disease, an ageing U.S. population, ageing plumbing systems and older infrastructure and predicted climate change (Reynolds, 2016). Most reported cases of LD have occurred in the summer but, LD outbreaks can happen at any time of the year. In USA, Legionnaires disease is considered as a nationally notifiable disease and is monitored by two national level surveillance systems: (i). Nationally Notifiable Diseases Surveillance System (NNDSS), (ii) Supplemental Legionnaires' Disease Surveillance System (SLDSS). All LD outbreaks are reported through the Waterborne Disease Outbreak Surveillance System (WBDOSS) and all the cases of LD should be reported to a local or state health department (McClung et al., 2018).

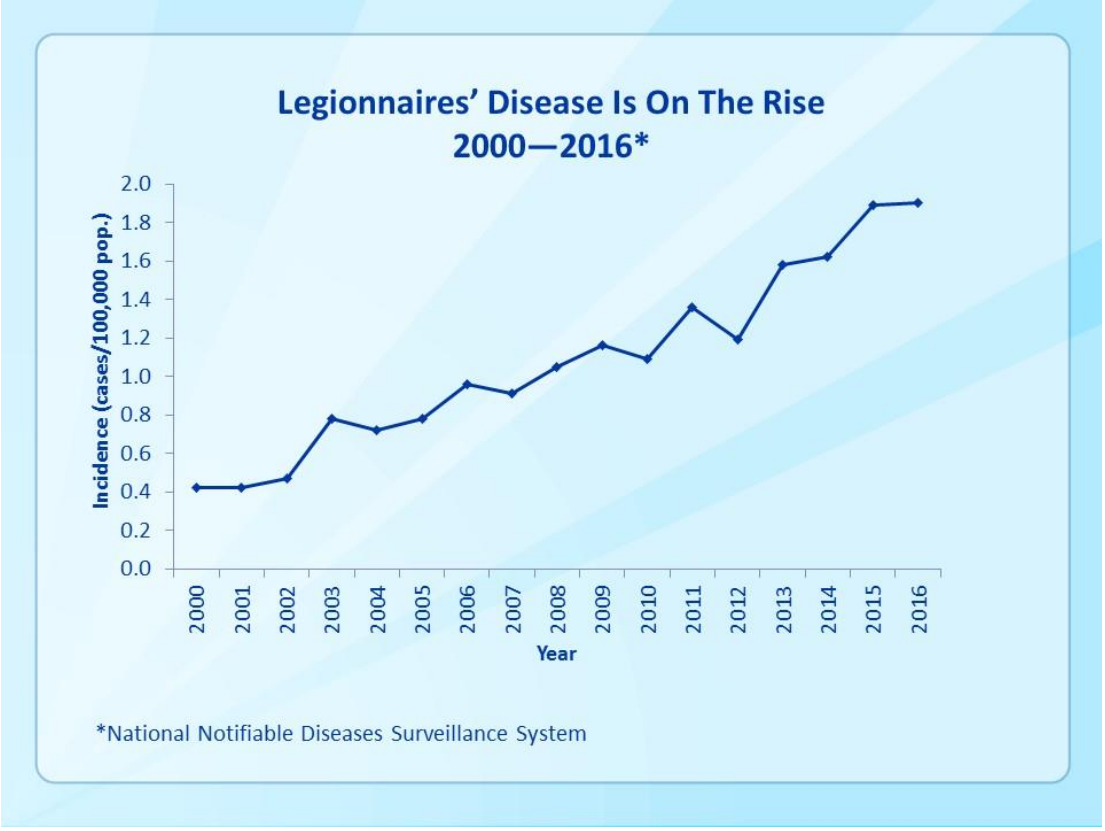


Figure 1.6 Report of National Notifiable Disease Surveillance System shows that the LD cases are on the rise since 2000. Source: (CDC, 2018d)

In Singapore, there are some reporting systems in place and during 2000-2009 period, 238 cases of LD were reported. These cases were reported sporadically and individually during each year. According to these limited data, annual incidence of community acquired LD cases have decreased to 0.16 cases per 100,000 in 2009 from 0.46 cases per 100,000 population in 2003 and travel related LD cases have increased to 27.3% during 2005–2009 from 6.2% during 2000–2004 ($p<0.0005$)(Lam et al., 2011).

There are some individual case studies also from Japan, where an outbreak of LD in 2002 infected 272 people of which 6 died. Further investigation identified the source of outbreak to a traditional bath house in southern Japan (BBC, 2002). An outbreak of LD was also reported in 2015 from Hong Kong's brand-new government headquarters. The water system was contaminated with *Legionella* bacteria at levels that were fourteen times higher than the acceptable level. Hong Kong's education secretary was diagnosed with Legionnaires' disease in 2015, and another report from Hong Kong's health officials report a 65 year male admitted in hospital with the symptoms of fever, cough with sputum and shortness of breath who was diagnosed with Legionnaires'

disease and died a few days after hospitalisation. Total 66 cases of LD were reported in 2015; however, the total number of Legionnaires' disease cases reported in 2016 was 51 (BBC, 2016; BBC, 2015; BBC, 2012).

In Asia, it is estimated that almost one million adult deaths occur every year due to CAP. Many of these deaths occur among the elderly, although an estimate reported 160,000 deaths occurred in the age group of 15–59. In Asia, there was no proper data or study on CAP until recently, and there is still no systematic review or study on CAP similar to the US and Europe (Peto et al., 2014). A number of observational studies of patients hospitalised with CAP have confirmed that 2 to 9% of CAP is contributed by Legionnaires' disease (Phin et al., 2014). Many studies have confirmed that the organism causing community acquired Legionnaires' disease in USA and Europe is the same in Southeast Asia. Therefore, the guidelines for controlling the disease and treatment of patients infected with *Legionella* bacteria in Asia should be the same as those used in Europe and the USA (Wattanathum, et al., 2003).

According to a study by Song and team, CAP caused by *Legionella* pneumophila in Asia appears to be relatively low and this study suggests that this could be due to difficulties in diagnosis of the causative pathogen. In contrast, Wattanathum and team identified that Legionnaires' disease was associated with CAP in a minimum of 8.2% of outpatients and 5.4% in hospitalised patients in Thailand (Wattanathum, et al., 2003; (Song et al., 2016). Inter-country variations are expected in Asia due to its large size, difference in climate diversity in geography and life style. In a study on CAP across Asia, 25% of CAP was attributed to LD in Manila, but in other Asian countries it was in the range of 0-5.3%. In addition, 32.4% of LD in Manila occurred in the month of January. Furthermore, an Asian surveillance study reported that Legionnaires' disease is more frequently associated with CAP among the urban population living in the east and south-east region of Asia (Peto et al., 2014; Ngeow et al., 2005).

Some studies on CAP from China reported that CAP remaining as a major public health issue in China and the reported cases were relatively higher than that reported in US and Europe. However, there are no specific studies reported from China on Legionnaires' disease (Zhu et al., 2018; Guan et al., 2010).

There are recent reports of *Legionella* outbreaks in the UAE, following recent government efforts to classify *Legionella* bacteria and LD as a reportable disease. Consequently any outbreaks of LD or positive detection of *Legionella* bacteria by

accredited laboratories must be reported to the government. In addition, enforcement authorities conduct spot inspections at various sites in order to collect water samples to check for the presence of *Legionella* to safeguard public health. However, they haven't established an emergency response system in case of an outbreak of Legionnaires disease (PHE-Dubai, 2017; UAE, 2017).

1.15.1 Summary of global LD situation

Legionnaires' disease is on the rise globally and is one of the predominant contributing factors of CAP. In many countries, LD is a nationally notifiable disease and LD outbreaks can happen at any time of the year. In USA, reported cases of LD have increased significantly; however, the actual cause of this increase is unclear (CDC, 2018a; Misch, 2016). Reported cases of LD in Europe also increased and this could be due to the climate change and changes in population structure (Beauté, 2017). In Singapore, LD reporting system and data are limited due to the limitations in diagnostic procedures; individual case studies were reported from Japan and Hong Kong (Phin et al., 2014; Lam et al., 2011; BBC, 2002; BBC, 2016; BBC, 2015; BBC, 2012). In Asia, there is no systematic study on CAP or LD; however, a recent study report from India demonstrated the presence of *Legionella* pneumophila serogroup 1 in the hospital water systems, and suggests LD could be the major cause of pneumonia among hospitalised patients (Chaudhry et al., 2017). Another study from China reported that 5.1% CAP cases in main land China is contributed by Legionellosis and *Legionella* pneumophila is the main causative agent for *Legionella* infections (Jiang et al., 2016). ECDC reported that an increase in LD cases in EU travellers returning from Dubai indicating the increase of LD cases in Middle East; recently, LD is classified as a reportable disease in UAE (ECDC, 2016; UAE, 2017).

1.16 Costs of LD to society

Most identified cases of LD are isolated sporadic cases where a source is never found. On the basis of the available information and comparative assessment, the source of outbreaks can be from home, hospital, work or other environments where *Legionella* bacteria is able to proliferate and survive. Some experts believe that this increase in LD is attributed to factors such as identification of new sources of infection, changes in diagnostic methods, and improved surveillance systems (Joseph, 2004; Gamage et al., 2018). Nevertheless, one of the most important concerns during a

Legionella outbreak (aside from public health), and one of the main reasons for preventative measures and monitoring, is the significant health-care costs associated with LD outbreaks (Lock et al., 2008) In addition, because of the complexity in identifying the source of an outbreak, and a general lack of necessary information, every confirmed case of *Legionella* is carefully investigated by public health authorities. The main aim of investigation is to identify the source of outbreak and to manage the risk of further infection. The costs involved in handling an outbreak and investigation of the source has rarely been considered, and there are no national guidelines in the UK on what reporting threshold (in terms of cost) is needed to initiate detailed investigations of *Legionella* outbreaks (Lock et al., 2008).

An economic evaluation of a single *Legionella* outbreak in South East London, (including epidemiological and environmental investigations, microbiological analysis and the staff time and resources provided by the 11 organizations responsible for managing the outbreak) revealed a total estimated cost of £455,856, although only £64,264 (14%) was spent on investigation and management of the outbreak compared with £391,592 (86%) spent on the hospital treatment of the infected patients (Lock et al., 2008).

Reports indicate that 261000 cases of community-acquired pneumonia have been diagnosed annually during 1992-1993 in the UK, costing £440.7 million to the National Health Service based on 1992/1993 prices (Guest and Morris 1997). Furthermore, two large-scale studies investigating the incidence of pneumonia in hospitals in England reported significant increases in admissions between 1998 to 2014 and 2002 to 2009, respectively (Quan et al., 2016). Moreover, 2012 NHS reports showed that the costs of pneumonia related admissions in hospitals account for £1,700 to £5,100 per each admitted patients (CRCAP, 2012). It is likely, therefore, that the current financial burden on the NHS, related to community-acquired pneumonia, is much higher than the 1992/1993 cost of £440.7 million (Feldman & Anderson, 2016). Moreover, if cases of pneumonia are treated with normal antibiotics without proper diagnosis for LD, the health consequences can be extremely serious, leading to long term morbidity, and creating additional cost burdens to the NHS (Ott et al., 2011; Guest & Morris, 1997). Indeed, a long term study carried out of 122 survivors of LD in the Netherlands found persistent symptoms of fatigue (in 75% of patients), neurologic symptoms (in 66%), and neuromuscular symptoms (in 63%) after 17 months despite the original diagnosis of LD (Lettinga et al., 2002).

According to a research report presented at the International Conference on Emerging Infectious Diseases in 2010, three common waterborne diseases (Legionnaires' disease, cryptosporidiosis and giardiasis disease) together cost $539 million annually in the USA (CDC, 2010). The cost analysis of these water-borne diseases poses physical burden as well as health care costs to thousands of infected people every year (CDC, 2010). Unfortunately, there are no well-documented data on the health care costs associated with these waterborne diseases. However, available information from insurance claims databases between 2004 and 2007, on the basis of the hospitalisation costs paid by insurers and direct costs to patients estimated total costs of confirmed diagnosis and hospitalisation for Legionnaires' disease to be approximately 101-321 million dollars (compared to 16-63 million dollars for giardiasis and 37-145 million dollars for cryptosporidiosis). Unidentified causes of illness (such as diarrhoea, cold and flu-like symptoms) were excluded from this annual estimate unless they were associated with prolonged hospitalisation periods (i.e. weeks). Consequently, the actual cost associated with these infections, especially Legionnaires' disease or community acquired pneumonia is likely to be much higher than the estimated cost in this study (Collier et al., 2012)

In addition to the costs of investigation, assessment, control and hospitalisation, there are additional costs to an organisation's overheads during an outbreak of LD, including system shutdowns, the costs of alternative arrangements and financial losses due to lost working days. There could be other costs to patients, their families and their employers due to the hospitalisation or absence from the family as well as work place (Lock et al., 2008).

1.17 Risk factors associated with Legionella bacteria in domestic water systems and control measures

1.17.1 Domestic water systems

Domestic water systems generally consist of a water supply source to the building (e.g. incoming city mains, bore well water), cold water storage, hot water storage, associated pipework, showers, water discharging taps and other outlets (HSE, 2016).

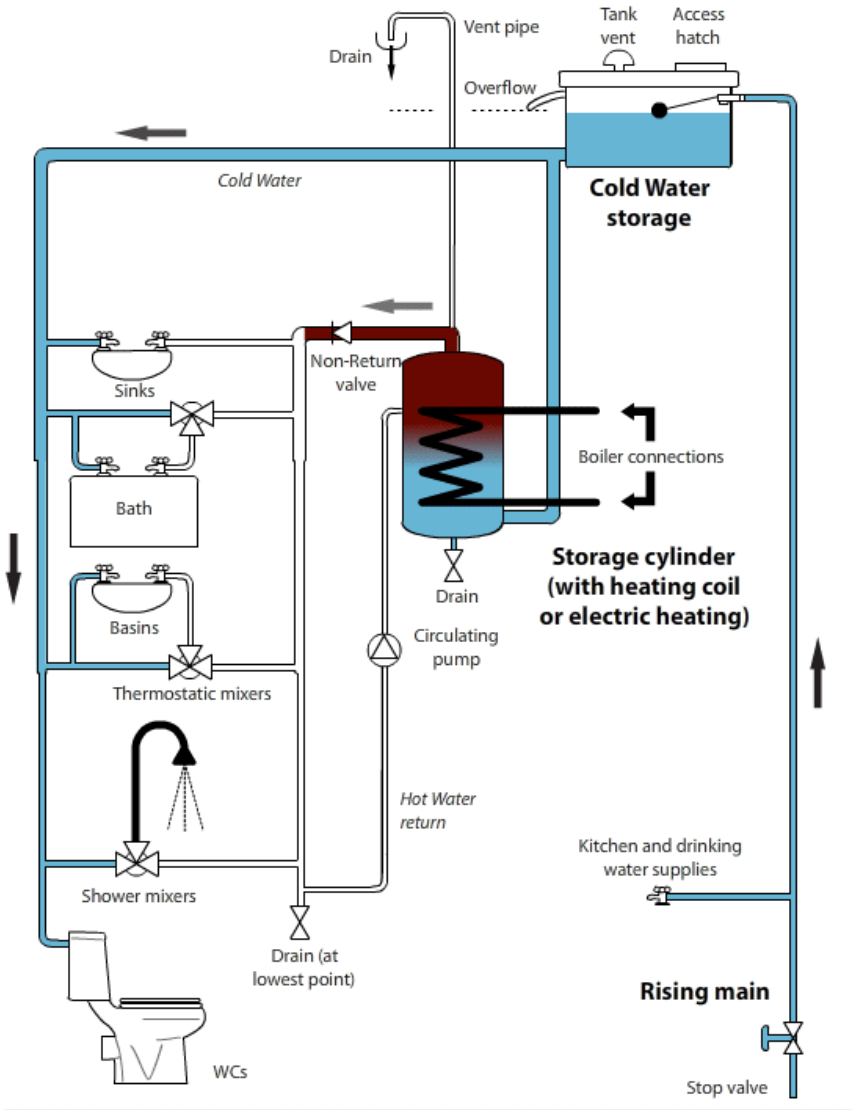


Figure 1.7 Typical domestic hot and cold water storage and pipework.
(Source: http://www.hse.gov.uk/legionnaires/hot-and-cold-water-storage.htm)

Domestic water systems have been identified as a major source of *Legionella* pneumophila (Cunha et al., 2016; Beer et al., 2015). *Legionella* can colonise and proliferate within domestic hot and cold water systems, particularly in showers and hot water systems (Figure 1.7), and be inhaled by humans during the water usage that produces aerosols (Hines et al., 2014; Lin et al., 2011). Many studies have confirmed that the mains water feeding our water systems derived from the water treatment plant is one of the major sources of *Legionella* bacteria (Waak et al., 2018; Özen et al., 2017; Parr et al., 2015). A study carried out in the United States during 2009–2010 reported that out of 6,868 Legionellosis cases reported to the Centre for Disease Control and Prevention, 84% were caused by *Legionella* pneumophila serogroup-1. LP

serogroup-1 has been isolated from a number of natural freshwater environments, and this can reach water treatment plants and subsequently escape disinfection (Donohue et al., 2014). Furthermore, biofilms found in water distribution systems (pipework and plumbing fixtures) contribute significantly towards *Legionella* proliferation within the water systems (Liu et al., 2006). A study carried out in Germany found that the household potable water systems as well as potable water systems in public buildings are contaminated most commonly with *Legionella* pneumophila (Kruse et al., 2016).

The mains water feeding domestic water systems produced from the water treatment plant is disinfected prior to distribution. Recent studies demonstrated that the disinfection techniques are effective in reducing the pathogens present in mains water but fails to eliminate them from the potable water (King et al., 2016; Kruse et al., 2016). Even though disinfection kills most of the pathogens in the mains water supply, the water can be re-contaminated during its transportation and distribution through the pipework as well as storage (Prest et al., 2016; Kilb et al., 2003). The quality of the water we obtain from the tap can differ markedly from that of the mains water supply (EWGLI, 2011). The long horizontal installation of pipework, the materials used for the pipework and fittings, dead-legs, thermal insulation of water storage system and their associated pipework, excessive water storage, internal condition of the storage tanks and their location can all affect the water quality (Brown et al., 2001). If suitable nutrients are present, and water temperatures are within the range of 20-45°C, *Legionella* proliferation may occur (HSE, 2013).

1.17.2 Effects of pipework and plumbing fixtures

The role of pipework and plumbing fixtures are significant in *Legionella* control (Rhoads et al., 2016).A study carried out on a hospital hot-water system in England consistently isolated *Legionella* pneumophila from the calorifier drain point at levels of 104cfu/L. The temperatures of water samples collected were 50°C or below. When the temperature of the calorifier was raised to 60°C (by shutting off the cold water feed to the calorifier), the *Legionella* count was reduced to a non-detectable level. However, 10 minutes after reopening the cold water feed to the calorifier the *Legionella* count reached previously detected levels (104cfu/L). Further Investigations confirmed that the cold-water supply to the calorifier was continually feeding *Legionella* to the hot water system despite disinfection of the cold water storage tank and hot water system (Farrell et al., 1990). This case illustrates the importance of the external water

distribution pipework system in distributing *Legionella* to domestic hot and cold water systems, where local conditions may promote proliferation (Casini et al., 2014).

A recent study on drinking water plumbing systems (DWPS) to identify the risk factors of *Legionella* proliferation reported that 807 potable water samples were collected from 9 separate buildings have had the presence of *Legionella* species including *Legionella* pneumophila (>100 cfu/100ml). This study identified pipework length proportion as the main contributing factor for *Legionella* contamination rather than temperature difference or water stagnation (Völker et al., 2016). Generally, in water distribution pipeworks, biofilm form on the interior of pipe walls which can harbour *Legionella* and other microorganisms such as *E. coli*, *Campylobacter* sp, *Pseudomonas aeruginosa*, *Mycobacterium* sp, *Aeromonas* sp, adenoviruses, rotaviruses, noroviruses, and parasitic protozoa. These micro-organisms can attach to pre-existing biofilms, and survive for longer periods depending on the ecology of the particular organism and the environmental conditions (Berjeaud et al., 2016).

Another study by Rhoads and team demonstrated that plumbing loops and restricted water circulation through long running pipework can encourage *Legionella* proliferation (Rhoads et al., 2016). Water distribution pipework design is one of the major contributing factors promoting *Legionella* growth within plumbing fixtures. In the case of hot water pipes that slowly mixed with the recirculating line; for example, a pipework feeding a shower head from a recirculating line on the floor or a kitchen tap feeding from a recirculating line running through the ceiling, can encourage *Legionella* growth due to the poor water flow, slow mixing and reduced water temperature resulted from the restricted water flow (Rhoads, 2017). The slow mixing creates an ideal temperature for *Legionella* proliferation even if the water heater(s) or calorifiers(s) operate at 60^0C and above. These types of plumbing or pipeworks with poor water flows are considered as dead legs or dead ends (HSE, 2014a). Dead legs and dead ends are a major concern in terms of *Legionella* control and is one of the major contributing factors of *Legionella* outbreaks identified in Chapter 5 of this thesis.

1.17.3 Deadlegs and dead ends

Deadlegs (Figure 1.8 A and B) and dead ends (Figure 1.8 C and D) always raise concerns within water distribution systems (Jjemba et al., 2015). Generally, 'a deadleg

or dead end is the part of a water pipework system which cannot be drained through a normal sanitary flushing' (ASHRAE Guideline, 2000). Dead ends (also known as blind ends) are normally a redundant length of pipe, closed at one end, through which water cannot flow (Figure 1.8 C). A dead leg is referred to a section of pipe leading to an outlet through which water flows, but the outlet is unused or infrequently used (Figure 1.8 D) (HSE Terminology).

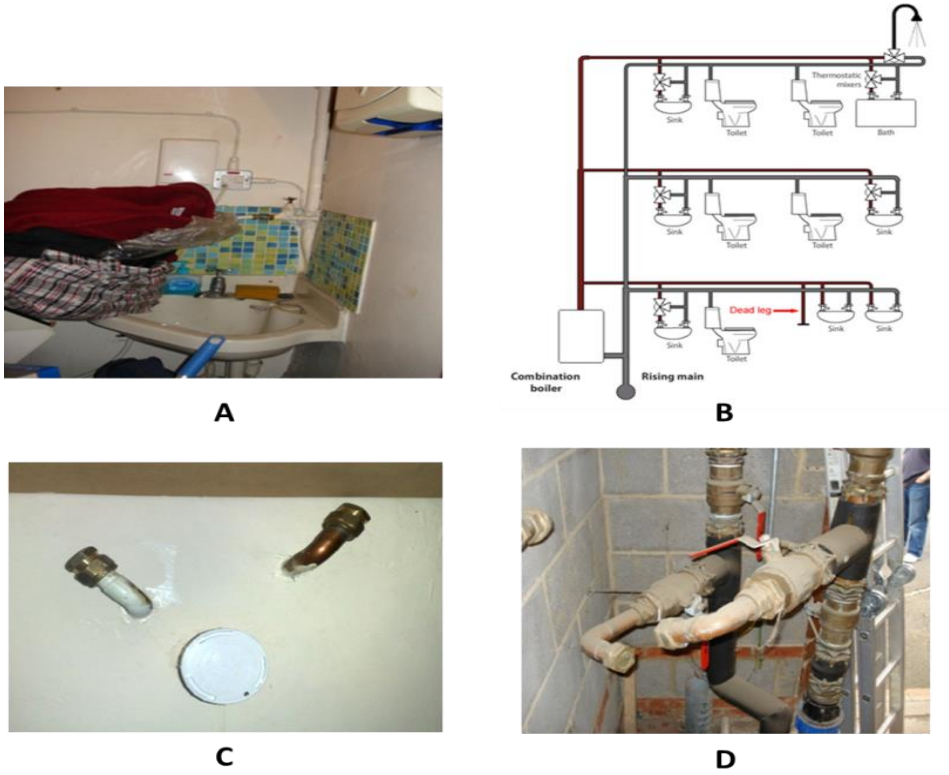


Figure 1.8 - A and B are deadlegs and C and D are dead ends, the examples of water stagnation that can be sources of *Legionella* contamination. (Source: A and D - own collection; B and C http://www.hse.gov.uk/legionnaires/hot-and-cold-terminology.htm**.**

The mains water derived from water treatment plants maintain the cold water temperature normally below 20°C at the point of distribution. However, cold water temperatures vary seasonally, and during extreme summer conditions the incoming mains temperature can exceed 20°C due to the heat transmission from increased ambient temperature. Nowadays, modern building specifications require an appropriate level of thermal insulation to keep the internal building temperature warm, and this may result in warming of the cold water pipes to above 20°C. In the event that the 'warm' cold water enters into a dead end or dead leg, biofilm formation

can easily occur, and eventually *Legionella* can proliferate as a result of possible stagnation (ACR, 2016). Similarly, hot water systems operating at 60°C are capable of delivering hot water at 50°C minimum to all related outlets in the building. However, when this hot water enters into a dead end or dead leg, the temperature will fall as a result of reduced water flow rate or possible stagnation. If the water temperature is between 20-45°C, and nutrients are available, *Legionella* proliferation can occur (Schofield, 1985).

Sometimes, domestic hot and cold water systems may not be in use for extended periods (e.g. holidays). During this time, cold water temperatures will rise to ambient temperature, and hot water in the system will radiate temperature to the surroundings. Either way, water temperatures can fall in the range of 20-45°C for a period of time which will favour *Legionella* proliferation (HSE, 2013a). On the other hand, it is common practice in many buildings to change the use of some areas. For example, the use of an existing toilet may be stopped, and the space is converted into a store room instead without isolating the hot and cold water pipeworks from the point of main distribution pipework (Figure1.6A). Consequently both the hot and cold water becomes stagnant within the associated pipework, and the variation in water temperature eventually results in optimal conditions for *Legionella* proliferation (Ciesielski et al., 1984).

A study reported that the presence of *Legionella* bacteria in flowing water as well as in stagnant water (sample from the dead end) of a drinking water distribution system found to be similar (Schwake et al., 2015). Contradictory to this report, many other studies reported that the poor water flow and water stagnation is an important contributing factor for the proliferation and multiplication of *Legionella* bacteria (Rakić, A 2018; Bédard et al., 2015; Boppe et al., 2016). From the view of a *Legionella* control practitioner with the first-hand experience, I agree with the reports that stagnant and poor flow situation can promote *Legionella* growth; the first report could be a standalone scenario and may be contributed by some other factors.

Traditionally, flushing of unused or infrequently used outlets and taps has been used as a remedial solution to avoid possible *Legionella* proliferation. However, normal sanitary flushing may not be sufficient to eradicate all the bacteria due to the restricted water flow within the dead legs. Furthermore, prolonged water stagnation contributes to biofilm formation where micro-organisms, including *Legionella*, can harbour. Usual

water stagnation in dead legs and dead ends can lead to biofilm formation within the pipework which can harbour micro-organisms (Green, 1993) as well as the protozoa in which *Legionella* can persist (Declerck et al., 2009).

Legionella proliferation within deadlegs or dead ends can result in contamination of the whole distribution system, and may result in human exposure during water usage (Greig et al., 2004). Figure 1.6A shows a wash room, but currently, water in this pipework is not in use as this room is used as a store room and this unused wash room is acting as a deadleg. In figure 1.6D, the provision of a by-pass connection from the incoming mains is capped off and the isolation valve installed is after 30 cm from the mains pipework. At present, this 30 cm pipework is acting as a dead end.

1.17.4 Galvanized steel pipework and fittings

Until around 1970, traditional domestic water systems were serviced with lead pipes and water tanks. However, once the health effects of lead poisoning in drinking water were realised, lead pipes were replaced with galvanised steel, copper and plastic (Quinn and Sherlock, 1990). Galvanised steel pipework has two major disadvantages when used in water distribution systems (Duruibe et al., 2007; Saby et al., 2005). Galvanized steel is corrosion resistant steel which has undergone a chemical processing to achieve its property. Steel is coated with a number of layers of zinc oxide which will act as a protective surface layer to protect the metal from corrosion. The most popular galvanisation method is hot-dipped galvanization and this process allows the zinc to bond permanently to the metal surface as well as within the steel itself which is incredibly beneficial to the steel fighting off rust. However, slow rusting can easily be caused by the reaction between iron in steel and oxygen as well as water which will lead to the deterioration of galvanised steel. In this slow deterioration process, the zinc layer will first become damaged, providing a longer life to the actual product (Dreulle, 1980). The zinc from the galvanised steel pipes mixes with drinking water and can lead to serious negative health impacts in terms of zinc poisoning (Duruibe et al., 2007). Several studies showed that the zinc contamination in drinking water as a result from the corrosion of distribution pipework poses serious threat to human health (Alam and Sadiq, 1989). Furthermore, corrosion products within the galvanised steel pipeworks can act as a better nutrient for microorganisms especially for *Legionella* pneumophila

(GROWTH-PROMOTING, 1998). A study into the effects of pipework materials on biofilm formation showed the highest rate of biofilm formation on steel pipes compared to copper and plastic (Yu et al., 2010). The presence of biofilm is a better indicator of microbial growth, as biofilms in many water supply systems form an environment in which different bacteria can become entrapped for long periods. Besides this, biofilms contribute to bio-corrosion, are a barrier to disinfection (organisms may survive deep within the biofilm) and possibly act as a reservoir for pathogenic and non-pathogenic microorganisms including *Legionella* pneumophila (Armon et al., 1997). Therefore, copper and plastic pipeworks and fittings have become more common in recent years.

1.17.5 Copper pipework and fittings

Copper has antimicrobial properties against bacteria, viruses and fungi (Warnes and Keevil, 2013; Salgado et al., 2013). Also, in recent years, antimicrobial action of copper against Gram-positive and Gram-negative bacteria and norovirus has been described in a number of studies (Bleichert et al., 2014). As a result of these advantages and easy to work with properties, use of copper and copper alloys has become widespread in water industry especially in water distribution network (Warnes et al., 2012). A study in USA hospitals demonstrated that the numbers of MRSA, Staphylococci, Gram-negative and vancomycin resistant enterococcus as well as the total number of bacteria were significantly lower when copper was introduced as the surface material (Schmidt et al., 2012). A similar study conducted in UK hospital also found that the isolation of several pathogens, such as, Escherichia coli, methicillin-resistant Staphylococcus aureus (MRSA), Clostridium difficile from copper surfaces was significantly lower than the samples from aluminium, plastic and chrome-plated surfaces. However, this study also demonstrated that copper was not effective in killing *Legionella* pneumophila (Giao et al., 2015). In contrast, many other studies (Warnes and Keevil, 2013; Salgado et al., 2013) have reported that copper has antimicrobial properties. These two reports are not contradictory to each other as other studies indicating that the microorganisms survive only a few minutes on copper surfaces depending on several other factors; copper corrosion produces toxic substances which inhibits microbial richness as well as copper pipes itself do not encourage bacterial community (Vincent et al., 2016; Fish et al., 2016). These reports indicate that the copper pipes are not effective in killing micro-organisms present in water but help to minimise the microbial proliferation and richness.

Copper is normally resistant to corrosion, but certain conditions can lead to surface corrosion within the water pipeworks; a process called 'cuprosolvency'. Cuprosolvency is directly related to the water quality within the distribution systems, especially water chemistry and temperature (Critchley et al., 2001). Recent studies have also found that micro-organisms are also involved in cuprosolvency, associated with the formation of biofilms within the internal pipework surface (Critchley et al., 2003). These biofilms are complex structures of micro-organisms with extra cellular products, organic and inorganic debris attached to surfaces (Bremer et al., 1992). Many studies have been conducted to investigate the causes of pitting corrosion in copper plumbing systems associated with biofilms. In all cases, corrosion was attributed to the biofilm itself, and corrosion products were microbial in origin (Arens et al., 1995). However, pitting corrosion is less significant in normal hard water with high organic carbon concentrations, but general corrosion is still present and pitting corrosion is particularly significant in the case of soft water (Lytle and Nadagouda, 2010). In general, accumulation of corrosion products and the presence of biofilm within the pipework are associated with an increased risk of *Legionella* proliferation (Liu et al., 2016; Walker et al., 1991). Despite the protective effects of copper on preventing biofilm formation, elevated copper concentrations in drinking water also have serious implications in terms of public health (Dietrich et al 2004). World Health Organisation (WHO) and European Union (EU) has adopted the guideline value of copper in the drinking water as 2mg/l and this threshold limit of copper is usually maintained in drinking water by water supply companies. However, corrosion of copper pipeworks and fittings together with copper leaching can increase the level of copper significantly high in drinking water (Water UK, 2006).

1.17.6 Plastic pipework and fittings

Plastic pipes and fittings are lightweight, non-corrosive, hard and resistant to chemical attack. Due to their availability in large lengths, elastic properties, reduced cost of handling, transportation and installation, they have become popular in the water industry (Andrady and Neal, 2009). Mainly, there are two types of plastic pipes are in use in terms of water and wastewater industry; (i) Poly Vinyl Chloride (PVC) and (ii) Chlorinated Poly Vinyl Chloride (CPVC) or High Density Poly Ethylene (HDPE) (Heim and Dietrich, 2007). PVC pipe and fittings are not suitable for pressured water and hot water as they are liable to crack, and reduces the strength in high temperatures. However, CPVC pipes can be used for hot water and potable water

distribution. CPVC pipes are in use to carry water for long distances without worrying about pressure loss, corrosion or scaling and are considered to be a better alternative to copper for potable water distribution (CPVC, 2017; Nesterchuk and Raisa., 2012).

Although plastic pipework and fittings have a number of positive advantages in water distribution network, many recent studies have demonstrated that synthetic plumbing materials adversely affect the water sensory aspects (i.e. taste and odour) and chemical water quality, as well as the significant negative impact in drinking water quality in terms of human health (Heim and Dietrich, 2007). A study published in Journal of Environmental Engineering reported that plastic pipework and fittings leach polyvinyl chloride and phthalates into drinking water, which may cause human health issues including reproductive and hepatic toxicity (Heim and Dietrich, 2007). Furthermore, hazardous dioxins can release into water from PVC pipeworks due to its heavy chlorine content and most of the dioxins are classified as carcinogens (Walter et al., 2011; Tomboulian et al., 2004; Rossi and Schettler, 2000). In the United States, the Centre for Disease Control (CDC) warned the general public that short term exposure to phthalates may not be toxic in adults, but infants and children should be protected and further reported that the long term exposure to phthalate is toxic in adults also (Schettler, 2006; Swan, 2008).

Moreover, Plastic pipes, cisterns and fittings can leach organic materials and chemicals (plasticisers) into the water and these can act as nutrients for micro-organisms in the water systems including *Legionella* bacteria (Lehtola et al., 2004). Another study on changes in water quality and biofilm formation in a pilot-scale water distribution system with copper and plastic pipe work showed, the formation of biofilm was faster in plastic pipes compared to copper pipes (Lehtola et al., 2004). The fundamental characteristics of biofilms are that they attached to a substrate and consists of a number of different bacteria co-adhered by physical appendages and extra-cellular polymeric substances. In order to form a biofilm, the essential requirements are the micro-organism themselves and a substrate; thus, a biofilm cannot form in the absence of one of these ingredients (Garrett et al., 2008). This indicates that plastic pipeworks and fittings can encourage the growth of micro-organisms in the water systems (Proctor and Hammes, 2015). Copper pipework and fittings are safer than any other plumbing materials that are used in installations, as many studies have shown copper has the effect in suppressing the micro-organisms such as *Legionella* pneumophila (Blanca et al., 2005).

1.17.7 Thermal insulation

Thermal insulation of the water distribution system especially water storage and pipework plays an important role in terms of *Legionella* control. Water temperature maintenance (e.g. hot water at 50^0C minimum and cold water below 20^0C) throughout the water system is vital to protect the system from the proliferation of *Legionella* bacteria (Bédard et al., 2016). As a *Legionella* control practitioner, presence of thermal insulation on water system and its condition is an important parameter to check while carrying out *Legionella* risk assessment in accordance with BS8580. In addition, thermal insulation has been identified as an important contributing factor in deciding a particular building as high risk or low risk category by using the risk predictive model based on statistical software PCA in Chapter 5 of this thesis.

Heat energy has the tendency to attain thermal equilibrium. As a result of this characteristic, heat always flows from warmer to cooler surfaces until the temperatures of both surfaces become equal. A material resistant to conduction, convection and radiation of heat can reduce the flow of heat from one surface to another, and these materials are generally known as thermal insulators (Yamamoto et al., 1985). The primary function of a thermal insulation is to protect the system from heat loss or gain. A good insulator is made from an opaque non-metallic material with a non-crystalline structure. Thermal insulating properties can be enhanced further by creating small air pockets inside the structure (Korjenic, 2011). There are different types of thermal insulation available commercially. However, the most commonly used types in the water industry are fiberglass insulation (figure 1.9 C), mineral wool, cellulose insulation, polyurethane foam and polystyrene (Styrofoam) (Al-Homoud, 2005).

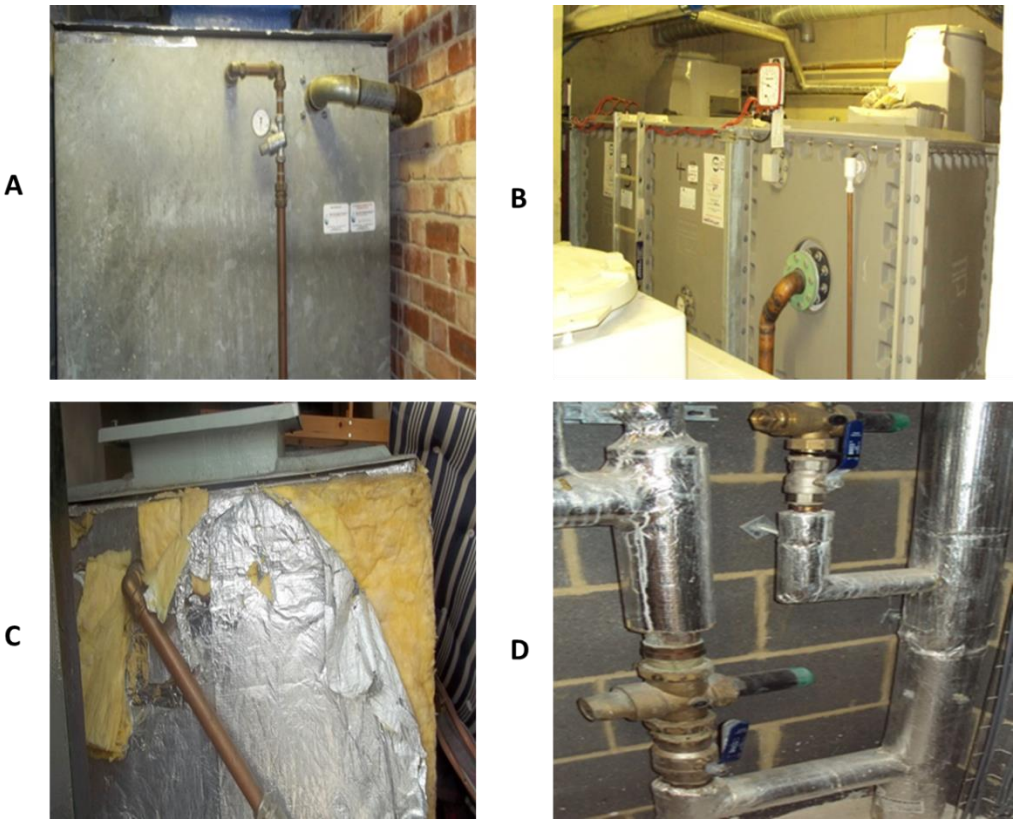


Figure 1.9 A: uninsulated metal cold water storage tank, B: modern pre-insulated (factory insulated) glass reinforced plastic (GRP) cold water storage tanks, C: fibreglass insulated tank and D: copper cold water pipes insulated with foil backed fibreglass insulation. (Source: own collection).

It is important that both the pipework and water storage system (i.e. cold water tanks and calorifiers) should be thermally isolated from the surroundings using an appropriate insulation (Figure 1.9b, 1.9d) (Plouffe et al., 1983). In the case of cold water, the mains water temperature at the distribution point is always less than 20°C. However, during transportation through the pipework, water temperatures can exceed 20°C if the ambient temperature is above 20°C (Hruba, 2009). Furthermore, domestic cold water storage tanks are normally located in the roof or loft space. Even at the beginning of the summer season, ambient temperatures can reach 20°C or above. In the event of a water storage tank without appropriate insulation (Figure 1.9a), thermal transmission can occur from the ambient warmer air into the stored cold water, and the increase in temperature of the stored water temperature may favour *Legionella* pneumophila proliferation (Goutziana et al., 2008).

A study carried out by Stout and colleagues on patient's drinking water facility within the hospital environment demonstrated that the incoming mains water

(municipal water) with all satisfactory parameters were supplied directly to the cold water storage tank feeding the drinking water facilities. Increased stored water temperature along with other adverse internal conditions of the storage tank encouraged the proliferation of *Legionella* bacteria, resulting in contamination of the entire system, including the drinking water facilities (Stout et al., 1985). Extreme winter weather can also freeze the cold water pipeworks in the loft space or roof, and may interrupt the entire building water distribution leading to temporary stagnation. Also, there are many incidents of pipes bursting when water freezes within the pipework due to the expansion below the freezing point 4°C (Westerberg, 1991). Appropriate thermal insulation is an essential requirement to keep the cold water storage and distribution system safe and free from *Legionella* pneumophila (Brundrett, 1994).

In the case of hot water storage, calorifiers or water heaters need to have proper thermal insulation to maintain appropriate operating temperatures. Otherwise, heat loss from hot water storage into the surrounding environment can adversely affect the operating temperature as well as the hot water distribution. If the hot water storage temperature falls below 60˚C, related hot water outlets could easily fall below 50˚C due to other heat losses throughout the distribution pipework (Lasheras et al., 2006). Moreover, even if the hot water storage operates at 60˚C, uninsulated distribution pipework can cause significant heat loss from the hot water distribution system, and water temperatures may fall below 50˚C. In both cases, water temperatures can fall in the favourable temperature range for the proliferation of *Legionella* bacteria (Darelid et al., 2002). A number of comparative studies on insulated and uninsulated hot water systems have shown that uninsulated hot water storage and pipework causes loss of water temperatures resulting in the reduction of operating temperatures that are optimal for *Legionella* proliferation (Rhoads et al., 2016; Clarke and Grant, 2010).

1.17.8 Cold water storage tanks

Cold water storage tanks are another important source of *Legionella* contamination in buildings. Many studies have reported that potable water is the most frequent source of exposure to *Legionella* bacteria (Garrison et al., 2016; Ryu et al., 2017; Seenivasan et al., 2005). It is vital to understand that in the practical world, potable water source in many modern or newly constructed buildings is mains break tank (Nowadays, it is common in the UK and other parts of the world that mains water

enters the building and stored in a tank located most commonly on the ground level or lower ground (basement) level of the building) (Figure 1.10 A). Water booster pumps connected to these mains break tanks pumps the potable water to the other parts of the building (Figure 1.10 B and C) (Practitioners practical experience). In addition, Drinking Water Inspectorate (DWI) suggests the use of outlets connected to the mains water supply for potable use; however if potable water comes from a storage tank it will be safe to use provided the tank is properly designed, correctly installed and kept in good condition (dwi, 2013). Another study by Ryu and colleagues reported that the newly built apartment's potable water, especially hot water was the source of *Legionella* infection and further recommended that effective monitoring of hot water in buildings should be considered (Ryu et al., 2017). Another important information to consider in this regard is the commonly used unvented hot water system cannot be fed directly from the incoming mains rather it can be fed only from a cold water storage tank located higher level than the hot water storage (HSE, 2014; See Chapter 3). Furthermore, Chapter 3 of this thesis, 'Specific Study on Cold Water Storage Tanks'; out of 15 cold water storage tanks surveyed, 7 was potable water tanks located in the lower ground or ground level. However, this important aspect of potable water storage is rarely studied.

A study on 134 roof-harvested rainwater tank samples using qPCR reported that *Legionella* was detected in all the tanks and warned opportunistic pathogens such as *Legionella*, canthamoeba spp, Pseudomonas aeruginosa, Mycobacterium avium and Mycobacterium intracellulare in tank water may present health risks from both the potable and non-potable uses (Hamilton et al., 2016). Another study conducted after a Legionnaires disease outbreak in Flint demonstrated that a corrosive potable water source caused high level of *Legionella* contamination in potable water distribution system (Rhoads et al., 2017). All these study reports indicates the importance of water storage tanks in buildings associated with microbiological contamination and *Legionella* outbreaks. In addition, one of the major contributing factors of *Legionella* contamination in building water system is identified as number of cold water storage tanks and their internal condition (see Chapter 5).

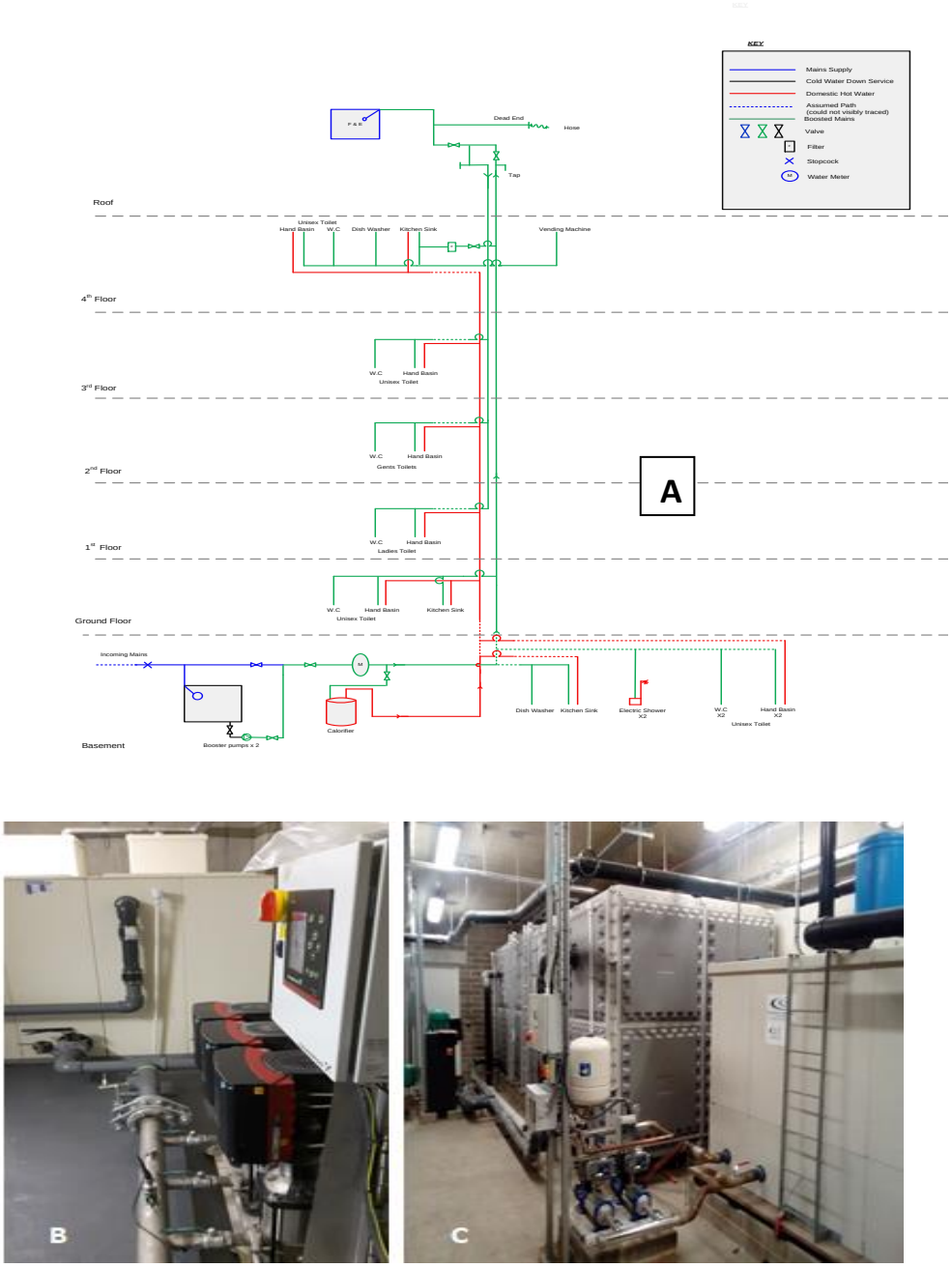


Figure 1.10. A – Schematic of a domestic hot and cold water system in W1T postcode (London) with mains break tank supplying the potable water; **B and C** are the typical examples of mains break tank located in the lower ground plant room connected to booster pump sets supplying potable water to the entire building. (Source: A-Own drawing; B and C own collection).

In some cases, water tanks are made with proper thermal insulation; however, other factors such as the construction method, materials used, tank's internal conditions, plumbing arrangements, internal water flow and tank location can contribute towards Legionella proliferation (Ciesielski et al., 1984). Water storage tanks with relatively lower storage capacity can be constructed as a single piece moulded tank which will be free from tiny internal joining gaps (Figure 1.11A), whereas, sectional tanks always have tiny gaps between the joinery and these can harbour micro-organisms if not properly maintained (Figure 1.11B) (Borella et al., 2005). This issue was addressed by using single piece plastic tanks. However, plastic tanks are not the best option for *Legionella* control due to chemical leaching, and less rigid properties (Abokifa et al., 2016; Rogers et al., 1994). Water safety regulations for portable water in England and Wales as well as Scottish Water Byelaws clearly states in the complete installation guidelines requirements of cold water services/storage tanks that the materials used for the manufacturing of cold water storage tanks should be rigid, as non-rigid materials can bend and become deformed during adverse weather conditions which may lead to cracks, breaks and even cause flooding in the entire building. On the other hand, the breaks and cracks on the non-rigid cisterns allow insects/vermin enter the tanks and contaminate the whole water system. (WRAS, 2015; Edwards et al., 2003)

A

B

Figure 1.11 Single piece factory insulated moulded GRP cold water storage tank **(A)** and sectional factory insulated GRP cold water storage tank **(B)**. (Source: own collection)

Cold water storage tanks constructed in galvanised iron or black steel get corroded during water storage; and corrosion products mainly iron oxide remains within the water tank for a long time. A study carried out by Conley and colleagues showed that the presence of certain metals such as potassium, iron and zinc can significantly encourage the growth of *Legionella* pneumophila even if their concentration is in lower level (2.29, 12.412 and 0.924 ppm respectively) (Conley et al., 1985). A recent study in USA demonstrated that iron is a key nutrient for *Legionella* growth in water systems (Rhoads et al., 2017). These findings indicate that metal corrosion products are important factors for the survival and growth of *Legionella* pneumophila (Carson and Mumford, 2010).

Mains water already contains a variety of minerals, in some cases relatively high amount of calcium and magnesium. These minerals can contribute to the formation of scale which can firmly adhere on the underlying surface of the water storage tank (Figure 1.12) (Donaldson and Grimes, 1988). The accumulation of scale presents a significant impact on biological contaminations within the water system such as providing habitat for bacteria colonies, including *Legionella*, and acting as essential nutrients for their metabolism (Bartram, 2007). Many studies on nutrients that encourage *Legionella* growth have reported scale nutrients to be one of the major contributing factors for *Legionella* proliferation (Schwake, 2013).

Figure 1.12 Scale accumulation on the tank wall (Source: own collection).

The suspended matter that settles at the bottom of a fluid is known as sediment. During water storage, tiny suspended solids and dissolved solids in the mains water will settle to the bottom of the tank as sediments (Figure 1.13). In metal tanks, corrosion products and sediments together encourage *Legionella* proliferation as they

act as nutrient sources (Qin et al., 2017). The relationship between sediments and Legionella growth in water systems was first established in 1982. In this study cultures taken from water samples from storage tanks with no sediments were found to contain very low numbers of bacteria. After a period of time to allow sediment accumulation, the same tanks produced a high count of bacteria, but once the sediments were removed by flushing the bottom of the tanks, no bacteria was found (Wadowsky et al., 1982). Later, many studies on water systems including cooling towers have confirmed that the sediments are responsible for Legionella proliferation and growth (Llewellyn et al., 2017; Springston and Yocavitch, 2017; Erdoğan and Arslan, 2016) (See Chapters 3 and 5). Sediments provide not only nutrients but also habitat for the survival of *Legionella* bacteria. Sediment in the pipework also provides the same habitat for *Legionella* bacteria as it would in the bottom of a cold water storage tank (Bothner, 2016). Stout and colleagues also reported the concentration of sediment to be directly related to the proliferation and multiplication of *Legionella* bacteria in the water system (Stout et al., 1985). *Legionella* risk associated with sediments can be minimised by more frequent monitoring and internal cleaning.

Figure 1.13 Sediments and corrosion at the bottom of a cold water storage tank (Source: own collection)

According to Health and Safety Executive's (HSE), approved code of practice legislation 8 (ACOP L8) 2013, water storage in any building should be limited to 24 hours usage; i.e. the maximum retention time of cold water storage should be maximum one day (HSE, 2013). Large tanks with excess storage capacity create conditions for water stagnation, which eventually leads to biofilm formation (Figure 1.14) which harbours many micro-organisms including *Legionella* pneumophila

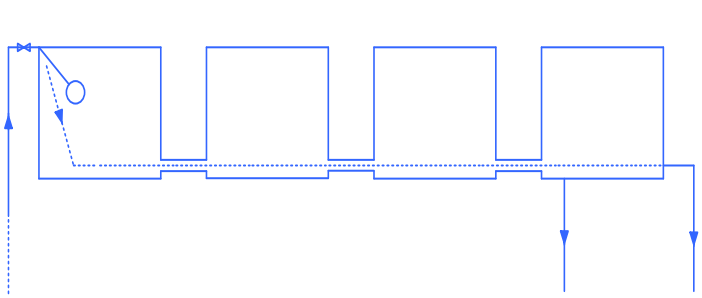
(Armon et al., 1997). If multiple cold water storage tanks are present in a building, they are usually linked together, either in parallel or in series. Multiple tanks connected in parallel (Figure 1.15b) often draws water equally from all the connected tanks (Figure 1.15d); in contrast, tanks connected in series (Figure 1.15a) discourage the internal water circulation thereby promoting water stagnation (Figure 1.15c). In this arrangement, incoming mains water is connected to the first tank only; all the other tanks acts as reservoir tanks, and the outlet is normally connected to the last tank in the series. This arrangement causes the water in the first and last tanks to replenish often, but the other tanks in between are affected by stagnation of the water on the upper layer. The number of outlets connected to each tank also plays a major role, as in some arrangements, most of the outlets would be connected to a single tank which may leave other tanks in the arrangement with less internal water movement.

Figure 1.14 Biofilm on water surface (Source: own collection)

Figure 1.15a

Figure 1.15b

Figure 1.15a Tanks connected in series and **Figure 1.15b** tanks connected in parallel (own collection)**.**



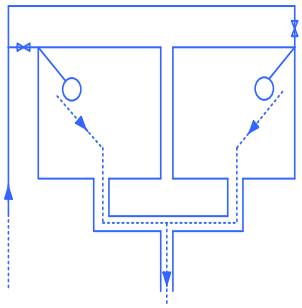


Figure 1.15c **Figure 1.15d**

Water flow indication schematic of tanks connected in series Figure 1.15c and water flow indication schematic of tanks connected in parallel Figure 1.15d.
(Source: own drawing).

The location of the cold water storage tank is also very important in terms of *Legionella* control. Normally, cold water storage tanks are located on the roof top, within the loft space of the building, on the ground floor or in the lower ground (basement) level. Tanks located on the roof top are mostly exposed to adverse weather conditions such as hot temperatures in the summer, freezing cold in the winter and heavy rainfall which can affect the quality of water stored inside the tanks. Appropriate thermal insulation can act as a barrier to these changes to a certain extent, and the provision of a tank housing will further help to maintain the water quality unaffected. Cold water storage tanks located within the loft space are more protected from adverse weather conditions due to well insulated building roof. However, some of the tanks in the loft space are tightly surrounded and sometimes partially covered by stored items that have been accumulated in the loft space. These items can restrict ambient air circulation and increase the ambient temperature, thereby affecting the stored water temperature if the thermal insulation provided to the tank is not appropriate (Figure 1.16a). Cold water storage tanks located on ground floor and in basement (lower ground) tank rooms are more protected against adverse weather conditions. However, these tank rooms are also often used as store rooms (practitioners site survey experience), and the stored items can create negative impacts in terms of ambient air temperature. Latest HSE guidelines for *Legionella* control in domestic water systems ACOP L8 2013 HSG 274 part-2 specify the need to maintain a minimum one metre clear space around the cold water storage tanks (Figure 1.16b) in order to rectify this issue.

Figure 1.16a. Single piece plastic tank without clear space around (Source: own collection)

Figure 1.16b. Pre-insulated GRP sectional tank with clear space around

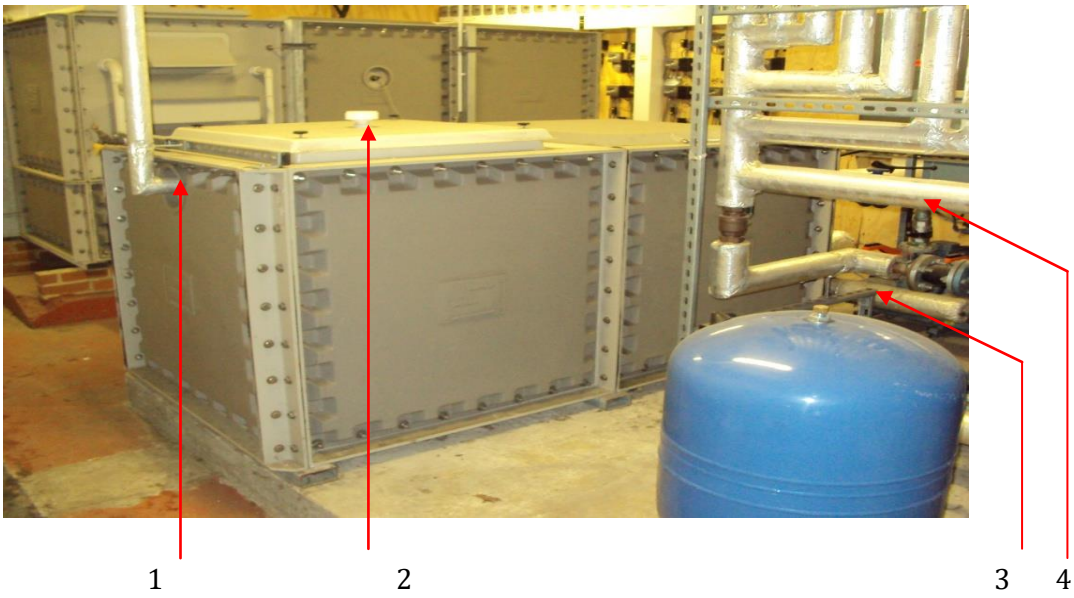


Figure 1.17 1 –inlet and 3 – outlet connected opposite to each other to encourage the tank's internal water flow, 2- is the screened breather vent to improve the internal air circulation of the water storage tank and 4 is the thermal insulation fitted to the pipework. (Source: own collection).

In the early part of 1980's, metal tanks were replaced by fibreglass tanks; but in early 1990's pre-insulated Glass Reinforced Plastic (GRP) tanks replaced metal and fibreglass tanks (ASHRAE Guideline, 2000). Even if the tanks are thermally insulated, poor internal air circulation can create humid conditions within the tank, resulting, eventually, in raised internal stored water temperatures leading to proliferation of *Legionella* bacteria (HSE, 2016a). In order to get rid of this possibility, the tanks should be fitted with a screened breather vent to improve internal tank air circulation, and to protect the stored water from insects entering the water storage tank (Figure 1.17 -2).

The plumbing arrangement of the water tank is also a very important factor in maintaining water quality. If the inlet (incoming mains) and outlets are on the same side, internal water circulation will be restricted and this could lead to the water stagnation within the storage tank. Current WRAS guidelines insist that the inlet and outlets of the storage tank should be fitted opposite to each other to enhance the internal water circulation (WRAS, 2015). Many water storage tanks were installed prior to the implementation of this guideline, with inlets and outlets fitted on the same side of the tank leading to internal water stagnation. However, this can be rectified by installing a sparge pipe to the tank outlet (Figure 1.18A). Size of the pipework is another factor as relatively larger inlet and smaller outlets could lead to the possible water stagnation (Stojek and Dutkiewicz, 2006).

A

B

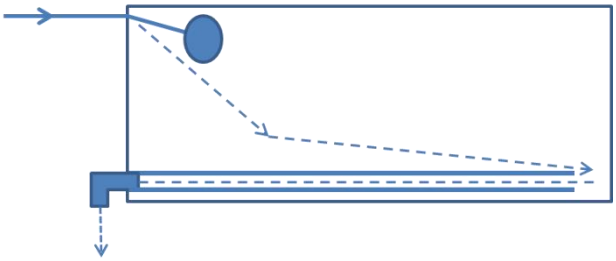


Figure 1.18A; Sparge pipe connected to internal of the tank outlet; B. water flow indication schematic when using a sparge pipe to the internal of tank outlet.
(Source: A - own collection; B- own drawing)

1.17.9 Hot water storage

Unlike other bacteria species, *Legionella* is considered as a thermo-tolerant bacteria which can withstand temperatures in the range of 50-60˚C for several hours (Bartram et al., 2007). These specialised characteristics allow *Legionella* to proliferate and

survive in hot water systems (Whiley et al., 2017). Allegra and colleagues (2011) proposed that *Legionella* strains can become heat resistant after being subjected to a cycle of heating treatments for a long time. One of the major findings of their study was that some strains of *Legionella* submitted to superheating in the environment for a long time were shown to develop resistance to high temperatures (Allegra et al., 2011). This phenomenon was demonstrated by the high proportion of culturable cells and 'not culturable but viable' (NCBV) cells still present after a 30-minute treatment at 70°C (Farhat et al., 2012). Further analysis showed that although *Legionella* diversity was reduced, pathogenic *Legionella* species (*Legionella* pneumophila and *Legionella* anisa) remained after the heat shock, and also after chemical treatments. The biofilm was not removed, and the bacterial community structure was transitorily affected by the treatments. It was concluded that eradication of *Legionella* requires a better understanding of the ecology of bacterial and eukaryal species associated with *Legionella*-containing biofilms (Farhat et al., 2012).

Main hot water storage in domestic properties is calorifiers, water heaters and instantaneous water heaters or point of use water heaters. Out of all these hot water storage systems, calorifiers are a major area of concern (HSE, 2014). A calorifier is a heat exchanger which heats water indirectly by circulating over a heating coil or multiple coils. Normally, the primary source of heat can be water or steam (heated by an external heat source such as a boiler), contained within a pipe immersed in the water. The hot water or steam within the coil do not mix or come in contact each other (DMME, 2015).

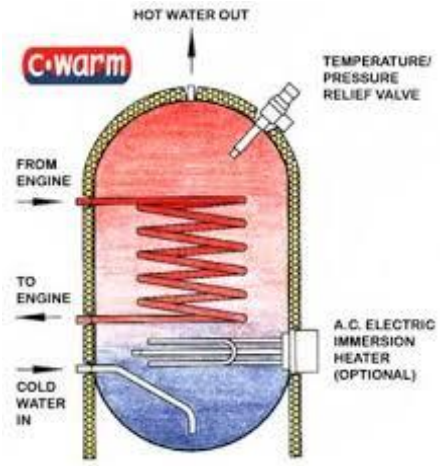


Figure 1.19 shows the internal coil structure and cold water feed and hot water generation within the calorifier.

(Source: http://www.tnorrismarine.co.uk/product/calorifiers)

The incoming cold water (typically less than 20 °C) enters at the bottom of the calorifier, (often gravity fed) from the cold water storage tanks and is mixed with hot water inside the calorifier and heated to the required temperature using the internal heated copper coils housed inside the calorifier (Figure 1.19). Mainly there are two types of calorifiers are in use; vertical calorifiers and horizontal calorifiers. In vertical calorifiers, the hot water or steam from external source enters the coil through the top pipework connection and passes through the inlet valve which is thermostatically controlled to avoid over heating or boiling of the stored water within the calorifier. In contrast, lower temperatures at the bottom of the calorifier, where cold water enters (Figure 1.19) together with the nutrient rich bottom area of the calorifier can support the abundant growth of *Legionella* bacteria. In the case of infrequently used hot water system, the operating temperature can fall below 50˚C which can further accelerate the growth of *Legionella* that is present at the bottom of the calorifiers (HSE, 2016).

Many studies have demonstrated that the domestic hot water system is a potential source of *Legionella* contamination (Ryu et al., 2017; Bédard et al., 2015; Rhoads et al., 2015). In Germany, 452 samples were collected from randomly selected single family residential flats to analyse for the presence of *Legionella*. All these flats were supplied with treated ground water from public water treatment works. Drinking water quality and parameters were within the satisfactory limits of German water regulations. Analysis results showed 12% of the flats with recirculating hot water system had maximum counts of *Legionella* which reached 100,000 cfu/100ml; out of the positive results 93.9% was *Legionella* pneumophila species and 71.8% was belonged to serogroup-1. This study also demonstrated that the volume of the hot water storage and intermittently raising hot water temperatures above 60ºC had no effect on *Legionella* count. Thus, this study reported that low temperature (below 46°C) in the hot water storage system to be a significant factor leading to the proliferation and intensified growth of *Legionella* bacteria (Mathys et al., 2008). The US Waterborne Disease and Outbreak Surveillance System (WBDOSS) has reported the increasing importance of LD contracted from individual residential hot water systems since its inclusion for monitoring which commenced in 2001 (Craun et al., 2010). A domestic hot water system was also shown to be responsible for an outbreak of LD originating from a residential block of flats in Copenhagen (Denmark) (Krojgaard et al., 2011).

In order to minimise the possibility of *Legionella* proliferation at the bottom of the calorifier, electric immersion heater is also introduced in recent years (Figure 1.19). In large calorifiers, an anti-stratification circuit is provided (Figure 1.20) to enhance the

internal water circulation, and to maintain the required hot water temperature throughout the hot water storage. A hot water return circuit is also necessary to maintain the hot water temperature throughout the system as this can encourage the water circulation when the water is not in use (Fields, 2002). These return hot water circuit (Figure 1.21) from the distribution pipework to the hot water storage (calorifier) also useful to eliminate the possible variation of hot water storage temperature and water distributing temperature (Rhoads et al., 2016). Provision of a circulating pump further encourages the hot water circulation to maintain the operating temperature throughout the hot water system (Laperriere et al., 1992). It is recommended in the latest HSE's ACOP L8 guidelines that the hot water return circuit should be fitted with circulating pumps (Figure 1.22), which can accelerate the recirculation and avoid possible water stagnation within the pipework (Boppe et al., 2016).

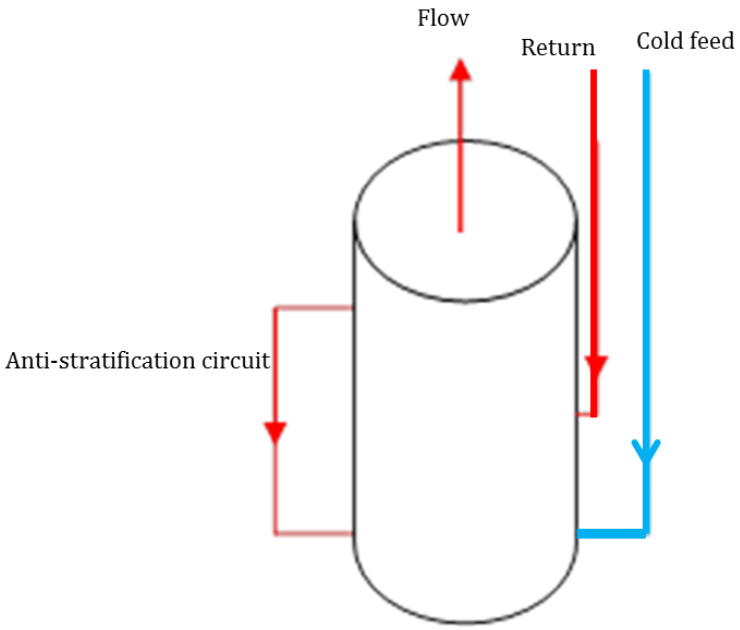


Figure 1.20 Schematic of large calorifier with anti-stratification circuit (Source: own drawing)

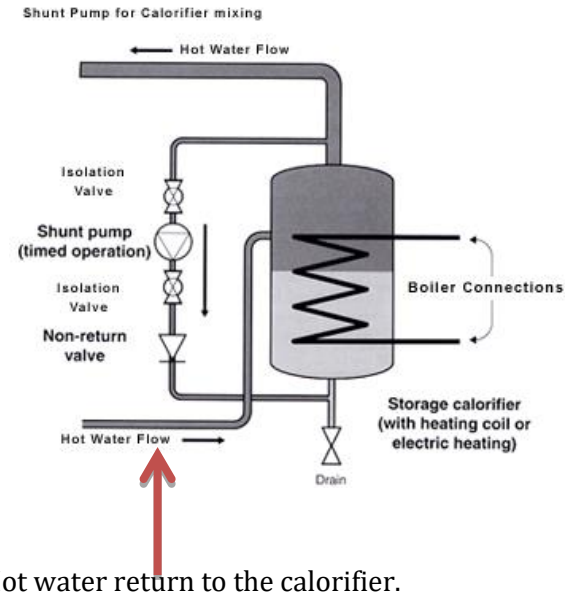
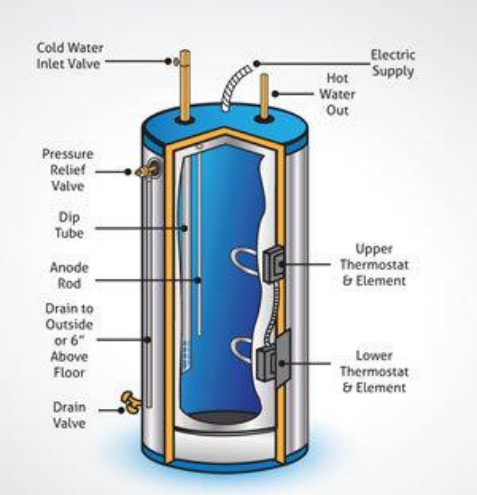


Figure 1.21 Hot water return to the calorifier.
(Source: http://www.tnorrismarine.co.uk/product/calorifiers)

Figure 1.22 Calorifier fitted with circulating pump on the return pipework to maintain the temperature throughout the hot water system. (Source: own collection)

Water heaters are another type of hot water storage system where *Legionella* can colonise and multiply. Water heater can be defined as an appliance usually powered by gas or electricity, that heats water and stored for domestic purposes. Both electric and gas fired water heaters are fed by direct mains water with the help of pressure regulating valves and non-return valves. Figure 1.23a is an electric water heater and

figure 1.23b is a gas fired water heater. In both the cases, water gets heated directly within the water heaters and stores the hot water ready to use.



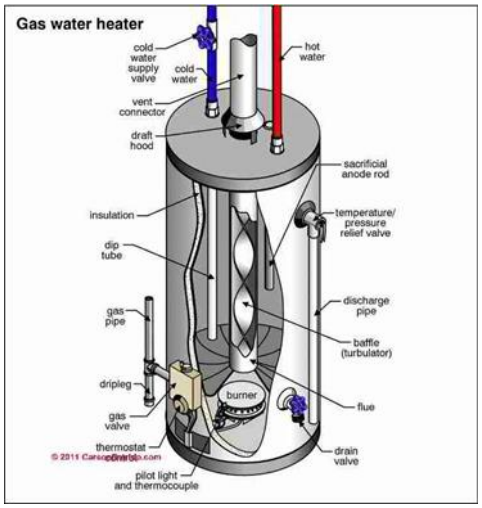


Figure 1.23a Electric water heater

Figure 1.23b gas fired water heater

(Source: http://www.engineeringapps.net).

Generally, electric water heaters operate by using one or two direct immersion heating elements controlled by thermostats, which heat the water within the water heater tank to the required temperature.

Gas fired water heaters also operate almost on the same principle. A thermostat located near the bottom of the tank is inserted into the side of the tank which senses the temperature drops below the desired set level, and this provides signal to the burner for ignition. When the desired temperature is reached, the thermostat shuts off the burner. Gas fired water heaters attain the desired temperature level about twice as fast as electric water heaters. Due to the provision of internal direct heating, these water heaters are much safer as compared to calorifiers in terms of *Legionella* proliferation.

Hot water storage such as calorifiers and water heaters should operate at 60ºC to attain a minimum of 50ºC at all related outlets. It is extremely important for calorifiers and water heaters to provide appropriate thermal insulation (Figure 1.24) as heat loss from these systems can reduce the stored hot water temperature which can lead to *Legionella* proliferation as well as more frequent or continues operation causes additional use of fuel, waste of energy and unnecessary contribution to emission (Johanssona et al., 2003).

Figure 1.24 Calorifier fitted with appropriate thermal insulation. (Source: own collection)

Another type of domestic water heaters exists, called 'reservoir type water heaters', which comprise of a cold water storage tank fitted on top of a water heater. This design poses a serious concern in terms of *Legionella* control. The heaters benefit owners in terms of energy savings, as the copper hot water tank below can heat the copper cold water tank above by convection. However, problems can occur if the cold water temperature falls within the range of 20-45ºC which is optimum for *Legionella* proliferation and multiplication. Furthermore, the possible presence of nutrients such as sediments and scale deposits within this cold water storage tank can further accelerate the *Legionella* growth and contaminate the entire hot water system in the event of heater operating at below 50ºC. This type of water heaters (Figure 1.25) are considered as high risk hot water systems in terms of *Legionella* control and their usage is discouraged, although they were previously promoted as energy saving water heaters over the last few decades.

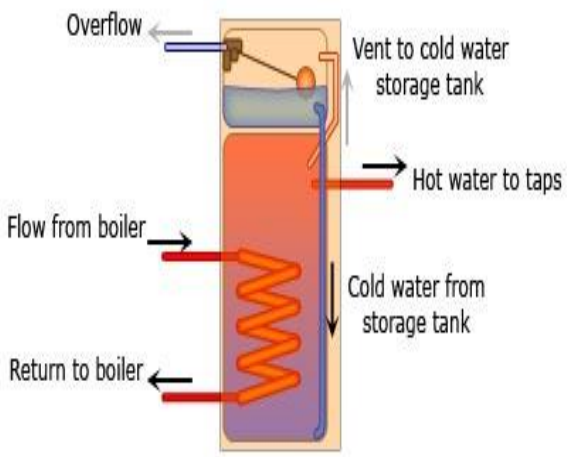


Figure 1.25 Reservoir type water heaters: A – internal structure and B – external view (Source: http://www.gasapplianceguide.co.uk/DirectandIndirectCylinders)

1.17.9.1 Other hot water sources

There are other hot water systems such as local water heaters, point of use water heaters (POU) and instantaneous water heaters.

A **B** **C**

Figure 1.26 A-Local water heater, B. Instantaneous water heater, C. Point of use water heater. (Source: own collection)

Small or local water heaters (Figure 1.26A) and the point of use water heaters (Figure 1.26B and C) are relatively low risk hot water systems as they operate with minimum water storage (EWGLI, 2011). These water heaters are comparatively safer than calorifiers and large water heaters in terms of *Legionella* proliferation. These water heaters are installed in situations where hot water usage is minimal, as they are

not designed for the hot water storage. However, *Legionella* can grow in these system also if they are operated at low temperatures (<50ºC). In general, these hot water systems are considered as low risk systems in terms of *Legionella* if they operate in the range of 50-60ºC at all times. These findings can be supported with a study carried out in Germany; analysis results of 452 hot water samples from domestic residences for the occurrence of *Legionella* bacteria showed 93.9% positive, in contrast, all the samples collected from local/instantaneous/POU water heaters were free from *Legionella* contamination (Mathys et al., 2008). However, intermittent operation of these systems can cause water temperatures to fall in the range of 20-45ºC which is the favourable temperature region for *Legionella* proliferation. Once *Legionella* proliferation is occurred within these systems, these organisms can move back towards the main flow stream and contaminate the whole system.

1.17.9.2 Mixed water sources

Showers and outlets fitted with thermostatic mixing valves are the main sources of mixed water in domestic properties. Favourable water temperatures, the presence of stagnant water, and scale deposits within the showerheads can create a nutrient rich environment for *Legionella* proliferation and growth (Spalekova and Bazovska, 2003). Showers (Figure 1.27) are considered as a major source of *Legionella* infection due to excessive aerosol generation during their use. Most showers are thermostatically controlled to provide with a comfortable showering temperature of 35-40ºC, which is the optimum temperature for *Legionella* proliferation. *Legionella* outbreaks in domestic properties have been traced back to contaminated showers; for example, during the summer of 1980, 58 people were infected with Legionnaires disease out of 5,000 guests stayed at the Rio Park Hotel. Epidemiological investigation reported that the outbreak was caused from infected showers as those who took shower in each morning had been contracted LD (Macfarlane and Worboys, 2012). Furthermore, many recent studies have identified that the showers are one of the major sources of *Legionella* contamination as well as potential LD outbreaks (Chinsembu, and Hakwenye, H (2010; Collins et al., 2017a; Prussin et al., 2017).

Vulnerable premises that accommodate vulnerable people such as hospitals, health care premises, schools and elderly homes are the main areas where thermostatic mixing valves (TMV's) are installed (Figure 1.28) to eliminate the risk of scalding.

TMVs are normally installed prior to the water usage point where cold water and hot water are mixed together in a ratio offset by the thermostat; normally in the range of 35-40ºC. Thus, water temperatures within TMV's are always in favour of *Legionella* proliferation, and scale deposits within the TMVs can provide nutrients for bacterial growth. Therefore, TMVs are categorised as high risk systems in terms of *Legionella* control and their usage has been discouraged in recent years. However, existing TMVs can be maintained with more frequent internal cleaning and disinfection with extensive monitoring (Murray, 1988).

Figure 1.27 Scaled showerhead
(Source: own collection)

Figure 1.28 TMV

1.18 Regulations for Legionella control

1.18.1 General control measures for *Legionella* bacteria

Legionella risk cannot be eliminated; but by taking appropriate control measures *Legionella* risk can be reduced. In the UK, HSE has implemented regulations and appropriate guidance to control *Legionella* in water systems (HSE, 2013). These include (a) avoiding water temperatures between 20 °C and 45 °C in the water system; (b) avoiding water stagnation within the water system; (c) avoiding the use of materials in the water system that encourage the proliferation of *Legionella* bacteria or any other microorganisms; (d) ensuring water system is clean and free from rust, scale, sludge, organic matter and biofilms (e) ensuring the water heaters or calorifiers are

operating at 60^0C to attain a minimum of 50^0C at all related hot outlets (f) ensuring the hot water is recirculated to avoid stagnation as well as maintain the temperature and (g) considering water treatment to control the proliferation of *Legionella* bacteria or to reduce the possibility of bacterial growth (h) monitoring any control measures in place (i) reducing aerosol generation and (j) keeping records of remedial actions taken including maintenance work (HSE, 2015).

World Health Organisation (WHO), United States Environmental Protection Agency (USEPA), American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE), European Centre for Disease Prevention and Control (ECDC) and UK Health and safety Executive (HSE) has provided specific guidelines to protect the public from *Legionella* infection.

1.18.2 WHO guidelines

The World Health Organization (WHO) had three principal documents providing guidance on *Legionella* control; Guidelines for Drinking-water Quality (WHO, 2004), Guidelines for Safe Recreational Water Environments (WHO, 2006), Guide to Ship Sanitation (WHO, 2007). In 2007, a review was conducted resulting in a specific document on *Legionella* control: *LEGIONELLA* and the prevention of Legionellosis (WHO, 2007). This 252 page document has addressed a number of shortfalls in previous guidelines and provided specific guidelines for healthcare and non-health care premises.

1.18.3 United States Environmental Protection Agency (USEPA) guidelines

The United States Centre for Disease Control and Prevention estimates that 8,000 to 18,000 people are hospitalised in the U.S every year with Legionnaires' disease. There are Federal guidelines and state guidelines in place to protect the public health in terms of Legionnaires disease (EPA 2017). In addition, there are ASHRAE (American Society of Heating, Refrigerating, and Air-Conditioning Engineers) guidelines to manage the risk of Legionellosis from Building Water Systems (ASHARE, 2015).

1.18.4 European guidelines

European Centre for Disease Prevention and Control (ECDC) and European Legionnaires' Disease Surveillance Network (ELDSNet) has provided specific guidelines on prevention and control of *Legionella* bacteria in water systems (ECDC, 2012; ECDC, 2011). The main aim of ELDSNet is to operate as a network to detect, control and prevent LD cases, clusters and LD outbreaks within EU/EEA countries and assist with detection and response even outside European countries. European Legionnaires' Disease Surveillance Network helps within the EU countries to share information and collaborate on Member State actions to ensure the residents of European countries are better protected from contracting Legionnaires' disease associated with travel within their own country or abroad (ELDSNet, 2017).

1.18.5 UK health and safety law

UK Health and Safety Executive (HSE) have implemented strict legislation under Control of substance hazardous to health (COSHH) regulations to control *Legionella* and protect the public from Legionnaires' disease. *Legionella* control procedures in the UK should be in accordance with Approved Code of Practice, Legislation 8 (ACOP L8). HSG Part 2 Published in 2014 gives practical advice on the legal requirements for the 'control of *Legionella* in hot and cold water systems (HSG, 2014).

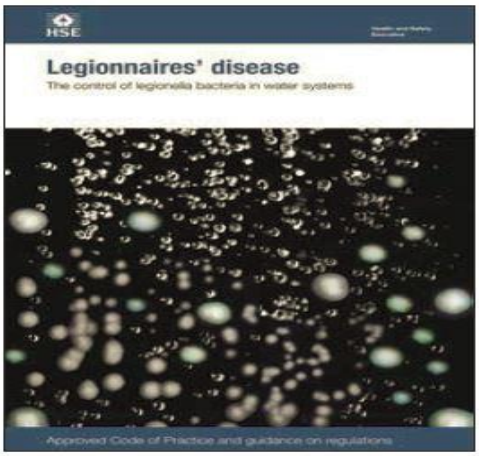
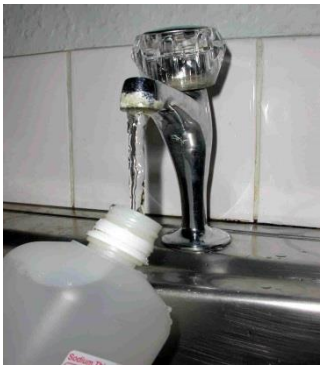


Figure 1.29 HSE's ACOP L8 legislation (latest) to protect the public from Legionnaires' disease. (Source: http://www.hse.gov.uk/pubns/books/l8.htm

ACOP L8 (Figure 1.29) gives the legislative requirements and guidance about risk of *Legionella* bacteria in the water systems and effective control measures. ACOP L8 also gives the guidance on the management of compliance with the relevant parts of Health and Safety at Work Place Regulations 1999 (HSE, 2014). ACOP L8 was revised in 2013 and a separate guideline was allocated for the control of *Legionella* bacteria in hot and cold water systems under the code HSG274 Part 2 published in 2014 (HSG, 2014).

There are two important steps to follow in *Legionella* control; first is to carry out a specific and detailed risk assessment to identify the possible risk factors in terms of *Legionella* and second is to establish a routine monitoring program on the basis of the risks identified. This monitoring program includes regular temperature testing, hot and cold water storage system inspection, water sampling for *Legionella* analysis and periodic cleaning of water systems (Stout, 1998). Temperature monitoring is needed to ensure the system is operating within the threshold limits as hot water temperature must be above 50^0C and the cold water temperature must be below 20^0C. Water storage systems inspection (e.g. water tank(s) are needed to ensure that the system is free from sediments, corrosion products, scale, water stagnation, other debris, sludge, slime, algae and biofilm as these can harbour *Legionella* as well as act as nutrients for bacteria (Geary, 2000).

Water sampling and analysis plays an important role in *Legionella* control as visual inspection cannot provide any estimate of microbiological contamination of the water system. There are specific guidelines outlined for water sampling in BS 7592:2008 (BS 7592:2008); water samples are collected in sterile bottles (Figure 1.30a) which are then sent to accredited laboratories to analyse for *Legionella* bacteria by culturing on Buffered Charcoal- Yeast Extract (BCYE) agar medium (Figure 1.30b).This standard diagnostic procedure can take up to eleven days to get the results and report (Ta et al., 1995). If *Legionella* is detected during the analysis, disinfection of the water system will be necessary (Figure 1.30c).



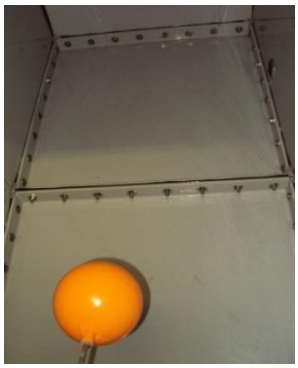


Figure 1.30a, Water sample collection for *Legionella* analysis in sterile bottles (supplied by UKAS accredited laboratory) in accordance with BS 7592:2008. **Figure 1.30b,** BCYE culture of *Legionella* bacteria from environmental water samples in UKAS accredited laboratory. **Figure 1.30c**, a sectional GRP tank after the cleaning and disinfection as water sample analysis of the stored water was identified with *Legionella* spp serogroup 1.
Source: Figure1.30a and c: own collection, Figure1.30b: https://www.alsglobal.com.

However, each year approximately 200-300 reported cases of Legionnaire's disease are reported in the UK every year, and there are hundreds of cases which are not reported. In 1981, WHO recommend all cases of pneumonia admitted to hospital be examined for *Legionella* infection, but in the current economic climate this is not practical and has not been followed. Thus the risk of Legionnaires' disease is increasing and subsequently the number of outbreaks is on the rise (Diederen, 2008). This increasing number of cases of LD could be resulted from public contracting *Legionella* infection from the domestic water systems which are not in-compliance with latest ACOP L8 guidelines (personal observation of Aji Peter as a *Legionella* control practitioner).

1.19 Remedial cctions in the event of positive Legionella detection

Once the laboratory analysis result confirms the system is infected with *Legionella* bacteria, disinfection of the water system may be necessary depending on the level of *Legionella* bacteria identified (HSG274Part2, 2014). There are a number of disinfection techniques available depending on the nature of the infected system.

1.19.1 Disinfection methods

There are a number of disinfection methods available but many of them are not been generally used (for example, membrane filtration), therefore, those are not reviewed here. Commonly used disinfection methods are thermal disinfection (normally for hot water system), chemical disinfection, Ultra Violet (UV) light disinfection, ozone disinfection, copper-silver ionisation disinfection and point of use filtration (Walraven and Chapman, 2016). Disinfectants used for portable water must be suitable for human consumption due to its chemical properties and concentrations. Additional disinfectants can be used for non-potable water systems as associated human health risks are no longer of concern (Werner et al., 2016; Blanc et al., 2005). In this research, I am mainly discussing about the domestic hot and cold water systems and therefore, other water systems disinfection methods are excluded from this review.

1.19.2 Thermal disinfection

Thermal disinfection (pasteurisation) has been widely used in hot water systems as *Legionella* cannot survive more than a minute at 70°C (Kruse et al., 2016; Callizoa et al., 2005). In this procedure, the calorifier/water heater need to be operated at 70°C and all the related outlets also should attain a minimum of 60°C. Any deadlegs or dead ends in the system need to be heated to 70°C by using trace heating techniques (Yang et al., 2016; Al-Sharif, 2006; Kima et al., 2002). All the outlets should be flushed for at least five minutes to ensure the water discharging temperature is 60°C or above.

The water system should be well insulated to achieve the best result of thermal disinfection. Once the temperature of all outlets reaches a minimum of 60ºC, the whole

hot water system should be kept at the same temperature for a minimum of one hour. After one hour, the calorifier's operating temperature is reduced to 60ºC and all the outlets are flushed until the temperature stabilises. At this stage, the system is considered to be free from *Legionella* and ready to use. However re-sampling is necessary to confirm that the hot water system is free from *Legionella* infection. In normal operation, hot water temperature should be in the range of 50-60ºC as above 62ºC presents a possible scalding risk (HSG274Part2, 2014). In contrast, many studies demonstrated that thermal disinfection is not effective compared to other popular disinfection techniques (Marchesi et al., 2016). A study conducted in a 1070 bedded hospital in Taiwan showed that pasteurisation performed over an eight-week period eliminated *Legionella* bacteria from the hospital ward taps and brought down the bacteria count from 80 percent to 25 percent in ICUs. However, *Legionella* growth rate increased significantly after two months, from zero to 15% in hospital patient wards, and from 25% to 93% in ICUs. A second round of thermal disinfection was carried out for a 2-day period, but no significant reduction was achieved during this treatment (Chen et al., 2005).

The main advantage of thermal disinfection is that, it generally does not require any special equipment and is easy to carry out (Ji et al., 2018; EPA, 2015). However, there are a number of disadvantages for this disinfection procedure as follows (Pūle, 2016; EPA, 2015; Campos et al., 2003):

(i) This is only applicable to hot water distribution system with heating efficiency.
(ii) It is labour intensive and time-consuming.
(iii) It requires significant amount of energy as well as manpower to perform the flushing.
(iv) It may not be effective in dead legs and dead ends where water circulation is relatively poor.
(v) Thermal disinfection is not effective within thermostatic mixing valves and mixed taps
(vi) Possible scalding risk during thermal disinfection is a significant and this risk increases in vulnerable premises such as hospitals, schools and elderly care homes.

(vii) The high temperature applied on the hot water distribution system during this method can damage pipes, valves and pump seals, resulting in leakage, valve and pump damage.

1.19.3 Chemical disinfection

Many studies have demonstrated that the chemical disinfection techniques are more effective in managing the risk associated with *Legionella* infected systems (Marchesi et al., 2016; CDC, 2016; Stalter et al., 2016). There are oxidising agents and non-oxidising agents as chemical disinfectants.

1.19.4 Oxidising disinfectants

Generally used oxidising agents are chlorine, bromine and iodine (Halogens), chlorine dioxide, potassium permanganate, hydrogen peroxide, ozone and chloramines. Out of all these oxidising agents, chlorine is the most widely used disinfectant; however, chlorine dioxide, ozone, hydrogen peroxide and chloramines are also popular in water treatment industry (Walraven and Chapman, 2016).

1.19.4.1 Chlorine

Chlorine and chlorine based compounds are oxidising disinfectants that can efficiently inactivate microorganisms including *Legionella* species during disinfection treatment, as well as helps to maintain the water quality throughout the distribution system. (Montes et al., 2014; McGuire, 2013). Chlorine is added to water as chlorine gas; sodium hypochlorite solution or dry calcium hypochlorite is used for this purpose. Chlorine as sodium hypochlorite is the most common form of disinfectant used in domestic water systems (Gillilland, 2014). Chlorine can be used in hot and cold water systems for in-line ongoing treatment (as a precautionary measure) as well as to the cold and hot water tanks or to the entire distribution system. Also, chlorine can use at high doses for emergency shock treatments (hyper chlorination) of domestic water systems (Borella et al., 2016). When chlorine is used as a disinfectant, the role of pH is

very important as chlorine exists as hypochlorous acid in the pH range of 6.4 to 7.6 and out of this pH range, it exist as hypochlorite ion (EPA, 2016a). Hypochlorous acid is comparatively a stronger disinfectant and oxidant than hypochlorite due to its fast reactive nature (Park et al., 2004).

The efficiency of chlorine disinfection is mainly depends on factors such as chlorine concentration, pH, temperature, contact time, buffering capacity of the water, types of microorganisms present in the water, concentration of organic matter in water, and presence of biofilm in the water system (McGuire, 2013); Le Dantec et al., 2002). Even though chlorine is widely used as a strong oxidising disinfectant against *Legionella*, there are significant negative impacts in chlorination. There are enough evidences that chlorination of hot and cold water systems can shorten the life of the installations and damage the entire water distribution system (Lytle and Liggett, 2016; Abigail et al., 2007). Last of all is the lack of long lasting effect which means chlorination cannot eliminate *Legionella*, but only can suppress the growth and also chlorine cannot penetrate in to biofilms (Percival and Williams, 2014).

1.19.4.2 Chlorine dioxide

Chlorine dioxide is relatively more powerful than chlorine in hot and cold water disinfection (WHO, 2016). Chlorine dioxide is a green-yellow water-soluble gas that can diffuse through cell membranes of micro-organisms, and this gas can be manufactured by mixing sodium chlorite and a strong acid such as hydrochloric acid or nitric acid. This can be used as the same way of doing chlorination and is more successful in the hot water system as chlorine dioxide can penetrate in to the biofilms, active in higher temperatures and in higher pH (Walraven and Chapman, 2016). Chlorine dioxide is active and is an effective disinfectant in a wide range of pH as compared to chlorine and is also considered as less corrosive than chlorine (Shirasaki et al., 2016). Some studies reported that chlorine dioxide reacts with polyethylene water pipes which resulted in serious damage to the pipework system (Yu et al., 2011). The amount of chlorine dioxide required for effective disinfection is depend on factors such as the complexity of biofilm within the system, pipework material and condition, population and type of micro-organism present, pipe size and length of the distribution system, water turnover rate and treatment goals such as *Legionella* control (WHO, 2016; Lin et al.,

2011). Maintaining total residual chlorine dioxide level of 0.1–0.5 PPM within the domestic water distribution system is usually sufficient to control *Legionella* bacteria, although higher levels may be necessary in a heavily contaminated system (HSE, 2014).

1.19.4.3 Ozone

Ozone is used as an effective disinfectant in domestic water systems (Xie, 2016). It is generated at the location of use, using either air or liquid oxygen and is then transferred into the water phase. When dissolved in water, unstable molecular ozone (O3) decomposes to hydroxyl radical, which is a stronger and reactive oxidizing agent than molecular ozone (Glaze, 1987). The efficiency of ozone disinfection is totally depending on the concentration of the oxidants and the reactivity of the contaminants with each oxidant (Gehr et al., 2003). Water temperature has direct impact on ozone disinfection as efficiency increases as temperature increases (Cho et al., 2003). In contrast, due to the fast decomposition of ozone in hot water, it is difficult to maintain a higher concentration level throughout the water system to control *Legionella* bacteria. Therefore, balance between higher inactivation rates and lower CT with increased water temperature need to be maintained during ozone disinfection (Ruiz et al., 2007).

1.19.4.4 Monochloramine

Monochloramine (NH2Cl) is an oxidising disinfectant and is active in controlling bacterial re-growth and controlling biofilms (Baron et al., 2015). The primary advantage of Monochloramine is that can penetrate biofilm, although excess ammonia contributes towards certain biofilm growth (Gomez-Alvarez et al., 2016). The main application of monochloramine in domestic water systems is for residual disinfection and it has a more persistent nature and stability in water system than chlorine (Taylor et al., 2000). When interacting with biofilm bacteria, monochloramine can actively penetrate; whereas chlorine cannot penetrate biofilm as well as it may get consumed through various reactions. Some studies have demonstrated that monochloramine was able to penetrate biofilms 170 times faster than equivalent amount of free chlorine in domestic water systems (Lee et al., 2011).

There are reports from studies that monochloramine can easily react with rubber and plastic materials which can create severe damages in pipework systems and pumps

(EPA, 2016a; Rogers et al., 2004). Monochloramine in water can adversely affect kidney dialysis and should not be present in dialysate water (Fairey et al., 2007; Tipple et al., 1990). Monochloramine can cause detrimental effects on fish species as water containing monochloramine cannot be used for aquariums (Tkachenko and Grudniewska, 2016; Altinok, 2004). Another risk factor is that monochloramine is capable to react with organic matters in the water to form a number of DBPs such as nitrosamines causing potential public health concerns (Yang et al., 2016a; Stalter et al., 2016; Vikesland et al., 1998).

1.19.4.5 Silver peroxide

Silver-peroxide is considered as an environmentally friendly water disinfectant mainly based on hydrogen peroxide and silver-nitrate (Marchesi et al., 2016; Pedahzur et al., 2000). The capability of silver-peroxide in eliminating micro-organisms such as bacteria, viruses, amoeba, mould, fungi and algae are well identified with its efficacy in controlling the biofilms (Kotze, 2015; Armon et al., 2000). Silver peroxide has been developed in 1970 and it offers hydrogen peroxide stabilisation; a long lasting efficient disinfectant. Silver peroxide differs from ordinary hydrogen peroxide because of the presence of antibacterial chemical, 'silver nitrate' (Pedahzur et al., 1997). When used as a water disinfectant, hydrogen peroxide activation stops when all the organic materials are oxidised. Once the oxidation process is stopped, the non-reacted hydrogen peroxide decomposes as water and oxygen. In contrast, silver-nitrate does not break down and become persistent in the domestic water system or remains in the environment (Ditommaso et al., 2016; Tofant et al., 2006; Pedahzur et al., 2000;Kim et al., 2004).

1.19.4.6 Non-Oxidising disinfectants

There are a number of non-oxidising organic disinfectants used against *Legionella* bacteria. They are amines, halogenated amides, halogenated glycols, aldehydes, heterocyclic ketones, thiocarbamates, organo-tin compounds and thiocyanates (Sanli-Yurudu et al., 2012). Most of these disinfectants are used in *Legionella* control of cooling tower systems and only rarely use in potable water

Legionella control; therefore, this review will not consider non-oxidising disinfectants (Reynolds-Clausen et al., 2018).

1.19.5 UV disinfection

As a water disinfection technique, UV is known to be an effective disinfectant due to its strong germicidal ability (Song et al., 2016). UV is capable of inactivating micro-organisms such as bacteria, viruses, protozoa, *Giardia lamblia* cysts or *Cryptosporidium* oocysts (Hijnen et al., 2006; Water Research, 2015). UV radiation inactivates microorganisms by altering their DNA in the cells and it does not remove organisms from the water (Giannakis et al., 2016; Liu et al., 1995). The efficiency of this process is mainly depending on exposure time, lamp intensity and water quality parameters (Water Research, 2015).

1.19.6 Copper silver ionisation method

Copper silver ionisation (CSI) method is found to be one of the most successful disinfection techniques against *Legionella* pneumophila (Walraven et al., 2016; Shih and Lin, 2010; Sabria and Victor, 2002). In contrast, some studies have demonstrated that CSI was not effective in the eradication of *Legionella* from the hot water system (Bédard et al., 2016; Garrison et al., 2016). Bédard and colleagues also reported that the operating temperature of the hot water system at the time of outbreak was in the range of 50-55°C. It is vital to understand that the hot water systems operating in this temperature region also can encourage *Legionella* proliferation if water stagnation or reduced water circulation arises anywhere in the system and sometimes promote *Legionella* growth if there is enough nutrients present within the water system (Barbosa and Thompson, 2016; Rhoads et al., 2016). In this method, water flows through a device with copper and silver electrodes connected to a low potential electricity supply. Positively charged copper ions (Cu 2+) and silver ions (Ag2+) are released in to the water and these ions establish electrostatic bonds with micro-organism's cell wall which is negatively charged (Parr, 2016; Vidic et al., 2002). These bonds create tension forces leading to altered permeability of the cell wall which

causes protein denaturation. This leads to cellular lysis and death (Kusnetsov et al., 2001; Rohr et al., 1999).

The main advantage of CSI system is easy to carry out installation and maintenance as well as very cost effective as compared to other disinfection techniques currently in use (Borella et al., 2016; Triantafyllidou et al., 2016). This procedure is efficient at any water temperature and this technique kills *Legionella* bacteria, rather than suppressing or preventing replication; thus eliminating the possibility of recolonization (Barbosa and Thompson, 2016; Lin et al., 1998a). Generally, it is recommended to maintain the copper ion level between 0.2 - 0.4 PPM and silver ions 0.02 – 0.04 PPM in domestic water systems (WHO, 2017). One of the main structural implications is that CSI can result in pipework corrosion (Loret et al., 2005). Copper and silver are heavy metals, and therefore it is desirable to reduce the quantity of these metals in domestic water especially in potable water to avoid various public health issues. (Lytle and Schock, 2008). Water distribution systems with copper pipework and CSI systems are considered as high risk water systems in terms of copper toxicity (Pettersson and Rasmussen, 1999; Zietz et al., 2003).

1.19.7 Point of use filtration

Advanced membrane filter technology have developed Point of Use (POU) water filtration systems capable of removing pathogenic microorganisms from domestic water (Alsbaiee et al., 2016). Microbiological contaminants including *Legionella* bacteria can be filtered using these technologies; and these filtration techniques include microfiltration (MF), ultrafiltration (UF), nano filtration (NF) and reverse osmosis (RO) processes. Point of Use (POU) filtration can be installed at specific taps and showerheads as a temporary measure providing physical barrier against water borne micro-organisms including *Legionella* pneumophila (Walraven and Chapman, 2016; Cervia et al., 2010). Hospitals and health care premises are benefitted with these devices to provide safe domestic water facilities for vulnerable patients by reducing the risk of pathogenic infections (Baron et al., 2014). The main risk factor with POU filters is the potential concentration of bacteria and foster growth of water borne pathogens within the filter. Any potential failures of POU filters can release high levels of pathogens and harmful micro-organisms to the water user. Also, there are

possibilities for filter to clog with scale, membranes foul and degradation by micro-organisms (EPA, 2015).

1.20 Challenges and shortfalls in current Legionella control strategy

Legionella can actively colonise and proliferate within domestic hot and cold water systems, and this presents one way for humans to come into contact with *Legionella* thereby increasing their risk of contracting the disease (Garrison et al, 2016). Consequently, the World Health Organisation (WHO), European Centre for Disease Prevention and Control (ECDC), European Legionnaires' Disease Surveillance Network (ELDSNet) and UK Health and Safety executive (HSE) have put in place guidelines to manage the *Legionella* risk and protect the public health (WHO, 2007; ECDC, 2012; ECDC, 2011; HSE, 2013). The UK Health and Safety Executive (HSE) have implemented strict rules under the 'Control of Substances Hazardous to Health' (COSHH) regulations to control *Legionella* and protect the public from LD by taking appropriate precautionary measures designed to reduce exposure to the bacteria. The latest guidance on the legal requirements to control *Legionella* bacteria in domestic hot and cold water systems HSG274 Part2 (published in 2014) has addressed many of the critical areas for practical *Legionella* control which was not addressed in WHO '*LEGIONELLA* and the prevention of legionellosis' (2007) and ECDC TECHNICAL DOCUMENT European Legionnaires' Disease Surveillance Network (ELDSNet) Operating procedures (2011). Despite the valuable role of the HSE ACOP L8 (2013) and HSG 274 Part2 (2014) in managing the risks of exposure to *Legionella*, there are concerns and short comings that must still be addressed and planned for.

1.20.1 Defining those who are most at risk

Legionella bacteria remain a continuous hazard to human health due to their specialised characteristics (Borges et al., 2012). In the latest HSE guideline HSG274 Part 2 (published in 2014), special consideration has been given to health care premises and care homes. This includes remedial action, such as investigation, for any detection of *Legionella* between 1 and 100 colony forming units (cfu)/l in domestic water systems.

Notably, however, there is no recommended action for the same level of *Legionella* detection in other premises such as residential complexes with communal hot and cold water systems; considered to be 'low risk' areas. Health care premises (such as hospitals and care homes) are deemed 'high risk' areas due to the high proportion of vulnerable groups with increased susceptibility to LD due to existing illness and/or impaired immune system (HSG274 Part2, 2014). Table 1.6 (an excerpt of HSG274 Part2) compares the guidance on remedial action in the event of a positive *Legionella* result in both high risk areas (hospitals and care homes) and low risk areas (residential homes). According to this guidance, a greater level of remedial action or investigation is necessary if *Legionella* is detected below 100 cfu/L in healthcare premises. However, in non-healthcare premises, the same result would require no remedial action or intervention of any kind, despite the possibility of occupancy by susceptible residents (see Chapter 2) (Quan et al., 2016).

WHO advice (2007) states a target level of <1000 cfu/L *Legionella* spp for patients with classical individual risk factors and <50 cfu/L *Legionella* spp in some areas for high-risk patients in healthcare premises, which is arguably too high (Table 1.7). However, similar to the HSE guideline HSG274 Part 2, there are no guidelines given for *Legionella* detection in non-healthcare premises. Furthermore, European guidelines recommend remedial action when Legionella are present between 1000 cfu/L and 10,000 cfu/L (Table 1.8) regardless of whether these are detected in healthcare or non-healthcare premises (ECDC, 2011; ECDC, 2017d). This variability in the stringency of control measures may further accelerate the number of Community Acquired Pneumonia (CAP) cases. A study carried out on the available European data collated between 2005 and 2012 indicated CAP to cost society around €10 billion annually due to hospitalisation and lost working days (Torres et al., 2013). Campese et al., 2015 have reviewed the current knowledge of Legionnaires' disease (LD) in France illustrated by the epidemiological situation in 2013. In the United States, the Environmental Protection Agency (EPA) also provides guidance on the control of *Legionella* bacteria in water systems. However, updated guidelines for the control of *Legionella* in Western Pennsylvania published in October 2014 state that 'in the absence of more definitive evidence or explicit U.S. federal guidance, guidelines from UK and EU can be considered' (UGCLWP, 2014).

Table 1.6 Comparison of actions to be taken following *Legionella* detection in water samples taken from hot and cold water systems in healthcare and non-health care premises.

Legionella bacteria (cfu/l)	Recommended actions	
	Healthcare premises	Non-healthcare premises
Not detected or up to 100 cfu/l	In healthcare, the primary concern is protecting susceptible patients, so any detection of *Legionella* should be investigated and, if necessary, the system resampled to aid interpretation of the results in line with the monitoring strategy and risk assessment.	No action
>100 cfu/l and up to 1000 cfu/l	Either: ■ If the minority of samples are positive, the system should be resampled. If similar results are found again, a review the control measures and risk assessment should be carried out to identify any remedial actions necessary or ■ if the majority of samples are positive, the system may be colonised, albeit at a low level. An immediate review of control measures and a risk assessment should be carried out to identify any other remedial action required. Disinfection of the system should be considered.	Either: ■ If the minority of samples are positive, the system should be resampled. If similar results are found again, a review of the control measures and risk assessment should be carried out to identify any remedial actions necessary or ■ if the majority of samples are positive, the system may be colonised, albeit at a low level. An immediate review of the control measures and risk assessment should be carried out to identify any other remedial action required. Disinfection of the system should be considered.
>1000 cfu/l	The system should be resampled and an immediate review of the control measures and risk assessment carried out to identify any remedial actions, including possible disinfection of the system. Retesting should take place a few days after disinfection and at frequent intervals thereafter until a satisfactory level of control is achieved.	The system should be resampled and an immediate review of the control measures and risk assessment carried out to identify any remedial actions, including possible disinfection of the system. Retesting should take place a few days after disinfection and at frequent intervals afterwards until a satisfactory level of control is achieved.

Source: http://www.hse.gov.uk/PuBns/priced/hsg274part2.pdf

Table 1.7 An example of WHO's recommendation for verification and corrective action for *Legionella* detected in samples taken from hot and cold water systems.

Process step	Indicator	Monitoring		Operational limit	Corrective action	
Verification	*Legionella* concentration in water	**What**	*Legionella* concentration	In areas for patients with classical Individual risk factors, target level of <1000 CFU/L *Legionella* spp. In some areas for high-risk patients, target level of <50 CFU/l *Legionella* spp.	**What**	Raising temperature, disinfection, restriction of water use, use of filtered water
		How	Employ documented, validated and quality controlled methods		**How**	Systematic search for failure in the system
		When	2 times/year(4 times/year in high risk areas)		**When**	Immediately
		Where	At the entry and at selected point of use sites		**Who**	Plumber (for pump) Building engineer (Calorifier)
		Who	Infection control officer or hospital hygienist			

Table 1.8 EU guidelines for required action following *Legionella* detection in hot and cold water systems.

Legionella bacteria (cfu/litre)	Action required
More than 1,000 but up to 10,000	Either: (i) If a small proportion of samples (10-20%) are positive, the system should be resampled. If a similar count is found again, then a review of the control measures and risk assessment should be carried out to identify any remedial actions; (ii) If the majority of the samples are positive, the system may be colonised, albeit at a low level, with *Legionella*. Disinfection of the system should be considered but an immediate review of control measures and a risk assessment should be carried out to identify any other remedial action required.
More than 10,000	The system should be resampled and an immediate review of the control measures and risk assessment carried out to identify any remedial actions, including possible disinfection of the system.

1.20.2 Detecting and quantifying *Legionella* to inform remedial actions

HGS274 recommends that water sample analysis for *Legionella* should only be performed in United Kingdom Accreditation Service (UKAS) accredited laboratories and most of these laboratories currently use culture methods as their standard analysis method (Gruas et al., 2013; Zhan et al., 2014). Quantitative data presented in the form of colony forming units per litre water (cfu/L) then form the basis for remedial action, if any, according to Table 1.6. Culture methods using Buffered Charcoal-Yeast Extract (BCYE) agar is still the 'gold standard' diagnostic procedure used (Collins et al., 2015). It can take up to 15 days for the full diagnosis and report to become available using the culture method, thereby increasing the likelihood of further exposure to *Legionella* in contaminated buildings following delays in taking preventative actions (Zhan et al., 2014; Pierre et al., 2017). In addition, many studies have demonstrated that quantification of *Legionella* using the culture method is not reliable as this always underestimates the population of *Legionella* in a given sample (Gruas et al., 2013; Whiley and Taylor, 2016; Dusserre et al., 2008; Delgado-Viscogliosi et al., 2009). An alternative rapid and sensitive testing method for quantifying *Legionella* bacteria in water is quantitative Polymerase Chain Reaction qPCR (Joly et al., 2006). qPCR is an efficient way to "amplify" (copy) specific small segments of DNA or RNA. PCR can therefore be used to detect and amplify genetic material recovered from environmental samples and present in small amounts over a million-fold within a few hours which is then sufficient for analysis (Higuchi et al., 1993).

Recent studies comparing culture methods to molecular biology approaches (qPCR), report large differences in *Legionella* count, with the BYCE Agar culture method often underestimating the presence of *Legionella* in around 50% of cases (Whiley and Taylor, 2016). Indeed, a sample recently analysed by ALcontrol Laboratories for Aqua Technologies produced a culture result < 100 cfu/l whilst the molecular determination by PCR reported 2448 genomic unit per litre (GU/L) (personal observation of the author; unreferenced, data not shown). Thus, significant challenges exist even today in reliably quantifying *Legionella* bacteria using culture methods due to the growth of other microorganisms and the presence of 'Viable But Non-Culturable' (VBNC) forms (Epalle et al., 2015; Gruas et al., 2013).

A comparative study of 3967 environmental samples analysed for *Legionella* using both culture methods and qPCR found large differences, with only 34% (n=1331) of samples testing positive with culture methods compared to 72% (n=2856) using qPCR (Whiley and Taylor, 2016). Culture methods may therefore (i) underestimate the presence of *Legionella* in samples (Dusserre et al., 2008), (ii) struggle to detect low concentrations of bacteria in environmental samples that fall within the regulatory framework, and (iii) introduce delays between monitoring and remedial action (Delgado-Viscogliosi et al., 2009). Inaccurate quantification of *Legionella* (especially at concentrations below 1000 cfu/L) and underestimation of viable bacteria in water samples (used for informing remedial action) raises important questions about the appropriateness of measures used in certain settings to protect human health, as well as the appropriateness of ''no action'' in non-healthcare premises where *Legionella* counts below 100 cfu/l are detected (Delgado-Viscogliosi et al., 2005; Dusserre et al., 2008).

The qPCR is however considered as an efficient diagnostic procedure for micro-organisms on the basis of DNA synthesis, there are many disadvantages in this method. Many studies reported that, qPCR often *overestimates* the concentration of *Legionella* bacteria as this method detects both viable and non-viable *Legionella* cells (Delgado-Viscogliosi et al., 2009). A recent study by Slimani et al., entitled 'Evaluation of propidium monoazide (PMA) treatment directly on membrane filter for the enumeration of viable but non cultivable *Legionella* by qPCR' has clearly reported that the limitation of qPCR technique because the PMA-qPCR quantification yielded was significantly overestimating the number *Legionella* bacteria by diagnosing non-viable cells (Slimani et al., 2012).

Another remarkable disadvantage of qPCR is that the amplification reactions can be affected by certain substances called inhibitors found in the environmental samples which can result in producing inaccurate results (Brooks et al., 2010). Calcium ions, rust, bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulphate (SDS), humic acids, tannic acid, melanin, different proteins such as collagen, myoglobin, haemoglobin, lactoferrin, immunoglobin G (IgG) and proteinases are some of the inhibitors possibly present in environmental samples (Schrader et al., 2012; Maiwald et al., 1994). However, this disadvantage can be addressed by measuring these inhibitors prior to analysis as well as diluting the DNA extract a further 10-fold (Miyamoto et al., 1997; Ballard et al., 2000). A study on environmental samples using qPCR techniques

demonstrated that naturally occurring inhibitors are a major threat for qPCR analysis of environmental samples, especially large volume water samples as the proportion of samples that could have been incorrectly reported as negatives was in the range of 0.3% to 71%, depending on water source. These reports indicates that the importance of measuring and addressing inhibition when reporting qPCR results as this raises serious concerns in the case of pathogenic micro-organisms present in environmental water samples (Gibson et al., 2012). Another study conducted on 133 fresh and marine water samples, serial dilutions were employed in addressing the identified inhibition along with four other internal controls (IC). The frequency and magnitude of inhibition was varied considerably among qPCR methods, but the performance was better when using an environmental master mix; however, fivefold dilution using DNA/RNA free molecular grade water was also effective in reducing inhibition in about 78% of samples (Cao et al., 2012).

Another reported disadvantage of qPCR is the difficulty in determining the cut-offs values. A study carried out on 223 hot water samples and 37 cooling tower samples using qPCR targeting the 16S rRNA, specific for the genus *Legionella* reported that PCR results were non-quantifiable for 2.8% of cooling tower samples and 39.1% of hot water samples; however, this was highly predictive in conventional culture methods for *Legionella* counts below 250 cfu/Litre. Furthermore, PCR cut-off values for identifying *Legionella* in hot water samples containing >103 cfu/Litre were determined separately in different laboratories. The cut-offs values were significantly different between the laboratories and had sensitivities from 87.7 to 92.9% and specificities from 77.3 to 96.5%. In addition, PCR cut-offs could not be determined for cooling tower samples and the results obtained were highly variable and often high for culture-negative samples. This study concluded by reporting 'quantitative *Legionella* PCR appears to be applicable to samples from hot water systems, but the positivity cut-off has to be determined in each laboratory' (Joly et al., 2006).

All these studies indicate that regardless of the current diagnostic procedures in place for *Legionella* detection, none of them provide accurate measurements to quantification of *Legionella* in environmental samples which is a barrier to effective monitoring and the protection of public health. Therefore, it is necessary to develop a more accurate, reliable and rapid diagnostic method for the determination and

quantification of live *Legionella* bacteria in water systems (Delgado-Viscogliosi et al., 2005).

Another issue that I have faced in my professional role as a practitioner in *Legionella* management and control are a lack of consistency in sampling protocols, sample preservation and sample transport conditions prior to analysis of a water sample at an accredited laboratory. If a sample is taken in a way that makes it unfit for purpose, (e.g. without immediate neutralisation of any biocides present), then the analysis result used for regulatory action will be unfit for purpose. This also applies to ensuring fit for purpose sample preservation and transport. Typically, sample bottles of 500 mL to 1000 mL should be suitable. Sampling guidance is available (ISO 19458:2006; BS 7592:2008). Ultimately, imprecision in the quantification of *Legionella* will negatively impact the effectiveness of enforcing authorities to recommend sensible practical guidelines for interpreting monitoring results.

1.20.3 Understanding the real extent of LD in society

The significant number of people classed as vulnerable/more susceptible/high risk residing in non-healthcare premises are not presently adequately protected by HSE ACOP L8 2013 and HSG 274 Part 2, combined with the possible role of changing external factors (e.g. climate change) in the UK, clearly indicates that a greater number of people are likely to be exposed to *Legionella* than presently thought. The latest report from the British Lung Foundation showed that 0.5-1.1% of adults in the UK get pneumonia each year (BLF 2014), and 0.1% of UK adults receive treatment for pneumonia every year by the National Health Service (NHS) (NHS, 2014). However, in most instances these cases are treated primarily based on their symptoms, and a specific diagnosis of the cause of the illness is not routinely made. Therefore, it is likely that the 306 cases of *Legionella* reported annually (PHE, 2014) are not a reflection of the true extent of Legionnaires Disease in the England and Wales. This findings can further be supported by a study in Germany led by The Competence Network for Community Acquired Pneumonia (CAPNETZ); thus reported that *Legionella* species was the causative pathogens in 3.8% of community-acquired pneumonia cases but only 3.7% in hospitalised patients related cases. *Legionella* pneumophila was the predominant species in both community acquired and hospitalised cases. According to

a recent clinical review of the diagnosis and management of pneumonia in the UK, 2-8% of all community acquired pneumonia cases are caused by *Legionella* pneumophila (CRCAP, 2012). It is vital to understand that the reported 2-8% underestimates the true incidence because it excludes pneumonia in immunocompromised groups or pneumonia as a pre-terminal event.

1.20.4 Shortcomings in the *Legionella* risk assessment

The first two important steps in *Legionella* control are (i) to carry out a detailed *Legionella* risk assessment to identify the possible risk factors, and (ii) establish a regular monitoring program on the basis of the highlighted risks (HSE, 2015). WHO guidelines, EU guidelines and HSE regulations all require a suitable and sufficient risk assessment and a regular review of the assessment to make necessary changes to keep the risk assessment up to date. There are proper guidelines given in HSG274 Part 2 to consider all aspects of *Legionella* control while carrying out a risk assessment. However, consideration of the type of population residing in buildings with communal domestic hot and cold water systems is presently missing. HSE ACOP L8 (fourth edition) published in 2013 has provided the guidelines to consider *when* to carry out a risk assessment review. This includes changes to the water system or its use, changes to the use of the building in which the water system is installed (e.g. a toilet and/or a wash basin is no longer 'in use' and rooms containing these items being used as storerooms instead), the availability of new information about risks or control measures, the results of checks indicating that control measures are no longer effective, changes to key site responsible personnel and a case of Legionnaires' disease or Legionellosis associated with the system (HSE, 2013). Within this guideline, I propose that the proportion of individuals in residential buildings with a weakened immune system, and/over 45 years of age, and/or suffering from existing illnesses are also important factors to consider (see Chapter 2). New legislation enabling the collection of health and demographic data on individuals in residential buildings by the Duty Holder for the purposes of risk management of LD may therefore be needed.

1.20.5 Summary of research gaps

Legionella bacteria are ubiquitous in the man-made environment and remain a serious threat to public health. Discrepancies in quantification of *Legionella*, shortfalls

in remedial action recommendations, omission of the ageing population residing in normal residential complexes when undertaking a risk assessment, and potential long term issues (such as climate change) means that practitioners must be ever more vigilant in protecting the public against the threat of Legionnaires' disease. The requirement of a more accurate, reliable and rapid standard sampling and testing method to quantify viable *Legionella*, and a review of remedial action recommendations is needed for the effective control of *Legionella* bacteria in domestic water systems.

1.21 Research aims

Following a critical review of the literature on the control of *Legionella*, this PhD research project, entitled 'Novel Approaches for Risk Management of *Legionella* bacteria in Domestic Water Systems' has identified a number of research gaps (from a practitioners perspective) in *Legionella* control, and has highlighted major risk factors that could lead to the proliferation and harbouring of *Legionella* bacteria in the domestic water systems. The following research aims have been identified:

1. Appropriateness of current guidelines to protect the ageing population: The present lack of consideration of the ageing population in the *Legionella* risk assessment guideline in residential settings highlights the possible need for a more focussed risk assessment strategy to manage the risk of *Legionella* infection in vulnerable individuals throughout society at large. In Chapter 2 the proportion of vulnerable occupants occupying typical London residential buildings is researched in order to establish the appropriateness of present measures detailed in HSE ACOPL8 to protect vulnerable people in society. New legislation enabling the collection of health and demographic data on individuals in residential buildings by the Duty Holder for the purposes of risk management of LD may be needed.

2. Appropriateness of current sampling methods: In Chapter 3 research is undertaken to investigate a possible shortfall in the Legionella risk assessment related to water sampling methods used for cold water storage tanks that inform regulatory actions. A thorough inspection of a number of cold water storage tanks from London properties was undertaken to see if changes in physical conditions within tanks can significantly affect the microbiological status of water samples collected at different locations from

within the same tanks. Water samples will be analysed by the same UKAS accredited laboratory using the 'gold-standard' culture method.

3. Rapid detection of live *Legionella*: Given the long time needed to obtain results using current culture methods, and the inability of current rapid methods to distinguish between live and dead *Legionella*, Chapter 4 attempts to develop a qPCR method to enable the rapid detection of live *Legionella pneumophila* in water samples. In order to develop the method, and avoid the need for culture, I will obtain RNA from live *Legionella* lenticules.

4. Finally, in my professional role as Technical Manager (Aqua Technologies Europe Ltd) I have access to risk monitoring data from hundreds of London buildings. The data contains information about known risk factors for *Legionella* proliferation. I will use these data to develop a predictive risk model to evaluate the likelihood of *Legionella pneumophila* occurring in residential buildings. The predictive power of the model will be validated using information from new client buildings (unknown status) that will be surveyed as part of routine monitoring.

CHAPTER TWO

SURVEY OF RESIDENTIAL BUILDINGS

2.1 Introduction

The first two important steps in *Legionella* control are (i) to carry out a detailed *Legionella* risk assessment to identify the possible risk factors, and (ii) establish a regular monitoring program on the basis of the highlighted risks (HSE, 2015). WHO guidelines, EU guidelines and HSE regulations all require a suitable and sufficient risk assessment and a regular review of the assessment to make necessary changes to keep the risk assessment up to date. There are proper guidelines given in HSG274 Part 2 to consider all aspects of *Legionella* control while carrying out a risk assessment. However, consideration of the type of population residing in buildings with communal domestic hot and cold water systems is presently missing. HSE ACOP L8 (fourth edition) published in 2013 has provided the guidelines to consider *when* to carry out a risk assessment review.

These include:

- changes to the water system or its use
- changes to the use of the building in which the water system is installed (e.g. a toilet and/or a wash basin is no longer 'in use' and rooms containing these items being used as storerooms instead)
- the availability of new information about risks or control measures
- the results of checks indicating that control measures are no longer effective
- changes to key site responsible personnel
- a case of Legionnaires' disease or Legionellosis associated with the system (HSE, 2013).

However, exposure of people over 65 years of age to *Legionella* in their residential homes, even at relatively low concentrations (and at levels that are deemed to be acceptable according to HSG274 Part 2), raises serious concerns about possible health implications of an increasing ageing residential population (Beauté, 2017). There are already clear warnings in the literature about the increasing burden of age-

related disease in UK society, with more than 6 million people aged 65 and above with long standing serious illness expected by 2030 based on current trends (LLUK, 2015).

In order to verify this concern, I conducted a survey of twenty residential building complexes in different London boroughs to gauge the extent of the 'high risk' occupants in residential buildings (Table 2.2).

2.2 Methods

This survey was administered through the specialised *Legionella* control company Aqua Technologies Europe Ltd. In order to gain access to these buildings and onsite documents, 50 building managers were contacted about the content and purpose of the survey so that they could make an informed judgment about whether they wished to participate. Any assurances, including confidentiality or anonymity, were also made explicit at this time. Twenty building managers agreed to participate in the study and signed the disclosure agreement with Aqua Technologies Europe Ltd. The necessary information required for the survey was provided by the building managers using onsite documents, and was independently verified with face to face communication with a small number of residents from each site. A structured questionnaire was used to carry out this survey as shown in Table 2.1.

Table 2.1 Structured questionnaire used for conducting the survey in 20 residential buildings in different London boroughs.

<table>
<tr><td colspan="3">Site details:</td><td colspan="3">Date of survey:</td></tr>
<tr><td colspan="6">Client details:</td></tr>
<tr><td colspan="3">Property Manager:

Contact details:</td><td colspan="3">Survey carried out by:</td></tr>
<tr><td colspan="2" rowspan="2">Property type:</td><td rowspan="2">Is there any communal cold water storage tank(s) on site</td><td>Yes</td><td rowspan="2">Is there any communal hot water system (eg; calorifiers/water heaters) on site</td><td>Yes</td></tr>
<tr><td>No</td><td>No</td></tr>
<tr><td>Total number of flats/apartments</td><td></td><td>Total number of residents/occupants</td><td></td><td>Total number of unoccupied flats/apartments</td><td></td></tr>
<tr><td colspan="3">Total number of residents aged 65 and above</td><td colspan="3"></td></tr>
<tr><td>Number of people(65+)with long lasting illness</td><td>Diabetes</td><td>Cancer</td><td>Asthma</td><td>Chronic Kidney Disease (CKD)</td><td>Number of people(65+)with other non-communicable disease (NCD)</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

2.3 Results

The survey was carried out successfully for 20 residential complexes from different boroughs in London. The total number of residents included in this survey was 5924, and the survey data which included the total number of flats in each development, the total number of residents, the total number of residents aged 65 and above, the number of people with long lasting illness such as diabetes, asthma, cancer and CKD and the number of people aged 65 and above with other non-communicable diseases is given in Table 2.2.

Table 2.2. Survey report of 5924 residents in 20 residential complexes from different boroughs in London for *Legionella* risk factors, including age and pre-existing long term illness.

No.	Post Code	Total number of flats/apartments	Total number of residents	Number and % (round figure) of residents aged 65 and above	Number of people(65+)with long lasting illness (diabetes, cancer, asthma & CKD*)	Number of people(65+)with other non-communicable disease (NCD)
1	TW3	48	142	18 (13%)	13	3
2	TW10	24	77	15 (20%)	14	0
3	KT1	30	102	21(21%)	18	3
4	W1	44	99	8 (8%)	5	0
5	NW1	96	256	57 (22%)	41	7
6	W5	25	79	17 (22%)	8	6
7	SE19	186	468	93 (20%)	62	10
8	SW17	645	1674	492 (29%)	320	45
9	E16	78	161	29 (18%)	14	4
10	NW8	28	77	20 (26%)	16	2
11	SW11	54	132	16 (12%)	9	4
12	SW9	28	67	8 (12%)	3	3
13	N2	64	157	29 (19%)	14	8
14	N3	58	139	14 (10%)	8	2
15	SW1P	54	128	23 (18%)	19	4
16	W1C	42	103	11 (11%)	5	2
17	E14	390	978	219 (22%)	145	27
18	W1J	32	68	13 (19%)	4	3
19	NW7	109	259	67 (26%)	43	9
20	SE20	244	710	129 (18%)	71	30

* Chronic Kidney Disease (CKD)

2.4 Discussion and conclusion

These buildings consisted of flats or apartments of varying size from 64 to 645 units. On average 18% of the residents were found to be 65 years of age or over (confirming Government statistics), and most reported suffering from impaired immune systems or underlying diseases such as diabetes, cancer, respiratory problems and kidney diseases making them susceptible to *Legionella* infection. Importantly, the ageing population tend to prefer these shared dwellings over detached or semidetached houses due to the increased security, variety of communal facilities (such as leisure centres, water features and gardens) which they can enjoy without responsibility for the day-to-day maintenance (Michael et al., 2009). However, the ageing residents within these complexes are as vulnerable to *Legionella* infection as care home residents due to continuous use of domestic hot and cold water systems (Lin and Yu, 2014).

Therefore, it is my opinion that residential complexes should also be categorised as "high risk" settings for Legionnaires' disease based on the percentage of the elderly and vulnerable population inhabiting each building. The proportion of individuals in residential buildings with a weakened immune system, and/over 45 years of age, and/or suffering from existing illnesses should be important factors to consider within the HSG274 Part 2 guideline (Reference to Chapter 1). New legislation enabling the routine collection of health and demographic data of individuals in residential buildings by the Duty Holder for the purposes of risk management of LD is therefore needed. In otherward, the people who are susceptible to Legionella (immunosuppressed and elderly) do not all reside in care homes, and therefore guidelines need to be more explicit about how to protect these vulnerable groups in residential settings.

CHAPTER THREE

SPECIFIC STUDY ON COLD WATER STORAGE TANKS

3.1 Introduction

Potable water is typically produced at water treatment facilities where incoming water is treated to remove pathogens and is disinfected before it leaves the treatment works (Betancourt and Rose, 2004). A small residual amount of chlorine is left in the water to maintain its quality as it travels through the network of mains and pipes that deliver this water to various residences. Despite this, treated water can become contaminated with microorganisms during transportation throughout the pipework network, and also during storage (Kilb et al., 2003; Volker et al., 2016). Long horizontal installations of pipework, the types of materials used for the pipework and fittings, deadlegs (isolated sections of piping) and excessive water storage or stagnation can all affect water quality and encourage the proliferation of many species of bacteria (Brown et al., 2001). The inner walls of the water distribution pipework may also become corroded, thereby enabling calcium and magnesium (already present in the water) to accelerate the process of scale formation within the water storage tanks (Li et al., 2016; Cramer, 2003). Ultimately, suspended solids and dissolved solids in the mains water, together with the rust (corrosion products attributed during transportation), form sediments at the bottom of the storage tanks which can act as nutrients for Legionella bacteria (Volker et al., 2016; Best et al., 1985; ASHRAE Guideline, 2000)

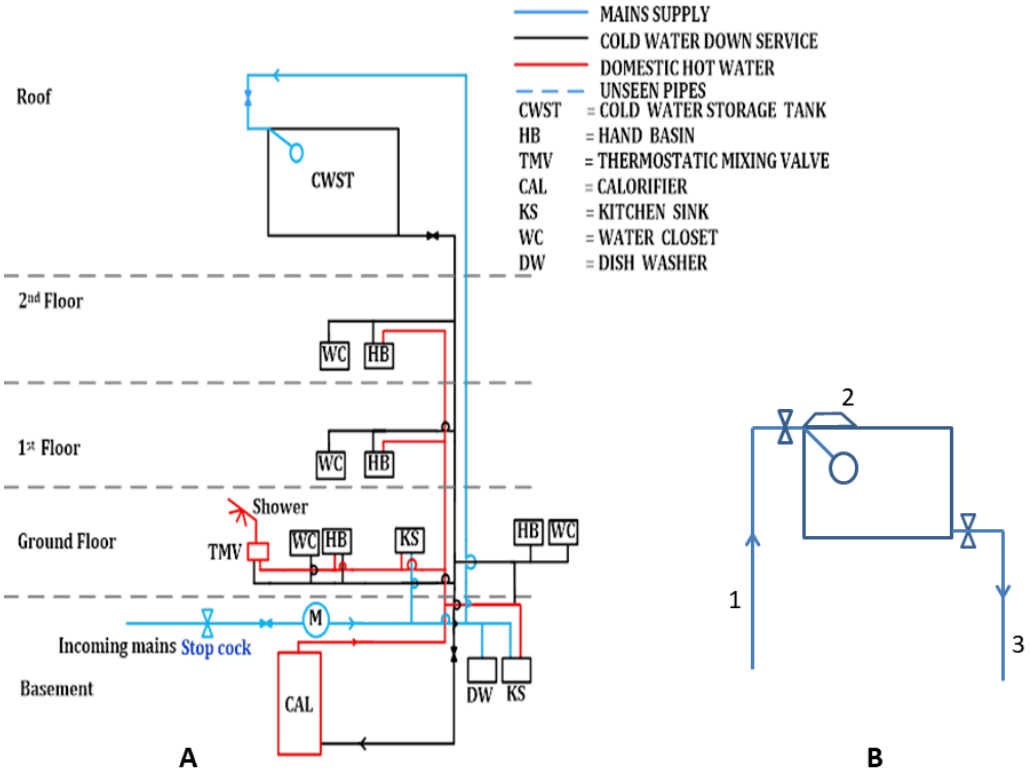


Figure 3.1 Schematic of a typical water system A) Schematic of typical gravity-fed water system found in may commercial and older residential buildings. B) Schematic diagram of a typical cold water storage tank as per WRAS Guidelines 1. Incoming mains with isolation valve connected to ball valve; 2. Inspection access hatch situated above the ball valve to assist with maintenance; 3. Outlet of the tank with isolation valve (own drawing).

The plumbing arrangement also affects the internal environment of the tank (Rogers et al., 1994). When inlet (incoming mains) and outlets are on the same side, internal water circulation may be limited leading to greater water stagnation within the tank. The combination of an oversized mains inlet pipe and a relatively small outlet can also lead to water stagnation (DWI, 2010; 2000), thereby increasing the stored water temperature as well as contributing to biofilm formation; a perfect breeding ground for *Legionella* bacteria (van der Kooij et al., 2017; Stojek and Dutkiewicz, 2006; Fisman et al., 2005).

In order to protect society against the harmful effects of exposure to pathogenic bacteria, many countries throughout the world have developed and adopted standards used for the evaluation of microbiological status of point-of-use and point-of-entry

potable water in buildings. The United States Environmental Protection Agency (USEPA) and the European Environment Agency (under the EU Water Framework Directive) have implemented monitoring and sampling strategies to ensure that the health of building occupants is protected from unabated proliferation of pathogenic bacteria (Storey et al., 2011; Figueras and Borrego, 2010). In order to ensure the water quality and maintain a healthy water system, routine water tank inspection and stored water sample analysis is necessary (DWI, 2014). Analysis of 'representative water samples' collected from any water system are an important tool in the armoury used to evaluate the human health risk posed by a particular water system (WHO, 1997). Cold water storage tanks are one of the most important elements of concern , being both the point-of-entry of potable water into buildings, and the reservoir of water used to supply the entire building (Anaissie et al., 2003). According to Water Regulations Advisory Scheme (WRAS), the water storage tank should have an access hatch above the mains inlet valve of the storage tank (Figure 3.1B) to enable routine internal inspection and water sampling (WRAS, 2015). In the UK, all water samples taken for microbiological assessment are taken, transported and analysed under UKAS accredited conditions as stipulated by Drinking Water Inspectorate (DWI) for compliance (DWI, 2016).

The majority of the properties in the UK have cold water storage tanks located in the loft space or on the roof (HSE, 2014a). These tanks usually feed cold water taps by gravity (with the exception of the kitchen cold water tap) and the hot water calorifiers (DWI, 2013)(Figure 3.1A). In modern buildings, cold water storage tanks are located in either the basement or on the ground floor of the building. These tanks are connected to booster pump sets to provide stored water to the entire building. Wherever their location, the internal condition of these storage tanks has a direct impact on the quality of stored water, even if the tanks are properly designed, correctly installed and kept in good external order (DWI, 2013). Factors such as the tank construction method, the materials used, plumbing arrangements, internal water flow and tank location (the ambient temperature around the tank) have a direct impact on the internal environment of the tank, and conditions may arise that encourage the proliferation of pathogenic bacteria, including Legionella (Ciesielski et al., 1984; Turetgen and Cotuk, 2007).

The key assumption used in current practice is that a small volume (500ml) of water taken from under the ball valve is representative of the entire tank (Practitioner's experience). Importantly, the inspection hatch above the ball valve is usually the only access point to the stored water in any cold water storage tank, even though, BS 7592:2008 suggests that the water sample should be taken from the furthest point from the ball valve. A representative sample is a small quantity of something whose characteristics represent (as accurately as possible) the entire batch (Lee et al., 2010). Obtaining a representative sample is the most important factor for a relevant description of the environment, especially when the result will be used for regulatory purposes (Ramsey and Hewitt, 2005). According to The DWI, 'samples must be taken from locations that are representative of the water source, storage facilities, distribution network and points at which water is delivered to the consumer (DWI, 2014). These points should include those that yield samples representative of the conditions at the most unfavourable sources or places in the supply system, particularly points of possible contamination such as unprotected sources, loops, reservoirs, low-pressure zones, ends of the system etc.'(WHO, 1997). Here we explore the possible limitations of assessing the actual risk factors within the water tank during present-day routine visual inspection and collection of a 'representative sample' used for microbiological analysis according to DWI.

3.2 Methods

3.2.1 Tank selection

Service engineers contracted for routine inspection and maintenance by a specialised water hygiene management company (Aqua Technologies Europe Ltd) were used to identify suitable tanks to include in the study. According to the Drinking Water Inspectorate (DWI) standards, cold water storage tanks must be completely sealed with the exception of one access point for inspection, monitoring and maintenance situated above the ball valve (DWI, 2013). Therefore, selection criteria for suitable tanks for inclusion in the study was based on (i) the ability to take a water sample from the normal sampling hatch (located above the ball valve) and from the far end of the tank (usually requiring disassembly of the tank lid with risk of structural damage), and (ii) permission being granted by the site manager to undertake the

additional investigation and sampling. Out of approximately 6000 cold water tanks surveyed over a 12 month period (July 2015-July 2016), only 15 suitable cold water storage tanks meeting the sample access criteria were identified by service engineers at various sites located in different London Boroughs. Permission was granted by site managers to gain access the far end of the tank and carry out the additional inspection work in every case.

3.2.2 Tank inspection

Surveyed tanks were constructed from various materials, including metal tanks (galvanised iron), metal tanks with internal butyl lining, fibreglass tanks, plastic tanks and modern GRP (glass reinforced plastic) tanks. The external dimensions of each tank were measured in metres using a standard tape measure in order to calculate the capacity of each tank. The location of each tank in the building was recorded together with the inspection and sampling date (Table 3.1). Temperature of (i) the mains water through inlet discharge (ball valve), (ii) the stored water just below the ball valve and (iii) of stored water at the far end of the tanks were also recorded using a Testo 925 Aktionsset Sensor type K digital thermometer (temperature range -50 up to +300°C). All the tanks surveyed had been cleaned and disinfected between 12-18 months prior to my inspection date, with visual inspections and water samples taken every six-months (with the exception of potable water tanks where inspections are carried out quarterly) as a part of the routine monitoring program. Internal visual inspection was carried out for all fifteen tanks; sedimentation level, presence of biofilm, presence of scale and corrosion level was recorded qualitatively as 'negligible', 'slight', 'moderate' and 'heavy', and these findings were converted into numerical data (Table 3.2). Based on current industry practice, the visual assessment must be carried out by qualified, experienced and adequately trained personnel in accordance to industry standards as no specific measure is provided for these assessments in any of the relevant guidelines. Tanks could only be accessed and inspected on one occasion as part of the routine service contract in place.

Table 3.1 Tank details of the fifteen tanks assessed, including tank dimensions, capacity, construction material, position and sampling date.

Tank Reference	Dimensions (Metres)	Volume (M^3)	Material	position	Sample Date
T1	3.0 x 1.5 x	5.4	Metal	Roof	Nov 15
T2	2.5 x 1.4 x	4.7	Metal	Roof	Dec 15
T3	4.0 x 2.0 x	16.0	GRP	Ground floor	Dec 15
T4	2.5 x 1.3 x	3.9	Fibreglass	Roof	Jan 16
T5	2.7 x 1.6 x	6.5	Metal	Roof	Jan 16
T6	6.0 x 2.0 x	36.0	GRP	Basement	Feb 16
T7	6.0 x 2.0 x	36.0	GRP	Basement	Feb 16
T8	1.9 x 0.8 x	1.2	Plastic	Roof	Mar 16
T9	3.2 x 2.0 x	9.6	Metal	Roof	Mar 16
T10	5.0 x 1.0 x	5.0	GRP	Ground floor	Apr 16
T11	4.0 x 4.0 x	32.0	GRP	Ground floor	Jun 16
T12	4.0 x 4.0 x	32.0	GRP	Ground floor	Jun 16
T13	4.0 x 1.7 x	10.2	Metal with butyl	Roof	July 16
T14	3.0 x 1.5 x	6.8	GRP	Basement	July 16
T15	1.0 x 0.7 x	0.6	Plastic	Roof	July 16

3.2.3 Water sample collection

Three water samples were collected from each of the tanks; one from the incoming mains (tank inlet), one from the tank just below the inlet ball valve (where routine sampling for pathogenic bacteria happens in practice), and one from the far end of the same tank (requiring dismantling on the tank). The samples for Legionella analysis were collected in 500ml sterile plastic bottles containing 0.69ml of 3.5% sodium thiosulphate and the samples for TVC, *E. coli*, coliforms and *Pseudomonas* analysis were collected in 500ml sterile plastic bottles containing 1ml of 18mg/L sodium thiosulphate (supplied by the UKAS accredited laboratory ALS Global) in accordance with BS 8554:2015 guidelines for the collection of water samples from hot and cold water services in buildings. All the samples were collected using a dipper, and the lid was closed tightly immediately after each sample collection. All the samples for Legionella analysis were kept in temperature controlled bags (6-18ºC depending on the ambient temperature) and all other samples for TVC, *E. coli*, coliforms and *Pseudomonas* were transported in polystyrene cooler boxes (6-8ºC) on their way to the laboratory. Water samples in temperature controlled bags were protected from heat

sources and sunlight during transportation to the laboratory. In all cases, the maximum time taken to deliver the samples to the laboratory was 4 hours from the point of collection of water samples.

3.2.4 Microbiological analysis

All water samples were analysed using standard UKAS protocols for TVC (3 days @ 22^0C), TVC (2 days @ 37^0C), *Pseudomonas*, *Escherichia coli* (*E. coli*), coliforms and *Legionella pneumophila* in a UKAS accredited laboratory (ALS Global, UK) under the same laboratory conditions within 12 hours of collection. In brief, the water sample for Legionella analysis (filtered with black nitrocellulose filters (Milipore), porosity of 0.45 µm, dimension of 47 mm), a 10-ml and 100-ml volume of the original water sample was filtered, and the filter membrane was then placed directly on the agar for culture. A separate 100µl of the water sample was also plated directly onto the agar. Reduction of other interfering bacteria present in the sample was undertaken by dividing the original water sample into further portions; one was heated at 50° ± 2°C for a period of 30 ± 2 minutes and another underwent acid treatment. All three portions were then plated separately onto BCYE agar with antibiotic supplements, Glycine Vancomycin Polymyxin Cycloheximide (GVPC), and incubated at 36°C for 10 days. Following incubation, an enumeration of morphologically characteristic colonies was made. The Legionella Spp. including Legionella pneumophila Serogroup1 and Serogroup 2-15 were then confirmed by MALDI-TOF MS protein profiling and the results were reported as cfu/L (Method statement for Legionella analysis in water samples, ALS Global).

TVC analysis was carried out using the pour plate method. Pour plates are prepared by pipetting 1ml of sample water into a sterile Petri dish and then adding molten agar and mixing gently by swirling the plate. Replicate sample plates are then incubated for 2 days at 37 °C and 3 days at 22 ºC. The results were reported as cfu/ml. In the event that samples could not be analysed immediately upon receipt by the laboratory, they were kept at a temperature between 2 - 8°C, in dark conditions until analysis was commenced (Method statement for TVC analysis in water samples, ALS Global).

Pseudomonas analysis was carried out by spread plate method. Spread plates were prepared by pipetting 100ml of sample filtered through a membrane filter (pore size 0.22–0.45 μm) onto the surface of the *Pseudomonas* agar base, supplemented with cetrimide, fusidic acid and cephaloridine (PCFC), and incubated at 30°C for 48 hours. The numbers of colony forming units were counted and then confirmed by a positive oxidase reaction. The results were reported as cfu/100ml. *E. coli* and coliforms analysis was also carried out by spread plate method using Membrane Lactose Glucuronide Agar (MLGA) plates, incubated at 35°C for 24 hours and any detection was reported as cfu/100ml (Method statement for *E. coli* and coliforms analysis in water samples, ALS Global).

All the samples were collected identically and analysed by the same UKAS accredited laboratory to ensure the consistency and accuracy of the results produced. Assay detection limits were 1 cfu/ml (TVC - 2 days at 37 °C, 3days at 22 ºC), 1cfu/100ml (*Pseudomonas*) and 20 cfu/L(*Legionella*), based on UKAS accredited methods (UKAS, 2015).

3.2.5 Data analysis and interpretation

Absolute values (in cfu/volume of water collected) reported by accredited laboratories (Table 3.3) are used to determine if remedial action is necessary. All the samples collected were tested specifically in UKAS accredited laboratory because the values reported by these laboratories are rarely questioned for their precision or reliability. The entire regulatory system is based on 'threshold' levels, that once exceeded instigate regulatory action through non-compliance with the standards. Results of any repeated tests by accredited laboratories as part of their sample analysis processes are not reported, and any measures of variability (such as SD) are also not reported. The crucial research question highlighted here is therefore not whether two samples taken at different ends of the tank are different (from a statistical standpoint), but whether samples taken at different locations in the same tank (e.g. the ball valve end where samples are routinely taken) might result in different regulatory actions compared to samples taken at a different location (e.g. the far end of the tank, where water is abstracted into the building). For this reason, absolute values from the accredited laboratory were used to determine if samples taken at different locations inside the tank were equivalent in terms of their compliance with regulatory thresholds.

3.3. Results

With few exceptions, the level of sedimentation and biofilm increased in quantity/severity between UBV and FE. Also, this occurred in both cold water and potable water storage tanks (Table 3.2). In contrast, the formation of scale and presence of corrosion was more uniformly found.

Table 3.2 Relative scoring: Relative scoring of sedimentation, biofilm, scale and corrosion levels in each tank, where negligible (not visible) = 0; Slight = 1; Moderate = 2; Heavy = 3 (P) shows that the tank is designated for potable water use.

Tank Reference	Sedimentation		Biofilm		Scale		Corrosion	
	UBV	FE	UBV	FE			UBV	FE
T1	0	1	0	1			1	1
T2	0	2	0	2			1	1
T3 (P)	0	1	0	1			0	0
T4	0	1	0	1			0	0
T5	1	2	0	2			1	1
T6 (P)	1	2	0	1			0	0
T7 (P)	1	2	0	1			0	0
T8	2	3	1	2			0	0
T9	2	3	1	3			1	2
T10 (P)	2	3	1	3			0	0
T11 (P)	1	3	0	1			0	0
T12 (P)	1	3	0	2			0	0
T13	1	3	1	3			0	0
T14 (P)	1	2	0	1			0	0
T15	0	0	0	0			0	0

The results of the water sample temperature taken at the time of sampling, and microbiological analysis by ALS Global are shown in Table 3.3. The data clearly shows that the microbiological status of the water decreases from incoming mains, to under the ball valve, and is worst at the far end of the tank.

Table 3.3 Water sample temperatures and analysis results (TVC, *E. coli*, coliforms, *Pseudomonas* and *Legionella pneumophila*) used to generate the figures 2-5. IM – incoming mains; UB – under the ball valve; FE – far end.

Sample Ref.	Sample temperature	TVC(3 days @ 22˚C) cfu/ ml	TVC (2 days @ 37˚C) cfu/ml	*Pseudomonas* spp (cfu/100ml)	*E. coliE. coli*	Coliforms	*Legionella* Spp cfu/L
T1 - IM	12.5	0	0	0	0	0	0
T1 - UB	12.5	0	0	0	0	0	0
T1 - FE	12.6	38	0	1000	0	0	0
T2 - IM	12.5	0	0	0	0	0	0
T2 - UB	12.5	0	0	0	0	0	0
T2 - FE	12.5	3000	23	0	0	0	0
T3 - IM	7	0	0	0	0	0	0
T3 - UB	8	0	0	5	0	0	0
T3 - FE	9	1100	0	500	0	0	0
T4 - IM	7	0	0	0	0	0	0
T4 - UB	7	9	0	3	0	0	0
T4 - FE	10	1300	85	27	0	0	0
T5 - IM	9.3	0	0	0	0	0	0
T5 - UB	9.8	97	6	12	0	0	0
T5 - FE	13.2	4200	165	1100	0	0	0
T6 - IM	8.4	1	0	0	0	0	0
T6 - UB	8.4	56	2	9	0	0	0
T6 - FE	12	2300	130	760	0	0	0
T7 - IM	8.5	0	0	0	0	0	0
T7 - UB	8.7	67	1	0	0	0	0
T7 - FE	13	1400	185	1390	0	0	0
T8 - IM	12.7	0	0	0	0	0	0
T8 - UB	12.9	50	0	7	0	0	0

T8 - FE	14.8	2900	170	500	0	0	0
T9 - IM	13.1	2	0	0	0	0	0
T9 - UB	13.3	64	13	17	0	0	0
T9 - FE	15.2	2500	430	960	0	0	0
T10 - IM	16.4	0	0	0	0	0	0
T10 - UB	16.7	1974	1140	83	0	0	0
T10 - FE	18.0	2362	1900	680	0	0	0
T11 - IM	17.7	0	0	18	0	0	0
T11 - UB	18.9	3700	2620	980	0	0	0
T11 - FE	20.2	5930	3170	3110	0	0	100
T12 - IM	18.4	12	0	0	0	0	0
T12 - UB	18.5	560	120	9	0	0	0
T12 - FE	19.1	2300	900	420	0	0	0
T13 - IM	19.0	10	0	9	0	0	0
T13 - UB	19.2	1670	840	290	0	0	200
T13 - FE	20.7	5400	3850	2200	0	0	800
T14 - IM	17.9	0	0	0	0	0	0
T14 - UB	19.2	169	1	5	0	0	0
T14 - FE	19.8	1400	285	490	0	0	0
T15 - IM	17.0	0	0	0	0	0	0
T15 - UB	17.0	10	1	0	0	0	0
T15 - FE	17.0	10	2	0	0	0	0

3.3.1 Effect of seasonality on stored water temperature

Figure 3.2 shows the temperature of the incoming mains (IM) water and storage water under the ball valve (UBV) and at the far end (FE) of fifteen tanks, recorded during routine inspection and sampling between November 2015 and July 2016. Seven tanks were situated on the ground floor or basement, and were connected to booster pumps to distribute potable water to the entire building. Eight tanks were located on the roof, with the purpose of distributing stored water to the calorifier(s) and cold water taps (with the exception of the kitchen tap). Storage water temperature varied with seasonality as expected, with water UBV temperatures as low as 7°C in December (winter time) rising to 19.2°C in July (British summer time). In all fifteen tanks, IM water temperature varied between 7-19°C (depending on the season), which was below the regulatory threshold of 20°C for both mains water and stored water (HSE, 2011). The stored water increased in temperature by 0.1°C to 3.6°C from UBV to FE in the majority of tanks. In ten tanks, this temperature difference was >1°C, and in 4 tanks the temperature difference was > 3°C. The smallest water temperature differences (0°C to 0.1°C) occurred in tanks below 6m^3 in either the winter (November/December) or summer (July), whereas the greatest temperature gradient differences occurred in large tanks (36m^3) sampled in February. Although UBV water temperatures were always below the regulatory threshold, the FE of Tank 14 reached the regulatory threshold limit (20°C), and Tanks 11 and 13 exceeded the regulatory threshold (20.2 and 20.7°C, respectively).

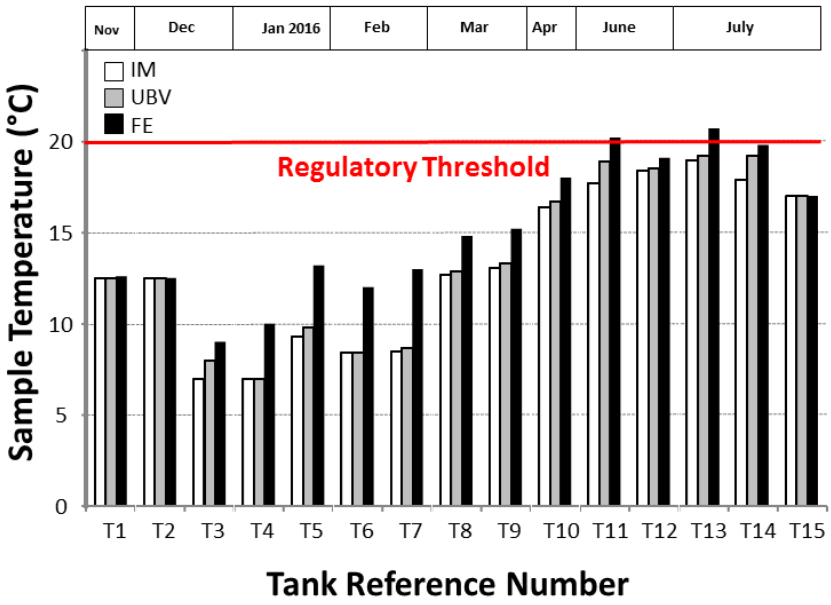


Figure 3.2 Sample temperature comparison: Sample temperature (°C) of the incoming mains water (IM), under the ball valve (UBV) and at the far end (FE) of fifteen operational cold water and potable water storage tanks in different London Boroughs recorded during routine inspection and sampling between November 2015 and July 2016. The red line shows the regulatory threshold of 20°C (acceptable limit) used for routine monitoring.

3.3.2 Microbiological status of water samples

Figure 3.3 shows the water sample analysis results for Total Viable Counts (TVC) incubated for 3 days at 22°C (Figure 3.3B) and for 2 days at 37°C (Figure 3.3A), respectively. The TVC is an estimate of the total number of viable individual micro-organisms (including bacteria, fungi and mould species) present in a set volume of sample, and provides a relatively rapid quantitative insight of the microbiological status of the sample. Fourteen of the tanks sampled showed increases in TVC between samples taken UBV and FE and incubated for 3 days at 22°C, with the exception of Tank 15 that had 10 cfu/ml at the both ends (Figure 3.3B). The biggest differences were observed in T2 and T3, where TVCs were 'not detected' in the UBV sample, but 3000 and 1100 cfu/ml were measured in the FE sample. T4 yielded 9 cfu/100ml in the UBV sample and 1300 cfu/ml at the FE, producing in 144-fold difference (2 orders of

magnitude) in TVCs between the UBV sample and the FE sample. The tanks sampled between November and March (with the exception of T4) showed a 40.2-fold (± 12.1 sd) mean increase in TVCs at the FE compared to UBV, and the tanks sampled in the summer had relatively higher TVCs in the UBV samples resulting in a 3.7-fold (± 2.5 sd) mean overall increase in TVCs at the FE. Although, thirteen out of fifteen tanks showed increased TVCs in FE samples compared to UBV samples incubated for 2 days at 37°C, only eleven (T2,T4,T5,T6,T7,T8,T9,T12,T13,T14 and T15) were greater. UBV samples of potable water incubated for 2 days at 37°C were found to greater only on two occasions (T10 and T11; figure 3A). Figure 3A shows that the incubation temperature of 37°C favoured growth of microorganisms in water samples collected in April and into the summer. In T10 (sampled April 2016) the potable water UBV sample yielding 1141 cfu/ml, whereas the FE sample was yielding 1900 cfu/ml.

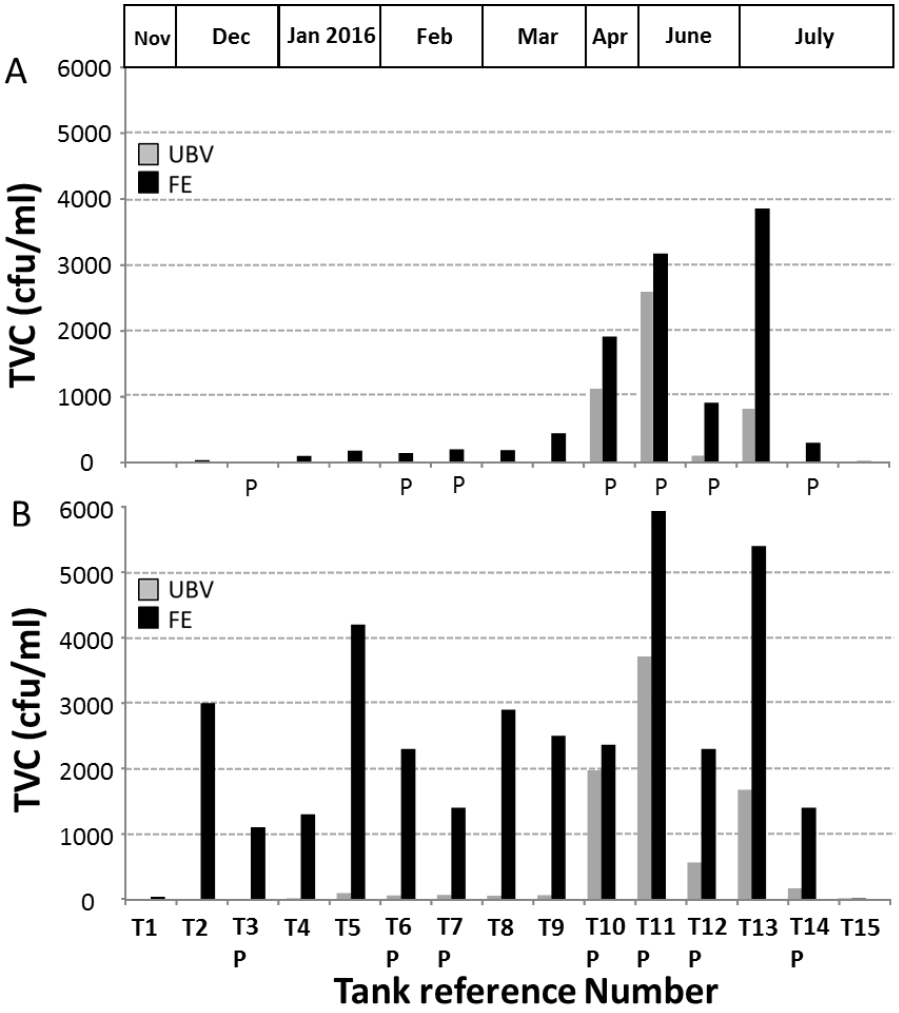


Figure 3.3 TVC analysis result comparison. Comparison of Total Viable Counts (expressed as colony forming units/ml) from water samples taken under the ball valve (UBV) and at the far end (FE) of fifteen independent cold water and potable water storage tanks located in different London Boroughs, and incubated at A) 37 °C for 2 days and B) 22 °C for 3 days. Tanks were sampled between November 2015 and July 2016. 'P' denotes tanks designated for potable water storage.

Figure 3.4 shows the results of analysis of the water samples for both *Pseudomonas* and *Legionella* species. The regulatory threshold for *Legionella* in tank water is 100 cfu/L. Figure 3.4A shows that most samples tested negative for *Legionella* in either UBV or FE samples. However, Tank 10 reached the regulatory threshold for *Legionella* (100 cfu/L) at the FE whereas *Legionella* was undetected under the ball valve. T12 also tested positive for *Legionella* in both UBV and FE samples, although the number of bacteria was 4-fold higher (800 cfu/L FE c.f. 200 cfu/L BVE) at the far end of the tank. *Pseudomonas* was regularly detected in tank water both in UBV and FE samples, with the exception of T2 and T15. In T7 (potable water) no *Pseudomonas* were detected in the UBV sample, whereas the FE sample yielded 1390 cfu/100ml. A similar finding occurred in T1, although this tank was not designated for potable water use. The UBV samples followed a seasonal trend, increasing from 7 cfu/100ml in March to a maximum of 980 cfu/100ml in June. In all tanks where *Pseudomonas* was detected in both UBV and FE samples, the number of bacteria in the FE sample was on average 54-fold higher (±39) and varied between 9-fold and 98-fold depending on the individual tank and the season of sampling.

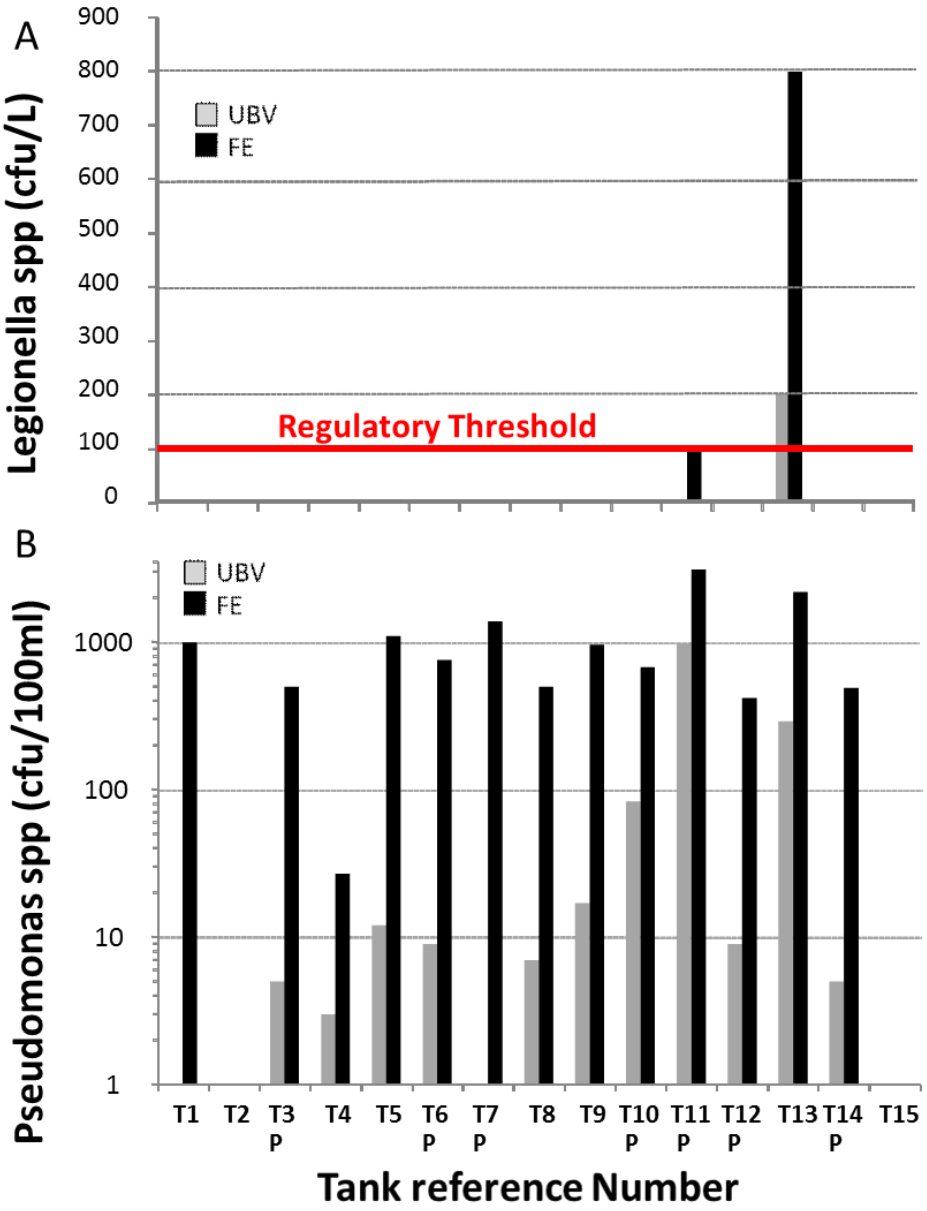


Figure 3.4 Comparison of *Legionella* and *Pseudomonas* analysis results. Comparison of (A) *Legionella* species (expressed as colon forming units/litre) and (B) *Pseudomonas* species (expressed as colony forming units/100ml) in water samples taken under the ball valve (UBV) and at the far end (FE) of fifteen independent cold water and potable water storage tanks located in different London Boroughs. Samples were taken between November 2015 and July 2016. 'P' denotes tanks that are designated for potable water use. The regulatory threshold for *Legionella* in tank water is 100 cfu/L.

Figure 3.5 shows a comparison of TVC and *Pseudomonas* species quantified in samples collected from both UBV and FE locations in potable water tanks at different times of the year. In all cases the UBV and FE samples provided a consistent course of action for TVCs incubated for 2 days at 37°C (10 cfu/ml), although T10 showed slight difference in the UBV sample (11 cfu/ml) compared to the FE sample (19 cfu/ml) (Figure 3.5A). In addition, in T12 the FE sample was 9 cfu/ml whereas the UBV sample was substantially lower (1 cfu/ml). In contrast, the levels of *Pseudomonas* in UBV samples was typically low (between 5 and 9 cfu/100ml) with the exception of T10 and T11 (83 and 980 cfu/100ml, respectively). All UBV samples, with the exception of T7, were detected with *Pseudomonas*. However, T7 showed much higher count in the FE sample (1390 cfu/100ml). There were consistently higher *Pseudomonas* counts in the FE sample relative to the UBV sample (Figure 3.5B).

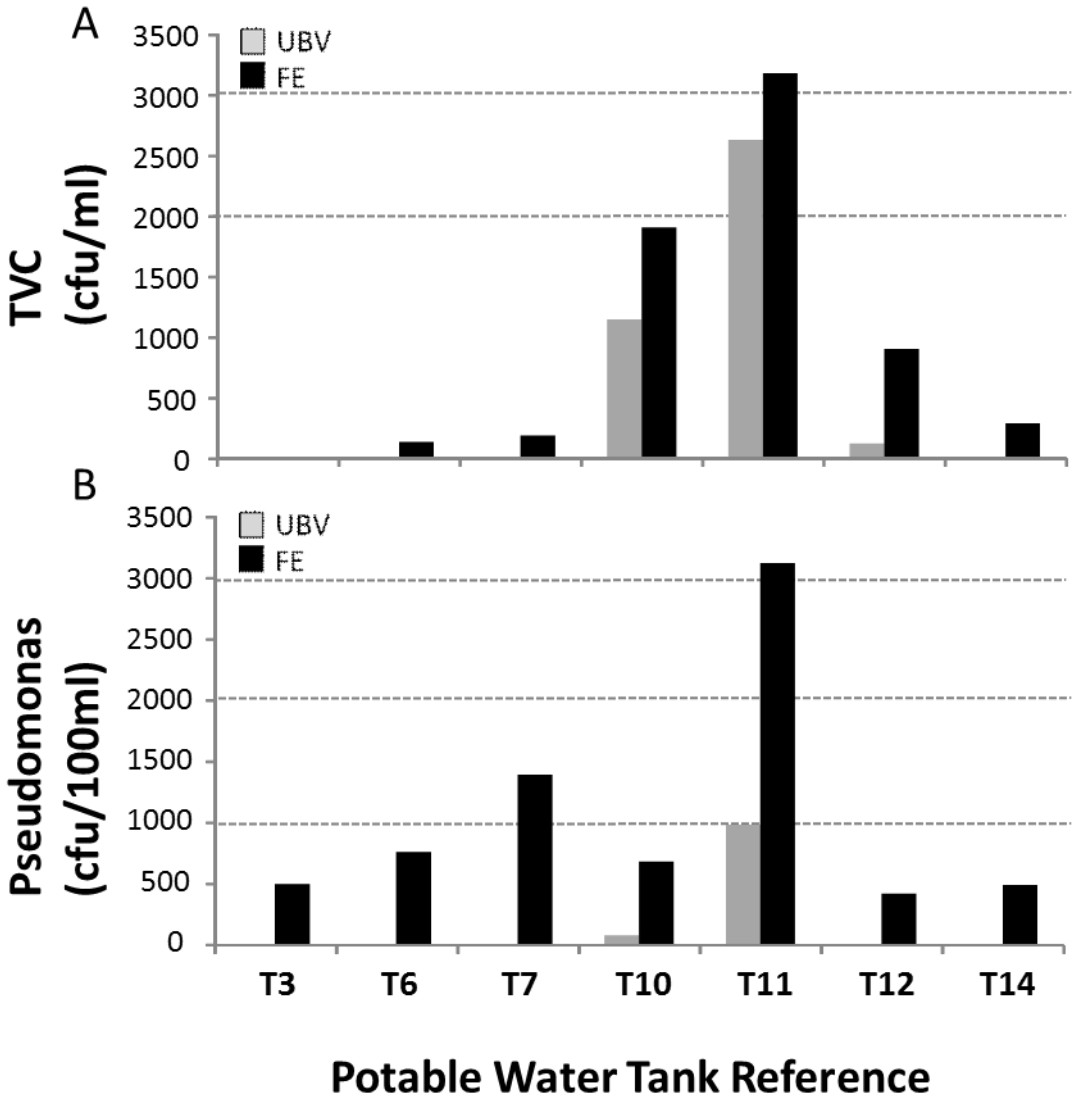


Figure 3.5 Comparison of TVC and *Pseudomonas* analysis results: Comparison of A) Total Viable Counts (TVC; 2 days incubation at 37°C) and B) *Pseudomonas* species (expressed as colony forming units/100ml) measured in water samples taken under the ball valve (UBV) and at the far end (FE) of potable water storage tanks located in different London Boroughs. Samples were taken between December 2015 and July 2016.

Table 3.4 Comparison of TVCs, *Pseudomonas* and *Legionella* (cfu/volume) from UBV and FE samples.

Tank	TVCs (cfu/ml)			*Pseudomonas* (cfu/100ml)			Legionella (cfu/L)		
	UBV	FE	RT	UBV	FE	RT	UBV	FE	RT
11(P)	2620	3170	N/A	980	3110	N/A	0	100	100 cfu/L
13	840	3850	N/A	290	2200	N/A	200	800	100 cfu/L

Boxes in dark red show instances where there is a disagreement in compliance from UBV and FE samples

(P) Denotes that the water was used for potable use.

RT denotes the regulatory threshold.

3.3.3 Visual inspection report

Visual inspection of the tanks showed a clear increase in the level of sedimentation with distance from the ball valve (Figure 3.6B and 3.7B), and presence of biofilm was also noted towards the far end of the tanks whereas under the ball valve, water appeared visibly clear (Figure 3.6A and 3.7A). The presence of a slight scale was noted in T1,T2,T8,T9,T10,T13 and T14, although it appeared to be similar at both ends of the tanks. There was evidence of slight corrosion throughout all the metal tanks, whereas in T9 the far end appeared as moderate (Table 3.2). In the case of one metre long plastic tank (T15), water was apparently clear without sedimentation, stagnation, scale and biofilm (Figure 3.7D), and a temperature of 17^0C was recorded for the incoming mains temperature and for the UBV and FE samples.

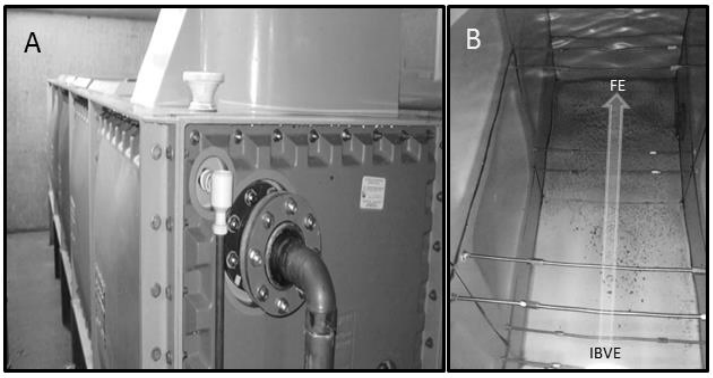


Figure 3.6 Long cold water storage tank: (A) Example of a 5 metre long GRP potable cold water storage tank showing the mains water inlet. (B) Sedimentation levels increase towards the far end (FE) of the tank from the inlet ball valve end (IBVE). The arrow shows the direction of mains water flow into the tank. Tank reference T10. Source: own collection

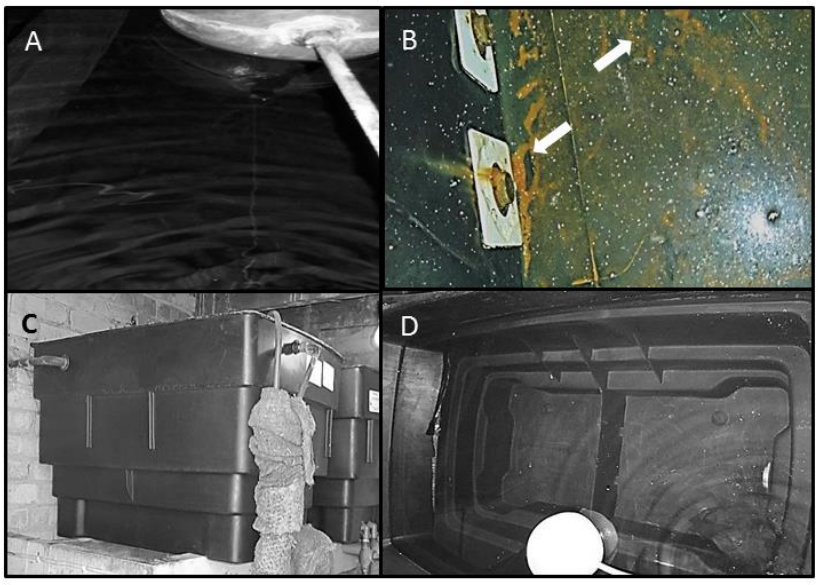


Figure 3.7 Comparison of long metal tank and small plastic tank: (A) 4 metre long metal cold water storage tank with butyl lining (T13) showing visibly clear stored water under the ball valve (A), compared to sediments and biofilm at the far end (B). (C, D) shows a 1 metre long plastic tank with visibly clear stored water (T15).

Source: own collection

3.4 Discussion

3.4.1 Factors affecting regulatory action decisions

Microbiological analysis of water samples taken from different cold water storage tanks throughout a year has shown large differences in microbiological concentration between UB and FE of the tanks. Furthermore, 2 out of the 15 tanks surveyed failed to trigger appropriate current regulatory action based on microbiological analysis (Legionella) of the water sample taken under the ball valve (n=15 tanks) compared to the far end sample using present-day standards (Table 3.4). Tanks that failed to trigger appropriate regulatory action were sampled in late spring and summer, suggesting warming temperatures to be an important factor in this response. Indeed, tanks 11 and 13 both exceeded the threshold for temperature at the far end of the tank, despite being complaint at the ball valve end. The variation in temperature of the incoming mains water was due to seasonal changes, whilst differences between UBV and FE samples were due principally to water stagnation towards the far end of the tanks (Lautenschlager et al., 2010).

3.4.2 Comparison of TVC analysis under the ball valve and at the far end of tanks

Most water contains microorganisms, and an estimation of their overall numbers provides important information used for system surveillance and water quality maintenance (Manuel et al., 2007). The Total Viable Count (TVC) is essentially a simple enumeration of all viable bacteria present in water (Allen et al. 2004). Microorganisms growing better in laboratory media at 22°C reflect environmental micro-organisms and can be used to plot seasonal variations. In contrast, microorganisms that grow at 37°C may represent those of faecal origin (Edberg et al., 2000). TVC analysis (3 days incubation at 22°C) of incoming mains samples taken from T6, T9, T12 and T13 (1, 2, 12 and 10cfu/ml, respectively) were below the HSE's former

Potable Water Standard limit of 100cfu/ml (HSE, 2002). Also, TVC analysis (2 days incubation at 37°C) of mains water was non-detectable and below the former regulatory threshold of 10cfu/ml (HSE, 2002) (This RT mentioned here is only for comparison as this regulation is outdated). Although the mains water and UBV temperatures recorded were almost identical, the temperatures recorded at the far end of the tanks were comparatively higher (Figure 2) thereby encouraging bacterial growth at the far end of most tanks (van der Kooij et al., 2017; Whiley et al., 2017; Schwake et al., 2016). According to a study carried out in USA, it was reported that bacteria in drinking water pose a health risk to all individuals, and especially patients with underlying health issues (Ashbolt, 2015). TVC analysis results (2 days at 37°C) for UBV and FE samples from T11p were much higher (2620 and 3170, respectively) than expected. These results clearly indicate that the samples taken from UB cannot represent the actual quality of the water that draws to the building outlets.

3.4.3 Comparison of *E. coli*, Coliforms and *Legionella* under the ball valve and at the far end of tanks

E. coli and coliforms analysis results were negative in all the samples tested, indicating that the water was likely to be free of pathogens associated with faeces (Kapperud, 1982). However, in all the six potable water tanks *Pseudomonas* was detected at levels ranging from 5 to 980 cfu/100ml for UBV samples and 420 to 3110 cfu/100ml for FE samples. The presence of both biofilm and sedimentation towards the far end of the tanks (see Table 3.2) may be responsible for this concentration of (Liu et al., 2016; Klausen et al., 2003; Al-Harbi, 2003). *Pseudomonas* is bacteria commonly found in soil and water and it can cause a variety of infections in immunosuppressed population and people with long-term lung diseases such as cystic fibrosis (BLF, 2018). There are a number of strains of *Pseudomonas* and *Pseudomonas* aeruginosa strain is the most common strain causing infections in humans (CDC, 2018). Some studies report that *Pseudomonas* growth in drinking water is probably related to its ability to create and colonize biofilms in plumbing fixtures (Moritz et al., 2010 ; Kennedy, 2012).

The presence of *Pseudomonas* and their associated biofilms is also a risk factor for other pathogenic bacteria, including *Legionella* (Falkinham III et al., 2015). This is illustrated here using the water sample analysis result from T13, where the TVC analysis and *Pseudomonas* counts were high in both cases, suggesting the higher risk of

Legionella proliferation. Analysis confirmed the presence *Legionella* in both UBV and FE samples (200 cfu/L and 800cfu/L, respectively) in T13. Changes to the internal environment of the tank from the ball valve end to the far end of the tank are supported by visual inspection reports produced at the time of water sampling (Table. 3.2) and additional photographic evidence included in this study (Figures 3.6 and 3.7). On the basis of the routine inspection report and the results presented, the increasing trend of microbial activity towards the far end of the tanks is likely due to increasing temperature, water stagnation, presence of biofilm and sedimentation with distance from the ball valve (Barko and Smart, 1986; Konishi et al., 2006).

3.4.4 Variation in water temperature UBV and FE of tanks

In this study, variations in water temperature from the mains supply end to far end of some tanks increase by as much as 3-3.6°C. Temperature is known to play an important role in the colonization of *Legionella* bacteria in water systems (Buse et al., 2017; Schwake et al., 2015; Borella, 2004). *Legionella* bacteria can survive and persist at temperatures between 6 and 63°C although proliferation is generally accepted to occur between 20-45°C and when suitable nutrients are available (HSE, 2013). Although the temperature of the mains water entering the tank was at, or below, 12°C in the winter months, the temperature at the far end of the tanks exceeded 12°C in almost all cases, with the exception of two tanks sampled in the winter (December and January). Therefore, the temperature of the stored water at the far end of the tank may reach optimum levels for *Legionella* proliferation if the water is not frequently replenished (Valero et al., 2017). Indeed, T11 and T13 both contained live *Legionella pneumophila* (100cfu/L and 800cfu/L, respectively), and had water temperatures at the far end that exceeded 20°C. According to Health Technical Memorandum Part B produced by UK Health Department in 2016, incoming mains water temperature can reach up to 25°C in the summer season (HTM, 2016) and the water temperature at the far end can reach well above 30°C; close to the maximum virulence temperature (37°C) for *Legionella* bacteria where it achieves maximum ability to infect and subsequently multiply (WHO, 2007).

In effect, there is a significant difference in the temperature of the stored water under the ball valve and at the far end of the tank which is influenced by seasonal

variables (Figure 3.2), resulting in clear differences in the microbiological quality of water samples taken from these two locations.

3.4.5 Water stagnation and microbiological growth

Water stagnation is recognised to be a major factor in water hygiene maintenance and management (Lipphaus et al., 2014). Stagnant water provides ideal conditions for microbiological growth to occur (ASHRAE, 2018). For example, overnight stagnation of drinking water in household taps was found to be associated with a 2-3 fold increase in microbial concentrations and changes to the bacterial community composition. However, after flushing the taps for 5 minutes, bacteria concentrations and water temperatures decreased to levels generally found in the drinking water network (Soderberg et al., 2004; Pepper et al., 2004). Visual observations of the water contained within the tanks investigated here also found evidence of surface water stagnation, due to a combination of slow outgoing of water from the bottom of the tank and poor internal water circulation (Manuel et al., 2007). Surface water stagnation is an important causal factor for biofilm formation, thereby creating a perfect breeding ground for pathogenic bacteria, including *Legionella* pneumophila (Declerck, 2010).

3.4.6 The role of biofilm as a source of bacterial contamination

Biofilms are known to be a major source of bacterial contamination, and are often responsible for recurrent contamination of water systems by *Legionella* pneumophila (Declerck et al., 2009). In natural environments, biofilms are typically described as complex, natural assemblages of various types of microorganism involved in a multitude of trophic and symbiotic interactions (Berlanga and Guerrero, 2016; Stoodley et al., 2002). Although biofilms often typically start in nutrient rich environments (where bacteria change from free-living planktonic cells to sessile surface bound cells state), their presence represents a protected mode of growth allowing different types of cells to survive in hostile environments for extended periods of time, and also to disperse to colonize new niches when environmental conditions change (NRM, 2004; O'Toole et al., 2000; Kurniawan et al, 2015). Once established,

biofilms can cause bio-corrosion of water storage and supply materials, and are a major cause of disinfection inefficiency, serving as reservoirs for various pathogenic and non-pathogenic microorganisms, including *Legionella* (Fish et al., 2016; EPA, 2007). Biofilm growth was consistently greater at the far end of the tank (Table 3.2). Indeed, only 25% of tanks surveyed had biofilms at the ball valve end and these were characterised as 'slight' and occurred in the spring and summer periods. In contrast, 90% of all tanks surveyed had biofilm at the far end of the tank (50% 'slight', 20% 'Moderate' and 21% 'severe') and these occurred throughout the year despite seasonal changes in incoming mains temperatures (Table 3.2). 11 out of the 15 tanks surveyed here were also noted to have surface water biofilms at the far end of the tank. Importantly, FE water samples had consistently higher levels of microbiological activity (TVCs and *Pseudomonas* counts). Therefore, analysis of water samples taken under the inlet ball valve (where samples are routinely taken for monitoring and regulatory compliance) is not representative of the actual overall condition of the stored water due to biofilm growth associated with water stagnation.

3.4.7 Microbiological impact of sediments at the FE of tanks

Tiny suspended and dissolved solids are carried in the mains water and settle in the bottom of water storage tanks to form sedimentary deposits (Liu et al., 2017; Gray et al., 2000). Corrosion products scale and sediments then act together as nutrients, encouraging *Legionella* proliferation (Qin et al., 2017; Douterelo et al., 2016). According to a study carried out by Veterans Administration Medical Centre and University of Pittsburgh, the presence of sediment in stored water enhances the survival of *Legionella pneumophila* directly by acting as a nutrient, but also indirectly by encouraging the growth of other environmental bacteria that interact with *Legionella* via nutritional symbiosis. The bacteria and sediments act synergistically, in combination, to improve the survival of bacteria, including *Legionella pneumophila* (Fabian et al., 2017; Stout et al., 1985). Here we found that eleven of the tanks with microbiological activity also had sedimentation at the far end of the tanks, whereas the bottom of the tank under the inlet valve was comparatively free from sediments (Table 3.2). As with the biofilms, sedimentation became more severe with distance from the inlet valve, suggesting that a greater risk of bacterial contamination would occur from the mains inlet to the far end of the tank. The sample collected from the far end of the

tank therefore more accurately represents the actual quality of the stored water entering the building system, and it is therefore vital to collect water samples from this location. Unfortunately, the far ends of most water storage tanks are sealed and completely inaccessible. Current WRAS guidelines, state that the inspection and sampling access hatch location should be above the inlet ball valve (Figure 3.1B and Figure 3.8) in order to facilitate maintenance of the inlet/ball valve (DWI, 2014).

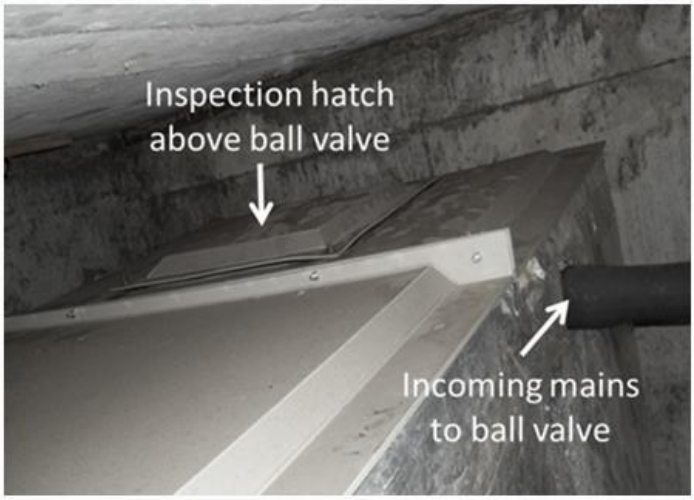


Figure 3.8 Position of inspection hatch and inlet on cold water storage tank: A typical 4 metre long metal cold water storage tank with internal butyl lining showing the position of the inspection hatch and mains inlet to the ball valve.

Source: own collection

3.4.8 Public health perspectives

Even in modern society, waterborne pathogenic bacteria continue pose a serious threat to human health. Legionnaires' disease (LD), caused by *Legionella pneumophila*, is just one of a number of potentially fatal diseases associated with water related infections (PHO, 2014). Importantly, the internal conditions of the tanks used to store water, and associated water quality parameters, will influence the rate of proliferation of *Legionella* bacteria within the water system, the risk of exposure to contaminated aerosols containing *Legionella* bacteria during normal water usage, and the likelihood of contracting in LD (HSE, 2014). Water temperatures between 20-45ºC

are known to encourage *Legionella* growth within water systems (FHG, 2014; Temmerman et al., 2006; Konishi et al., 2006) and studies in United States and Europe have confirmed that stored cold water temperature is likely to rise above 20ºC in summer, consistent with greater numbers of community acquired Legionnaires disease (Worrall 2015; Brandsema et al., 2014). We found that incoming mains water of ~ 20ºC could reach 22-23ºC at the FE of tanks greater than 1 metre in length in the summer. *Legionella* proliferation (if present) is likely to occur quickly in the warmer and nutrient rich water at the far end of the tank, where it is then abstracted for use in the building. The number of reported cases of Legionnaires' disease in the USA follows a seasonal trend, being much higher during the summer season (Prussin II et al., 2017), and the seasonal prevalence of LD appears to be worsening, possibly as a result of climate change (Worrall, 2015). Indeed, the number of reported LD cases in The Netherlands was unusually high in the summer of 2010, associated with warmer and wetter climatic conditions (Brandsema et al., 2014). Furthermore, the outcome of these studies agrees with official statistics on LD published by both US and UK government agencies (Figure 3.9). Given the disparity between measurements taken at different end of the tanks, we propose that monitoring at the far end of cold water storage tanks would provide a more accurate and relevant indication of microbiological contamination, enabling appropriate precautions to be taken to protect the public from water pathogenic diseases, including Legionnaires' disease.

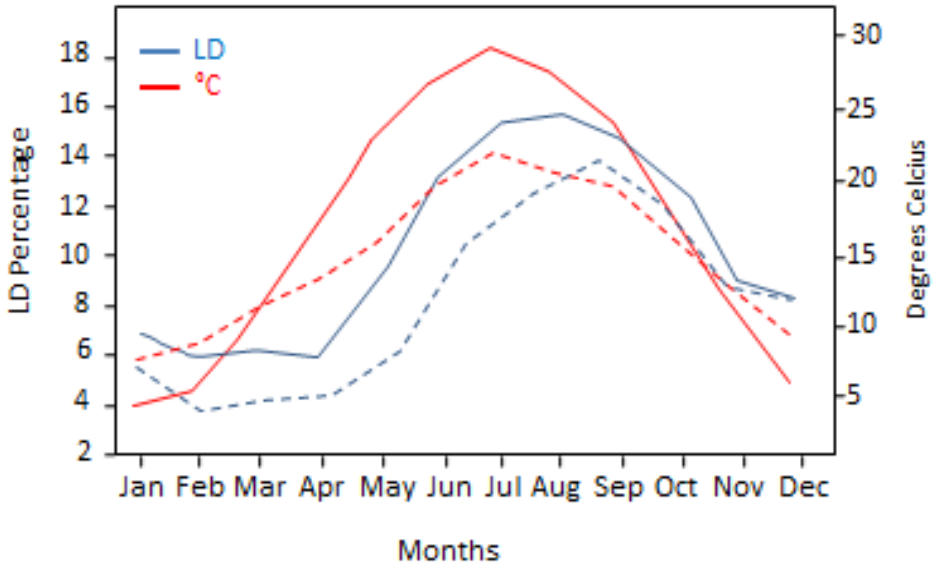


Figure 3.9 Comparison of LD cases in the USA and UK: Average percentage of LD cases occurring in the United States and UK annually by month. U.S census data 2000-

2009 and UK census data from 2015 and 2016 relative to seasonal high temperatures (District of Colombia and average UK temperature). Census data reported to Centres for Disease Prevention and Control (CDC) through the National Notifiable Disease Surveillance System (NNDSS) and a Supplemental Legionnaires Disease Surveillance System (SLDSS). UK data on Legionnaires disease was acquired from Public Health England reports. Solid line represents USA and dashed line is the UK.

Source: Created using the data from CDC and PHE.

3.5 Recommendations and conclusions

Public health risks from exposure to pathogenic bacteria in buildings, and strategies used to control pathogenic bacteria must be constantly reviewed and revised (Whiley, 2016). It is generally assumed that water sample analysis will provide a representative view of the microbiological status of the entire tank in order to inform risk management strategies. Here we report significant differences in the microbiological status of water samples collected under the ball valve (where there is easy access, and where samples are routinely taken for regulatory compliance) compared to the far end of the tank where water typically enters the building but where sampling access is constrained. In order to control *Legionella* and maintain water hygiene standards, it is vital that a representative sample is collected as part of routine monitoring.

According to our results we recommend that there should be an additional access hatch at the far end of water storage tanks for internal inspection and water sampling. Large disparities in water quality parameters were noted in water samples taken from opposite ends of tanks greater than one metre in length. In order to comply with current WRAS guidelines, any cold water storage tank over 1000 litres capacity should have a screened warning pipe and a screened overflow (DWI, 2014). In the same way, we propose that new water storage tanks of similar capacity should contain an additional inspection and sampling access hatch at the far end of the tank, and this requirement could be imposed through appropriate national and international guidelines.

3.6 Further work

The number of tanks surveyed in this study was limited due to difficulties in accessing the far end of the storage tanks. Further study with increased number of water storage tanks would be beneficial to understand the findings of this study in depth. Thus, the regulating authorities can be convinced and the proposals made in the recommendation (3.5 of this thesis) can be implemented for better monitoring and maintenance of cold water storage systems.

CHAPTER FOUR

DEVELOPING A qPCR METHOD FOR THE DETECTION OF LIVE *LEGIONELLA*

4.1 Introduction

Legionella bacteria remain as a serious threat to public health especially among immunosuppressed and elderly population (Alsehlawi et al., 2016). *Legionella* bacteria are the main causative agent of community acquired and hospital-acquired pneumonia, with sporadic cases and outbreaks of LD reported worldwide (Cameron et al., 2016). Sporadic cases of LD can be treated effectively and outbreaks of LD can be controlled more efficiently if there is a rapid reliable diagnosis method to identify all the species and strains of *Legionella* bacteria. The diagnostic methods currently in use are neither fully reliable nor standardised (Whiley and Taylor, 2016; Mercante and Winchell, 2015).

4.1.1 Unreliability of diagnostic methods

There are different diagnostic methods available to detect the presence of *Legionella* in environmental samples as well as clinical samples. Culture based diagnosis methods using Buffered Charcoal-Yeast Extract (BCYE) agar is still the 'gold standard' diagnostic procedure used to detect *Legionella* bacteria (Andreozzi et al., 2016; Cunha et al., 2016). However, culture is time consuming due to the slow growth rate of *Legionella* species, and this delay in getting the analysis results increases the likelihood of exposure to *Legionella* in contaminated systems (Zhan et al., 2014; Delgado-Viscogliosi et al., 2009). This method is further complicated by difficulties in isolating *Legionella* species in samples due to the abundant background growth of other micro-organisms, which also can inhibit *Legionella* growth (Diaz-Flores et al., 2015; Toplitsch et al., 2018). Furthermore, *Legionella* species may be protected within amoebae and remain undetected (Berjeaud et al., 2016;; Greub and Raoult, 2003). In addition, the standard culture methods employed for the isolation of *Legionella* species were primarily developed for the detection of *Legionella* pneumophila; therefore, this method is much less sensitive in detecting other *Legionella* species causing similar diseases. For these reasons, it is widely recognised that standard culture methods significantly underestimate the presence of *Legionella* in samples (Dietersdorfer et al., 2016).

Moreover, culture based diagnosis methods have very poor sensitivity and struggle to detect low concentrations of bacteria in environmental samples that fall within the regulatory framework (Delgado-Viscogliosi et al., 2009). A comparative study on different diagnostic procedures reported that only 20% of all the known *Legionella* positive samples were reported as positive by culture methods, and the major factor for this failure was reported to be the overgrowth by competing micro-organisms on the culture medium (Díaz-Flores et al., 2015). These drawbacks of conventional culture methods for *Legionella* detection can have a significant negative impact on the results and interpretation in terms of underestimating the actual health risk. Inaccurate quantification and underestimation of *Legionella in samples (*especially at concentrations below 100cfu/L), due to poor sensitivity, and delays in diagnostic procedures associated with conventional culture methods raises serious concerns about the reliability of current methods used to protect public health against Legionnaires' disease (Conza et al., 2013; Mercante and Winchell, 2015; Díaz-Flores et al., 2015).

There are various clinical methods to diagnose *Legionella* bacteria such as direct fluorescence antibody staining, serological diagnosis and *Legionella* pneumophila urinary antigen tests (UATs). However, these procedures (with the exception of UATs) are considered unreliable with poor sensitivity and specificity. (Maurin et al., 2010). As an alternative solution, PCR based diagnostic techniques have been developed in recent years as rapid, efficient and sensitive alternatives to culture methods in the diagnosis of all *Legionella* species in clinical and environmental samples (Avni et al., 2016; Cunha et al., 2016).

4.1.2 qPCR diagnostic method

A real-time polymerase chain reaction, also known as quantitative polymerase chain reaction (qPCR) is a laboratory technique that monitors the amplification of a targeted DNA molecule during PCR. DNA replication occurs when a DNA sample and a DNA polymerase, primers, nucleotides and other reagents are added in laboratory condition and the reagents facilitate the required reaction to copy the DNA code. qPCR can be used not only to detect the presence of specific bacteria in a given sample, but also to monitor the amount of bacteria in terms of Genomic Unit (GU)(Kralik and Ricchi, 2017; Thermofisher, 2016). GU is a measure of the number of genomes present, with the

genome being the genetic blueprint of an organism consisting of both genes and all non-coding DNA packaged in chromosomes (Joly et al., 2006).

4.1.2.1 DNA (Deoxyribonucleic acid)

Deoxyribonucleic acid (DNA) is the genetic material present in every cell of organisms and mostly it is located within the cell nucleus (Alberts et al., 2002; Bowater, 2001). In 1869, a German scientist named Frederich Miescher was the first person to observe DNA. Later in 1953, James Watson, Francis Crick, Rosalind Franklin and Maurice Wilkins figured out the structure of DNA, and established that DNA can carry biological information in living organisms. In1962, they were awarded the Nobel Prize in medicine for the same discovery (Dahm, 2005).

4.1.2.2 RNA (Ribonucleic Acid)

RNA is a biological macromolecule with a number of different functions. Messenger RNA (mRNA) is transcribed from DNA to act as a template for protein synthesis - carried out by ribosomes, which includes ribosomal RNA (rRNA) and associated proteins. Amino acids required for protein synthesis are delivered to the ribosome on transfer RNA (tRNA) molecules to be assembled into proteins using the mRNA as a template. There are non-coding RNAs such as tRNA and rRNA, small nucleolar RNAs (snoRNA), short interfering RNAs (siRNA), microRNAs (miRNA) and piwi-interacting RNAs (piRNA) which are functional RNA molecules that do not translate into proteins ((Tan and Yiap, 2009).

4.1.2.3 Importance of extraction and analysis of RNA

Although DNA is double stranded and RNA is a single stranded, both contain a sequence of nucleotides that carry genetic information. RNA analysis by hybridization technologies, such as RT–PCR, northern blotting, RNA-sequencing and microarray analysis can provide almost accurate indication of an organism's gene expression profile. However, RNA is relatively unstable compared to DNA due to the presence of ribonucleases (RNases; enzymes that rapidly degrade RNA molecules). RNases are,

however, stable, effective in very small quantities, difficult to inactivate and do not require cofactors. Therefore, RNA isolation and analysis requires specialised techniques (Tan and Yiap, 2009). One of the most valuable techniques used in recent micro-biological research is the nucleic acid amplification and detection using qPCR (Bustin, 2010).

4.1.2.4 qPCR diagnostic method for *Legionella* bacteria

An alternative diagnostic method for quantifying all *Legionella* species and serogroups present in water samples is quantitative Polymerase Chain Reaction (qPCR) (Whiley and Taylor, 2016). This method is an efficient way to "amplify" (copy) a specific small segment of DNA in order to make it detectable (Kralik and Ricchi, 2017). This process can be considered quantitative, if the product is detected in the exponential phase of amplification when the amount of product detected is still proportional to the amount of starting material. By using a fluorescent marker within the reaction and following the increase in fluorescence in "real-time," the exponential phase of the reaction can be identified and samples compared, either to a control reference gene or to a quantitative standard (Valones et al., 2009).

4.1.2.5 The working procedure of qPCR method

The qPCR technology generates multiple copies of a gene, or section of DNA, from a sample of genomic DNA (Garibyan and Avashia, 2013). Using this technique, rapid DNA amplification resulting in millions of copies of target DNA enables detection and identification of specific gene sequences. Under controlled laboratory conditions, small segments of DNA are produced by enzymes called DNA polymerases which add complimentary deoxy-nucleotides (dNTPs) to a piece of single-stranded DNA (ssDNA) called the 'template'. Smaller pieces of DNA, known as primers are used as a starting point for the polymerase reaction. Primers are small synthetically-produced pieces of DNA (oligomers), between 15 and 30 nucleotides long and are designed from short DNA sequences at the very ends of the gene being amplified. During the PCR process, the DNA being amplified is denatured by heat in order to separate the double stranded DNA. Upon cooling, the primers then bind to the ssDNA template and create a place for the polymerase reaction to begin. The polymerase then extends the primer using the DNA as a template, until the temperature is increased again to denature the newly formed double stranded DNA. Performing multiple cycles leads to a doubling of the

amount of template after each cycle and an exponential amplification of the desired product. PCR was developed from the discovery of thermophiles and thermophilic polymerase enzymes so that the polymerases maintained structural integrity and functionality even after heating at high temperatures (Robertson and Phillips, 2008).

The reaction process involved in the PCR technique is as follows (Garibyan and Avashia, 2013; Butler, 2012):

- Denaturation: A sample containing DNA template, primers, polymerase enzyme and dNTPs is heated to 94-98˚C to denature the DNA template.

- Annealing: Following denaturation, the sample is cooled to a moderate temperature in the range of 48-72˚C, which facilitates the binding of the primers to the single-stranded DNA templates.

- Elongation: In this step the sample is heated to 68-72˚C for elongation of the desired template from the primers. The DNA polymerase uses the original single strand of DNA as a template to attach complementary dNTPs to the 3' ends of each primer and form a section of double-stranded DNA in the region of the gene of interest.

During the PCR reaction, this process of denaturing, annealing and elongation are repeated 30-40 times; increasing exponentially the number of copies of the desired gene in the sample (Figure 4.1) and the entire PCR assay can be completed in 1-2 hours' time. The number cycles required is based on the amount of DNA target material at the beginning, and the number of copies of the PCR product required (Biorad, 2015). The denaturing step in each cycle stops the elongation process of the previous cycle. The elongation step duration can be made longer or shorter depending on the size of the desired product (determined by the distance of the binding sites of the primers on the target DNA) and as the PCR progresses, the majority of new DNA copies will be the size of the section of gene between the primers as they would have been generated from products of both primers.

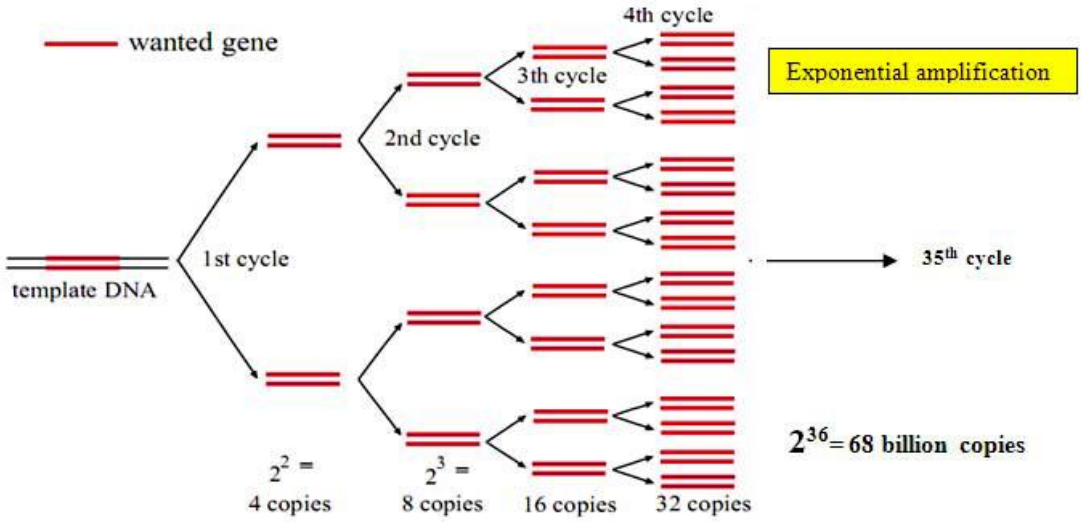


Figure. 4.1 In a typical PCR reaction, number of copies of DNA obtained after each cycle.
Source: http://vlab.amrita.edu/?sub=3&brch=186&sim=321&cnt=1

The probe is a fluorescently dye-labelled oligonucleotide (25–40 nt) that forms a hairpin structure; the 5' and 3' ends have complementary sequences of 5–6 nucleotides enabling the probe to form a hairpin-like structure that specifically hybridizes to a 15–30 nucleotide section of the target DNA. The fluorescent reporter molecule is attached to the 5' end, and a quencher molecule is attached to the 3' end of the probe. Thus, in the absence of suitable template, the formation of the hairpin structure brings the fluorescent reporter and quencher together, and no fluorescence is emitted. qPCR relies on the specific and efficient amplification of target DNA, and the binding of the fluorescent probe enables the visualisation and quantification of PCR products. SYBR Green is the most commonly used DNA-binding dye for qPCR as this binds non-specifically to double-stranded DNA (dsDNA). SYBR Green exhibits relatively less fluorescence when it freely exists in solution; however, fluorescence increases up to 1,000-fold once it binds to dsDNA. Thus, the fluorescence signal becomes proportional to the amount of dsDNA in a reaction and also increases as more PCR product accumulates during amplification. As DNA amplification progresses exponentially, so does the fluorescence signal, thereby making it possible to calculate the original amount of target DNA (Biorad, 2015; NEB, 2018).

Generally, Taqman primers are designed to have an annealing temperature in the range of 55 - 60°C and the TaqMan probe should have a melting temperature (Tm) 5-10°C higher than that of the primers. The probe is typically <30 nucleotides and should

not contain a G at its 5' end as this could quench the fluorescent signal even after the hydrolysis. Also, a sequence within the target that has a GC content of 30-80% is preferable, meaning that the probe also contains a high GC content for complimentary binding. The reporter and quencher selection is an important aspect of designing a TaqMan probe. When designing singleplex reactions, use of FAM-labelled probes are recommended because they perform well and are readily available and inexpensive. Furthermore, they can be detected by most of the instruments currently in use (Sigma, 2017; Smith and Osborn, 2009; Nadkarni et al., 2002).

4.1.2.6 General interpretation of qPCR results

In order to understand the qPCR results, examination of a typical sample amplification plot is useful (Figure 4.2). In this plot, the PCR cycle number is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in each sample, is shown on the y-axis.

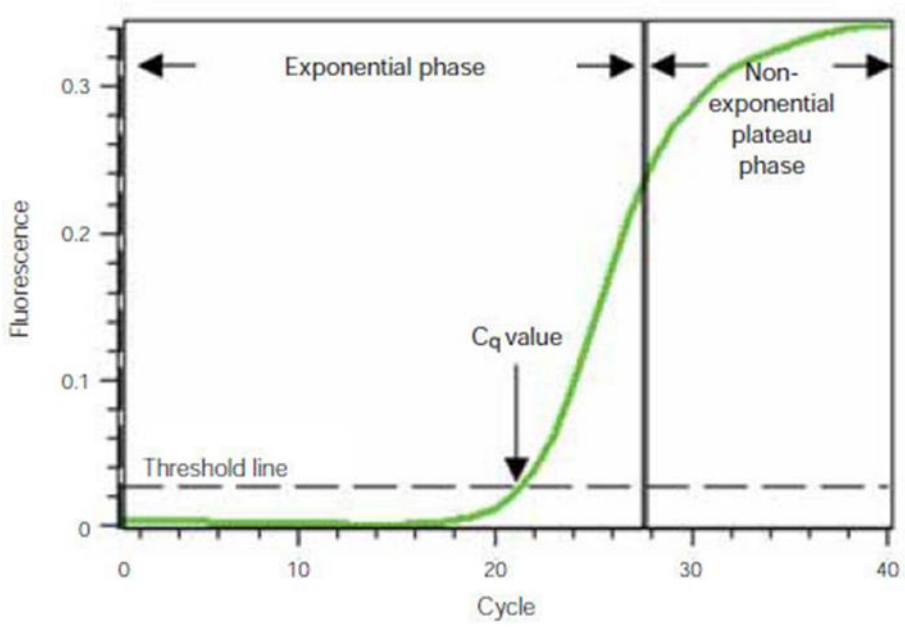


Figure 4.2 Typical qPCR amplification plot showing number of cycles, exponential phase and non-exponential phase.
Source:http://www.bio-rad.com/en-ch/applications-technologies/what-real-time-pcr-qpcr

The qPCR amplification is characterised by two separate phases; an exponential phase (first phase), followed by a non-exponential phase (second phase). In first phase, the amount of PCR products almost doubles in each cycle. As the reaction progresses, the reaction components are consumed and become limited, and the reaction slows down and enters the plateau phase. At the beginning, fluorescence remains at background levels, until the amplified products accumulate enough to produce a detectable fluorescent signal. The cycle number at which this fluorescent signal is seen produces is called the 'threshold cycle' (CT or Cq). When the reagents are not limited and CT values are measured in the exponential phase, this can be used to calculate an accurate amount of template present in the reaction. The amount of template present at the beginning of the amplification reaction determines the CT, and if the amount of template present at the beginning of the reaction is large, only few amplification cycles are required to accumulate enough amplified product to produce a detectable fluorescent signal above the background levels. In other words, more amplification cycles are required to produce fluorescent signal above back ground levels if the amount of template product is small at the beginning of the reaction; such a reaction would have late CT values. This relationship of amplification cycle and the amount of template present forms the basic principle of the quantitative aspect of real time PCR (Biorad, 2017; Biorad, 2006). I will now describe a PCR technique that was used to amplify complimentary DNA (cDNA) synthesised from RNA extracted from live *Legionella* cells.

4.1.2.7 Advantages and disadvantages of current qPCR diagnostic method

One of the most valuable techniques used in recent micro-biological research is nucleic acid amplification and detection (Brahmadathan, 2016). Scientists in many areas of research (such as micro-biology, biotechnology, basic science, medicine, diagnostics, forensic science etc.) rely on these techniques for a wide range of applications (Bustin, 2010). Qualitative nucleic acid detection is sufficient for some applications; however, many other applications demand a quantitative approach (Schoch et al., 2002). Current PCR methods used to identify *Legionella* species in water samples are specifically designed to detect DNA. However, a serious disadvantage of DNA based amplification methods is that they cannot differentiate between the viable and non-viable *Legionella* cells, which results in an overestimation of the presence of

viable *Legionella* bacteria in any sample under investigation (Cancino-Faure et al., 2016). This is because amplification of DNA occurs in both live and dead *Legionella* cells in the sample (Polo-López et al., 2017). Therefore, rapid detection of *Legionella* by qPCR is suitable for monitoring changes in *Legionella* concentration in a water system, but cannot be used for the determination of live *Legionella* bacteria in water samples (Díaz-Flores et al., 2015). Furthermore, risk assessment by qPCR diagnosis of *Legionella* in water systems without any background information (such as water treatment program and duration of treatment) may be inappropriate as false positives may occur due to the presence and detection of persisting dead cells left over from previous disinfection treatments. On the other hand, a low number of bacteria detected by qPCR is considered to be a strong indication for the absence of risk in terms *Legionella* bacteria (Díaz-Flores et al., 2015).

In the current qPCR diagnosis method for *Legionella* detection, there is no consensus on its application (e.g. how and when it should be applied) and there is no standardised interpretation used for reporting the results (Collins et al., 2017; Shih and Lin, 2006). Therefore, although the PCR diagnostic method is considered to be a useful technique for identifying the likely presence of *Legionella* species in water samples, it cannot be used for positive prediction. Despite this, it can effectively be used for negative prediction in routine water system monitoring, and to check the effectiveness of water treatment programs in place (Krojgaard et al., 2011; Tronel and Hartemann, 2009; Díaz-Flores et al., 2015). qPCR data is reported to practitioners in genomic units per litre (GU/L). However, a specific explanation of how these data relate to the conventional gold standard has still not been established (Whiley and Taylor, 2016). Indeed, the sensitivity of qPCR methods (a measure of the true positive detection rate) varied widely between laboratories (being in the range of 87.7-92.9%) and specificity (a measure of the true negative rate) was found to differ in the range of 77.3-96.5%. Furthermore, qPCR cut-off value for cooling tower samples was unable to determine due to the large variability in results. In contrast, qPCR cut-off value for hot water samples was determined, but the results obtained from each laboratory were significantly different (Joly et al., 2006).

In order to overcome the problem of overestimation of *Legionella* bacteria caused by detecting non-viable cells in clinical and environmental samples, a new qPCR diagnostic method was developed in recent years to detect viable cells by including

photoactivatable DNA intercalators; either propidium monoazide (PMA) or ethidium monoazide (EMA) (Ditommaso et al., 2015). EMA and PMA are capable of penetrating the membrane of damaged (unviable) cells and then bind to DNA molecules, thus inhibiting the amplification of DNA from dead cells. This results in the amplification of unbound DNA from viable cells (Zhong et al., 2016). This rapid detection method showed comparable specificity and sensitivity in detecting viable *Legionella* in water samples compared to current gold standard 'conventional culture analysis' (Li et al., 2015; Ditommaso et al., 2014). Compared to EMA, PMA has increased specificity for dead cells. Many studies have reported that this diagnostic method (qPCR combined with PMA) is highly successful in detecting viable *Legionella* in water samples compared to conventional culture analysis (Scaturro et al., 2016; Ditommaso et al., 2015). In contrast, a study reported that PMA concentrations in the samples should be high enough to compensate for other compounds present in environmental samples. This raises concerns due to the increased toxicity of PMA at higher concentrations. Thus, this study concluded that PMA cannot be regarded as an appropriate method for detecting viable *Legionella* cells in environmental water samples (Taylor et al., 2014). There are other remarkable disadvantages in the use of this method, as comparison of the results from different laboratories is complicated by the use of different sample concentrations, light exposure times and PMA exposure protocols. In addition, the efficiency of PMA varies depending on each experimental condition, which raises concerns about the reliability of the results (Bonetta et al., 2017).

Inhibitors found in the environmental samples can interfere with qPCR amplification reactions resulting in inaccurate results (Brooks et al., 2010). A study on environmental samples using qPCR techniques demonstrated that naturally occurring inhibitors are a major threat for qPCR analysis of environmental samples as between 0.3% to 71% of samples being incorrectly recorded as being negative depending on the water source. These reports illustrate the importance of measuring and addressing inhibition when reporting qPCR results used to monitor pathogenic micro-organisms present in environmental water samples (Gibson et al., 2012). This disadvantage can be addressed by measuring these inhibitors prior to analysis, as well as diluting the DNA extract a further 10-fold (McKee et al., 2015; Cao et al., 2012). A study conducted on 133 fresh water and marine water samples, used serial dilutions and 4 internal controls to address the identified inhibition. The frequency and magnitude of inhibition varied considerably depending on the qPCR method used, but assay performance was better

when using an Environmental Master Mix. However, a five-fold dilution using DNA/RNA free molecular grade water was also effective in reducing inhibition in about 78% of samples (Cao et al., 2012).

In summary, none of the current diagnostic procedures used for *Legionella* detection can reliably quantify *Legionella* in environmental samples, and this poses a serious problem in the effective monitoring and protection of public health. Therefore, more accurate, reliable and rapid diagnostic methods for the determination and quantification of *Legionella* bacteria in water systems are needed (Cristovam et al., 2017; Delgado-Viscogliosi et al., 2005).

4.2 Aim and objectives of this research

4.2.1 Aim

The aim of this research is to develop a method for the detection of viable *Legionella* and quantification using qPCR techniques. Distinguishing between viable and non-viable bacterial cells poses a significant challenge in microbiological diagnostics using qPCR (Alvarez et al., 2013). qPCR is a highly sensitive and quantitative method of detecting bacteria; but it does not distinguish between viable and non-viable bacteria, because, this method detects the organism's DNA (Chen and Chang, 2010). But, since DNA is persistent in the environment for a long period of time (and potentially even after cells have lost viability), DNA-based detection and quantification methods often over estimate the number of viable cells or provide false positive results in the absence of viable cells (Ditommaso et al., 2014). For example, in terms of *Legionella* control in domestic hot water systems, a qPCR test is more likely to show positive results even after the *Legionella* contaminated hot water system has been adequately disinfected due to the persistence of genomic material (DNA) from dead cells in the system (Nocker and Camper, 2006; Delgado-Viscogliosi et al., 2009).

However, RNA is only found in viable cells and, unlike DNA, quickly disappears from non-viable cells due to its short half-life period Cangelosi and Meschke, 2014). . RNA can be detected and quantified by PCR if it is first converted to complementary DNA (cDNA) using the enzyme reverse transcriptase (RT) (Xu et al., 2017; Wang et al., 2016). With this knowledge, this research aimed to extract total RNA from *Legionella pneumophila* species and convert it into cDNA, in order to detect and quantify the concentration of live *Legionella pneumophila* based on the amplification of the 23S gene. The longer-term aim of this research is to develop a laboratory-based assay that could be applied by UKAS-accredited laboratories to detect and quantify live *Legionella* in real environmental samples.

4.2.2 Objectives of this research

In order to establish a qPCR method to detect live *Legionella*, it was necessary to:

i) Validate a method to obtain live *Legionella*, and heat-killed *Legionella* samples.

ii) Validate RNA extraction and cDNA synthesis protocols on *Legionella*.

iii) Validate PCR primers/probes (used in DNA assay) on *Legionella* cDNA and a separate DNA positive control.

iv) Test whether viable/nonviable *Legionella* cells can be distinguished with RT-qPCR assay.

v) Establish a relationship between qPCR results and *Legionella* culture analysis results (cfu/L).

4.2.3 Exclusions of the research

In this PCR assay the 23S gene primers were developed to detect the *Legionella* genus, and were used to amplify cDNA from *Legionella pneumophila* alone. No other *Legionella* species were tested for validation of the primers/probes for cross reactivity. Testing and validation of this assay with environmental samples was not carried out.

4.3 General Methodology

4.3.1 MIQE guidelines

The PCR Assay developed in this chapter was conducted following the the Minimum Information required for the publication of qPCR Experiments (MIQE) guidelines formulated by Bustin and colleagues in 2009. MIQE guidelines describe the minimum information necessary for conducting and evaluating qPCR experiments with increased consistency and transparency. There are nine major components to consider, including: experimental design, sample preparation, nucleic acid extraction, reverse transcription, qPCR target information, qPCR oligonucleotides, qPCR protocol, qPCR validation and data analysis (Bustin et al., 2009).

4.3.2 Design of primers and probes

Taqman based primers and probes were used in this qPCR assay. The primers (short nucleic acid sequences) provide the starting point for DNA synthesis DNA primers are designed in the laboratory to bind to known sequences of the 5' and 3' strands of the target single stranded DNA, enabling DNA replication (and amplification) to occur (Smith and Osborn, 2009).

The design of primers and probes is one of the most important requirements for qPCR experiments. The probe design and chemistry are largely personal choices as there are a number of options to consider prior to their selection and design (Navarro et al., 2015). The probes and primers used in this assay were taken from the peer reviewed research article entitled 'Design and implementation of a protocol for the detection of *Legionella* in clinical and environmental samples' (Nazarian et al., 2008) and are listed in Table 4.1. Primer and probe sequences for the *Legionella pneumophila* specific 23S rRNA gene and the *Legionella pneumophila* specific TaqMan probe were designed and optimised using 6-carboxy-fluorescein (FAM) as the fluorescent reporter on the 5′ end, and the *Legionella* genus specific TaqMan probe was designed with VIC as the fluorescent reporter on its 5′ end. All probes were designed with 6-carboxytetramethylrhodamine (TAMRA) as the quencher dye on the 3′ end of the probe (Nazarian et al., 2008).

Table 4.1 Primers and probes used in this qPCR assay

Primer or probe	Nucleotide sequence (5′-3′)	Gene detected
Leg23SF forward	CCCATGAAGCCCGTTGAA	23S rRNA (92 bp)
Leg23SR reverse	ACAATCAGCCAATTAGTACGAGTTAGC	
Lsp23SP VIC-TAMRA	TCCACACCTCGCCTATCAACGTCGTAGT	

Leg23SF forward, product code: SY150613449-096, Leg23SR reverse, product code: SY150613448-091 and Lsp23SP VIC-TAMRA, product code: HA10101976-002 was purchased from SIGMA ALDRICH UK and Taqman Environmental Master Mix 2.0 was purchased from Fisher Scientific UK Ltd.

4.3.2.1 Assay targeting 23S rRNA gene

Legionnaires' disease (LD) are predominantly caused by the *Legionella* species *Legionella pneumophila*, although an increasing number of other *Legionella* species have been reported to cause human diseases (Refer Chapter 1 section 1.3). There are no specific clinical presentations to LD; therefore accurate diagnosis of the causative species is necessary. The 23S rRNA gene is common to all prokaryote species. Although the primers of this qPCR assay are designed specifically to target 23S rRNA gene of the *Legionella genus,* they were only tested here on *Legionella pneumophila* (Llewellyn et al., 2017).

4.3.2.2 TaqMan assays

TaqMan assays (also known as the 5'-nuclease assay) employ sequence-specific primers as well as a sequence-specific fluorescently labelled oligonucleotide probe known as the TaqMan probe. This probe consists of a fluorescent reporter (fluorophore) at the 5' end and a quencher at the 3' end (Taqman, 2017). Taq polymerase was originally extracted from bacteria that tolerate (i.e. replicate) in high temperatures of 75–80°C. In PCR, the ability of the Taq polymerase enzyme to withstand high temperatures without denaturing is critical due to the requirement of high temperatures for denaturing (or separating) the dsDNA prior to copying. Same as

in any other PCR, TaqMan also requires a DNA template, a polymerase, two primers (5' and 3') that are specific to the region being amplified, and a unique, sequence-specific probe.

Fluorophores are small fluorescent molecules attached to oligonucleotides in order to function as probes in qPCR technology. There are two types of fluorophores known as reporter (donor) and quencher (acceptor). When a reporter fluorophore absorbs energy from light sources, it attains an excited state and the emission of energy from fluorophore as fluorescence causes the returning of the same to the ground state. This emitted light from the reporter has a lower energy, lower frequency and a longer wavelength than the absorbed light. This light can be transferred to an acceptor fluorophore. If both fluorophores are within a specific distance (10 to 100Å), the transfer of excited-state energy from a reporter to a quencher is described as Fluorescence Resonance Energy Transfer (FRET). There are two different FRET mechanisms, (i) FRET-quenching; in this mechanism the electronic energy of the quencher is dissipated as heat because the acceptor molecule is non-fluorescent, and (ii) FRET mechanism; the transferred energy is emitted as fluorescence because the acceptor molecule is fluorescent (Navarro et al., 2015).

During the PCR reaction, Taq polymerase moves towards the template strand and adds nucleotides to the 3' end from the primers to produce a complementary strand of DNA. The probe cannot be extended by Taq polymerase as Taq polymerase does not contain a free hydroxyl. In this instance, the reporter dye on the 5' end of the probe generates fluorescent light proportional to the amount of DNA produced and the 3' end of the probe is a quencher. While the probe is intact, the reporter dye is in very close proximity to the quencher. If both fluorophores are within a specific distance (10 to 100Å), the transfer of excited-state energy from the reporter to the quencher occurs, and is known as Fluorescence Resonance Energy Transfer (FRET). In this way the fluorescence emitted by the reporter on the probe in quenched due to the energy transfer by fluorescence resonance. However, when Taq reaches the fluorescent probe, Taq partially displaces and cleaves the probe by removing it from the DNA template in addition to its polymerase activity. This causes separation of the reporter from the quencher, thereby generating a permanent increase in fluorescence correlating with DNA doubling (Figure 4.3). Separation of the reporter from the quencher and cleavage of the probe from the target allows primer extension to continue to the end of the template strand. Therefore, the probe does not inhibit the overall PCR process. As the

reporter dye molecules are cleaved from their respective probes with each PCR cycle, an increase in fluorescence intensity proportional to the amount of amplicon produced is observed (Taqman, 2017).

The major advantages of TaqMan chemistry are the requirement for specific hybridization between probe and target in order to generate a fluorescent signal. The probes can be labelled with different reporter dyes allowing amplification and detection of two distinct sequences in one reaction tube and elimination of post-PCR processing reduces assay labour and material costs. However, synthesis of different probes is required when amplifying distinct sequences in the same reaction (Taqman, 2017).

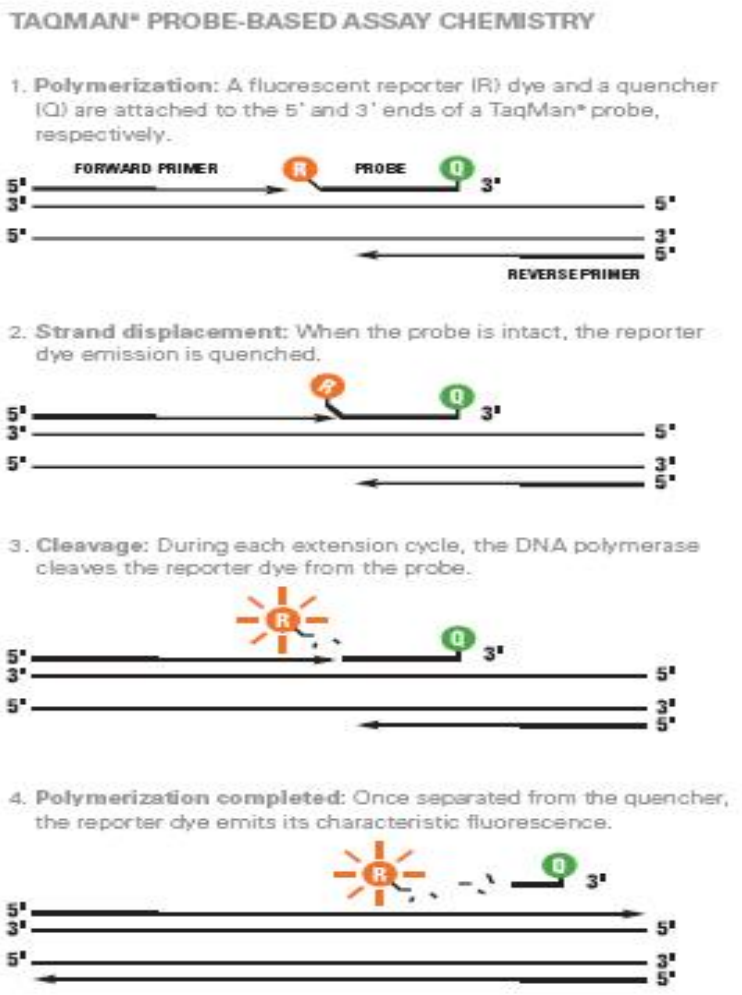


Figure. 4.3 1. An oligonucleotide probe is constructed containing a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET). 2. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. 3. This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal, and removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. 4. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

(Source: https://www.biosyn.com/tew/taqman-vs-sybr-green-chemistries.aspx).

4.3.2.3 Primers/probes validation

A known amount of *Legionella* DNA standard supplied by Pall GeneDisc Technologies (France), was used to prepare five serial dilutions that were amplified (targeting 23S rRNA gene) to generate the standard curve for the validation of primers and probes used in the assay (see 4.3.3).

4.3.3 Positive control

The inclusion of a positive control in the qPCR is necessary to ensure that the assay is performing optimally, thereby eliminating the chance of false negatives. Commonly used positive controls are external DNA or RNA carrying a target sequence of interest. If these positive controls are assayed in separate PCR wells from the experimental samples, these external controls determine whether the RT or PCR reaction conditions are optimal or not. Furthermore, positive controls can be used to assess whether the experimental samples contain inhibitors that affect reverse transcription or PCR reactions (Qiagen, 2017; Nolan et al., 2013).

4.3.3.1 Preparation of standard solutions for positive control

The positive control in this qPCR assay contained a known amount of *Legionella* pneumophila DNA (equivalent to 250,000 GU in 6 µl– which is the volume used in the Gene Disc assay) supplied by Pall GeneDisc Technologies, France. The initial standard was serially diluted a further four times in 10-fold steps and were amplified alongside

four 10-fold dilutions of cDNA synthesised from RNA extracted from a *Legionella* lenticule (See 4.3.16).

In brief, the 2ml micro-tube containing diluent was thawed at room temperature and mixed by vortexing for five seconds. The micro-tube containing frozen dehydrated standard *Legionella* pneumophila DNA was centrifuged for five seconds followed by the addition of 200 µl of diluent. Without shaking or vortexing, the standard *Legionella* pneumophila DNA in micro-tube with diluent was allowed to stand for a minimum of 60 minutes at 5°C ± 3°C. This micro-tube containing 250,000 GU/6 µl was labelled as 5. Another four 1.5 ml micro-tubes were prepared and labelled as 1, 2, 3 and 4. 180 µl of diluent was then dispensed into each empty micro-tubes. Micro-tube 5 (containing 250,000 GU/6 µl) was vortexed for 5 seconds, then briefly centrifuged after which 20 µl of *Legionella* pneumophila DNA was pipetted from '5' into micro-tube '4'. The micro-tube '4' was vortexed for 5 seconds and centrifuged shortly. 20 µl of *Legionella* pneumophila DNA from '4' was pipetted into micro-tube '3' followed by five seconds vortexing and short centrifugation. Again 20 µl of *Legionella* pneumophila DNA from '3' was pipetted into micro-tube '2'; vortexed for 5 seconds and centrifuged shortly. Finally, 20 µl of *Legionella* pneumophila DNA from '2' was pipetted into micro-tube '1' followed by five seconds' vortexing and short centrifugation. Thus, the 10-fold dilution series of five standard *Legionella pneumophila* DNA samples was prepared. The GU present in each 6µl volume are as follows:

1- 25 GU (Log value -0.00001)

2- 250 GU (Log value -0.0001)

3- 2500 GU (Log value -0.001)

4- 25000 GU (Log value – 0.01)

5- 250000 GU (Log value – 0)

The Genomic Units (GU) correspond to the final amount of DNA in each PCR well based on a volume of 6µl used in the PALL GENE DISC system. These standard DNA solutions can be stored up to 72 hours at 5 ˚C ± 3 ˚C. The standard dilution series were amplified in triplicate to generate the PCR data to calculate the slope and qPCR assay efficiency.

4.3.4 *Legionella* lenticules

Legionella pneumophila bacteria used in all experiments were extracted from *Legionella* lenticules. Each lenticule contains bacteria from a pure culture preserved in a tablet format (LENTICULE disc) with a self-indicating silica gel desiccant. Manufacturing and testing of the bulk reference material was done by Public Health England (PHE). Preparation and retesting of the final product ready to use for customers (which includes filling, packaging and distribution) was done by Sigma-Aldrich (12821 *Legionella pneumophila* Lenticule discs, 500-50,000cfu, product code: 101768252). On receipt, the mylar bag containing the plastic vials with the lenticule discs was stored unopened at -20^0C.

4.3.4.1 Handling *Legionella* lenticules.

The plastic vials containing the lenticule discs were removed from the freezer, and left for 10 minutes to reach ambient temperatures (15-20°C).Lenticule discs must not be refrozen and must be used within one hour after reaching ambient temperature. Ringers solution was prepared by dissolving Sodium Chloride (NaCl - 7.2 gram)Potassium Chloride (KCl – 0.37 gram) and Calcium Chloride ($CaCl_2$ - 0.17 gram) into 1 litre reagent grade water and adjusting the pH between 7.3 – 7.4 using Sodium Hydroxide (NaOH) solution. This solution was filtered through a 0.22µm filter and autoclaved for sterilisation. The bacterial culture medium was prepared by adding 10µl of Ringers solution to 390 µl of deionised water in a 2ml sterile plastic tube and one lenticule was added directly to the solution by capping and inverting the tube, after which it was allowed to stand for 10 minutes at room temperature (15-20^0C) to ensure the lenticule had dissolved completely. After 10 minutes, the bacterial culture was vortexed three times for 30 seconds and left at room temperature for five minutes to allow any air bubbles to disperse. A short spin was carried out in centrifuge to get rid of any remaining air bubbles that had formed during vortexing.

4.3.5 *Legionella* culture

A total 6 *Legionella* lenticules were used to prepare 6 solutions (1 lenticule dissolved in 400 µL Ringers solution) of *Legionella pneumophila*. Three of the solutions (Triplicate) were heated to 80°C on a Dri-block (hot plate) for one hour and then kept for 2 hours at room temperature (22°C). These heat killed *Legionella* solutions, along with the three untreated samples, then were made up to 500 ml with deionised water for culture analysis. All the samples were analysed at a UKAS accredited laboratory by culture on BCYE agar (Ref. Chapter 3, section 3.2.4).

4.3.6 General precautionary measures

RNases are very active and generally stable enzymes that do not require cofactors to function, and small amounts are sufficient to destroy RNA (Moelling and Broecker, 2015)). Therefore, it was extremely important to eliminate any possible RNase contamination from plasticware. Care is also needed to avoid introducing RNases into the RNA sample during purification procedures. The following precautionary measures were employed to create and maintain an RNase-free environment whilst working with RNA. Dust particles, bacteria and moulds (being the most common sources of RNase contamination) was minimised by ensuring that the laboratory space was thoroughly cleaned before and after use; vinyl gloves were used while handling reagents and RNA samples to prevent RNase contamination from hands as well as from dusty laboratory equipment. Gloves were changed frequently and all the sample and reagent tubes were closed whenever possible in order to avoid contamination. The purified RNA was kept on ice for downstream applications.

In order to remove RNase contamination from laboratory equipment, such as Gilson pipets, a commercially available RNase removal solution was used. These were thoroughly rinsed with 0.1 M NaOH, 1 mM Ethylenediaminetetraacetic acid (EDTA) followed by RNase-free water to ensure that they were free from RNase contamination (Lever et al., 2015; Farrell, 2012). Pre-treated sterile, disposable polypropylene tubes were used because no pre-treatment was required for these tubes to inactivate RNase. Virkon solution(Dupond) was prepared by dissolving 1 tablet in 500ml of tap water to be used as the disinfectant during the entire procedure in all experiments. All the steps up to RNA extraction were carried out in the Biological Safety Cabinet (BSC) as *Legionella* bacteria was considered inactive only after the lysis step. A FFP3-face fit tested mask was used for work with *Legionella*.

Six different experiments were carried out during this research. The protocol employed during RNA extraction and DNA extractions are same in all eight experiments. However, two different kits were used for cDNA synthesis and three different protocols were followed in genomic DNA elimination.

4.3.7 RNA stabilisation

Additional materials required to carry out this protocol, included RNAprotect Bacteria Reagent and RNeasy Protect Bacteria Kits, Ringers solution, dry hot plate and face fit masks. Two volumes of RNAprotect Bacteria Reagent was added to one volume of bacteria culture (i.e. 800µl of RNAprotect Bacteria Reagent was pipetted into 2ml tube containing 400µl of the bacterial culture). The volume of the tube must be 4-times that of the bacterial culture. After addition of RNAprotect Bacteria Reagent to the bacterial culture, the tube was capped and mixed immediately by vortexing for 5 seconds, and then allowed to stand for 5 minutes at room temperature (15–25°C) to achieve immediate stabilization of RNA. After five minutes, the bacterial culture was centrifuged for 10 minutes at 5000 x g. After centrifugation, the pellet may not be easily visible due to an interaction between the cells and the stabilization reagent that causes a change in the optical density of the cells. The supernatant was decanted, and the remaining liquid was removed by gently dabbing the inverted tube onto a paper towel. Any remaining supernatant was not removed by pipetting, as this may lead to loss of the bacterial pellet. Pellets can be stored at –15 to –30°C for up to 2 weeks or at –70°C for up to 4 weeks if needed. The pellets were thawed at room temperature (15–25°C), in readiness for RNA extraction and purification.

4.3.7.1 RNA extraction

This protocol involves enzymatic lysis followed by mechanical disruption recommended for Gram-negative bacteria and easy-to-disrupt Gram-positive bacteria grown in minimal media.

Equipment and reagents required for this protocol included Lysozyme, Tris and EDTA for preparing TE buffer, TissueLyser system (TissueLyser II, QIAGEN), acid washed glass beads, 2 ml Safe-Lock tubes, RNeasy Kits, β-mercaptoethanol (β -ME) (stock

solutions are usually 14.3 M), RNeasy Mini Kit, RNeasy Midi Kit and RNeasy Protect Bacteria Midi Kit (QIAGEN), Ethanol (96–100%) and ethanol (80%).

1. 10µl β-mercaptoethanol was added per 1ml Buffer RLT from RNeasy Protect Bacteria Midi Kit, and mixed gently. Buffer RLT is stable for 1 month after the addition of β-mercaptoethanol. However, the protocol recommends that you only make up what is required for immediate use. 350µl was required per sample in this experiment.
2. 100µl of TE buffer prepared by mixing 10mM TrisCl, 1mM EDTA and adjusted the pH to 8.0 using NaOH solution) containing 15 mg/ml lysozyme was required per sample.
3. 25–50mg acid-washed glass beads (150–600µm diameter) were required per sample. These were weighed out in a 2 ml Safe-Lock tube for use in step 7.

Freshly prepared of previously thawed bacterial pellets (see '4.3.7 RNA stabilisation') were prepared for use in step 4.

4. 100µl of TE buffer containing lysozyme was added to the sample and mixed by vortexing for 10 seconds.
5. The sample was incubated at room temperature (15–25°C) for 10 minutes. During incubation, the sample was vortexed for 10 seconds every 2 minutes. (Vortexing can be avoided if the incubation can be carried out on a shaker–incubator).

   Note: Since the RNA is stabilized, the incubation time can be extended without affecting the procedure, and may increase the RNA yield.
6. After the incubation at room temperature for 10 minutes, 350µl of Buffer RLT was added to the sample and vortexed vigorously for 5–10 seconds.

Note: It is important to ensure that β-meracaptoethanol is added to Buffer RLT before use and the pellet is thoroughly re-suspended in Buffer RLT.

7. The suspension was transferred into a 2 ml Safe-Lock tube containing the acid washed glass beads prepared in step 2 and the cells were disrupted in the TissueLyser for 5 minutes at maximum speed.

8. After the completion of step 7, the samples were centrifuged for 10 seconds at maximum speed and the supernatants were transferred into new 2 ml tubes.

9. 220µl of RNA free, 96–100% ethanol was added to the supernatant in each 2 ml tube, and mixed gently by pipetting. (Centrifugation is not recommended at this stage).

   Note: After the addition of ethanol, a precipitate may form but this will not affect the RNeasy procedure.

The following steps were completed before starting with the main protocol of RNA Purification Procedure:

a. Buffer RPE was supplied as a concentrate (RNeasy Protect Bacteria Midi Kit) and 4 volumes of ethanol (96–100%) was added before using it for the first time, as indicated on the bottle, to obtain a working solution.

b. DNase I stock solution was prepared before using the RNase-Free DNase Set for the first time. The solid DNase I (1500 Kunitz units) was dissolved in 550µl of RNase-free water provided. In order to avoid loss of DNase I RNase-free water was injected directly into the stock vial using an RNase-free needle and syringe. The solution was mixed gently by inverting the vial as vortexing is not recommended at this stage.

c. In order to achieve long-term storage of DNase I (for up to 9 months), the stock solution was removed from the glass vial, and divided into single-use aliquots (10µl), and stored at –15 to –30°C. Thawed aliquots can be stored at 2–8°C for up to 6 weeks, however, refreezing of the aliquots after thawing is not recommended.

4.3.7.2 RNA purification

1. The lysate from step 9, including any precipitate that may have formed, was transferred into an RNeasy Mini spin column placed in a 2 ml collection tube supplied with the kit. The lid was closed gently, and centrifuged for 15 seconds at 8000 x g. After the centrifugation, the flow-through was discarded and the collection tube was reused in step 2. In the event of lysate exceeding 700 µl, centrifugation of successive aliquots through the spin column is recommended. The flow-through should be discarded after each centrifugation.

2. 10µl of DNase was added to 70µl Buffer RDD (supplied in the kit) for each sample, i.e., 10µl DNase from DNase I stock and 70µl Buffer RDD makes 80µl solution. Aliquots of frozen DNase from the DNase I stock (see above) were thawed until became clear. A short centrifuge was carried out before mixing with Buffer RDD. The solution was mixed gently by inverting the tube, and centrifuged briefly to collect residual liquid from the sides of the tube.

3. 80µl of DNase I was added to the sample directly to the RNeasy spin column membrane, and incubated at room temperature (20–30°C) for 15 minutes.

   It is important to ensure to add the DNase I incubation mix directly to the RNeasy spin column membrane as DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

4. After incubation, 350µl of Buffer RW1(from the kit) was added to the RNeasy spin column, and then centrifuged for 15 seconds at 8000 x g. The flow-through was discarded along with the collection tube.

6. RNeasy Mini spin column was placed in a new 2 ml collection tube (supplied) and 500µl Buffer RPE was then added to the RNeasy Mini spin column. The lid was gently closed before centrifuging for 15 seconds at 8000 x g to wash the spin column membrane. The flow-through was discarded, and the collection tube reused.

7. A second 500µl aliquot of Buffer RPE was added to the RNeasy Mini spin column. The lid was gently closed before centrifuging for 2 minutes at 8000 x g to wash the spin column membrane.
   This long centrifugation ensures that no residual ethanol is carried over during elution as this may interfere with downstream reactions. After centrifugation, the RNeasy Mini spin column was carefully removed from the collection tube which was discarded avoiding touching the elute, otherwise, carryover of ethanol may occur.

8. The spin column was placed in a new 2 ml collection tube, and centrifuged at full speed for 1 minute.

9. The RNeasy Mini spin column was then placed in a new 1.5 ml collection tube

(supplied) and 30µl RNase-free water was added directly to the spin column membrane. The lid was closed gently, and centrifuged for 1 minute at 8000 x g to elute the RNA (Eluted RNA can be kept at -80°C).

4.3.8 DNA extraction

DNA extraction in this research was carried out as follows using QIAamp DNA extraction MiniKit (50), Ref. No. 5130.

1. The live bacterial culture (Sample A) and heat killed Legionella (Sample B) were centrifuged for 10 minutes at 5000 x g and the supernatant was decanted. Any remaining supernatant was removed by gently dabbing the inverted tube once onto a paper towel. The bacterial pellets were proceeded with DNA extraction and purification.

2. The bacterial pellet was re-suspended in 180µl ATL buffer, supplied with the QIAamp DNA Mini Kit (50).

3. 20µl of proteinase K was added to the sample, mixed by vortexing, and incubated at 56°C (water bath) for 3 hours until the tissue is completely lysed. The samples were occasionally vortexed during incubation to disperse the sample.

   Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of ATL Buffer. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 hrs. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

4. After lysis, the samples were briefly centrifuged to remove any drops from the inside of the lid.

5. 200µl Buffer AL was added to the sample, mixed by pulse-vortexing for 15 seconds, and incubated at 70°C for 10 minutes. The 1.5 ml micro-centrifuge

tube was briefly centrifuged to remove drops from inside the lid.

Note: It is essential that the sample and AL Buffer are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of AL Buffer, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

6. 200µl ethanol (96–100%) was added to the sample and mixed by pulse-vortexing for 15 seconds followed by brief centrifugation of the 1.5 ml micro-centrifuge tube to remove the drops from inside the lid.

Note: It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application. Do not use alcohols other than ethanol since this may result in reduced yields.

7. The mixture from step 6 (including the precipitate) was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed and the tubes were centrifuged at 6000 x g for 1 minute. The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded. Each spin column was closed to avoid aerosol formation during centrifugation.

Note: It is essential to apply all of the precipitate to the QIAamp Mini spin column. Centrifugation is performed at 6000 x g to reduce noise, and centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

8. The QIAamp Mini spin column was carefully opened and added 500µl Buffer AW1 without wetting the rim, closed the cap and centrifuged at 6000 x g for 1 minute. The QIAamp Mini spin column was placed again in a clean 2ml

collection tube (provided), and discarded the collection tube containing the filtrate.

9. The QIAamp Mini spin column was carefully opened and 500µl of AW2 Buffer was added without wetting the rim. The cap was closed and the tube was centrifuged at full speed (16,000 x g) for 3 minutes.

10. The QIAamp Mini spin column was placed in a new 2 ml collection tube (not provided with the kit) and the old collection tube with the filtrate was discarded. The tube was centrifuged at full speed (16,000 x g) for 1 minute to eliminate the chance of possible AW2 Buffer carryover.

11. The QIAamp Mini spin column was placed in a clean 1.5 ml micro-centrifuge tube (not provided with the kit), and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 200µl of AE buffer was added, after which the tube was incubated at room temperature for 1 minute, followed by centrifugation at 6000 x g for 1 minute.

12. The resulting DNA (pellet) was stored in the freezer at -20°C.

The DNA pellets were not diluted for PCR amplification. All other procedures for PCR amplification were as described in Experiment 1, including master mix preparation. The cDNA synthesised from RNA extracted from completely heat killed Legionella lenticule (plus any residual genomic DNA in the sample; if dead, there should theoretically not be any cDNA) was also amplified with DNA extracted from full live lenticule and completely heat killed lenticule for comparative study purpose.

In this experiment, a different protocol was tested for genomic DNA elimination in Sample C, but cDNA synthesis was undertaken as described in Experiment 4.

4.3.9 RNA quantification

4.3.9.1 Gel electrophoresis

The overall quality of extracted RNA including RNA yield may be assessed by electrophoresis on a denaturing agarose gel (Ream et al., 2016). In this qPCR assay, gel electrophoresis experiment was conducted as follows:

0.5g of agarose powder was weighed in a paper plate and mixed with 50ml of Tris-borate-EDTA (TBE) buffer and added RNase-free water to make up the solution to100 ml in a 200ml conical flask. The mixture was heated in a microwave by swirling the flask occasionally until the agarose is completely dissolved in TBE. The clear agarose solution was cooled to 65–70°C in a water bath and 3µl of GelRed Nucleic Acid Stain was added and gently mixed by swirling. Once mixed, the whole mixture was poured into a gel tray to prepare a gel with dimensions 10 x 14 x 0.7 cm. A comb was inserted into the gel immediately after pouring the gel into the tray by ensuring that there is enough space between the bottom of the comb and the gel tray (0.5–1.0 mm) to allow proper well formation and avoid sample leakage into the gel tray. The gel was allowed to set for 30 minutes whilst ensuring that there was no air bubbles trapped in the gel or between the wells.

An electrophoresis tank was cleaned with detergent solution, thoroughly rinsed with RNase-free water followed by a gentle rinse with ethanol and allowed to dry. The gel and comb were placed in the electrophoresis tank which was filled with TBE gel running buffer, ensuring approximately 1 mm of liquid above the surface of the gel. The comb was then removed carefully from the gel which was left to equilibrate for 30 minutes. 5µl of RNA loading buffer was added to 3µl of each RNA sample followed by a short centrifugation. The sample was then heated at 72°C for 2 minutes in a water bath and directly kept on ice. The heat killed samples were applied to the wells of the gel in the electrophoresis tank by inserting the pipette tip deep into the well and slowly expelled the liquid. The electrodes were connected and the power supply turned on (100 V). The gel was run for 25 minutes, after which it was placed under a UV light source in a BioDoc-It Imaging System. The GelRed in the gel allows visualization of the RNA with UV light.

4.3.9.2 Nanodrop concentration check

RNA yield from each dilution (Refer section 4.4.11) was also checked using a Nanodrop One spectrophotometer (Thermofisher, 2017) to ensure that the RNA extraction procedure was successful and the concentration of RNA (in ng/µl) was recorded. The samples do not need to be diluted before measurement due to the wide range detection

with small sample volume, and reading can be taken in a single measurement in less than 30 seconds. No other reagents or accessories are required for this measurement (Wieczorek et al., 2012). Blanking of the machine was carried out using 1 µl of blanking solution (provided by the manufacturer) after which1 µl of each purified and undiluted RNA sample (prepared from 1, ½ , ¼ and 1/8 lenticule) was dispensed directly on to the Nanodrop sensor .

4.3.10 gDNA elimination and cDNA synthesis (various approaches)

4.3.10.1 Using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR.

gDNA elimination and cDNA synthesis was carried out using ThermoFisher SuperScript III First-Strand Synthesis SuperMix for qRT-PCR, product number: 11752050. The following protocol has been optimized for generating first-strand cDNA for use in two-step qRT-PCR. An incubation temperature of 50°C for 30 minutes is recommended as a general starting point; higher temperatures (up to 60°C) may be used for difficult templates.

1. Two tubes of 0.2ml were prepared; One for the sample with Reverse Transcription (RT) and the other for the same sample without RT.

Table 4.2 Reagents used for RT and NRT samples.

	Samples with RT (RT)	**Samples without RT(NRT)**
RT Reaction Mix	10 µl	10 µl
RT Enzyme Mix	2 µl	-
RNA	8 µl	8 µl
DEPC-treated water	-	2 µl

2. The tube contents were gently mixed by flicking followed by a short centrifuge.

The following procedures were carried out in a PCR machine.

3. The samples were incubated at 25°C for 10 minutes to allow the reverse transcriptase to convert RNA to cDNA in the sample and then the temperature was raised to 50°C for 30 minutes as the enzyme is more active at 50°C and can provide higher specificity with higher yield of full length cDNA (up to 12 kb in length).
4. The reaction was terminated by heating at 85°C for 5 minutes to heat-inactivate the reverse transcriptase, and then chilled on ice.
5. 1µl of *E. coli* RNase H was added to each sample and incubated at 37°C for 20

minutes. (*E. coli* RNase H is an endoribonuclease which degrades the RNA strand of RNA/DNA hybrid molecules and produces ribonucleotide molecules with 5-phosphate and 3-hydroxyl termini. RNAse H is inactive against single or double-stranded RNA molecules).

The synthesised cDNA was then diluted in the ratio of 18µl deionised water (DW) and 2µl cDNA followed by a short centrifuge. Diluted cDNA was stored at –20°C until use.

4.3.10.2 Using QIAGEN DNA elimination kit

The genomic DNA elimination reaction was carried out on ice as follows using QIAGEN RNeasy Plus Micro Kit (50), product No. 74034. gDNA Wipeout Buffer (2µl) and Template RNA from viable or heat killed lenticules were (12µl) mixed in a 0.2ml tube, incubated for 2 minutes at 42°C, and then placed immediately on ice. Reverse transcription master mix was prepared on ice as follows:

Table 4.3 QIAGEN genomic DNA elimination reaction mix followed by cDNA synthesis with RT to look at cDNA yield and without RT to look at effectiveness of genomic DNA removal. Sample A is heat killed; whereas Sample B is live (untreated).

Component	Sample B, with RT(µl)	Sample B, without RT (µl)	Sample A, with RT(µl)	Sample A, without RT (µl)
RT Master mix	1	-	1	-
RNase free water	-	1	-	1
RT Buffer	4	4	4	4
RT Primer Mix	1	1	1	1
Template RNA (live or heat killed)	14	14	14	14

All the samples were incubated in a PCR machine, 15 minutes at 42°C followed by 3 minutes at 95°C and chilled on ice. Synthesised cDNA was diluted in the ratio of 18µl DW and 2µl cDNA followed by a short centrifugation and kept at -20°C.

4.3.10.3 Using amplification grade DNaseI

Amplification Grade DNaseI, product code AMP-D1 supplied by Sigma Aldrich UK is used for gDNA contamination removal. Amplification Grade DNase I (Deoxyribonuclease I) is an endonuclease isolated from bovine pancreas that digests double- and single-stranded DNA into oligo- and mono-nucleotides and this kit is suitable for eliminating DNA from RNA preparations prior to RT-PCR (DNASE1, Sigma Aldrich).

1µl of DNAseI and 3.5µl RNAse free buffer (Sigma-Aldrich) were added to 30µl RNA and incubated 10 minutes at 37°C in a water bath. After the incubation, 3.5µl of 3M (mol) Sodium Acetate, 100 µl ice cold 96-100% ethanol and 1µl GlycoBlue was added to the sample and kept at -20°C overnight for 14 hours. The sample was then centrifuged at 4°C for 30 minutes at 12000g and resultant pellets were washed with 100µl ice cold 70% ethanol and centrifuged again for15 minutes at 4°C and 16000g. The supernatant was removed and the pellets were air dried for approximately 10 minutes. The pellets were then dissolved in 25µl RNAse free water ready for cDNA synthesis as described in in Experiment 4.

4.3.11 TaqMan mastermix preparation

TaqMan Master Mix was prepared using the kits (TaqMan Environmental Master Mix 2.0, Ref. No. 4396838 supplied by Life Technologies, UK, Probes SF (Product code: SY150613449-096), SR (Product code: SY150613448-091) and SP (Product code: HA10101976-002) are supplied by Sigma Aldrich) in the following basic ratio:

10µl TaqMan

0.5µl Probe SF

0.5µl Probe SR

0.2µl Probe SP

6.8µl Deionised Water (DW)

Samples were prepared in triplicate; therefore, the volume of master mix was prepared for 8 PCR wells (3 for RT, 3 for no RT, 1 for NTC and 1 extra as a precautionary measure to avoid shortage due to the possible loss during pipetting and well filling).

Taqman - 80µl

Probe F - 4.0µl

Probe R - 4.0µl

Probe P - 1.6µl

DW - 54.4µl

4.3.12 qPCR amplification methods

PCR plate wells were filled with 2µl of diluted cDNA sample and 18µl of Taqman master mix. One well was filled with 2µl deionised water and 18µl TaqMan master mix as no template control (NTC) to look for general reagent contamination. One sample was with Reverse Transcription (RTcDNA) and the other was without RT (purified RNA prior to cDNA synthesis) to look for genomic DNA contamination. After filling the required number of PCR wells, the whole plate was sealed and spun downed to eliminate air bubbles and all the samples were amplified in triplicate.

4.3.13 qPCR assay conditions

This qPCR assay was performed with primers and probes targeting the *Legionella pneumophila* 23S gene, and both primers and probes were optimised to a Taqman assay. Bio-Rad CFX96™ real-time PCR detection system was used to amplify the cDNA, Hard-Shell 96-Well Semi-Skirted PCR Plates and seals used were manufactured and supplied by Biorad Systems. The PCR wells are filled with samples (Refer section 5.3.4) containing primers, probes, TaqMan environmental master mix and diluted cDNA (2µL) template with RT reaction mix for a total volume of 20 µL. 2µL of cDNA from the total cDNA yield (30 µL) was diluted with 18µL of RNA/ DNA free molecular grade water

supplied by Sigma Aldrich. For the positive control, a known amount of *Legionella pneumophila* DNA standard was added to tubes containing the same amount of primers, probes, and TaqMan environmental master mix. In order to check for genomic DNA contamination, NRT samples containing the same amount of primers, probes, and TaqMan environmental master mix, but without RT reaction mix were also included. Differences in volumes in the various samples and controls were adjusted with RNA/ DNA free molecular grade water as necessary. The thermal cycling process consisted of 95 °C for 10 minutes as an initial incubation, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute representing standard TaqMan conditions (Nazarian et al., 2008).

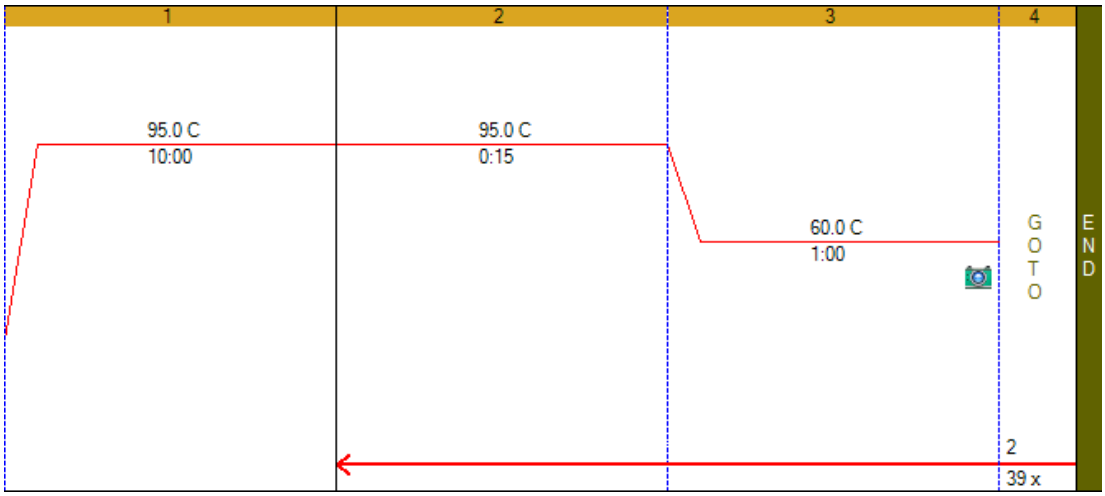


Figure. 4.4 Representation of the overall amplification reaction employed in this assay.

4.3.14 Inhibition monitoring

Accurate qPCR results depend on a variety of factors including the quality of the reagents, experiment design, sample quality, and the presence of inhibitors. High protein levels and other chemical contaminants that persist after cDNA preparation can cause PCR inhibition. Inhibition in a qPCR assay can be monitored by performing a serial dilution of synthesised cDNA templates (Svec et al., 2015). In this assay, qPCR data from four dilutions of synthesised cDNA were used to generate inhibition plots. The cDNA samples were amplified in triplicate and were assayed for inhibition and the average of proportionate amplification (C_T) values used to generate the inhibition plot.

4.3.14.1 qPCR assay optimisation

The efficiency of a PCR assay can be defined as the fraction of target molecules that are copied in a single PCR cycle (Svec et al., 2015). A 100% efficient qPCR assay is expected to yield a 10-fold increase in PCR amplicon in every 3.32 cycles during the exponential phase of amplification (log2 10 = 3.3219). The slope of a standard curve of qPCR amplification is commonly used to calculate the efficiency of a qPCR reaction. A qPCR standard curve is graphically represented as a semi-log regression line plot of CT value against log of input nucleic acid. A slope of the standard curve –3.32 demonstrate that the PCR reaction efficiency is 100%. Slopes more negative than –3.32; e.g. –3.8 indicate that the PCR reaction efficiency is less than 100% and slopes more positive than –3.32; e.g. –2.7 indicate possible pipetting errors (Nolan et al., 2013).

In this qPCR assay, a known amount of *Legionella* DNA standard (between 250,000 GU and 25 GU in 6 ul) was used to calculate the efficiency (E) of the qPCR reaction using the formula E = (10 –1/slope –1) × 100.

4.3.14.2 Assay limitations

Legionella pneumophila is classified as a Hazard Group 2 organism, which can cause human disease and may be a hazard to laboratory workers as well as other people entering the laboratory. I was therefore instructed to conduct my *Legionella* research in only one laboratory following approval of the risk assessment by the university. RNA extraction, purification and qPCR process were therefore conducted in the same laboratory, resulting in low levels of genomic DNA contamination. However, by taking maximum possible precautions, this contamination was minimised, and I was successful in eliminating this contamination in few of the experiments I conducted at the end of this research project. There are slight variations in amplification (C_T values) of cDNA in some of the experiments with the same concentration of bacterial culture; this variation is likely to be caused by pipetting errors of small volumes. In addition, validation of this assay with environmental samples was not possible due to time constraints.

4.3.15 Result interpretation in this assay

In order to make data interpretation and analysis more understandable, scatter plot is produced in each experiment using either number of cycles and fluorescence from the amplification reaction or CT values and percentage of dilution.

4.3.16 Method development

4.3.16.1 Experiment 1

In this experiment, RNA was extracted from a *Legionella* lenticules using Qiagen RNeasy Mini Kit (50) (Refer 4.3.7 - 4.3.7.2), Ref. No. 74104, QIAGEN GmbH, Germany and cDNA was synthesised from purified RNA using Invitrogen superscript cDNA synthesis kit supplied by Thermofisher Scientific, UK (Refer 4.3.10.1). TaqMan master mix was prepared as shown in 4.3.11. The synthesised cDNA was amplified using the 23S primers. Five different protocols were needed in this experiment and each protocol required different kits and reagents (Refer 4.3.7 - 4.3.7.2). The following materials were required to carry out all the protocols: Sterile RNase-free pipet tips, suitably sized tubes and micro-centrifuge or centrifuge with appropriate rotors, disposable gloves, vortexer and shaker–incubator.

4.3.16.2 Experiment 2

Aim of this experiment was to determine the effectiveness of heat killing step to kill *Legionella.* This experiment was conducted to compare amplification reactions from cDNA synthesised from RNA extracted from live (viable) and heat killed (confirmed by culture analysis) *Legionella* lenticules. RNA extraction and purification procedures were same as in Experiment-1 (Refer 4.3.7 - 4.3.7.2). However, a different DNA elimination kit (QIAGEN) was used for gDNA elimination and cDNA synthesis (Refer 4.3.10.2). This protocol change was introduced due to presence of gDNA contamination identified with NRT samples in Experiment-1.

4.3.16.2.1 Heat killing and amplification of *Legionella*.

Two *Legionella* lenticules were dissolved separately in 400µl culture medium for each (2 x 400) as explained in experiment -1, and labelled as 'sample A' and 'sample B'. Sample A was heated on a hotplate at 80˚C for one hour and then left at room temperature (20°C) for two hours to allow the RNA to degrade. RNAprotect Bacteria Reagent was added to Sample B and RNA extraction was carried out as described in Experiment 1. The RNA pellets were stored at -20°C until needed. After the completion of two hours of wait, RNAprotect Bacteria Reagent was also added to Sample A, followed by RNA extraction (Refer 4.3.7.1). RNA purification was undertaken simultaneously for both samples (Refer 4.3.7.2). Genomic DNA elimination and cDNA synthesis was carried out simultaneously using QIAGEN DNA elimination kit (Refer 4.3.10.2).

TaqMan master mix was freshly prepared for 13 wells as explained in 4.3.11; and all the PCR procedures were same as in Experiment 1 and the samples were amplified in triplicate.

4.3.16.3 Experiment 3

Aim of this experiment was to look at (i) the extent to which genomic DNA from a full lenticule (live and dead) could interfere with the qPCR if not removed and (ii) how well genomic DNA is removed. Three separate lenticules were used in this experiment. One for DNA extraction from live *Legionella*, one for DNA extraction from a completely heat killed *Legionella* and the third one for cDNA synthesis and extraction from completely heat killed *Legionella*. Heat killing, RNA extraction, purification, and cDNA synthesis was carried out as described in Experiment 2. However, a new protocol was trialled for genomic DNA elimination in the third lenticule due to the persisting nature of genomic DNA contamination following RNA extraction (Refer 4.3.10.3). This is the first experiment involving DNA extraction in this research and was carried out using QIAamp DNA extraction MiniKit (50), Ref. No. 5130 (Refer 4.3.8).

4.3.16.3.1 Sample preparation for DNA and RNA extraction

Three *Legionella* lenticules were dissolved in 400µl culture medium in 1.5 ml tubes as described in Experiment 4, and labelled as Sample' A, B and C. Sample A and B was used for DNA extraction and Sample C was used for RNA extraction. Sample A was used for DNA extraction from live *Legionella*, sample B was used for DNA extraction from completely heat killed *Legionella* and sample C was used for RNA extraction and cDNA synthesis from completely heat killed *Legionella*. Two water baths were heated: one to 56°C and the other to 70°C. Buffer AE was equilibrated to room temperature for elution and buffers AW1 and AW2 have been prepared by adding ethanol as per shown on the bottle in the kit. DNA was extracted from sample A and B using the method outlined in 4.3.8. The DNA pellets were not diluted for PCR amplification. All other procedures for PCR amplification were as described in Experiment 1, including master mix preparation. The cDNA synthesised from RNA extracted from completely heat killed *Legionella* lenticule (plus any residual genomic DNA in the sample; if dead, there should theoretically not be any cDNA) was also amplified with DNA extracted from full lenticule (live *Legionella*) and completely heat killed *Legionella* lenticule for comparative study purpose.

In this experiment, a different protocol was tested for genomic DNA elimination in Sample C (Refer 4.3.10.3), but cDNA synthesis was undertaken as described in Experiment 2.

4.3.16.4 Experiment 4

This experiment was primarily aimed at establishing a relationship between cDNA amplification curves (C_T values) and colony forming units per litre (cfu/L) reported in conventional culture methods used for diagnosing *Legionella* bacteria in water samples. The RNA extraction, purification, genomic DNA elimination and cDNA synthesis was performed as described in Experiment 3. Two full *Legionella* lenticules were used in this experiment for RNA extraction; one for total (100%) live detection and the other for preparing five different dilutions as 50%, 25%, 12.5% and 5%. The RNA extracted

from lenticule (full live) and the dilutions series were amplified separately due to the persisting nature of gDNA contamination. In addition, the amplification of dilution series was also aimed to identify the lower detection limit of cDNA amplification. Another set of samples were prepared in the same dilution (all in triplicate) for standard culture analysis in a UKAS accredited laboratory (Refer Chapter 3.2.4) for comparing the results of both qPCR and gold standard culture.

4.3.16.4.1 Preparation of dilution series for qPCR

Two *Legionella* lenticules were dissolved in 400µl Ringer's solution as described in Experiment 1 and labelled as Sample A and B. Sample A was used for full live detection and Sample B was used to prepare 5 dilutions of bacterial culture as 50%, 25%, 12.5%, 5% and 1%; i.e. 200 µl bacterial culture, 100µl bacterial culture plus 100µl deionised water (DW) made 200µl, 50µl bacterial culture plus 150µl DW, 20µl bacterial culture plus 180µl DW and 2µl bacterial culture plus 198µl DW respectively. Two volume of RNAprotect Bacteria Reagent was added to one volume of bacteria culture; i.e. pipetted RNAprotect Bacteria Reagent into 2ml tubes as 400µl, 200µl, 100µl, 40µl and 4µl respectively for 50%, 25%, 12.5%, 5% and 0.5% bacterial culture. All other procedures followed were same as described in Experiment 2; allowing for differences in sample number.

Taqman Master Mix was prepared for 38 samples using the kit in the following basic ratio and all the samples were amplified in triplicate.

4.3.16.4.2 Preparation of samples for standard culture analysis.

Six separate samples of 'full lenticule' were prepared by using six *Legionella* lenticules from the same batch as follows:

Three *Legionella* lenticules were dissolved separately in 400µl of Ringers solution as described in Experiment 1. Each bacterial culture was then transferred into a separate 500 ml sterile plastic bottle (supplied by UKAS accredited laboratory for *Legionella* sampling) by pipetting and made up to 500ml by adding deionised water. Three samples were labelled as Sample A, B and C. In addition to these samples, the other three lenticules were used to prepare five dilutions (50%, 25%, 12.5%, 5% and 0.5%) in triplicate were made up in 500ml deionised water. All the samples were stored in

temperature-controlled bags, protected from heat sources and sunlight, during transportation to the laboratory. All eighteen *Legionella* samples in water were then analysed using standard UKAS protocols for *Legionella pneumophila* at a UKAS accredited laboratory (ALS Laboratories Limited) under identical laboratory conditions, within 12 hours of preparation (Ref. Chapter 3, section 3.2.4).

4.3.16.5 Experiment 5

This experiment was designed to investigate the source of persisting gDNA contamination identified in Experiments 1-4 and to study the efficiency of qPCR response to a dilution series of cDNA extracted from RNA. In order to avoid all possible sources of gDNA contamination, a separate lab space (isolated from the lab space where RNA extraction and purification had taken place) was used to prepare the master mix and PCR well filling. Furthermore, all the probes/primers, TaqMan Mastermix and RNA/DNA free molecular grade deionised water was newly purchased and unopened until using in this experiment. All other procedures followed were exactly same as in Experiment – 2. The dilution series was of 10 fold 4 dilutions (1, 0.1, 0.01 and 0.001) of bacterial culture.

4.3.16.5.1 qPCR response to a dilution series

One *Legionella* lenticule was dissolved in Ringers solution as described in Experiment 1 and used to prepare 10-fold dilution series 1, 0.1, 0.01 and 0.001 as 1, 2, 3 and 4 respectively and these 4 dilutions of the bacterial culture is shown in Table 4.4.

Table 4.4. Four dilutions (1, 2, 3 and 4) of bacterial culture with proportionate RNAprotect Bacteria Reagent.

	1	**2**	**3**	**4**
Bacterial culture (µl)	400	40	40	40
RNAprotect Bacteria Reagent	800	80	80	80
Deionised water (µl)	0	280	280	280

Two volumes of RNAprotect Bacteria Reagent were added to one volume of bacteria culture (see Table 4.4) and left for 2 minutes to allow RNAprotect Bacteria Reagent to work before adding deionised water. In order to eliminate the persisting gDNA contamination in previous experiments, newly purchased unopened molecular grade deionised water was added to the bacterial culture as shown in Table 4.4. Other reagents such as probes/primers and TaqMan Mastermix used was also newly purchased and the Mastermix was prepared in a separate lab space. All other procedures followed were exactly same as in Experiment 2; the only difference being the number of samples used. All the samples were amplified in triplicate.

4.4 Results and discussion

4.4.1 Primer/probe validation output

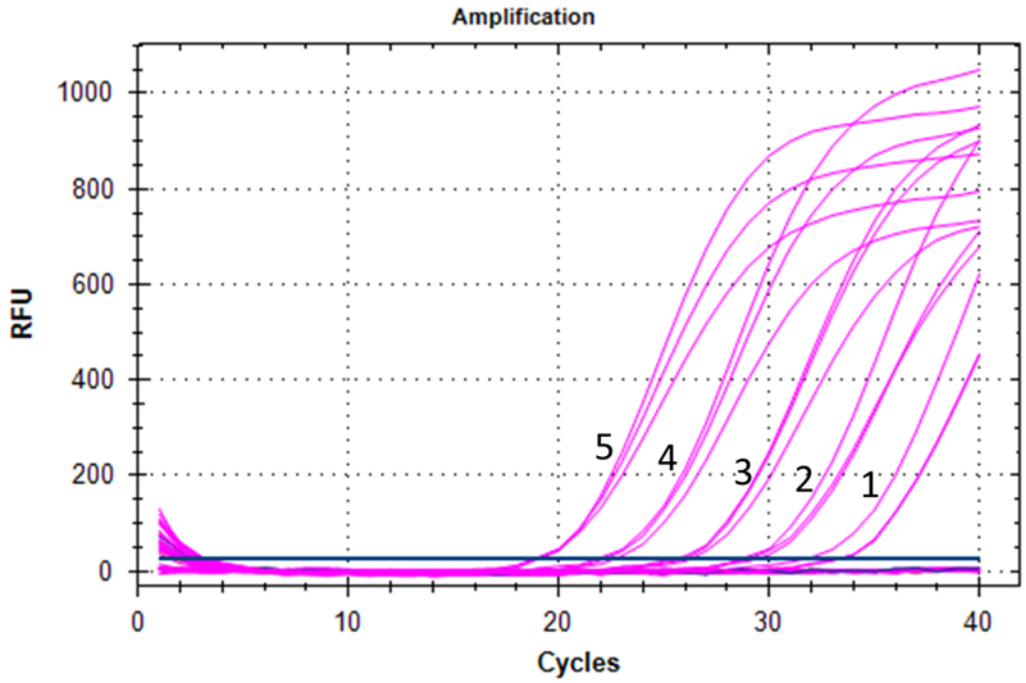


Figure. 4.5 Amplification of 23S rRNA gene from five samples of standard 10 fold dilutions of *Legionella pneumophila* DNA showing efficient and proportionate amplification of targeted gene (23S rRNA) from DNA confirming the primers/probes are optimal.

Standard 10-fold dilutions of 5 samples of known amount (250,000, 25,000, 2,500, 250 and 25 GU's in 6µl respectively) of *Legionella* pneumophila DNA were amplified by targeting 23S rRNA gene. All the 5 concentrations were amplified successfully and

generated the standard curves as expected (Figure 4.5). This result indicates that the efficiency of primers/probes used in this assay is optimal, and confirmed the successful amplification of targeted 23S rRNA gene.

4.4.2 Inhibition monitoring output

Synthesised cDNA from a full *Legionella* lenticule dilution series (Refer 4.3.16.5.1) was used to perform this experiment. The cDNA samples were amplified in triplicate and the amplification was successful (Figure 4.6). The amplification data (Table 4.5) was used to generate the inhibition plot (Figure 4.7).

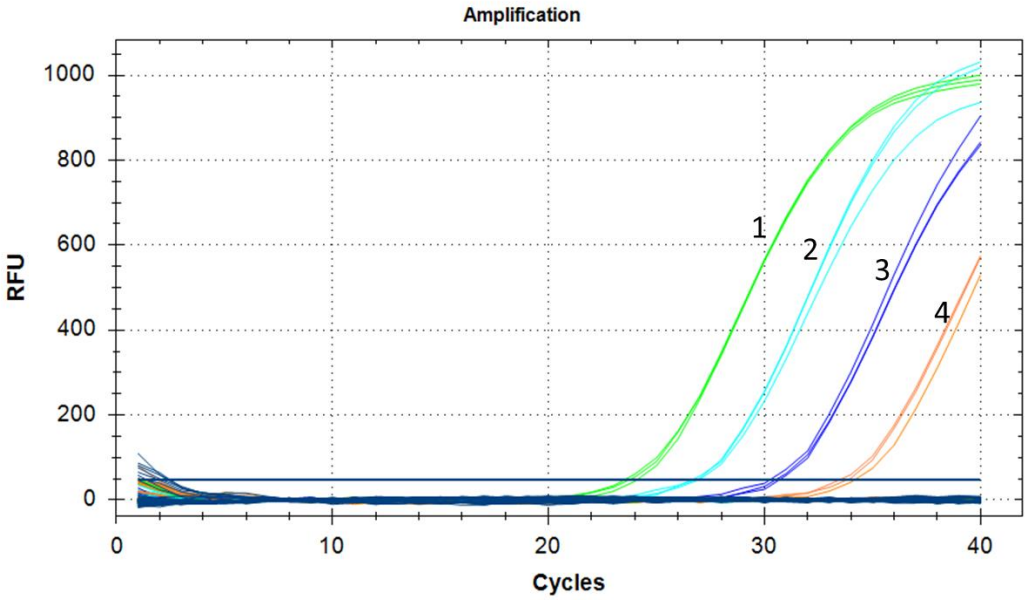


Figure 4.6 Amplification curves from 4 samples of 10 fold dilutions of cDNA from one *Legionella* lenticule.

Table 4.5 Diluted cDNA amplification data used to generate the inhibition plot.

Sample	C_T -1	C_T -2	C_T -3	Average C_T value	Sample quantity	Log (sample quantity)
1	24.09	23.73	23.51	23.77	1.0000	0.00
2	26.95	26.8	26.62	26.79	0.1000	-1.00
3	30.51	30.68	30.25	30.48	0.0100	-2.00
4	33.47	33.82	34.17	33.82	0.0010	-3.00

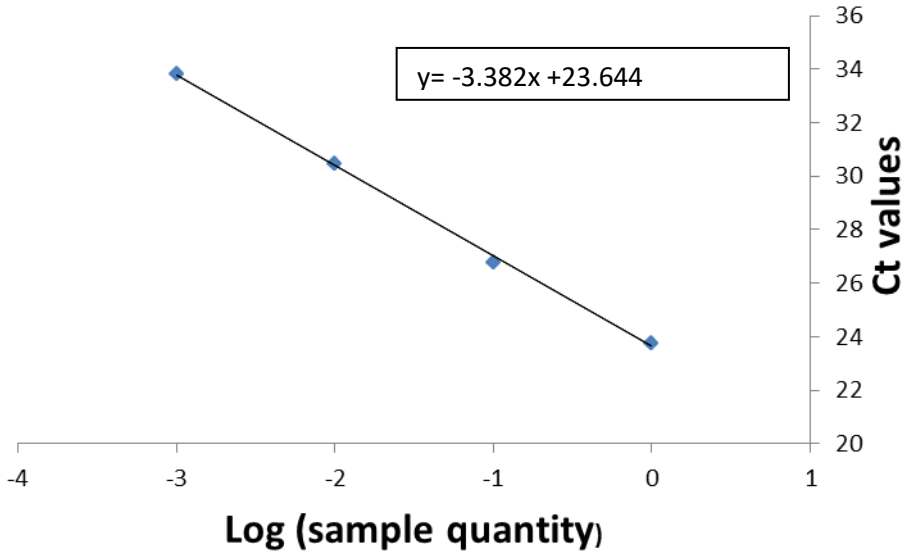


Figure 4.7 Inhibition plot generated from cDNA dilutions demonstrating that no inhibition in this qPCR assay. Dilution factor is 10, slope is -3.382, R^2 is 0.9986 and efficiency is 97.55%.

Efficiency of qPCR assay is calculated using the formula E = (10 −1/slope −1) × 100. The inhibition plot (Figure 4.7) showed 97.55% efficiency, slope - 3.382 and R2 0.9986. A slope −3.3 with 100% efficiency is the clear indication of no inhibition. In this assay, the slope -3.382 with 97.55% efficiency ruled out the possibility of any inhibition (Svec et al., 2015).

4.4.3 Positive control output

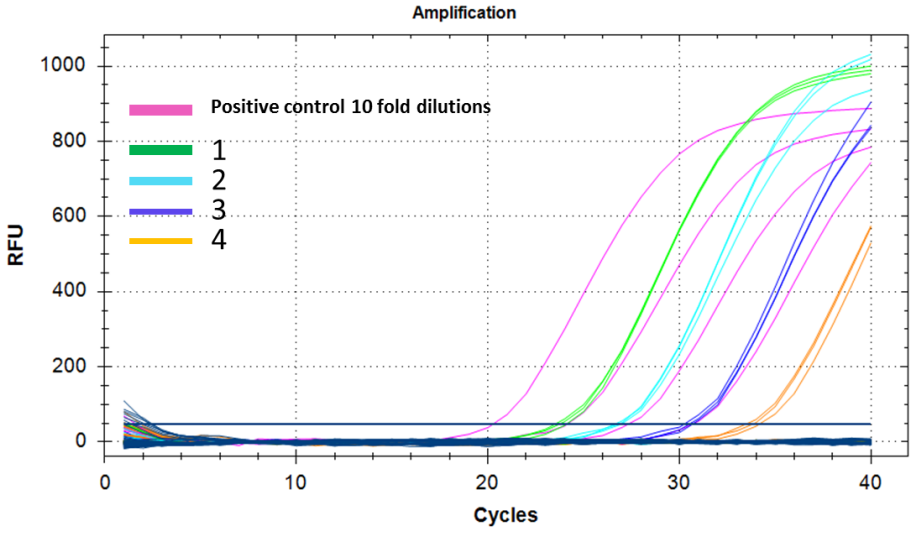


Figure 4.8 Amplification curves of 4 x 10-fold dilutions of cDNA synthesised from RNA extracted from a *Legionella* lenticule and 4 x 10-fold serial dilutions of a *Legionella pneumophila* DNA positive control 250,000, 25,000, 2,500, 250 GU's per 6µl of sample.

Table 4.6 Four 10-fold dilution series of cDNA and positive control amplification data used to generate the Figure 4.8. Dilution series of cDNA was amplified in triplicate.

Sample	**5**		**4**		**3**	**2**
Positive control	**20.26**		**24.14**		**27.32**	**30.66**
Sample quantity	**1.0000**		**0.1000**		**0.0100**	**0.0010**
Log (sample quantity)	**0.00**		**-1.00**		**-2.00**	**-3.00**
Sample **cDNA dilution**	**CT -1**	**CT -2**	**CT -3**	**Average CT value**	**Sample quantity**	**Log (sample quantity)**
1	24.09	23.73	23.51	23.77	1.0000	0.00
2	26.95	26.8	26.62	26.79	0.1000	-1.00
3	30.51	30.68	30.25	30.48	0.0100	-2.00
4	33.47	33.82	34.17	33.82	0.0010	-3.00

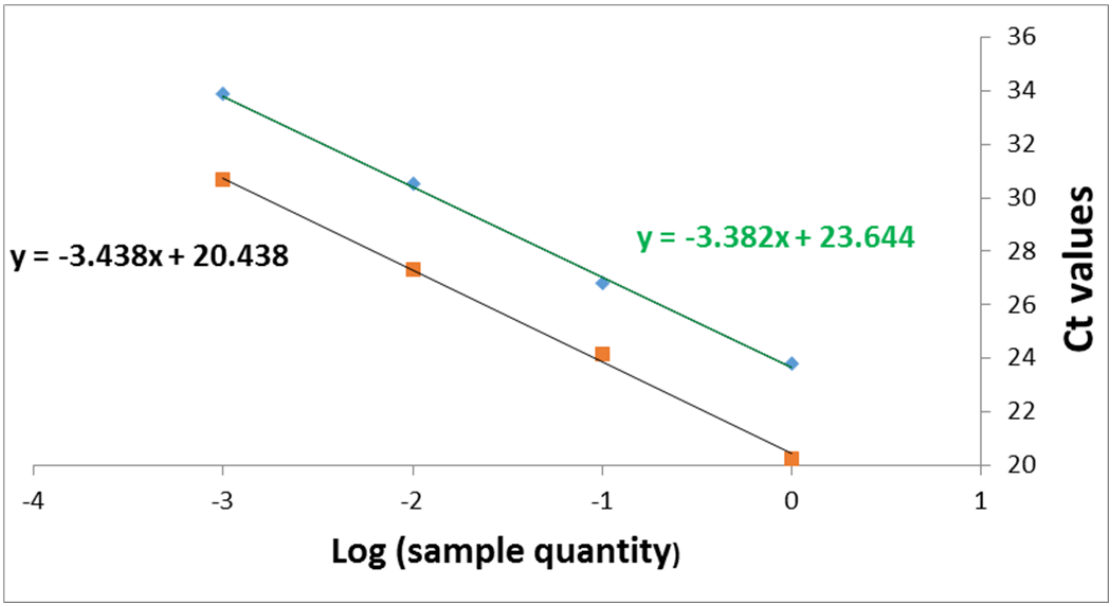


Figure 4.9 Scatter plot showing efficient amplification of cDNA and the DNA positive control diluted in 10-fold steps. For the positive control, the slope is -3.438, R^2 is 0.9981 and efficiency is 95.37% demonstrating that the PCR reactions are optimal and no inhibition has occurred. cDNA (green line) is as shown in Figure 4.7.

4.4.4 qPCR assay optimisation

Amplification data of 23S gene from 10-fold dilutions of five dilution series (concentration 250,000, 25,000, 2,500, 250 and 25 GU's in 6µl) of known amount of standard (*Legionella* pneumophila DNA) used for the assay optimisation (Refer 4.4.1). Corresponding amplification data is shown in Table 4.7.

Table 4.7 qPCR data of standard curve generated from the amplification of 10-fold five dilution series of standard *Legionella pneumophila* DNA.

Sample	C_T -1	C_T -2	C_T -3	Average C_T value	Sample quantity	Log (sample quantity)
1	18.90	18.87	19.04	18.93667	1.0000	0.00
2	22.63	21.83	22.16	22.2066	0.1000	-1.00
3	26.24	26.03	25.46	25.91	0.0100	-2.00
4	29.06	29.38	28.68	29.04	0.0010	-3.00
5	33.33	33.33	31.73	32.7866	0.0001	-4.00

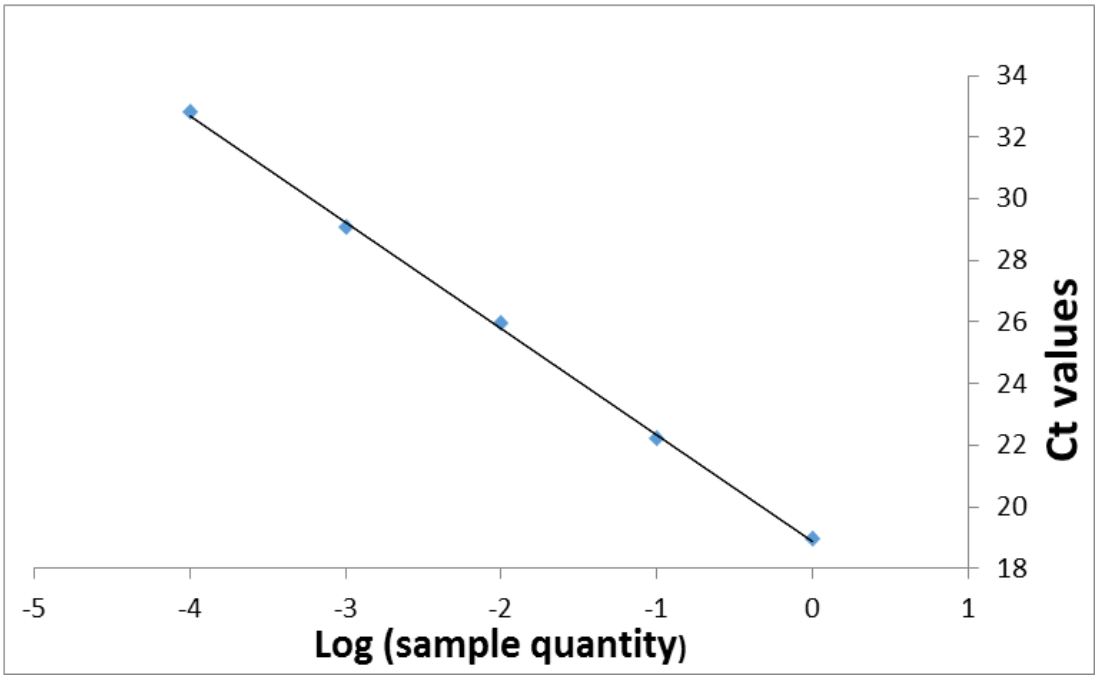


Figure 4.10 qPCR standard curve plot of C_T values of 10 fold dilution series of five standard *Legionella* pneumophila DNA samples against log of input nucleic acid showing slope -3.4533, R^2 0.9993 with qPCR assay efficiency 94.79%.

qPCR assay optimisation was conducted by amplifying 10 fold dilution series of five standard *Legionella* pneumophila DNA samples (Figure 4.5). 23Sr RNA gene amplification was proportional to the dilution series of standard DNA (Figure 4.10). Amplification data from this experiment (Table 4.7) was used to generate a qPCR standard curve plot of C_T values against log of input nucleic acid is shown in figure 4.11. The slope calculated is -3.4533, R^2 0.9993 and the qPCR assay efficiency calculated using the formula E = (10 –1/slope –1) × 100 is 94.79%.

4.4.4.1 Assay optimisation including probes/primers

Successful amplification of 23S rRNA gene from standard 10-fold dilutions of known amount of *Legionella* pneumophila DNA using 23S primers and probes clearly demonstrated that the primers and probes used in this assay are optimal. In addition, successful amplification of cDNA synthesised from RNA dilution series prepared from full *Legionella* lenticule was with qPCR efficiency of 97.55%. The slope calculated was -3.382 and R^2 was 0.9986. This slope with 97.55% efficiency indicating that there was no inhibition in this qPCR assay (Svec et al., 2015). Furthermore, amplification of positive control (standard known amount of LP DNA) along with cDNA synthesised from dilution series of RNA demonstrated a slope -3.438, R^2 0.9981 and 95.37% efficiency. This slope and efficiency from positive control amplification indicates that the RT reaction and qPCR reactions in this assay are optimal with no inhibition in the PCR process as the acceptable range of qPCR efficiency is between 90 and 110%. (Reyneke et al., 2016). In addition, graph generated from C_T values of 10-fold dilution series of five standard *Legionella pneumophila* DNA samples (Figure 4.10) showed slope -3.4533, R^2 0.9993 with qPCR assay efficiency 94.79%. This qPCR efficiency percentage further confirms that the qPCR reactions in this assay are optimal. Many studies have demonstrated that the use of positive controls in qPCR provides a number of advantages such as quality assurance, confirming the functionality of the reaction components, to assess the efficiency of the qPCR assay, to ensure that there is no inhibition taking place, for assessing reaction specificity for genotyping assays and furthermore, it is essential when measuring low copy numbers (Reyneke et al., 2016; Zhang et al., 2016 Svec et al., 2015).

4.4.5 RNA quantification

4.4.5.1 Agarose gel analysis output

Agarose gel analysis for RNA from four concentrations was conducted; but no visible bands were available on the gel due to the insufficient amount of RNA that was extracted from *Legionella* bacteria.

4.4.5.2 Nanodrop concentration check output

Nanodrop concentration check output for four different concentrations of RNA was as follows:

100% - 1.6 ng/ µl
50% - 1.3 ng/ µl
25% - 1.7 ng/ µl
12.5%- 0.5 ng/ µl

The RNA concentration reported by the Nanodrop was not consistent with the initial concentration of bacteria used. Therefore, this data was not used to quantify RNA concentrations in lenticules, but serves as evidence of RNA recovery from lenticule samples.

The purified RNA concentration obtained from Nanodrop was not used to quantifyi RNA due to the apparent inconsistency in quantification (i.e. 100% - 1.6 ng/ µl, 50% - 1.3 ng/ µl , 25% - 1.7 ng/ µl and 12.5%- 0.5 ng/ µl). NanoDrop One (Thermofisher, 2017) spectrophotometers were designed to specifically measure absorbance using small sample volumes and can measure absorbance in the wavelength range of 190–840nm and requires only in the range of 0.5–2µl of sample for an accurate measurement. However, for RNA, the Nanodrop instrument's lower detection limit is 2ng/µl (Wieczorek et al., 2012). In this assay, the purified RNA concentration from *Legionella* bacteria was below the lower detection limit of Nanodrop instrument. Therefore, the Nanodrop output/concentration check was not considered further in this assay, but serves as positive detection only.

4.4.6 *Legionella* culture enumeration

Standard culture analysis of six *Legionella pneumophila* samples prepared from 6 full lenticules (3 heat killed and 3 untreated) found positive culture of all three untreated (not heated) samples in the range of 8500-10,000 cfu/L, whereas heat killed *Legionella* samples were negative for culture. Thus, the same method was used to prepare the heat killed *Legionella* samples throughout this research.

4.4.7 Results of Experiment 1

The aim of this experiment was to extract RNA from two *Legionella* lenticules using Qiagen RNeasy Mini Kit. The amount of RNA extracted was checked using Nanodrop; however, the concentration level showed by Nanodrop was below the lower quantitative limit of the equipment and was therefore not considered to be reliable. Thus, a number of different experiments were needed to check and ensure that RNA extraction was successful. cDNA was synthesised from purified RNA using Invitrogen Superscript III and then amplified using 23S primers. RNA extraction, purification, genomic DNA elimination procedure, cDNA synthesis and PCR amplification were done simultaneously for both the lenticules.

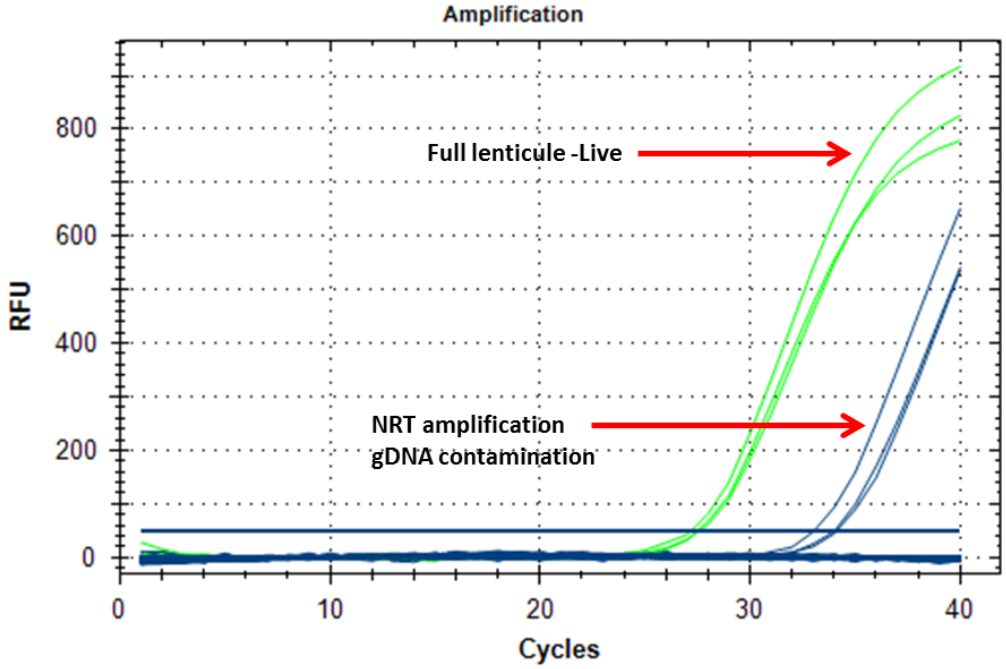


Figure 4.11a qPCR amplification plots for 23S rRNA gene from RNA extracted from a full *Legionella* lenticule; with and without RT. Green represents the amplification from live RTcDNA, blue represents the NRT amplification (genomic DNA contamination).

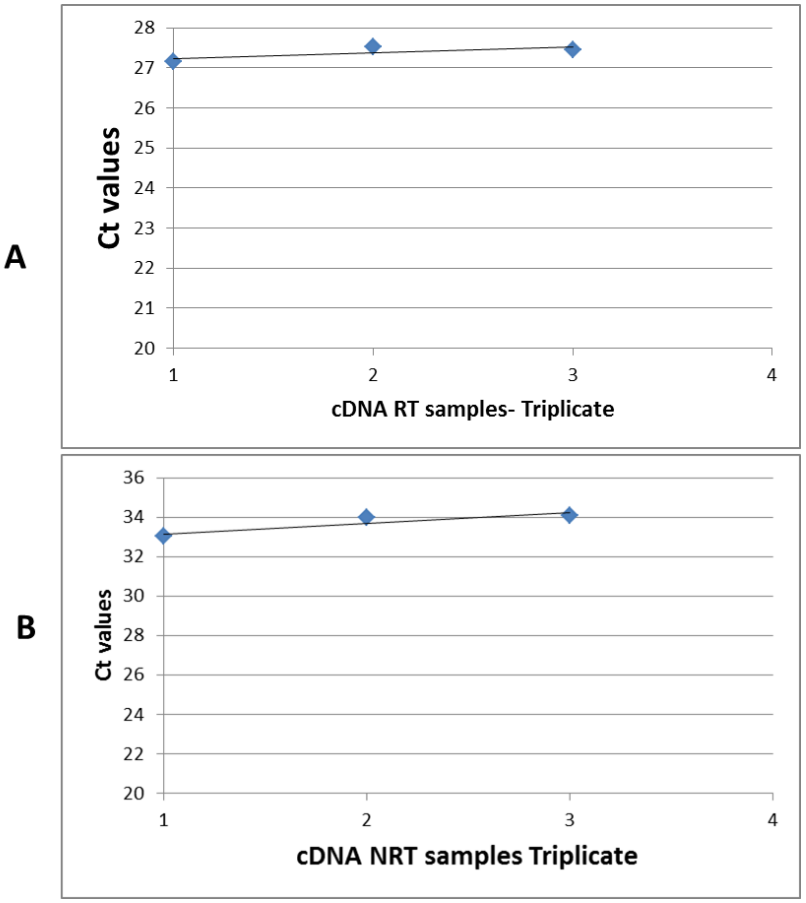


Figure 4.11b C_T values of triplicate qPCR samples used for 23S gene amplification from RNA extracted from live lenticule; RT represents the qPCR response to samples in which RNA has converted to cDNA. NRT samples contain RNA without conversion to cDNA. All samples may contain small amounts of genomic DNA.

Figure 4.11a show the amplification curves for 23S gene from RNA extracted from whole *Legionella* lenticule. One full lenticules were used in this experiment and its amplification is expressed in scatter plots as A and B (Figure 4.11b). The cDNA made from RNA extracted from a full lenticule was amplified in triplicate for RT samples. However, there are responses from NRT samples also and this can be resulted from the genomic DNA contamination with NRT samples. The corresponding number of cycles of amplification (C_T) values is shown in Table 4.8.

Table 4.8 C_T values corresponding to each amplification curve in experiment-1.

RT cDNA	NRT
27.15	33.01
27.53	33.98
27.44	34.09

Mean C_T values of the RT amplification cycle for lenticule was 27.37 which was lower than the NRT mean C_T value of 33.69 (a difference of 6.32). In these samples, the 23S gene was amplified from cDNA indicating that 23S rRNA was present in the live sample. However, amplification responses from NRT samples occurred suggesting that a low level of genomic DNA contamination occurred in the samples.

The aim of Experiment-1 was to check the possibility of extracting RNA from *Legionella* lenticules using the Qiagen RNA extraction kit, synthesise cDNA and amplify it using the 23S primers. The results clearly indicate that the experiment was successful. Furthermore, the results of all remaining experiments 2-5 confirmed that the RNA extraction from live *Legionella* lenticule using Qiagen RNA extraction mini kit is possible and amplification using 23S primers of the resulting cDNA is achievable. The cDNA amplification from full *Legionella* lenticules in Experiments 1, 2, 4 and 5 shows slight variation as the corresponding mean C_T values are 27.37, 27.38, 22.94 and 23.77 respectively and this could be due to the difference in bacterial concentrations between the lenticules (different lenticules from the same batch), the loss of RNA during the purification and genomic DNA eliminations procedures and possible pipetting errors (Refer section 4.3.10 and 4.3.14.3). Mean of these C_T values (mean C_T values from experiments 1, 2, 4, and 5) is 25.36 (±2.01). The lenticules used in these experiments were from the same batch (and should therefore be almost identical in number of bacteria) and quantification of *Legionella* pneumophila bacteria in each lenticule was also carried out using standard culture analysis. The mean concentration of *Legionella* from three lenticules from the same batch was 4950 cfu/500ml (±676sd). This variation in culture analysis result is also due to small differences in number of bacteria present in each individual lenticule; and cumulative errors introduced to individual samples during the culture process.

My results suggest that the success of experiments in this type will depend to a certain extend on the kits used and methods involved. Several studies have demonstrated that RNA loss can occur during different RNA extraction and purification

methods (Honsvall and Robertson, 2017; Faucher et al., 2010). Another study on reverse transcription and PCR amplification of purified RNA demonstrated that RNA purification using Qiagen columns resulted in improved quality and purity of RNA yields, but resulted in an overall loss of RNA product (Chomczynski and Sacchi, 1987). PCR analysis result also showed that the RNA extracted by QIAGEN-kit was of the highest quality compared to all other extraction kits used (Belder e al., 2016; Nuyts et al., 2001). However, van der Zee and colleagues in Netherlands reported that the columns supplied with the Qiagen extraction kit itself failed to eliminate *Legionella* genomic DNA contamination. In that study, three different batches of columns were used as supplied in the Qiagen kit. Twenty columns of each batch were tested and with one batch, all 20 columns tested were negative; in contrast, all the columns in the other two batches were contaminated with *Legionella* DNA (van der Zee et al., 2002). This possibility was also addressed by using columns and reagents from different batches in Experiments (QIAGEN RNeasy Protect Bacteria Midi Kit in Experiment 1, QIAGEN QuantiTect Reverse Transcription and DNA elimination kit in Experiments 2-4 and Sigma-Aldrich Total RNA purification kit in Experiments 5 and 6), but the genomic DNA contamination remained unchanged.

4.4.8 Results of Experiment 2

In this experiment, two *Legionella* lenticules were used; one for live detection (Sample B) and the other is heat killed (Sample A). RNA extraction, RNA purification, cDNA synthesis and cDNA amplification were carried out simultaneously to compare the two samples. RNA extraction and purification procedures were as described in Experiment-1, although a different gDNA elimination kit (QIAGEN) was used for genomic DNA elimination and cDNA synthesis due to the low-level amplification (Average CT value 33.69) from NRT samples.

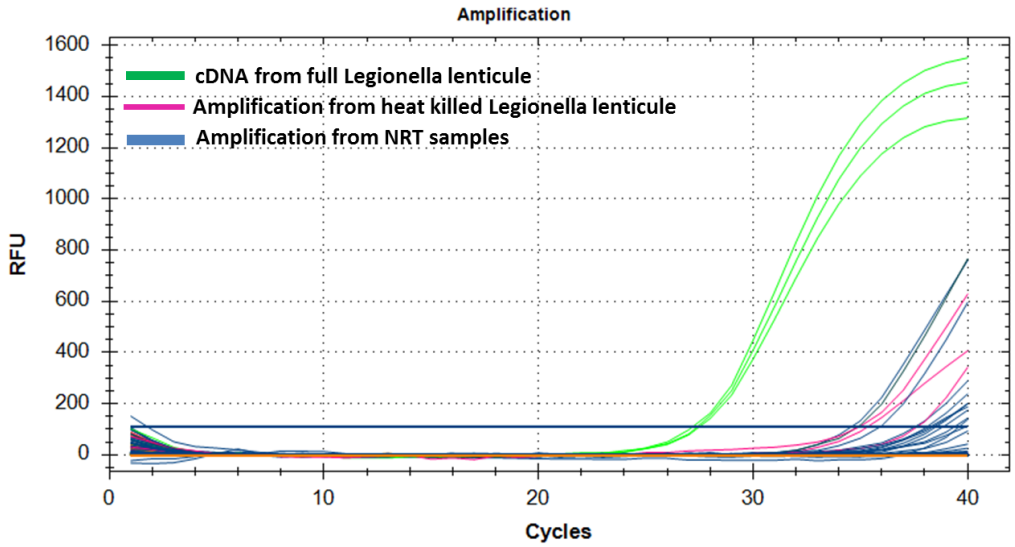


Figure 4. 12a Amplification of cDNA made from RNA extracted from full lenticule (live *Legionella*) and fully heat killed *Legionella* lenticules. Green represents the cDNA amplification, red represents the amplification from heat killed sample (genomic DNA contamination), blue represents the amplification from NRT samples (genomic DNA contamination) and low level responses from master mix and empty wells.

Amplification of cDNA made from RNA extracted from the live *Legionella* lenticule showed early amplification at a mean C_T value of 27.38 (Figure 4.12a). The same process from the fully heat killed *Legionella* lenticule yielded a mean C_T value of 36.01; the difference in mean C_T values between live and dead is 8.63. The corresponding number of amplification cycles is shown in Table 4.9. The difference in these C_T values demonstrates detection of live *Legionella* compared to the amplifications from the heat killed *Legionella* cells. The amplification response from the heat killed cells is of a similar level to the no RT controls (Figure 4.12b), indicating the presence of genomic DNA contamination with the RNA product. These results confirm the detection of RNA from live *Legionella* bacteria. The amplification product detected in the dead cells occurs around the same level as that found in the background control, that the product is most likely contaminating genomic DNA. These results further confirm that the denaturing process employed in this experiment was successful, as heat killed bacteria did not appear to produce measureable levels of RNA.

Table 4.9 C_T values corresponding to each amplification curve in Experiment-2.

Full Lenticule – live *Legionella* (RT) -1	**Full Lenticule – live *Legionella* (NRT) -2**	**Heat killed *Legionella* lenticule (RT) -3**	**Heat killed *Legionella* lenticule (NRT) -4**
27.21	34.61	35.00	37.58
27.41	34.90	35.37	38.13
27.52	35.36	37.67	38.56

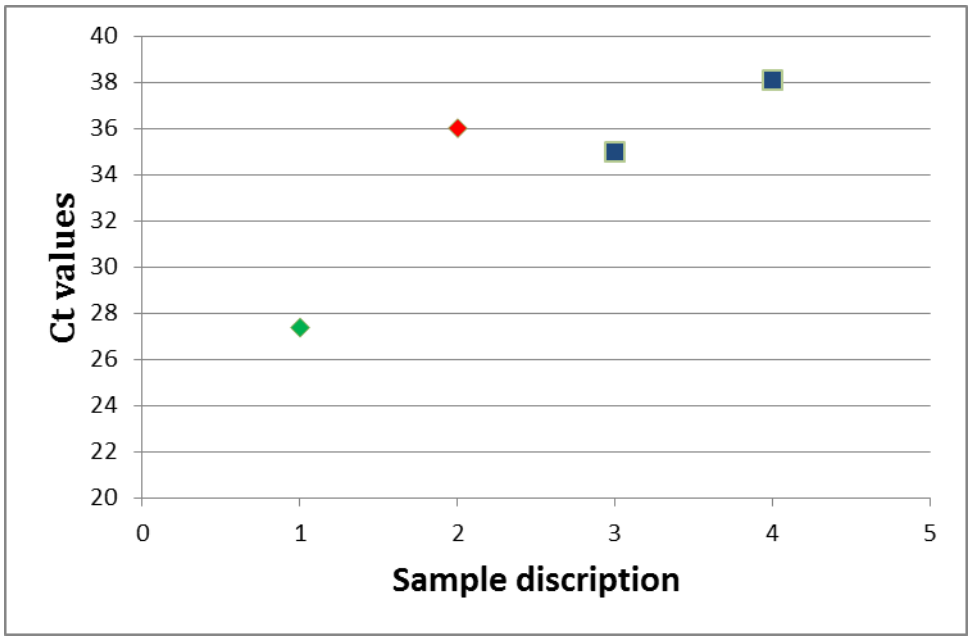


Figure 4.12b. Scatter plot representation of C_T values corresponding to live sample (1), heat killed sample (2) and comparison of genomic DNA contamination amplification with both live and heat killed samples (3) and NRT(4).

In this experiment, a different cDNA synthesis kit (QIAGEN) was used which includes two genomic DNA elimination steps, both in the RNA specific gDNA wipeout step. However, no significant improvement was achieved in eliminating gDNA contamination. The source of this persisting gDNA contamination could be the laboratory conditions where RNA extraction, cDNA synthesis and qPCR amplification is carried out in the same laboratory space (Refer section 4.3.10).

In Experiment- 2, cDNA synthesised from RNA extracted from a full *Legionella* lenticule (live) and a full lenticule (heat killed) was amplified using the 23S primers. Amplification from live sample was similar as in Experiment -1 and the mean of C_T

values from triplicates of live sample is 27.38 whereas with the dead sample it was 36.01. No amplification was expected from the heat killed lenticule sample in Experiment -2. However, there was a low level amplification (mean C_T 36.01) from the heat killed lenticule sample which was close to that seen in the NRT sample in experiment-1. Furthermore, amplification from live lenticules in Experiment -1 and Experiment -2 were almost identical which confirms the result achieved in Experiment -1. In contrast, the similar C_T values observed in heat killed lenticule sample (NRT samples from experiment 1 and 2) confirmed the presence of genomic DNA contamination within RNA samples in both the experiments. This statement is supported by a study report which says negative Reverse Transcription control (NRT) is necessary to include in all RT-qPCR experiments to test for genomic DNA contamination as such control contains all the reaction components except for the reverse transcriptase. RT amplification should not occur in this control, and if any PCR amplification is seen from this control, it would be derived from contaminating genomic DNA (Laurell et al., 2012; Bustin and Nolan, 2004).

The different DNA elimination protocol used in Experiment-2 did not lower the genomic DNA contamination. The results from Experiment-2 indicated that there was no RNA present in the heat killed sample; and the culture analysis results of three separate samples are also supporting this finding as no *Legionella* was detected from heat killed *Legionella* samples analysed in UKAS accredited laboratory using standard protocol (Refer Chapter 3, section 3.2.4) and there by confirming that (i) the heat killing method was successful in killing the *Legionella* that was present in the lenticule and (ii) RNA extraction and amplification method employed was also effective. However, the consistent lower level amplification from heat killed samples as well as in NRT samples suggests that genomic DNA contamination is persistent with RNA, and is present in almost similar level in all experiments.

Although sources of contamination are unclear, genomic DNA contamination in RNA samples is known to occur from external sources, including dust particles carrying bacteria and moulds. In addition, laboratory equipment, plasticware and other consumables such as reagents and deionised water can become the source of contamination when shared. In order to eliminate the possibility of contamination from the laboratory, all possible precautions were taken, such as more frequent changing of gloves, cleaning of work area using commercially available RNase removal solution, and the use of sterile disposable polypropylene tubes and pipette tips while carrying out all

these experiments. A study carried out in United States to compare three different methods of RNA extraction from Gram-positive bacteria: i) An acid–phenol extraction protocol ii) The "RNeasy mini kit" from QIAGEN iii) The "SV Total RNA Isolation System" from Promega reported that the QIAGEN-kit produced the greatest amount of RNA with the highest purity (Nuyts et al., 2001). Another possibility of this genomic contamination can be the laboratory conditions as all the protocols in the entire assay (RNA extraction, purification, cDNA synthesis and qPCR process) was carried out in the same lab space, though it is not recommended (Refer section 4.3.14.3).

4.4.9 Results of Experiment 3

The aim of this experiment was to compare the PCR amplification of genomic DNA extracted from a live *Legionella* lenticule, genomic DNA extracted from a completely heat killed *Legionella* lenticule and cDNA synthesised from RNA extracted from a completely heat killed *Legionella* lenticule. Three separate lenticules were used in this experiment as one for genomic DNA extraction from live *Legionella* lenticule, the other for genomic DNA extraction from completely heat killed *Legionella* lenticule and the third one for RNA extraction from completely heat killed *Legionella* lenticule. In this experiment (only for the third lenticule; RNA extraction from completely heat killed sample) a different genomic DNA elimination protocol was employed by using a different kit; however, RNA extraction, purification, and cDNA synthesis was carried out exactly same as in Experiment 2. The QIAamp DNA extraction kit (see Section 4.3.8) was used for genomic DNA extraction from live and completely heat killed *Legionella* lenticule.

In this experiment three samples were amplified for comparative study as genomic DNA extracted from a fully live *Legionella* lenticule, genomic DNA extracted from a completely heat killed *Legionella* lenticule as well as cDNA produced from RNA extracted from a completely heat killed *Legionella* lenticule. Amplification plots showed almost similar amplification for total genomic DNA concentration in fully live and fully heat killed *Legionella* lenticules as expected due to the presence of similar numbers of bacteria regardless of viability. In addition, amplification of cDNA synthesized from RNA extracted from completely heat killed *Legionella* lenticule showing only lower level of amplification as there should be no RNA or cDNA present in this sample (Figure 5.14a). However, the lower level response is likely a consequence of incomplete

removal of gDNA by DNAse treatment. This experiment further confirms that RNA extraction and cDNA amplification achieved in previous experiments (Experiments 1 and 2) is successful. In contrast, as described in previous results, low level amplification showing from the sample of completely heat killed *Legionella* lenticule is due to the persistent gDNA contamination even after RNA purification and gDNA digestion or elimination treatments.

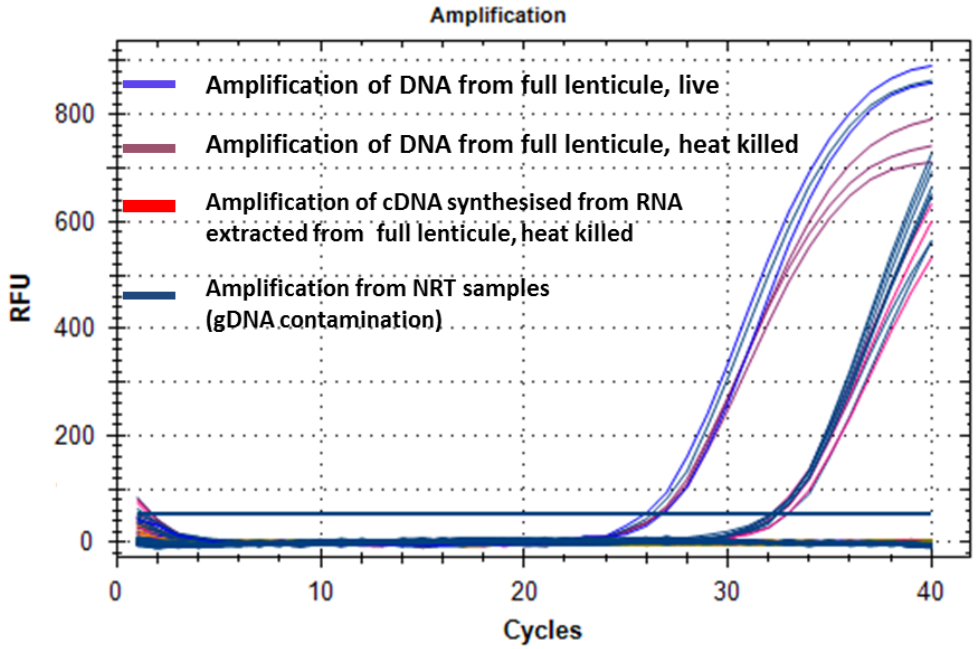


Figure 4.13a Amplification plots showing successful extraction and amplification of genomic DNA from full live *Legionella* lenticule and completely heat killed *Legionella* lenticule along with amplification of cDNA synthesized from RNA extracted from completely heat killed *Legionella* lenticule, further confirming that the RNA extraction is successful.

The C_T values corresponding to each of the amplification and their mean are given in (Table 4.10). In this experiment, triplicates (technical replicates) of each sample produced almost consistent curves (C_T values) and the C_T value for RT in the heat killed lenticule was similar to genomic DNA contamination is also similar as in previous experiments.

Table 4.10 The C_T values corresponding to each of the amplification and their mean values with NRT amplification values for comparison.

Sample description	Full live *Legionella* lenticule DNA		Full heat killed *Legionella* lenticule DNA		Full heat killed *Legionella* lenticule cDNA	
	RT	**NRT**	**RT**	**NRT**	**RT**	**NRT**
C_T1	25.96	32.18	26.63	32.45	32.15	32.10
C_T2	26.26	32.43	26.53	32.10	32.45	32.47
C_T3	26.79	32.24	26.73	32.45	32.93	32.95
Mean	**26.33**	**32.28**	**26.63**	**32.33**	**32.51**	**32.50**

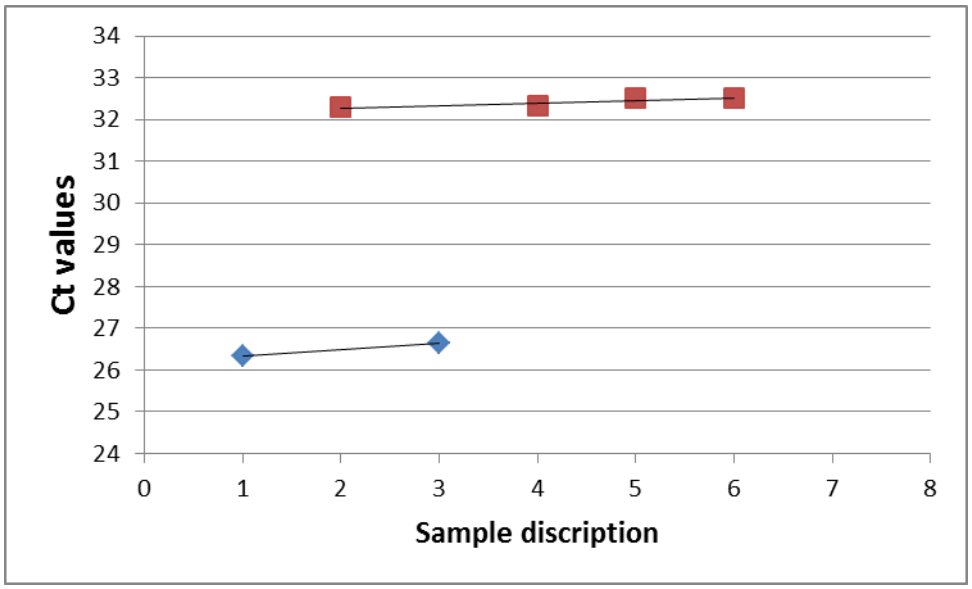


Figure 4.13b Scatterplot representations of C_T values obtained from the RT amplification of total genomic DNA from full *Legionella* lenticule (Live), completely heat killed *Legionella* lenticule and cDNA produced from completely heat killed *Legionella* lenticule. 1 and 3 are DNA RT samples, 5 is cDNA RT sample and 2, 4 and 6 are corresponding NRT samples. This plot clearly demonstrating that the heat killed *Legionella* sample does not contain any RNA, but the amplification produced is from the similar level of gDNA contamination that is present in all NRT samples.

The mean C_T values of genomic DNA amplification from full live *Legionella* lenticule and completely heat killed *Legionella* lenticule is 26.33 and 26.63 respectively. The difference of mean in these two values is 0.3 which is unlikely to be significantly different; although the same C_T values were expecting for these amplifications (Figure 4.13a). Experimental error during genomic DNA extraction and purification may have caused for this slight difference in C_T values. Amplification of cDNA produced from completely heat killed *Legionella* lenticule (Mean C_T values of RT cDNA 32.51 and NRT cDNA 32.50) shows almost similar level of amplification which is caused by the presence of genomic DNA contamination as described in previous experiments (Figure 4.13b). Despite of this negligible variation in C_T values and low level of genomic DNA contamination, the results from this experiment further confirms that the methods used for extracting RNA, cDNA synthesis and cDNA amplification in Experiments 1and 2 is successful.

4.4.9.1 Amplification differentiates the concentration of DNA and RNA

The results from Experiment-3 showed similar levels of DNA amplification from fully live and completely heat killed *Legionella* lenticules. This was expected as both live and dead samples would contain similar numbers of *Legionella* bacteria. Low level RT amplification in completely heat killed *Legionella* lenticules suggests gDNA may occur in RNA samples despite DNAse treatment.

The mean C_T values of DNA recovered from a full lenticule (live *Legionella*) and a completely heat killed *Legionella* lenticule were 26.33 and 26.63, respectively (mean C_T value of 26.48(±0.15sd). Several studies on nucleic acid have demonstrated that the concentration of DNA in a bacterial cell is identical regardless of their viability (Cangelosi and Meschke, 2014; Barbau-Piednoir et al., 2014); therefore, the result obtained from Experiment -3(almost similar amplification of DNA extracted from full live and full heat killed *Legionella* lenticule) is to be expected. Furthermore, the mean C_T value of total RNA concentration in a fully heat killed *Legionella* lenticule was 32.51 although no amplification was expected from this sample due to the absence of RNA in the sample. However, the mean C_T value of all NRT samples, two DNA samples and one cDNA sample was 32.44(±0.07sd) indicating the presence of a low and consistent amount of level of persisting genomic DNA contamination in all samples. Therefore,

this study confirms that the apparent low level amplification of cDNA synthesized from RNA extracted from heat killed *Legionella* lenticules in all the experiments is most likely a consequence of persisting genomic DNA contamination. In addition, these results further confirm that RNA extraction from live *Legionella* in lenticules is successful, and that the amplification of cDNA from live *Legionella* lenticules exceeds that which occurs due to contamination by gDNA.

4.4.10 Results of Experiment 4

Aim of this experiment was to establish a relationship between cDNA amplification curves (C_T values) and standard culture analysis results, colony forming units per litre (cfu/L). The RNA extraction, purification, genomic DNA elimination and cDNA synthesis was exactly same as described in Experiment -3. Two full *Legionella* lenticules were used in this experiment for RNA extraction; one for total (100%) live detection and the other for preparing five different dilutions as 50%, 25%, 12.5%, 5% and 0.5%. The RNA extracted from full lenticule (live *Legionella*) and the dilution series were amplified separately to minimise the overlapping of cDNA amplification curves of dilution series with gDNA amplification. This experiment also aimed to identify the lowest cut off value of cDNA amplification. In addition, 18 samples were prepared in the same dilution (all in triplicate) using six full *Legionella* lenticules for standard culture analysis and sent to a UKAS accredited laboratory for standard culture analysis (Refer Chapter 3.2.4). PCR amplification results were compared with gold standard culture analysis results.

4.4.10.1 Comparison of C_T values with culture analysis results (cfu/L)

Experiment -4 was mainly aimed to find out two important factors in this assay; firstly what is the lower detection limit of cDNA amplification and the secondly to compare culture analysis results of the RNA dilution series with C_T values corresponding to the same dilution series. The mean of standard culture analysis result of three full (100%) *Legionella* lenticules was 9900 cfu/L (±1353sd). In addition, the culture analysis results of three different dilutions, (50%, 25%, and 12.5%) were 5000cfu/L (±360sd), 2166cfu/L (±208sd) and 566cfu/L (±208sd). Culture analysis was unable to detect any

Legionella at 5% and 1% dilution suggesting that the culture method used here could not detect *Legionella* in 500ml samples at concentrations lower than 500cfu/L.

The mean C_T values of cDNA amplification for full lenticule (100%) and dilution series, 50%, 25%, 12.5% and 5% was 22.94, 30.26, 31.54, 33.12 and 35.89, respectively. A comparison of standard culture analysis results and C_T values of the similar dilution series were carried out as 9900 cfu/L ≃ 22.94, 5000cfu/L ≃ 30.26, 2166cfu/L ≃ 31.54 and 566cfu/L ≃ 33.12 as shown in the figure 4.14. The C_T value for 12.5% dilution was 35.89, but culture analysis for corresponding dilution was non-detected. A linear relationship is observed in standard culture analysis; proportionate reduction in *Legionella* count (cfu/L) as dilution increases from 100% to 25% (Figure 4.14) and a similar trend was also observed in cDNA amplification from the same dilution series (Figure 4.14).

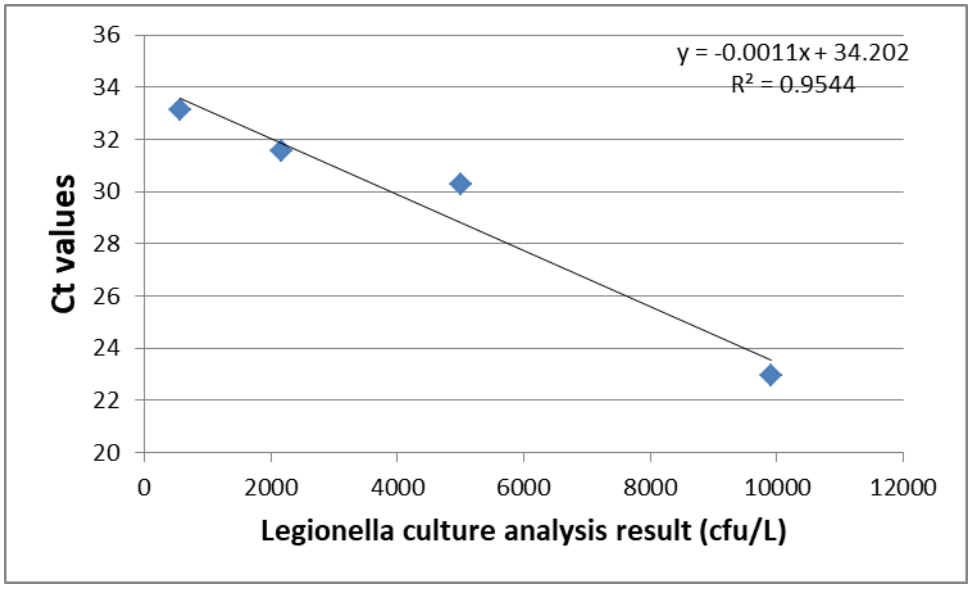


Figure 4.14 A Linear relationship between the number of *Legionella* analysed by standard culture analysis (cfu/L) in dilution series 100%, 50%, 25% and 12.5% and amplification of cDNA extracted from RNA of the similar dilution series.

PCR amplification was successful for cDNA made from 10% dilution series; although it was at the same level of genomic DNA contamination, so that it is impossible to distinguish the signal due to the cDNA. On the basis of this results presented, it is estimated that in the event of successful elimination of genomic DNA contamination from RNA yield, amplification of cDNA from 1% dilution also could be identified; and thus the lower detection limit can be established. In contrast standard culture analysis was unable to

detect any *Legionella* even in 10% dilution. It is widely understood that this is one of the major unreliability factors in culture diagnostic procedure as it struggles to detect lower concentrations of bacteria in environmental samples (Cunha et al., 2016). In a comparative study on *Legionella* diagnostic procedures such as conventional culture analysis, real time PCR, viability labelling and immune-detection (solid-phase cytometry) demonstrated that culture analysis failed to detect lower concentration of *Legionella* bacteria (>100cfu/L) as well as VBNC *Legionella*. Furthermore, this study also examined the amplification of DNA from dead bacterial cells resulting in overestimation of the presence of bacteria by real time PCR techniques (Scaturro et al., 2016).

Conventional culture diagnostic procedure for *Legionella* cannot detect lower concentration as well as VBNC *Legionella* and real time PCR based on DNA amplification detects viable and non-viable *Legionella* from environmental samples. A recent study from the UK also reported that there is a serious data gap in understanding how to use data from *Legionella* analysis using qPCR (Collins et al., 2017). But, this laboratory based assay on the amplification of synthesised cDNA from RNA extracted from live *Legionella* confirms that live detection of *Legionella* is possible and it can be applied in environmental water sample analysis without detecting non-viable cells. This method would be of great advantage in interpretation and understanding of qPCR techniques for *Legionella* control.

Total six cDNA samples were synthesised from RNA extracted from full lenticule (live *Legionella)* and five different dilutions were prepared. All samples were amplified in triplicate and the PCR amplification result was proportionate to the bacteria culture dilution series up to 5%; however, amplification corresponding to 0.5% RNA dilution was not detected. The amplification curve from 5% shows a C_T at approximately the same level of amplification from the genomic DNA contamination and it is therefore difficult to identify (Figure 4.15). Otherwards, the signal from the cDNA is swamped by the contamination at this level. In this experiment, all the dilution triplicates produced consistent amplification (Figure 4.15) but, later amplification from NRT and master mix as seen in figure 4.15 is caused by persisting genomic DNA contamination. This amplification did not significantly affect the quality of the experiment; although the 5% dilution amplification curve was overcrowded with these curves. The C_T values corresponding to each dilution with the mean values are given in Table 4.11.

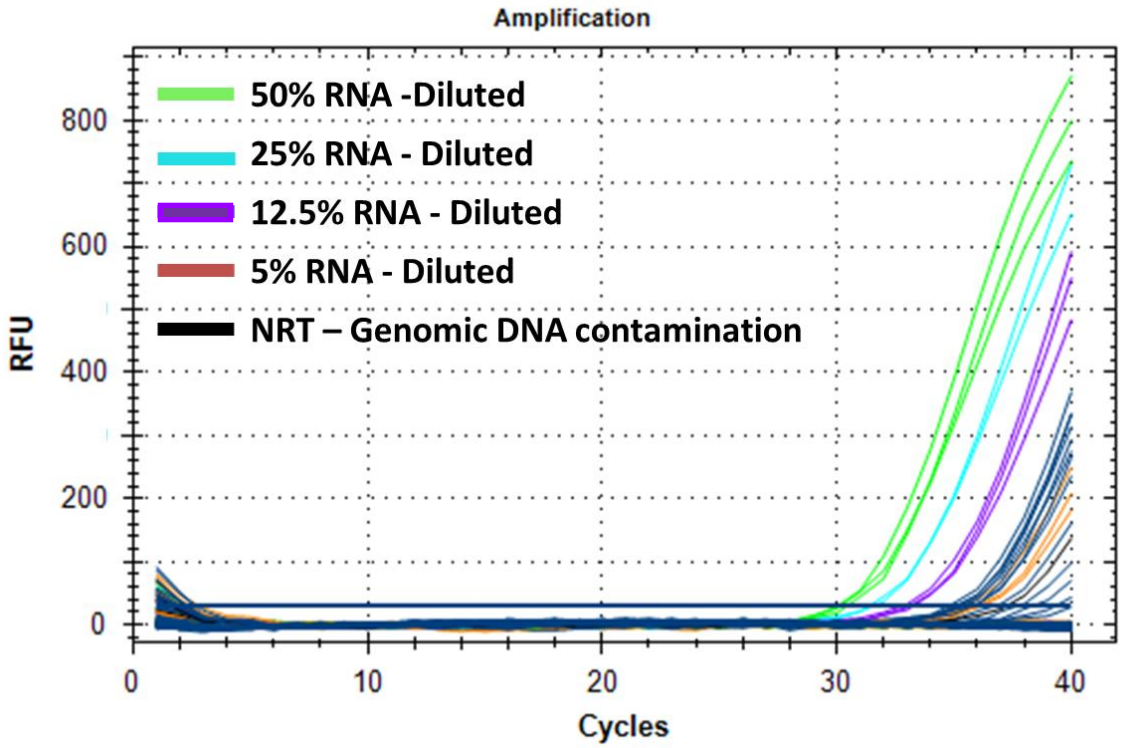


Figure 4.15 Amplification of cDNA synthesised from RNA extracted from dilution series 50%, 25%, 12.5%, 5% and 0.5% from full live *Legionella* lenticule showing the proportionate reduction in amplification.

Table 4.11 The C_T values corresponding to 100% (full live *Legionella*) and each RNA dilution along with mean C_T values.

The C_T values produced from 100% cDNA concentration, 50% cDNA concentration, 25% cDNA concentration, 12.5% cDNA concentration and 5% cDNA concentration is differed but, one of the triplicates did not amplify from 25%. Therefore, the mean value is calculated from two of the C_T values produced. In the case of 12.5% and 5% dilution, all the triplicates were amplified as shown in the Table 4.11. The C_T value of NRT also differed proportionately up to 12.5% dilution; NRT amplification from 5% dilution was almost similar to that of 12.5% dilution and 0.5% dilution was not amplified at all.

Concentration	Full lenticule (100%)		50%		25%		12.5%		5%		0.5%	
	RT	NRT	RT	NRT	RT	NRT	RT	NRT	RT	NRT	RT	NRT
	23.19	32.90	30.20	36.92	31.44	38.66	33.31	39.22	35.54	39.81	-	-
	23.01	32.44	30.09	36.78	31.64	38.80	32.89	38.99	36.24	-	-	-
	22.64	32.78	30.49	37.01	-	38.79	33.16	39.61	35.90	39.92	-	-
Mean	**22.94**	**32.70**	**30.26**	**36.90**	**31.54**	**38.75**	**33.12**	**39.27**	**35.89**	**39.86**	-	-

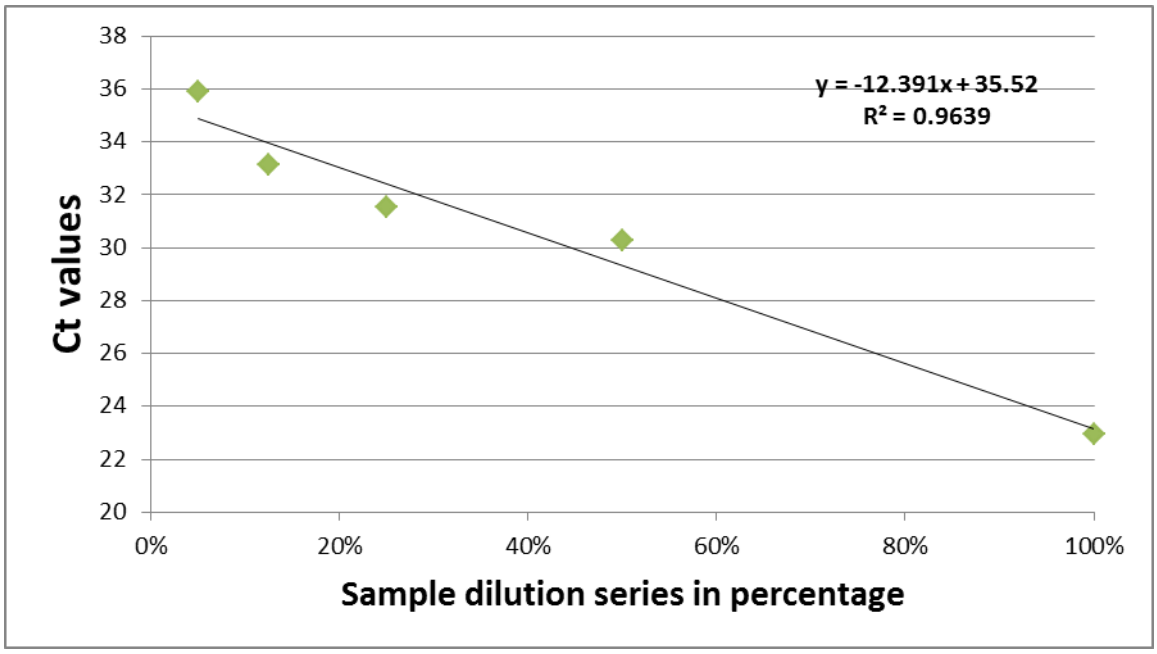


Figure 4.16 Scatter plot representation of amplification of cDNA (mean C_T) synthesised from RNA extracted from full lenticule (Live *Legionella* (100%) and dilution series 50%, 25%, 12.5%, and 5% from full lenticule (live *Legionella)* showing the proportionate reduction in amplification.

One full *Legionella* lenticule and five dilutions (all in triplicate) were analysed in UKAS accredited laboratory 'ALS Global' by using standard culture methods to establish a quantitative relationship with C_T curves. The results received from the laboratory are given in Table 4.12.

Table 4.12 Full *Legionella* lenticule and dilution series 100%, 50%, 25%, 12.5%, 5% and 0.5% culture analysis results received from UKAS accredited laboratory with the mean of triplicates.

Sample description	***Legionella* pneumophila (cfu/500ml)**	**Mean (cfu/500ml)**	**Mean (cfu/L) Calculated**
Full lenticule sample (100%) - A	4250	4950	9900
(100%) - B	5600		
(100%) - C	5000		
50% - 1	2650	2500	5000
50% - 2	2300		
50% - 3	2550		
25% - 1	1050	1083	2166
25% -2	1000		
25% -3	1200		
12.5% - 1	200	283	566
12.5% -2	400		
12.5% -3	250		
5% - 1	Not detected	-	-
5% -2	Not detected		
5% -3	Not detected		
0.5% - 1	Not detected	-	-
0.5% -2	Not detected		
0.5% -3	Not detected		

Standard culture analysis showed a full *Legionella* lenticule dissolved in 500ml deionised water contains live *Legionella* pneumophila concentration of 9900cfu/L (mean of three *Legionella* lenticule results); in addition, 100%, 50%, 25% and 12.5% dilution showed 9900cfu/L, 5000cfu/L, 2166cfu/L and 566cfu/L, respectively and 12.5% and 0.5% showed as non-detected. This culture result was compared with corresponding qPCR amplification plots (Figure 4.16). Standard culture analysis could not detect the bacteria count in 5% and 0.5% dilutions; in contrast, 5% dilution was clearly detected and amplified in qPCR, although amplification of 0.5% dilution was not clearly visible as this is mixed with the amplification from persisting genomic DNA contamination or swamped by the contamination.

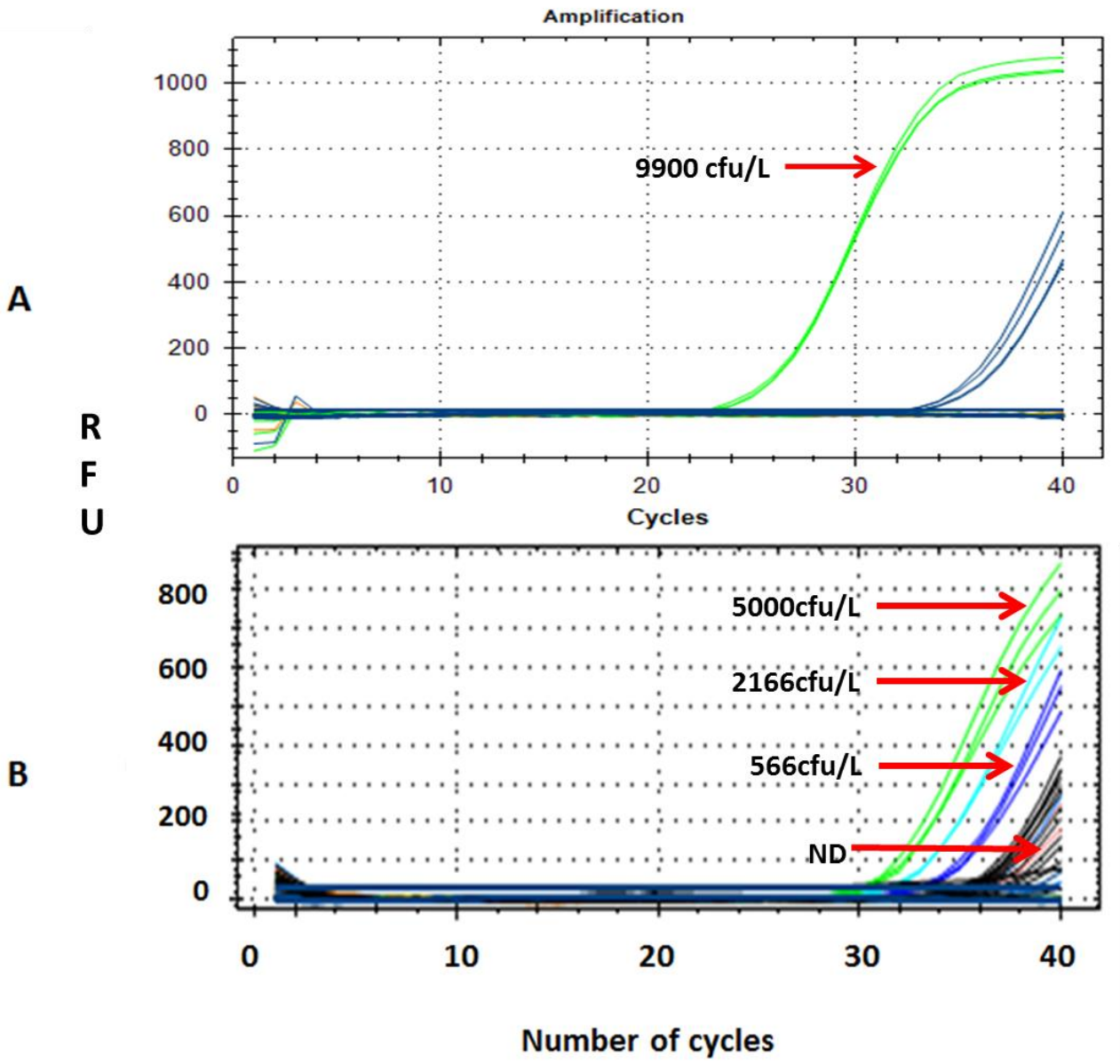


Figure 4.17 Comparison of culture analysis results with qPCR amplification plots from RNA extracted from full lenticule (100% live *Legionella)* 'A', and dilution series 50%, 25%, 12.5% and 5% 'B'. Black curves in 'A' and 'B' represents the gDNA amplification from NRT samples.

4.4.11 Results of Experiment 5

This experiment was designed to investigate the source of gDNA contamination as well as to confirm the qPCR response to a dilution series, i.e. to ensure that a proportionate increase in RNA dilution resulted in a proportionate reduction in amplification of cDNA.

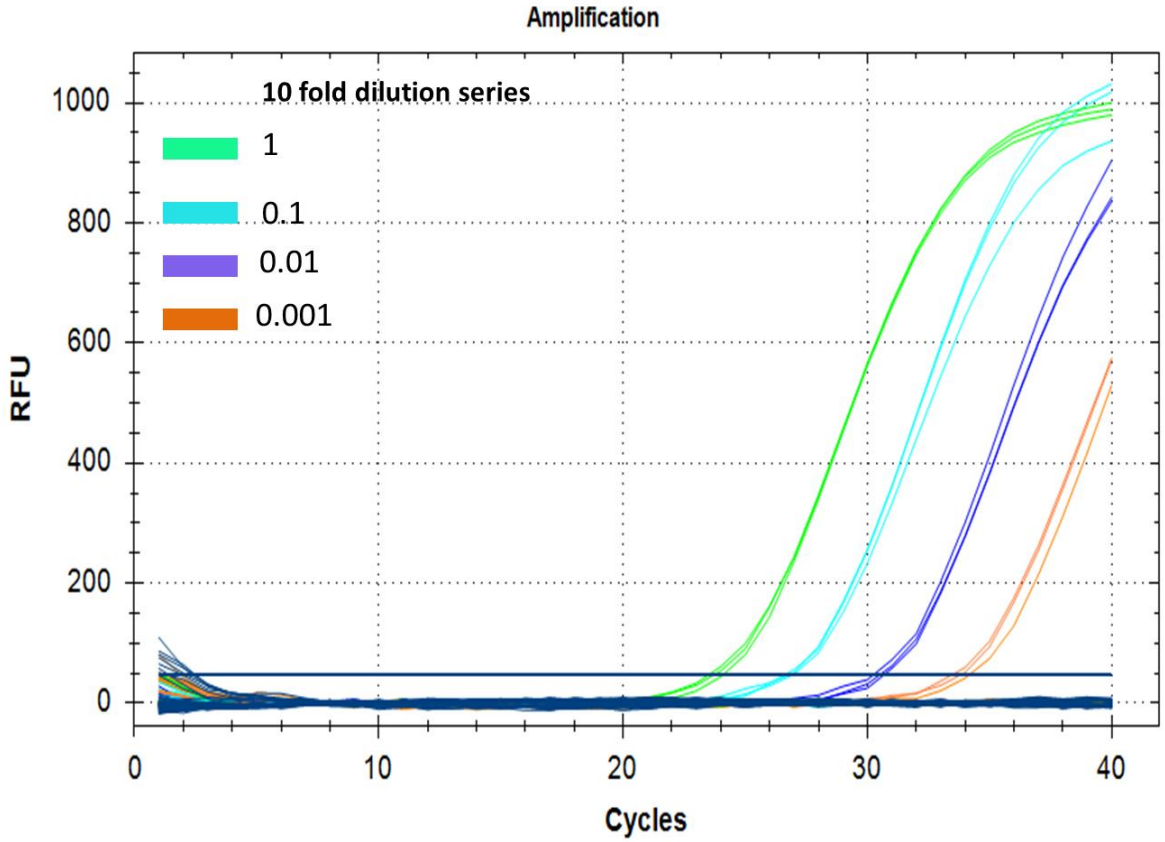


Figure 4.18a Amplification of cDNA from RNA extracted from a bacteria culture of 10 fold dilution series 1, 0.1, 0.01 and 0.001 as 1,2,3 and 4 respectively in triplicate. Plot shows no gDNA contamination from any of the wells as well as proportionate increase in C_T values with increase in bacteria culture dilution.

Table 4.13 qPCR amplification data of four 10 fold dilution series of cDNA with mean C_T values used to generate the scatter plot.

Sample	C_T -1	C_T -2	C_T -3	Average C_T value	Sample quantity	Log (sample quantity)
1	24.09	23.73	23.51	23.77	1.0000	0.00
2	26.95	26.8	26.62	26.79	0.1000	-1.00
3	30.51	30.68	30.25	30.48	0.0100	-2.00
4	33.47	33.82	34.17	33.82	0.0010	-3.00

This purpose of this experiment was to investigate the source of persisting gDNA contamination identified in Experiments 1-4, and to study the efficiency of the qPCR response to a dilution series of cDNA extracted from RNA. This experiment showed no gDNA contamination from any of the qPCR wells including master mix. This was the first experiment in this assay without any gDNA amplification occurring in the NRT samples. The gDNA contamination was persisting in all previous experiments even after implementing maximum precautionary measures including the use of different gDNA elimination treatments. This experiment further confirms that the source of gDNA contamination in previous experiments was either from the lab space used for RNA extraction or from the old stock reagents such as primers/probes, TaqMan master mix and deionised water.

The entire triplicate produced consistent amplification and C_T values proportionate to dilution series (Figure 4.18A). This amplification further confirmed the successful extraction of RNA from live *Legionella* cells (Figure 4.18A). The amplification cycle of each dilution and their mean C_T values was consistent with the proportionate reduction in concentration of synthesised cDNA (Table 4.13). Furthermore, a linear relationship was also observed in cDNA amplification depending on the concentration of each dilution series as the concentration decreases, the C_T values increases proportionately (Figure 4.18B). In this experiment, the dilution factor is 10, the slope calculated is -3.382, R^2 is 0.9986. The efficiency of the qPCR assay is calculated using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$ and found to be 97.55%. Any qPCR assay with 97.55% efficiency is considered as robust and the data generated is reliable.

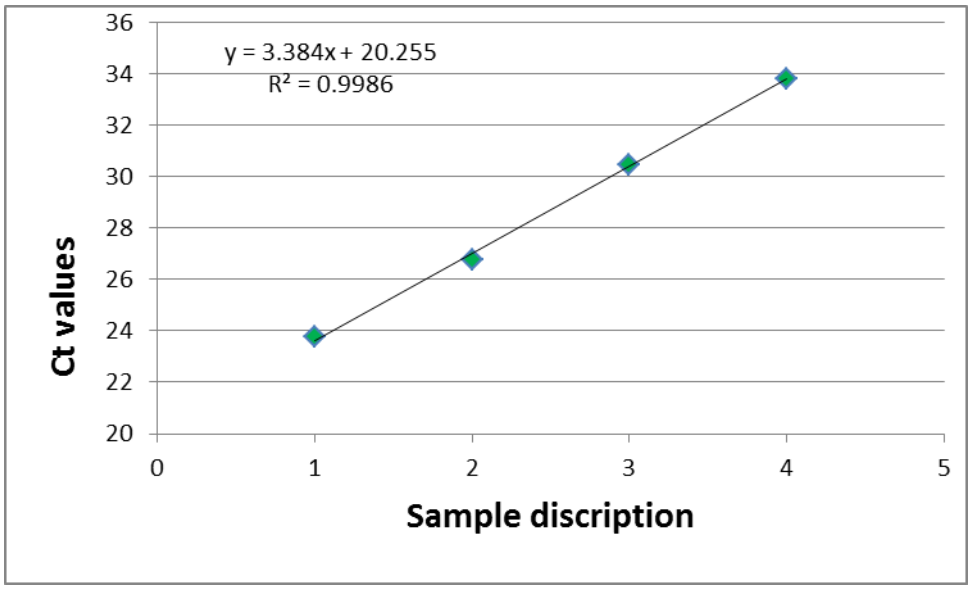


Figure 4.18b Scatter plot generated from the cDNA dilution amplification data demonstrating a linear relationship with C_T values and increase in dilution. Samples 1, 2, 3 and 4 are log sample quantity 0, -1, -2 and -3 respectively).

The results from experiments 4 and 5 showed that cDNA amplification was proportionate to the dilution of RNA and viability of the bacteria. In Experiment 4, the dilution series was in percentage; the mean of C_T values corresponding to dilution series 100%, 50%, 25% and 12.5% was 22.94, 30.26, 31.54 and 33.12, respectively (Table 4.11). The mean of CT values corresponding to 10 fold dilution series (1, 0.1, 0.01 and 0.001) in Experiment 5 was 23.77, 26.79, 30.48 and 33.82 (Table 4.13), respectively. These CT values clearly show a proportionate reduction in amplification of cDNA, indicating the amplification is proportional to the RNA concentration in each sample. Various studies on DNA amplification using PCR have demonstrated that CT values are proportionate to the concentration of genomic DNA templates (O'donnell et al., 2016; Zhong et al., 2016). Indeed a linear relationship between DNA amplification cycles (CT values) and concentration of DNA templates is observed (Lv et al., 2016; Gudnason et al., 2007).

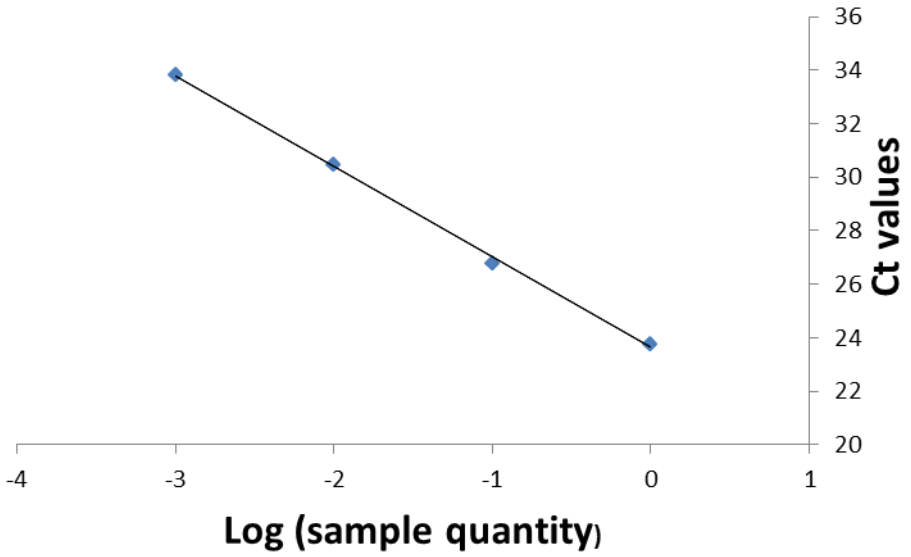


Figure 4.19 Linear correlations between C_T values and sample RNA concentration. C_T values increases as RNA concentration decreases in Experiment 5.

Experiment-5 showed a linear relationship with RNA concentration and PCR amplification (C_T) values (Figure 4.19). The slope was calculated from the scatter plot generated from corresponding PCR data; with dilution factor 10, the slope is -3.382, R^2 is 0.9986 and efficiency is 97.55%. A slope and efficiency in this range indicates that the assay is reliable (Svec et al., 2015).

A 'SWOT' analysis also has been carried out to assess the strength and weakness of this assay (Table 4.14).

Table 4.14. SWOT analysis of the PCR assay based on the cDNA amplification from RNA extracted from live *Legionella* bacteria.

Strength	**Weaknesses**
1. Live *Legionella* detection is possible. 2. Do not detect non-viable *Legionella* cells. 3. Detects VBNC *Legionella* bacteria 4. Detects lower concentrations of *Legionella* bacteria compared to conventional culture analysis. 5. No risk of underestimation of bacterial population as in culture analysis. 6. No overestimation of bacterial population as in current real time PCR technique. 7. Accurate result can be available in 3-4 hours' time. 8. Delay in between water sampling and remedial action implementation can be avoided.	1. Genomic DNA contamination can affect the quantification. 2. Lower detection limit could not be identified due to the presence of genomic DNA contamination. 3. During RNA extraction and purification, there are possibilities for RNA loss from the samples, which can adversely affect the quantification. 4. Possibility of contamination during RNA extraction and purification. 5. Inhibitors present in the environmental water samples may affect the reaction.
Opportunities	**Threats**
1. Accurate live detection of *Legionella* bacteria in domestic hot and cold water systems can significantly reduce the risk of Legionnaires' disease. 2. Accurate detection will help the responsible people to take appropriate action to control *Legionella*; thus a significant reduction can expect in the number of community acquired pneumonia (CAP) cases. 4. Early stage accurate detection of *Legionella* in water systems can help to take precautionary measures and can avoid frequent disinfection works which can make significant financial savings in terms of *Legionella* control and water hygiene maintenance. 5. Possibility of the approval from the regulating government authorities such as HSE, DEFRA, EA and private experts. 6. Potential commercial benefit from laboratories in the field of environmental water sample analysis for *Legionella* worldwide. 7. This live detection assay can be implemented in other areas of micro-biological analysis.	1. Commercial laboratories in the field of *Legionella* testing may not entertain the new method due to the legal implications in the event of possible failure. 2. Regulating authorities may not support/approve the new method. 4. Building managers may take a negative attitude towards the new method when comparing with the analysis cost of standard culture method.

4.5 Conclusion and recommendation

4.5.1 Conclusion

The results presented in this assay clearly indicate that the live detection of *Legionella* bacteria is possible with real time PCR techniques, although accurate quantification of live bacteria still presents some challenges. This assay confirms that RNA extraction from *Legionella* lenticules using the Qiagen RNA extraction kit, cDNA synthesis and amplification of cDNA using the 23S primers was successful.. Precautions and care must be taken to avoid possible genomic DNA contamination, especially when dealing with primers, probes and TaqMan Mastermix. Further research is needed in setting up the lower detection limit for quantification. Further validation of this method with for detecting live *Legionella* bacteria in real environmental water samples in still needed. However, this method looks promising in the field of *Legionella* control as well as in many other areas of water microbiology.

4.5.2 Future work and Recommendations

- Investigate the sensitivity of the method from 100 cfu/L and below (real world applicability).
- Validate the primers/probes used in this assay with other *Legionella* species to confirm the specificity of the assay to detect all the *Legionella* species.
- Establish the relationship between cfu/L and C_T value more accurately.
- Lenticule standards with accurate levels of bacteria should be developed to support development of a rapid qPCR kit. These could serve as positive control whilst providing quantitative information (cfu/L)
- Investigation will need to identify the possible interfering substances (inhibiting substances) when dealing with real environmental samples and develop methods to get rid of the possible inhibitors.
- Carry out further validation of the method after the above mentioned refinement.

- Carry out the test use of this novel approach to detect live *Legionella* in real-world samples (environmental water samples) and compare it with Gold Standard (culture).
- PCR assay that targets the 23S-5S rRNA intergenic spacer region allows detection of all *Legionella* species and discrimination of *Legionella* pneumophila from other *Legionella* species. The 23S rRNA gene was used as a target to detect all *Legionella* spp., and the mip gene was targeted for the specific detection of L. pneumophila in this multiplex Taqman real-time PCR assay.

CHAPTER FIVE

DEVELOPMENT OF AN EVIDENCE BASED RISK MANAGEMENT MODEL

5.1 Introduction

One of the important steps in *Legionella* control is to carry out a suitable and detailed *Legionella* risk assessment in order to then establish a regular monitoring program on the basis of the identified and possible risk factors (HSE, 2013). Qualified and trained individuals carry out the risk assessments using the guidelines BS 8580:2010, after which a decision is made about the nature and extent of the ongoing monitoring programme (HSE, 2013). However, human errors that affect decisions about monitoring occur, and consequently, low and medium risk buildings can mistakenly fall into a high risk category, resulting in a significant increase in monitoring intensity and sampling frequencies. This could add additional financial burdens to building management companies in terms of *Legionella* control. Equally as serious are events where a 'high risk' building is placed into the 'low risk' category, so that water facility users in any particular building face the unabated and hidden threat of Legionnaires disease from their domestic water systems. In order to help address such inconsistencies in decisions about *Legionella* control caused by human errors or subjectivity a standard uniform model (tool) is needed to categorise any building purely on the basis of identified risk factors in terms of *Legionella* control.

Indeed, professional discussions with building managers responsible for water hygiene management and water treatment, and Legionella control experts in the industry, has confirmed this to be a major gap in Legionella control and management; a standard platform based on a statistical model would be of a great advantage in addressing this serious concern. Whilst discussing this data gap with my second supervisor (Professor Xiaohui Liu, Director of The Centre for Intelligent Data Analysis Department of Computer Science, Brunel University London) it became apparent that Principle Component Analysis was the best statistical approach to develop this risk predictive model.

5.2 Aim

The main aim of this research was to integrate existing building monitoring data (including information on *Legionella* status) into a statistical approach known as Principal Component Analysis (PCA) in order to develop an evidence-based risk management system (model) for the better understanding of the risk category of any domestic building in terms of *Legionella* bacteria. If successful, human errors during *Legionella* risk assessments (practioners, responsible personals and industry experts are aware of this human errors, but rarely studied and documented) and can be minimised and decisions related to establishing monitoring programmes can be improved; better protection from Legionnaires disease as well as significant financial savings can be achieved by avoiding unnecessary monitoring and water sampling for domestic water systems. In addition, the impact of changes to building water systems can be modelled for their likely impact on Legionella status prior to work being carried out.

5.3 Methodology

The primary approach was to use routine building monitoring data to identify and rank the common risk factors that could lead to the proliferation and harbouring of *Legionella* bacteria in the domestic water systems, and to integrate these into a risk predictive model. The Risk Predictive Model was developed using a statistical approach known as Principal Component Analysis (PCA). Principal Component Analysis is one of the most frequently used multivariate data analysis methods. It is a projection method as it projects observations from a p-dimensional space with p variables to a k-dimensional space (where $k < p$) so as to conserve the maximum amount of information from the initial dimensions. PCA dimensions are also called axes or Factors. If the information associated with the first 2 or 3 axes represents a sufficient percentage of the total variability of the scatter plot, the observations could be represented on a 2 or 3-dimensional chart, thus making interpretation much easier. PCA can thus be considered as a Data Mining method as it enables easy extraction of information from large datasets. The study and visualization of the correlations between variables can be used to limit the number of variables to be measured and

these factors can be used in modelling methods such as linear regression, logistic regression or discriminant analysis. PCA enables the visualisation of observations in 2 or 3 dimensional spaces and helps identifying uniform or typical groups of observations (xlstat.com/pca).

PCA aims to present multivariate information in a correlation matrix to highlight the relationship among combinations of variables in a data set. The higher the percentage of variance that the model manages to explain, the more valid the model will be (xlstat.com/pca). The contribution of each composite variable (i.e. a new variable created by combining 2 or more individual variables together - known as a 'component') to the explained variance of the model can be described as a percentage, enabling users to eliminate unwanted variables whilst maintaining the optimal number of components needed for maximum predictive power. However, once the principle components that explain the variance have been established, the percentage contribution of each component to variance becomes somewhat arbitrary (and therefore is not considered in this model), as PCA emphasises the strong patterns, or relationships, in the data (shown in figure 5.0).

Figure 5.0 is an example of PCA plot generated in the study of *Trypanosoma brucei* infections in mice. The black dots represents the number of uninfected mice; blue dots shows the changes after the first day of infection, green dots represents the 3rd day of infection and red dots represents the 4th day of infection. PCA was able to distinguish between the different categories of mice using a common set of variables, thereby enabling categorisation of the infected and non-infected mice on the basis of the input measurements. The same concept was applied in the development of *Legionella* risk predictive model, except instead of mice we are using buildings.

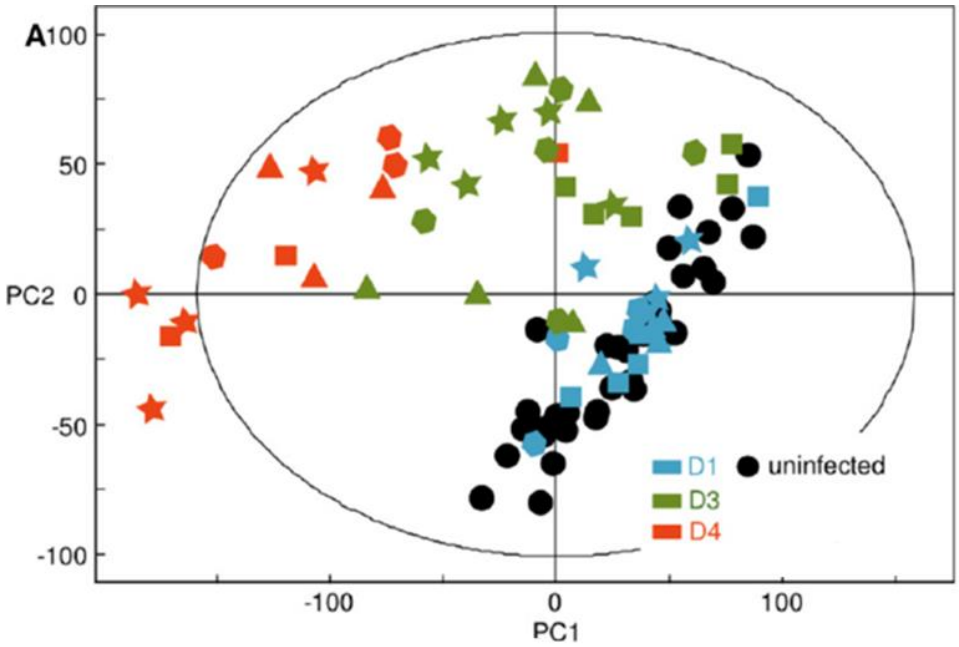


Figure 5.0 Principal component analysis (PCA) plots derived from a study of urinary nuclear magnetic resonance (NMR) spectra of mice; pre-infection (black), after 1 day of infection (blue), 3rd day of infection (green) and 4th day of infection (red). Source: Li et al., 2011.

Six main steps were used to develop this basic model as follows:-

5.3.1 Compilation of risk monitoring data

Any building with communal water storage tank(s) or a communal hot water system in the UK comes under HSE's legislation ACOP L8 2013 HSG 274 Part 2(HSG, 2013). In order to comply with this legislation, such buildings must undertake a risk monitoring assessment by an independent qualified engineer. As a part of my profession, I have access to such risk monitoring data from hundreds of London buildings, and randomly selected 180 sites of known *Legionella* status to compile the raw risk monitoring data (data was available for the past 6 years). From these data, I identified 60 infected sites, and therefore randomly selected the same number of non-infected sites for comparison and development of the model.

These data (present as monitoring forms completed by hand) included the age of the building, size, usage, total number of cold water storage tanks, actual cold water

storage, actual cold water requirement, percentage of excess water storage, total number of calorifiers, total number of communal showers, status of existing *Legionella* risk assessment (if any), total number of deadlegs in the systems, inspection frequency, temperature monitoring frequency, number of *Legionella* positive results in the past six years, tank's internal condition at the time of positive *Legionella* detection, condition of the thermal insulation of the tank(s) and pipework, water sample temperatures, season of positive results, remedial action used and the re-sampling result. The compiled risk monitoring data is shown in Appendix-1

5.3.2 Data entry

Initially monitoring data from 180 sites was compiled and from these data, I identified 60 infected sites, and randomly selected another 60 non-infected sites for comparison and development of the initial PCA model. Throughout the model development, a number of new sites (of unknown *Legionella* status) were also used to validate the predictiveness of the model, including sites from outside of London to investigate the applicability of the model nationally. Data presented throughout this Chapter can therefore vary in sample size.

The risk monitoring data compiled from the monitoring survey forms were systematically transferred to a spread sheet, and colour coded to identify different usage of the buildings (e.g. Residential, Commercial, and Care Home). Colour coding was also used to gain a more wholistic impression of the compiled monitoring data when split into *Legionella* positive and negative sites. This helped develop impressions and hypotheses about the data, including the likely relative importance of different risk factors to the overall *Legionella* status of the sites.

Table 5. 1a Numerical codes for age of the building, usage and availability of the risk assessment.

Age of the building	Code	Building category (usage)	Code	Risk Assessment available	Code
2000+	1				
1900 – 1999	2	Residential	1	Yes	1
1800 - 1899	3	Mix use (Residential and commercial)	2	No	2
Older than 1800	4	Commercial	4	N/A	0
		Care Home	4		

Table 5.1b Numerical codes for temperature monitoring frequency and sampling frequency.

Temperature Monitoring frequency	Code	Sampling frequency	Code
Monthly	1	Monthly	1
Quarterly	2	Quarterly	2
Half yearly	3	Half yearly	3
Annually	4	Annually	4
N/A	0	N/A	0

Table 5.1c Numerical codes for internal tank(s) condition and thermal insulation of the tanks and pipework.

Tank internal condition	Code	**Thermal insulation of the tank(s) and pipework**	Code
Visibly clean	1	Satisfactory	1
Corrosion	2	Not satisfactory	2
Sedimentation	3	Pipework not insulated	3
Corrosion and sedimentation	4	Uninsulated tank	4
Scale and sedimentation	5	Uninsulated tank and pipework	5
Stagnant water with bio film	6	N/A	0
Scale, sedimentation, and corrosion with bio film	7		
N/A	0		

Table 5.1d Numerical codes for annual occupancy rate and temperature recording during routine inspection visit.

Occupancy rate (%)	**Code**	**Temperature**			
100	0	**Hot**	**Code**	**Cold**	**Code**
90-100	1	60	0	<12	0
80-89	2	58-59	1	12-13	1
70-79	3	55-58	2	14-16	2
50-69	4	50-54	3	17-18	3
<50	5	48-49	4	19-20	4
		45-47	5	21-24	5
		<45	6	25+	6

Table 5.1e Numerical codes for seasonality.

Season	**Code**
December, January and February	1
March and April	2
May and November	3
June and October	4
July, August and September	5

All sites reported with *Legionella* positive results were then sorted so that they appeared as a group at the top of the spread sheet, followed by the non-infected sites. In order to maintain a consistent approach, risk factors (non-numerical data) were numerically coded according to severity (into categorical data), where a value ≤1 represents a low risk and higher numbers denote increasing levels of risk with respect to *Legionella* proliferation. These are shown in Appendix-2. The numerical codes assigned are shown in the Tables 5.1a-e. Table 5.1a shows the numerical codes for age of the building, usage and current status of the risk assessment, Table 5.1b shows the numerical codes for temperature monitoring frequency and sampling frequency and Table 5.1c shows numerical codes for internal tank(s) condition and thermal insulation of tank(s) and pipework. Increasing numbers represent a decrease in the health e.g. visibly clean – 1, presence of corrosion -2, presence of sedimentation– 3, presence of corrosion and sedimentation – 4, scale and sedimentation -5, stagnant water with bio film -6, scale, sedimentation, and corrosion with bio film -7.

5.3.3 Identification and ranking of the most common risk factors for *Legionella* contamination/proliferation.

In order to identify and rank the most important risk factors for *Legionella* occurrence within the monitoring data (see Appendix 2) I compared whether the various factors differed statistically between *Legionella* infected and non-infected sites. Out of 180 London sites in the spread sheet -1, 60 sites had *Legionella* positive results during the past six years and these 60 sites were compared against another 60 sites which had never had any reported *Legionella* positive results. A number of different tables (see Appendix- 3) and graphs (e.g. Figure 5.1) were prepared to summarise the data from the infected sites and to identify the contributing factors. The mean and standard deviation of each factor from both categories was calculated and the sum of mean and standard deviation was used to generate the box plots with error bars (e.g. Figure 5. 2 and Figure 5.3).

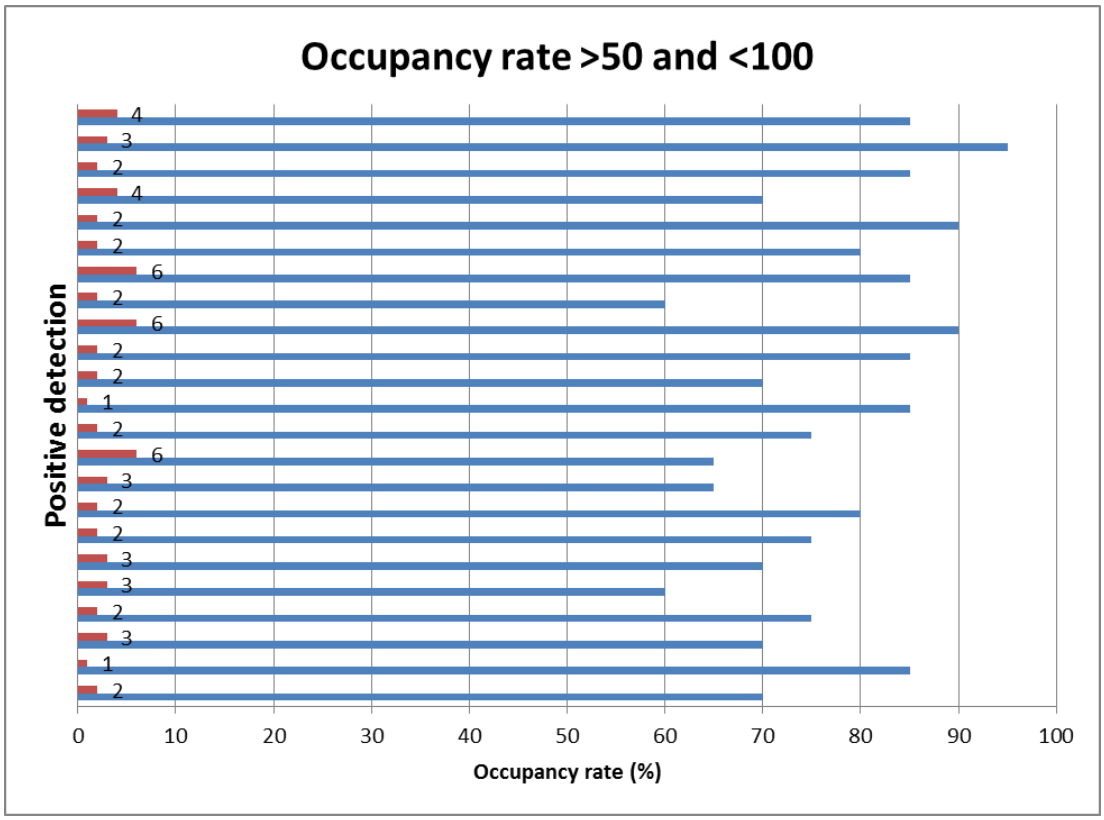


Figure 5.1 Graph prepared from the table comparing the average building occupancy rate (%) >50 and <100 (blue bar) and the total number of *Legionella* positive detection per building (red bar) in the past six years. The minimum occupancy rate documented was 55%, hence the graph shows the occupancy rates between 50 and 100%.

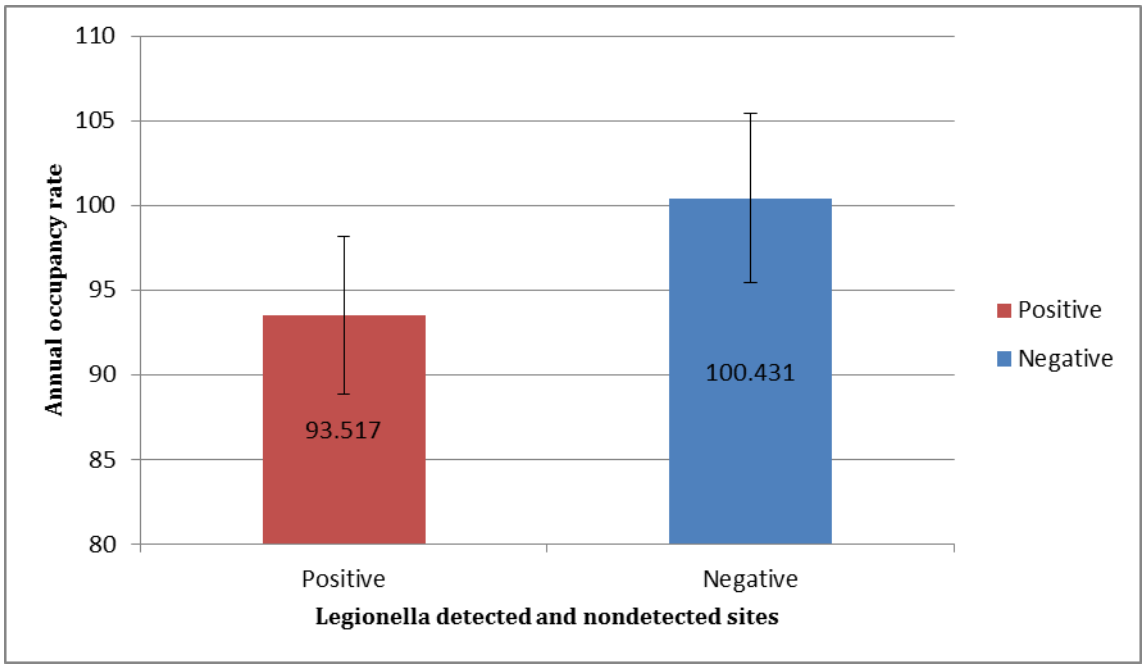


Figure 5.2 Comparison of the annual occupancy rate of *Legionella* detected (n=60) and non-detected (n=60) sites using categorical data (see Table 5.1a) and this was significantly different (P=0.0091) with 95% confidence.

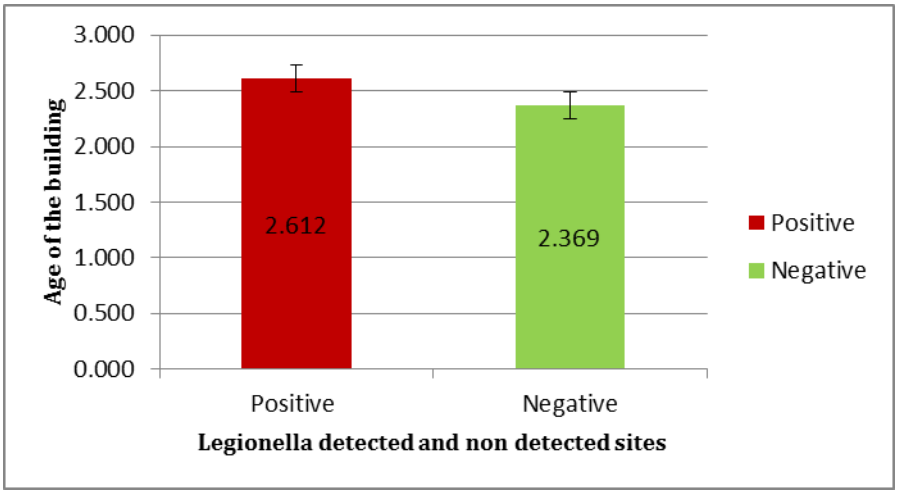


Figure 5.3 Comparison of the age of the buildings from *Legionella* detected (n=60) and non-detected (n=60) sites using categorical data (see Table 1a). Negative sites were on average slightly younger builds than positive sites, but this was not significantly different (P=0.056) with 95% confidence.

Comparative statistics between the infected buildings and non-infected buildings were carried out by calculating the 'p-value' to determine which factors significantly contributed to differences between these two groups. A parametric 't-test' was carried out on quantitative data for each factor to identify the most common risk factors using XLSTAT software. Calculated p-values for the most common risk factors are given

below (Table 5.2a). Computed p-values for all these factors are lower than the significance level alpha =0.05, indicating that these parameters differ significantly with 95% confidence between infected and non-infected sites. Calculated p-values for the less common (rarely-contributing factors) are given in (Table 5.2b)

Table 5.2a Calculated p-value for the most common contributing risk factors.

Contributing factor	p-value
Occupancy rate	0.0091
Number of water tanks	0.0005
Number of calorifiers	0.0033
Tank's internal conditions	0.0001
Thermal insulation of the tank(s) and pipework	0.0002
Excess water storage (%)	0.0001
Number of deadlegs	0.0055
Hot water temperature	0.0003
Cold water temperature	0.0001
Seasonality (summer)	0.0001
Number of showerheads	0.0014
Number of *Legionella* positive detection	0.0042

Table 5.2b Calculated p-value for the less common (rarely-contributing factors) risk factors.

Contributing factor	p-value
Age of the building	0.0860
Usage of the building (Residential, commercial, care home etc.)	0.1010
Legionella risk assessment	0.0623
Size of the building	0.2230
Number of TMV's in the building	0.0713
TVC analysis	0.0581

5.3.4 An assessment of the effectiveness of temperature monitoring.

A small comparative study was carried out between sites with effective temperature monitoring regimes in place and sites without effective temperature monitoring (frequency of less than monthly) to determine whether this approach is effective at managing *Legionella*. (It is a normal practice in the industry to carry out the temperature monitoring of hot and cold water systems; daily, weekly, fortnightly, monthly, quarterly or rarely six monthly depending on the risk highlighted in the water system risk assessment carried out in accordance with BS8580:2010. In this assay, monthly (or more frequent) temperature monitoring frequency is considered to be effective temperature monitoring). Ten sites were randomly selected in total; five with effective temperature monitoring (daily to monthly monitoring) and five without effective temperature monitoring (half yearly only). This information was systematically arranged in a table for convenience (Table 5.3).

Table 5.3 Randomly selected sites with regular temperature monitoring in place and without regular temperature monitoring in place.

Building No.	Age	Size	Occupancy rate (%)	Number of tanks	Number of calorifiers	LP * positive results	LP detected sample	Temperature monitoring frequency
1	68	1	85	2	1	2	Cold tap	Half yearly
2	213	4	80	2	3	2	Cold tap	Half yearly
3	76	4	78	2	1	1	Hot tap	Half yearly
4	68	5	72	2	1	2	CWST	Half yearly
5	83	1	90	1	1	0	NA	Half yearly
6	213	12	98	2	2	1	Hot tap	Monthly
7	193	3	95	1	1	0	NA	Weekly
8	73	9	100	4	2	0	NA	Daily
9	73	7	100	3	2	0	NA	Daily
10	7	8	100	2	1	0	NA	Daily

*LP-*Legionella* pneumophila.

5.3.5 Development of a predictive risk model to determine the likelihood of *Legionella* contamination in buildings.

A predictive risk model for *Legionella* contamination in buildings was developed on the basis of the risk factors identified in the spread sheet, risk ranking and the

comparative study of the effectiveness of temperature monitoring. The risk model was developed with the help of the statistical software XLSTAT. Principal Component Analysis (PCA) was carried out with twelve selected variables (identified as important risk factors by statistical comparisons of infected and non-infected sites – described above). Using this approach I then combined various factors together within a range of PCA plots in order to identify the best combination of factors needed to discriminate contaminated sites from non-contaminated sites.

5.3.6 Validation of the predictive risk model with new client buildings of unknown *Legionella* contamination status.

In order to validate the predictiveness of the *Legionella* risk model, 66 site visits to new client buildings in London were undertaken in order to test the ability of the developed model to correctly determine the likely risk (high or low) of *Legionella* contamination. A separate spread sheet was prepared and the site survey data was converted into numerical data as before (Appendix-4). This numerical data was then used to carry out the PCA method in order to categorise the sites into 'high risk' buildings and 'low risk' buildings. Finally, the results of this PCA (i.e. *Legionella* positive or negative) were compared to historical monitoring data (available on site) to determine the accuracy (predictive power) of the developed model.

5.3.7 Validation of the predictive risk model with sites outside of London

In order to validate the risk predictive model with out of London buildings, 9 site visits were carried out in Manchester, Darlington and Nottingham to test the predictive power of the developed model in building water system with different water quality parameters (e.g. water in Darlington buildings was found to be softer compared to water in London buildings). A separate spread sheet was prepared for these properties and the site survey data was converted into numerical data in the same was as for the London buildings (Appendix-5). This numerical data was then used to generate the PCA plot in order to categorise the sites into 'high risk' buildings and 'low risk' buildings. As described in the London building validation, PCA results of these out of London buildings were compared to historical monitoring data (available on site) to determine the accuracy (predictive power) of the developed model which was developed using data from London buildings.

5.4 Results

5.4.1 Risk factors

Most common risk factors for *Legionella* contamination identified by statistical comparisons were annual occupancy rate, number of water tanks, number of calorifiers, tanks internal condition, thermal insulation of the tanks and pipework, excess water storage, number of deadlegs, hot water temperatures, cold water temperatures, seasonality (especially summer), number of showerheads and number of *Legionella* positive results. Separate statistical comparison was also carried out for *Legionella* positive results with hot water system alone and cold water system alone. Factors contributing to *Legionella* positive results in the hot water system were annual occupancy rate, number of calorifiers, number of deadlegs, number of showerheads, number of *Legionella* positive results and water temperatures. In contrast, the factors contributing to *Legionella* contamination within the cold water system were annual occupancy rate, number of water tanks, tanks internal condition, thermal insulation of the tanks and pipework, excess water storage, number of deadlegs, water temperatures, number of *Legionella* positive results and seasonality (especially summer).

5.4.2 Effectiveness of temperature monitoring

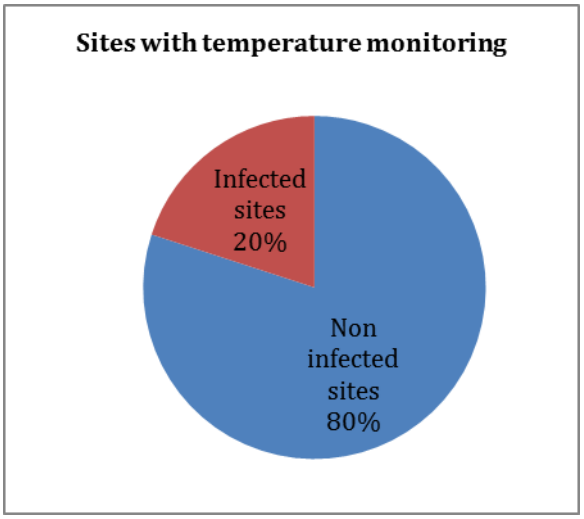
An assessment of the effectiveness of temperature monitoring was carried out for 10 sites of which five had daily/weekly/monthly temperature monitoring and five without daily/weekly/monthly temperature monitoring. One of five sites with regular temperature monitoring, had a single report of a *Legionella* outbreak in last six years (Table 5.5). Of the other five sites without weekly or monthly temperature monitoring, 4 sites had reported *Legionella* positive (total 7) in the last six years (Table 5.4). A summary of this assessment is shown in Figures 5.4a and 5.4b.

Table 5.4 Sites without regular temperature monitoring, 7 positive Legionella detections in the past 6 years.

Building No.	Age	Size	Occupancy rate (%)	Number of tanks	Number of calorifiers	LP positive results	LP detected sample	Temperature monitoring frequency
1	68	1	85	2	1	2	Cold tap	Half yearly
2	213	4	80	2	3	2	Cold tap	Half yearly
3	76	4	78	2	1	1	Hot tap	Half yearly
4	68	5	72	2	1	2	CWST	Half yearly
5	83	1	90	1	1	0	NA	Half yearly

Table 5.5 Sites with regular temperature monitoring, only one positive Legionella detection in the past 6 years.

Building No.	Age	Size	Occupancy rate (%)	Number of tanks	Number of calorifiers	LP positive results	LP detected sample	Temperature monitoring frequency
1	213	12	98	2	2	1	Hot tap	Monthly
2	193	3	95	1	1	0	NA	Weekly
3	73	9	100	4	2	0	NA	Daily
4	73	7	100	3	2	0	NA	Daily
5	7	8	100	2	1	0	NA	Daily



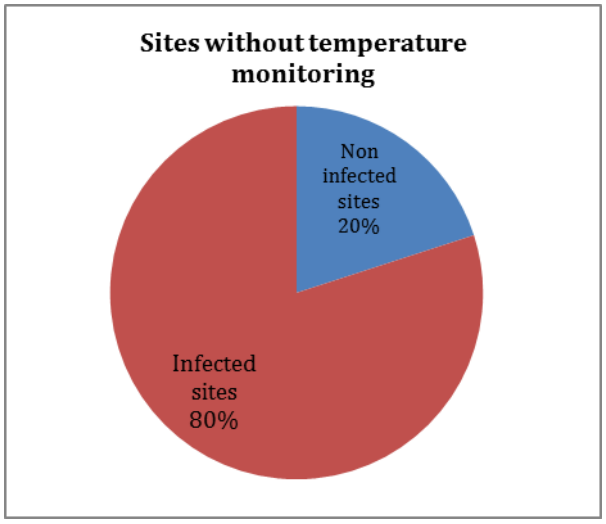


Figure 5.4a **Figure 5.4b**

Figure 5.4a shows the percentage of infected and non-infected sites where regular temperature monitoring was in place and figure 5.4b shows the percentage of infected and non-infected sites where regular temperature monitoring was not in place.

5.4.3 *Legionella* risk predictive model

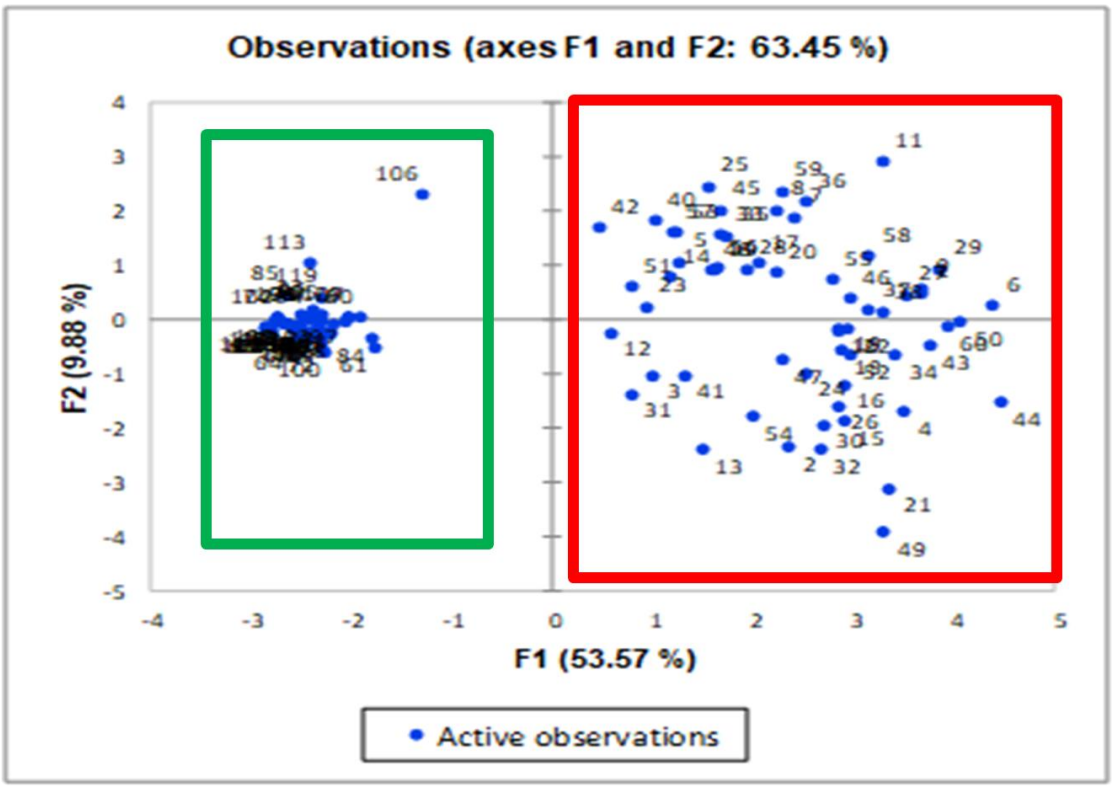


Figure 5.5 PCA for 60 infected and 60 non-infected sites. Red box represent the infected sites and the green box represent the non-infected sites.

The PCA approach was able to accurately separate buildings into low risk and high risk categories, where all the high risk buildings (*Legionella* positive) are on the right hand side of the Y-axis and all the low risk (negative for *Legionella*) buildings are on the left hand side of the Y-axis. PCA has separated 60 infected sites on the RHS of the Y axis and 60 non-infected sites on the LHS of the Y-axis. Therefore, 100% of the infected sites fell on the RHS of Y-axis and 100% of the non-infected sites fell on the RHS, i.e. all the 60 non-infected sites were correctly identified as being negative being on the LHS of the Y-axis and all of them made a cluster (Figure 5.5).

5.4.4 Validation of the risk model with new client buildings.

The PCA *Legionella* Risk Predictive Model was then applied to 66 new client buildings and the results are shown in (Figure 5.6). As per the PCA, 32 sites were found to be in the high risk category and 34 sites were in the low risk category. Real historical data available from each site confirmed that 32 buildings (building numbers 1-32) had *Legionella* positive detection in the past whereas the remaining 34 (building numbers 33-66) had no previous record of *Legionella* positive detection. Therefore, the *Legionella* Risk Predictive Model has correctly identified all previously contaminated sites as being 'high risk' (100% accurate for contaminated sites) and in the same way the model has correctly identified all non-contaminated sites as being 'low risk' (100% accurate for non-contaminated sites).

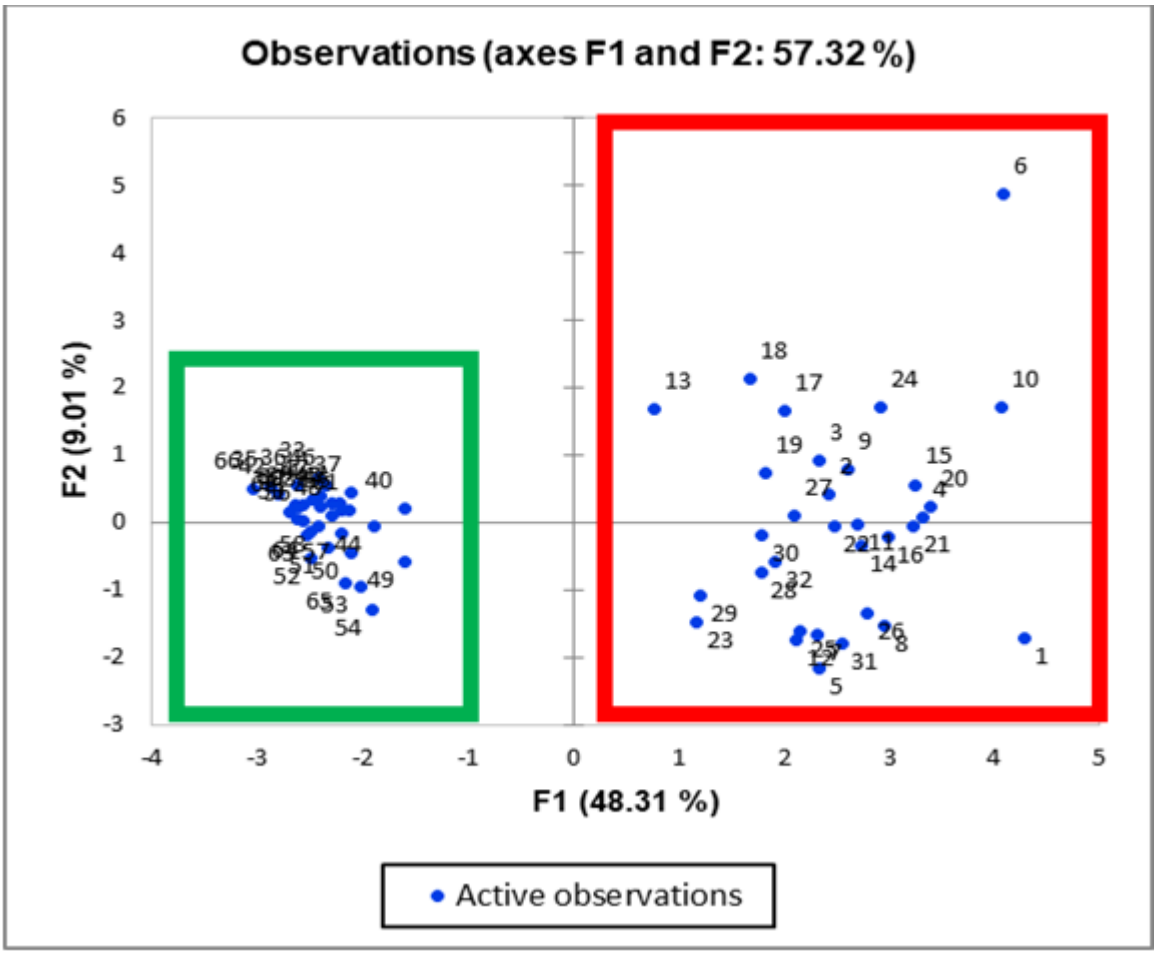


Figure 5.6 PCA for 66 new client buildings. Green box represents 34 sites that were previously found to be negative for *Legionella*, whereas the red box represents 32 sites where *Legionella* was reported previously.

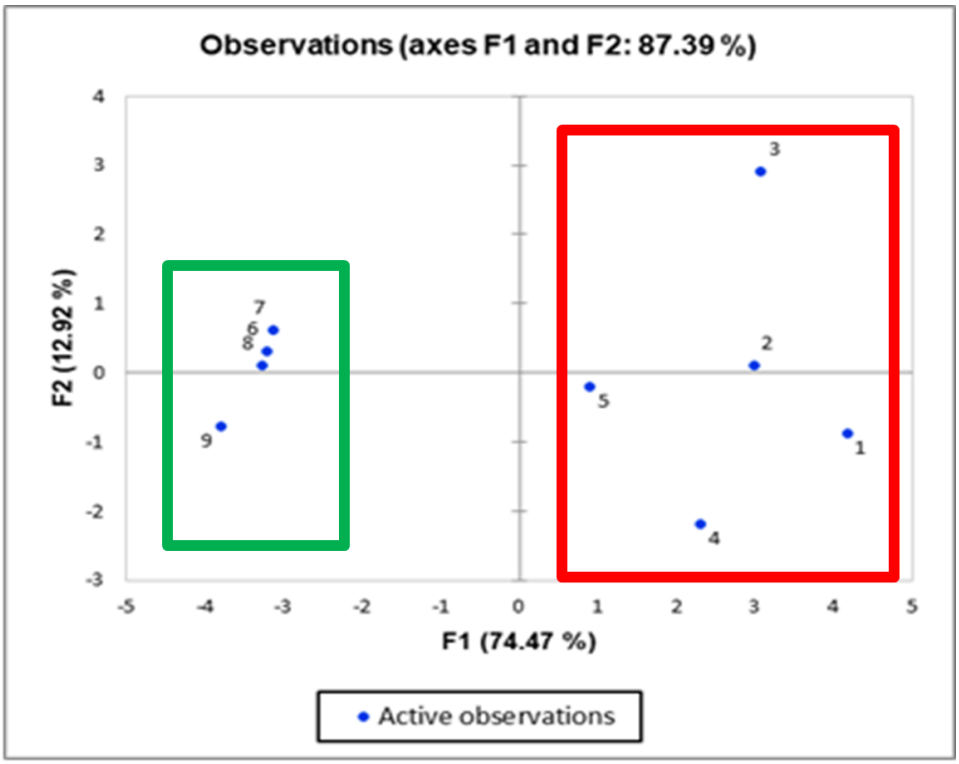


Figure 5.7 PCA for 9 new out of London buildings. Green box represent 4 sites that were previously had no *Legionella*, whereas the red box represents 5 sites where *Legionella* positive detection was reported previously.

5.4.5 Validate the application of the risk predictive model with out of London buildings.

The PCA based *Legionella* Risk Predictive Model was applied to 9 new buildings in Manchester, Darlington and Nottingham to test the predictive power of the model in building water system with different water quality parameters compared to water in London buildings and the results are shown in (Figure 5.7). As per the PCA, 5 sites were found to be in the high risk category and 4 sites were in the low risk category. Real historical monitoring data available from each site confirmed that 5 buildings (building numbers 1-5) had *Legionella* positive results in the past whereas the remaining 4 (building numbers 6-9) had no previous record of *Legionella* outbreak. Therefore, the *Legionella* Risk Predictive Model has correctly identified all previously contaminated sites as being 'high risk' (100% accurate for contaminated sites) and all non-contaminated sites as being 'low risk' (100% accurate for non-contaminated sites) regardless of geographical location of water systems and water quality parameters.

5.5 Discussion

5.5.1 Identification of risk factors

Out of 18 selected possible risk factors that can contribute towards Legionella proliferation in domestic water systems, 12 were identified as the most common risk factors using t-test and comparative studies. Mainly buildings can be categorised as residential, commercial, mixed use (residential and commercial) and industrial. Block of flats, apartments, houses and care homes comes under the 'Residential' category. Office complexes, hospitals, schools, universities etc. are considering as 'Commercial' buildings. Industrial units and factories come under the 'Industrial' category (Health Protection Agency, 2009). Out of these three categories, residential buildings, especially residential accommodation, are more susceptible to *Legionella* infection. The main reason for this is water stagnation within the pipework, which occurs when some of the individual flats/houses within the blocks are unoccupied (Rakic et al., 2011). Water stagnation within this pipework can last up to several months if the owner or occupying tenant is a rare visitor to the UK. In the other categories, there will be somebody responsible for periodically flushing all the outlets and carrying out temperature tests to comply with HSE's ACOP L8 legislation. Thus, the occupancy rate in residential buildings is a major contributing factor for *Legionella* proliferation. The building water storage facility is always designed for a 100% occupancy rate. As the occupancy rate goes down, water becomes stagnant within the storage facility (cold water storage tank and calorifier) as well as within the pipework to the unoccupied flats (Feazel et al., 2009). This was confirmed in a study that was carried out in a newly constructed building by Srivastava and team in 2009. The occupancy rate was only 71% of the original design, and thus led to the water stagnation and heat gain to the cold water system. *Legionella* serogroup-1 was cultured from the cold water sample (Srivastava et al., 2009).

The number of water tanks, the number of calorifiers and excessive water storage also cause the same effect, as all these lead to water stagnation which eventually encourages *Legionella* proliferation (HSE, 2015). Ciesielski and colleagues carried out a study in four infected hot water storage tanks in a hospital, where two of the tanks were in regular use but other two were not in use. *Legionella* counts were significantly

reduced in the active tanks within a short period of time during usage whereas in the stagnant tanks, the bacterial count had increased significantly (Ciesielski et al., 1984).

A tank's internal condition is another important contributing factor. Sedimentation, scale, corrosion in case of the metal tank, presence of mould and biofilm are the main components affecting the internal condition of the water tanks. Each of these can act as the nutrient for *Legionella* and thus encourage the proliferation (Ohno et al., 2003). During a study in 2009, Declerck found the presence of biofilm can encourage the growth and spreading of *Legionella* bacteria (Declerck, 2010). The number of deadlegs is another contributing factor, as water will be stagnant within the deadlegs enabling sites for *Legionella* proliferation (Springston and Yocavitch, 2017; Yu et al., 2006). Thermal insulation and water temperature is related each other. Thermal insulation to the hot/cold water tanks and pipework prevents the heat exchange with the surroundings. In the case of hot water storage (calorifier), operating temperature could be 60^0C. If the calorifier and pipework is fitted with proper thermal insulation, hot water can be delivered to the furthest outlet with almost the same temperature. If the thermal insulation is not appropriate, the water temperature can fall below 50^0C due to ambient heat loss during transportation through the pipework (Bagh et al., 2004).

In the case of water tanks, the ambient temperature is a major factor to determine the stored water temperature. If the water storage tank is thermally isolated, it is very unlikely that the stored water temperature will exceed 20^0C unless the incoming mains water temperature is above 20^0C. In any case, if the water temperature falls in the range of 20-45^0C, the water system is considered to be 'high risk'. In the same manner, the season can influence the stored cold water temperature. In summer, mains water temperature is always close to 20^0C and the ambient temperature reach up to 30^0C. In this case, if the thermal insulation is not appropriate, the stored water temperature could reach as the same as ambient temperature which is in favour of *Legionella* proliferation. Thus, season is a major contributing factor to the *Legionella* proliferation (Doelman, 2012). According to Rakic and colleagues, seasonal changes, especially the approach of summer, can accelerate the proliferation of *Legionella* bacteria within water systems (Rakic et al., 2011).

Showers are one of the major sources of *Legionella* proliferation and also been identified as a route for spreading infectious diseases including Legionnaires' disease (De Filippis et al., 2017; HSE, 2016). Mainly there are two types of showers; mixed showers and electric showers. Mixed showers work by mixing hot and cold water from

separate pipes to generate warm water where the temperature is somewhere in between. Cold water temperature is normally below 20ºC and hot water temperature is above 50ºC and the mixed water discharging through the showerhead normally fall in the range of 35-40ºC (Dimitriadi and Velonakis, 2014). This is an ideal temperature for *Legionella* proliferation. Moreover, scale from the hot water as well as the cold water can easily be deposited within the showerheads and can use as a nutrient for *Legionella* bacteria (GROWTH-PROMOTING, 1998). Another concern is the stagnant water within the showerheads as this water stagnation with favourable temperature can significantly encourage the proliferation of *Legionella* and can accelerate the multiplication if enough nutrients are present. Generally, showers generate plenty of aerosols as infectious microorganisms can spread into the surrounding atmosphere and get contacted with susceptible hosts. In the case of infrequently used showers, this risk increases significantly as the users can directly inhale the concentrated infectious dose of this pathogen while using the shower after the break (Ditommaso et al., 2010).

Electric showers pose a slightly lower risk in terms of *Legionella* compared to mixed showers; however, the showerhead of electric showers also can act as a breeding ground and harbour the Legionella bacteria in the same way as of a mixed shower. Moreover, in both mixed and electric showers, discharging water temperatures are in favour of Legionella and the rate of aerosol generation is also same (Euser et al., 2016; EPA, 2016).

The number of positive *Legionella* detection in any particular building is a clear indication of the potential risk in terms of Legionnaires disease. Most often a *Legionella* outbreak is an after effect of water system non-compliance and possible hidden factors of non-compliance accelerate the frequency of *Legionella* positive results. Thus, the increased number of positive results becomes one of the clear indicators of the potential risk in terms of Legionnaires disease (HPS, 2014).

The less or non-contributing factors for *Legionella* proliferation was also identified using t-test and comparative studies. These are age of the building, building usage, *Legionella* risk assessment, size of the building, total number of TMV's in the building, and TVC analysis of water samples. Calculated p-value for these factors (Table 5.2b) indicated that these parameters do not differ significantly with 95% confidence between infected and non-infected sites. However, a *Legionella* risk assessment

normally highlights the potential risk factors and helps to reduce the risk in terms of *Legionella* proliferation. In contrast, the risk reduction is not dependant on the presence of a *Legionella* risk assessment; rather implementation of the remedial actions recommended in the risk assessment is needed to mitigate the potential risks identified.

5.5.2 Assessment of the effectiveness of temperature monitoring

Sites with regular temperature monitoring were found to be safer than the sites without temperature monitoring. Historical data shows that only one of five sites, i.e. 20% of the total sites had *Legionella* outbreak when regular temperature monitoring was in place. In contrast, 80% of the sites without regular thermal monitoring had *Legionella* positive results. *Legionella* can multiply only in the temperature range of 20-45^{0}C. In the event any water system operating temperature fall in to this range, it can be rectified if there is a regular temperature monitoring regime in place, which will eliminate the possibility of *Legionella* proliferation due to the favourable temperature.

A study on cold water was carried out in a newly commissioned hospital building by Srivastava and team in 2010. Cold water discharging temperature was above 20^{0}C and *Legionella* pneumophila serogroup -1 was detected during outlet sample culture. Increased cold water temperature contributed to this proliferation as the water system was newly commissioned and was free from all other possible contributing factors (Srivastava et al., 2011). Another study by Darelida et al., on hot water in a district general hospital shows that the temperature maintenance and monitoring can effectively control *Legionella* and reduce the possibility of positive Legionella detection (Darelida et al., 2002).

5.5.3 *Legionella* risk predictive model

On the basis of the significance of the contributing factors, PCA categorically separates the sites into 'high risk' buildings and 'low risk' buildings. Infected buildings are on the RHS of Y-axis and non-infected buildings are on the LHS of Y-axis. Out of 120 buildings, 60 infected buildings fell on the RHS of the Y-axis and 60 non-infected buildings fell on the LHS of the Y-axis which indicates that the *Legionella* risk predictive model is 100% accurate. Among infected sites, most of the parameters were

satisfactory in some of the buildings, however, 2 or 3 contributing factors were not in compliance and PCA was able to identify those non-compliances while distinguishing the sites.

For example, building number 11 has an 85% occupancy rate, 23% excess water storage and has had one *Legionella* outbreak in the past six years. There is no deadleg in this building and all other factors are satisfactory. However, PCA successfully identified this site as 'high risk' category. Similarly, building number 25 has a 100% occupancy rate, no excess water storage; but there was one deadleg and there were three *Legionella* positive results from the showerheads in the past six years. PCA was successful in identifying this building as a high risk building. However, exclusion of this variable (number of past positive results) did not affect the predictive power of the model; for some of the other sites where there was no historical data available but, on the basis of the other contributing factors, the model was successful in predicting the *Legionella* risk in those particular sites. Thus the PCA based *Legionella* risk predictive model gives more accuracy and confidence in categorising the risk in terms of *Legionella* bacteria.

Even if the hot and cold water system is operating fully in compliance with the precautionary measures, showerheads need to be considered as a separate system. Hot and cold waters mix together prior to their discharge through the showerhead and this water temperature always will be in favour of *Legionella* proliferation. Moreover, calcium and magnesium precipitates (scale) from the hot water can easily accumulate within the showerheads and this can be the nutrient for *Legionella* bacteria once it has proliferated. Proceedings of the National Academy of Sciences of the United State of America conducted a study in 45 showerhead sites across United States in 2009 and they concluded that the showerheads present significant potential exposure to *Legionella* pneumophila (Feazel et al., 2009). Showerheads in any buildings need to be treated as a separate system and regular cleaning, de-scaling and flushing is highly necessary (Delia et al., 2007). On the basis of these findings and in comparison with infected and non-infected buildings, the accuracy of the assessment by PCA risk predictive model is 100%.

5.5.4 Validation of the risk predictive model with new client buildings

Table 5.6 SWOT analysis of the new PCA *Legionella* risk predictive model

Strength	**Weaknesses**
1. High risk buildings can be identified quickly. 2. Once the data is collected, the risk status of buildings can be identified in a single analysis. 3. 100% accuracy in risk determination. 4. High risks buildings can be treated with more care and attention and thus reduce the possibility of *Legionella* positive results. 5. Water sampling frequency can be reduced or avoided for low risk buildings to save money. 6. Human errors present in current risk categorising can be eliminated and better consistency can be achieved in assessing the actual risk of any building.	1. Detailed data collection is necessary for the use of this risk predictive model and not all data will be available for some buildings. 2. Data need to be converted into numerical values and this should be uniform. Small error while converting the data into the numerical value can affect the results. 3. The model is not a replacement for routine monitoring. The presence or absence of *Legionella* cannot be confirmed until water sample analysis has been carried out using current diagnostic methods.
Opportunities	**Threats**
1. Current *Legionella* control method, treating all the buildings in the same way can be altered by giving more attention to the high risk buildings. Thus, significant cost savings can be achieved. 2. Very high possibility of the development of an accurate Risk Predictive Model and anybody involved in the *Legionella* control can use this tool without expert help. 3. Significant financial savings can be achieved with reduced water sampling frequency and less number of water samples. 4. Possibility of the development of dedicated software to enable building managers to carry out their own risk assessment more efficiently and accurately. 5. Possibility of the approval and support from the regulating government authorities and private experts. 6. Significant commercial benefit from the possible high potential marketing of proposed software in the *Legionella* control sector worldwide. 7. Building managers can assess the possible impacts of remedial work to buildings on *Legionella* risk before commissioning work.	1. Building managers responsible to control *Legionella* in their premises may not entertain the new method due to the legal implications in the event of possible failure. 2. A minor error in categorising the risk buildings can lead to the *Legionella* outbreak and serious health impacts. 3. Regulating authorities may not support/approve the new method. 4. Public attitude towards the new method could be negative due to the diversion from the traditional water sampling method (current industry practice is to carry out water sampling as the part of a routine monitoring program regardless of high risk or low risk building) and the existing fear of Legionnaires' disease.

While applying this *Legionella* Risk Predictive Model with 66 new client buildings, 32 sites (buildings numbers 1-32) fell in high risk category and the remaining 34 sites (buildings numbers 33-66) fell in low risk category. On comparing this result with the real historical data available from the site showed that the buildings 1-32 had *Legionella* positive results in the past. This shows that the tool is 100% accurate for contaminated sites; and in the same way, non-infected buildings 33-66 fell into the low risk category which further confirms that the tool is 100% accurate for non-contaminated sites also. A 'SWOT' analysis has been carried out to assess the strength and weakness of the Risk Predictive Model (Table 5.6).

5.6 Conclusions

The PCA *Legionella* Risk Predictive Model shows accuracy and promise in categorising buildings into high and low risk. Risk category of any building in terms of *Legionella* proliferation can be identified in a single analysis and the buildings that fall into high risk category can be monitored with additional care. In addition, the human errors present in current risk assessment can be eliminated and better consistency in Legionella risk assessment can be achieved. However, detailed collection of the historical monitoring data can be practically difficult in some of the buildings (buildings with no/limited historical data) and human expertise may be required in such cases to collect the accurate data to use with the model. More validation from other parts of the world is needed to increase the confidence in the method and achieve a global perspective. There is a high possibility for this model to incorporate into a handheld device (e.g. a mobile app) which may be easy to use for practioners involved in the practical *Legionella* control in the real world.

CHAPTER SIX

GENERAL DISCUSSION

6.1 General discussion

Legionnaires' disease continues to present a serious threat to public health. This is especially true for the immunocompromised elderly population. An increasing number of elderly people (many with immunocompromised) are choosing to live in large privately-owned individual residential complexes Importantly, these people have less protection against Legionnaires' disease compared to people of similar age and health that reside in health care premises or care homes (Totaro et al., 2017; Whiley, 2016). A survey that I undertook of 20 residential complexes from different boroughs in London has confirmed the presence of this vulnerable elderly population who, despite being as susceptible to Legionella as care home residents, are still offered less protection in terms of LD.

Water-borne bacteria, found in cold water storage tanks, are causative agents for various human infections and diseases including Legionnaires' disease. Regular microbiological monitoring of tank water is undertaken as part of the risk management strategy to control pathogenic bacteria including *Legionella.* I have investigated the appropriateness of water sampling strategies currently used to protect the public from exposure to pathogenic bacteria. Domestic water samples taken from different locations within 15 tanks in London properties between December 2015 and July 2016 were analysed for TVCs, Pseudomonas and *Legionella* at an accredited laboratory. Despite seasonal differences in water temperature, I found 100% compliance at the ball valve end. 40% of the tanks exceeded the regulatory threshold for temperature at the far end of the tank in the summer months. Consequently, 20% of the tanks surveyed showed significant difference based on microbiological analyses of the water sample taken under the ball valve (n=15 tanks) compared to the far end samples. Typical water samples collected for routine monitoring will underestimate the microbiological status of the water entering the building. Many studies on cold water distribution system have reported that the water distribution system promote colonisation of domestic water with a number of microbial species causing serious risk to public health (McClung et al., 2017; Kulinkina et al., 2016; Kilvington et al., 2004). In order to address this serious concern, I propose that water storage tanks should be redesigned to allow access to the

far end of tanks for monitoring purposes, and that water samples used for compliance should be taken at the point at which water is abstracted for use in the building. Implementation of this proposed method will significantly improve the water hygiene and reduce the risk of pathogenic bacterial infections from domestic water systems.

Evidence suggests that current detection methods for *Legionella*, by culture and quantitative polymerase chain reaction (qPCR) show large disparities in the detection and quantification of bacteria in water samples, raising concerns about the reliability of measures needed to safeguard public health. Many studies have reported that all the diagnostic methods currently in use for *Legionella* detection are weak and cannot be completely reliable (Polo-López et al., 2017; Peci et al., 2016; Whiley, 2016; Ditommaso et al., 2014). However, some studies have suggested that the use of qPCR with improvements is the best way of monitoring *Legionella* to protect the public health (Lee et al., 2011). Under this circumstances, development of qPCR based diagnostic method for the live detection of *Legionella* bacteria in water samples will be a milestone in *Legionella* control and water hygiene management industry. Despite the requirement of further research for result interpretation and establishing a relationship with standard culture analysis (cfu/L), this research was highly successful in live detection and quantification of *Legionella bacteria using qPCR* techniques. Refinement of this developed method can provide accurate, reliable and rapid quantification of viable bacteria present in any given environmental water samples. Recent studies on the lack of correlation between *Legionella* diagnostic methods highlights the necessity of an acceptable standardised rapid method for quantification of *Legionella* bacteria and that will be sufficient for risk assessment and management of this life threating human pathogen (Whiley and Taylor, 2016).

According to the current *Legionella* risk assessment strategy, qualified and trained individuals carry out the risk assessments using the specific guidelines BS 8580:2010. On the basis of identified risk factors during the risk assessment survey, individuals making the decision of risk category of the buildings on their practical experience and establishing a monitoring programme for ongoing maintenance of the water system surveyed (HSE, 2013). There are lots of disparities in this decision making as one individual risk accessor can put a high risk building in low risk category on the basis of his own experience; but at the same time another risk accessor may put the same building in high risk category followed by his own assessment. In my personal experience as a *Legionella* control manager; this is quite normal in the current practice

of *Legionella* risk assessment (personal observation of Aji Peter). However, occurrence of human errors whilst making the decision is normal but, categorising a low risk building into a high risk category would result in a significant intensification of monitoring and water sampling frequencies thereby adding additional financial burdens to building management companies in terms of *Legionella* control. On the other hand, placing a high risk building into the low risk category would put water facility users in hidden threat of Legionnaires disease from their domestic water systems.

The tool developed in this research 'evidence based risk predictive model' showed 100% accuracy in predicting the *Legionella* risk in any building and its validation with 'London buildings' and 'out of London buildings' confirms that it is powerful in any region with different water quality parameters. Therefore, this model can be used as an easy tool and a standard platform for the risk assessment in any domestic water system. Moreover, this model would be of great advantage in eliminating possible human errors when making a decision to categorise the buildings in terms of *Legionella* risk. The possibility of integrating the predictive model into a handheld device (or as an App) would increase its commercial potential as well as providing another 'tool' in the armoury for the *Legionella* control management sector worldwide.

However, there are certain limitations for this model as detailed *Legionella* risk monitoring data is required for the effective prediction by this model, and this may be practically difficult on occasions due to the lack of necessary monitoring data in some buildings. In such cases, expert *Legionella* control practitioners help may be required for the collection of accurate data of any particular building.

CHAPTER SEVEN

CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORKS

7.1 Conclusions

Legionnaires' disease caused by Legionella bacteria remain a serious threat to human health. Discrepancies in quantification of *Legionella*, non-reliability of microbiological analysis of water samples collected from cold water storage tanks under present water sampling strategy, shortfalls when undertaking a *Legionella* risk assessment, potential long term issues such as climate change and global warming etc. point to the need for more accurate, reliable and rapid standard diagnostic methods to quantify viable *Legionella* in domestic water systems. Review of the regulatory guidelines of representative sample collection in view of achieving the microbiological status of the entire cold water tank in order to inform risk management strategies is very important to control *Legionella* and maintain water hygiene standards in domestic water systems. Last of all, development of a standard reliable *Legionella* risk assessment tool is vital for *Legionella* control and management in order to categorise the buildings on the basis of the actual risk factors present in each individual buildings. These three major shortfalls in *Legionella* control and management have been addressed in my research through development of a reliable and rapid method to detect live *Legionella* in water samples, the shortcomings of present-day sampling regimens in tackling the microbiological threat of pathogenic bacteria (including *Legionella)* in cold water storage tank was unveiled, and 'an evidence based risk predictive model' that can predict the *Legionella* risk in any domestic water system has been developed.

7.2 Recommendations

a. Live detection of *Legionella* bacteria using PCR technique is possible, although accurate quantification of viable bacteria still presents some challenges. However, this assay confirms that RNA extraction from live *Legionella* present in lenticules using the Qiagen RNA extraction kit, cDNA synthesise and amplification of cDNA using the 23S primers was successful.

b. Typical water samples collected for routine monitoring will underestimate the microbiological status of the water entering the building. Therefore, the water storage tanks should be redesigned to allow access to the far end of tanks for monitoring purposes, and that water samples used for compliance should be taken at the point at which water is abstracted for use in the building. Such measures will be increasingly important in protecting the vulnerable ageing population, especially given the tendency of the elderly to congregate in large residential blocks that fall outside the stringent regulations of hospitals and care homes.

c. The PCA *Legionella* Risk Predictive Model shows accuracy and promise. More validation from other parts of the world is needed to increase the confidence in the method and achieve a global perspective. There is very high possibility for the development of dedicated software/mobile app to carry out the *Legionella* risk assessment more efficiently and accurately. Significant commercial benefit can be achieved from the possible high potential marketing of proposed software in the global *Legionella* control sectors.

7.3 Future work

- Investigate the sensitivity of the method from 100 cfu/L and below (real world applicability).
- Establish the relationship between cfu/L and C_T value more accurately.
- Lenticule standards with accurate levels of bacteria should be developed to support development of a rapid qPCR kit. These could serve as positive control whilst providing quantitative information (cfu/L)
- Investigate to identify the possible interfering substances (inhibiting substances) and develop methods to get rid of the possible inhibitors.
- Carry out further validation of the method after the above mentioned refinement. Carry out the test use of this novel approach to detect live *Legionella* in real-world samples (environmental water samples) and compare it with Gold Standard (culture)

- More validation of PCA based '*Legionella* Risk Predictive Model' with water system having different water quality parameters from other parts of the world need to be carried out to ensure the predictive power of the model.
- Development of dedicated software/mobile app as tool to carry out the *Legionella* risk assessment.

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	Approx Year Built	Size	Usage	Approximate Annual Occupancy rate (%)	No of CWST	No of Calorifiers	Building Name	RA	Remedial Works	Monitoring Programme
1	1937	6 storeys	Residential Development	51	8	5	40 – 41 Chester Square	Yes	N/A	Temperature monitoring & Sampling
2	1930	5 storeys	Residential Development	71	7	3	Frognal Estate, London, NW3 5HG	No	Yes	Temperature monitoring & Sampling
3	1930's	6 Storeys	Mixed use, Residential and Commercial	75	6	3	28-56 Parkside, London, SW1X 7JP	Yes	N/A	Temperature monitoring & Sampling
4	1945	4 storeys	Residential Development	55	4	4	2-12B Gladstone Court, Anson Road, NW2 4LA	No	No	Temperature monitoring & Sampling
5	1900's	4 storeys	Residential Development	78	12	0	Denham Court, 114 Kirkdale, London, SE26 4BE	No	Yes	Temperature monitoring & Sampling
6	1900	7 storeys	Residential Development	72	8	1	14 Cadogan Gardens	Yes	Yes	Temperature monitoring & Sampling
7	1940's	8 Storeys	Large Residential Development	75	6	3	Arthur Court	Yes	N/A	Temperature monitoring & Sampling
8	1940	2 storeys	Residential Development	79	8	3	Waverley Court, 34-37 Beaumont Street, London, W1G 6DH	No	Yes	Temperature monitoring & Sampling
9	2000's	4 storeys	Mixed use, Residential and Commercial	55	9	4	Cross & Pillory House	No	Yes	Temperature monitoring & Sampling
10	2000's	2 storeys	Residential Development	54	4	2	Hampton Court, Hampden Road, London, N10 2HN	Yes	Yes	Temperature monitoring & Sampling
11	2003	22 storeys	Residential Development	85	11	2	Electron Tower, Blackwall Way,Leamouth, E14 9GW	No	No	Temperature monitoring & Sampling
12	2000's	5 storeys	Care Home	87	16	2	Ealing Eventide Homes Ltd, Downhurst, 76 Castlebar Road, Ealing, W5 2DD	Yes	Yes	Temperature monitoring & Sampling
13	1800's	4 storeys	Care Home	72	14	2	Wolsley House	08/08/2012	Yes	Temperature monitoring & Sampling
14	1940's	8/9 Storeys	Residential Development	75	12	2	One Princes Gate	Yes	Yes	Temperature monitoring & Sampling
15	1800's	2 storeys	Residential Development	79	10	3	The Lock House	No	Yes	Temperature monitoring & Sampling
16	1937	8 Storeys	largest privately owned block of flats under one roof in Europe	72	4	2	Du Cane Court	Yes	Yes	Temperature monitoring & Sampling
17	1945	7/8 storeys	Residential Development	61	7	2	Regent Park House, Park Road, London, NW8 7JP	Yes	no	Temperature monitoring & Sampling
18	1900's	4 storeys	Mixed use, Residential and Commercial	63	6	3	118A Cholmley Gardens, Fortune Green Road, NW6 1AA	No	Yes	Temperature monitoring & Sampling
19	1940's	5 storeys	Residential Development	15	3	4	Ashworth Mansions, Elgin Avenue, W9 1JP	No	Yes	Temperature monitoring & Sampling
20	1930's	7 storeys	Residential Development	62	3	4	Cumberland Mansions, London SW5	No	Yes	Temperature monitoring & Sampling
21	1800's	8 storeys	Large Residential Development	75	30	0	Bryanston Court II, 137 George Street, London, W1H 7HD	No	N/A	Temperature monitoring & Sampling
22	1920-1930'	5 storeys	Care Home	79	6	4	42 Newstead Road	07/08/2012	Yes	Temperature monitoring & Sampling
23	1800's	7 storeys	Residential Development	77	5	2	Cheyne Place	Yes	Yes	Temperature monitoring & Sampling
24	1940's	8 storeys	Mixed use, Residential and Commercial	55	2	2	86-89 Piccadilly	No	N/A	Temperature monitoring & Sampling
25	2000's	5 storeys	Residential Development	100	3	3	The Chambers,3 Constable Close,Friern Barnet, London,N11 3GW	No	No	Temperature monitoring & Sampling
26	1940	5 storeys	Residential Development	55	5	3	Greenwich Heights	Yes	Yes	Temperature monitoring & Sampling
27	1930	3 Storeys	Residential Development	36	5	3	Bernersmede	No	Yes	Temperature monitoring & Sampling
28	1930	5 storeys	Care Home	15	4	2	146-148 New Cross Road	10/10/2011	Yes	Temperature monitoring & Sampling
29	1800's	2/3 Storeys	Care Home	20	3	5	Tilehurst Lodge, 142 Tilehurst Road, Reading, Berkshire, RG30 2LX	No	Yes	Temperature monitoring & Sampling
30	1930's	2 storeys	Care Home	72	9	1	58 Halls Road, Tilehurst, Reading, Berkshire RG30 4EX	No	Yes	Temperature monitoring & Sampling
31	1940's	Single storey	School	82	13	2	Aldwickbury Preparatory School, wheathampstead Road Harpenden AL5 1AD	No	Yes	Temperature monitoring & Sampling

	Approx Year Built	Size	Usage	Approximate Annual Occupancy rate (%)	No of CWST	No of Calorifiers	Building Name	RA	Remedial Works	Monitoring Programme
32	1800's	7 storeys	Residential Development	78	14	2	Hillside Court, 409 Finchley Road, London, NW3 6HQ	16.02.2011	no	Temperature monitoring & Sampling
33	1800's	3 storeys	Residential Development	75	21	1	69-76 Church Crescent, Muswell Hill, London, N10 3NE	No	Yes	Temperature monitoring & Sampling
34	1900's	2 storeys	Care Home	78	16	0	Whitefriars Nursing & Dementia Home, 9 Dormers Wells Lane, Southall, UB1 3HU	26.04.10	Yes	Temperature monitoring & Sampling
35	1900	5 storeys	offices	79	11	3	One Vincent Square, London, SW1P 2PN	24.04.12	Yes	Temperature monitoring & Sampling
36	2000's	3 Storeys	Residential Development	62	4	4	Bouton Place, Waterloo Terrace, N1 1TR	No	Yes	Temperature monitoring & Sampling
37	1994	6 Storeys	Residential Development	65	12	5	Meath Crescent, London, E2 0QA	19.11.2010	Yes	Temperature monitoring & Sampling
38	1940	4 storeys	Residential Development	10	6	2	Sheridan House, Wincott street ,London, SE11 4NY	Yes	Yes	Temperature monitoring & Sampling
39	1920	7 storeys	Mixed use, Residential and Commercial	56	7	2	My Base,130 Webber Street, SE1 0QL,london	No	Yes	Temperature monitoring & Sampling
40	1910	3 storeys	Commerical	20	10	1	Kent County Council Library and History Centre	No	Yes	Temperature monitoring & Sampling
41	1937	7/8 storeys	Residential Development	75	5	0	Coin Street Neighbourhood Centre, 108 Stamford Street, South Bank, London SE1 9NH	29.11.11	Yes	Temperature monitoring & Sampling
42	1900's	5 storeys	Residential Development	79	4	2	West Gate House, 661 London Road, Isleworth, Middlesex, TW7 4AS	No	Yes	Temperature monitoring & Sampling
43	1937	4 storeys	Residential Development	55	5	3	Avanti Court,Bittons, Kingston upon Thames, KT1 2AN	Yes	Yes	Temperature monitoring & Sampling
44	1800's	3 Storeys	Residential Development	70	24	6	51-55 Marlborough Hill, St.Johns Wood, London, NW8 0NG	Yes	Yes	Temperature monitoring & Sampling
45	1940's	4 storeys	Residential Development	56	7	4	Ranelagh Mansions, 319 New Kings Road, London, SW6 9TJ	Yes	Yes	Temperature monitoring & Sampling
46	1945	7 storeys	Residential Development	61	6	3	West Bourne Corner, 2 Chepstow Road, London, W2 5AH	Yes	Yes	Temperature monitoring & Sampling
47	1800's	3 storeys	Residential Development	75	13	3	Christchurch House, Christchurch Rd, Lambeth, London, SW2 3UA	No	Yes	Temperature monitoring & Sampling
48	1994	4 storeys	Residential Development	79	8	4	Emmaus UK, Hill End Lane, St Albans, Herts, AL4 0FE	No	Yes	Temperature monitoring & Sampling
49	1940	6 Storeys	Residential Development	70	30	0	Cathcart House	No	No	Temperature monitoring & Sampling
50	1920	4 storeys	Residential Development	20	6	4	Shepherd House	Yes	Yes	Temperature monitoring & Sampling
51	1930	6 storeys	Residential Development	71	3	2	21/22 Queensgate Gardens	Yes	Yes	Temperature monitoring & Sampling
52	1900's	2 storeys	Residential Development	75	7	2	41/43 Cadogan Square	Yes	Yes	Temperature monitoring & Sampling
53	1800's	4 storeys	Residential Development	78	8	2	26/27 Egerton Crescent	No	Yes	Temperature monitoring & Sampling
54	1930's	6 Storeys	Residential Development	85	4	2	Ashburn House	No	Yes	Temperature monitoring & Sampling
55	1945	3 Storeys	Residential Development	70	4	2	63-72 Harvard Court	Yes	Yes	Temperature monitoring & Sampling
56	1800's	4 storeys	Residential Development	72	6	2	73-82 Harvard Court	Yes	Yes	Temperature monitoring & Sampling
57	2000's	7 storeys	Industial Development (Factory)	75	10	2	Henkel Ltd	No	Yes	Temperature monitoring & Sampling
58	1994	7-9 storeys	Residential Development	55	3	2	Oyster Wharf	Yes	Yes	Temperature monitoring & Sampling
59	1920	2 storeys	Residential Development	60	7	2	21/23 Cadogen Gardens, London, SW3 2EW	No	Yes	Temperature monitoring & Sampling
60	1900	5 storeys	Residential Development	72	5	3	Hylton House,34 The Ridgway, Sutton, SM2 5JU	No	Yes	Temperature monitoring & Sampling
61	1930	4 storeys	Commercial	92	2	2	J&P, 16 Hanover Street, London, W1S 1YL	Yes	Yes	Temperature monitoring & Sampling
62	1900's	3 Storeys	Residential Development	98	3	1	Gladbeck Heights, 5 Gladback Way,Enfield, EN2 7FG	Yes	Yes	Temperature monitoring & Sampling
63	1937	4 storeys	Residential Development	100	2	0	City Gate home	Yes	Yes	Temperature monitoring & Sampling

	Approx Year Built	Size	Usage	Approximate Annual Occupancy rate (%)	No of CWST	No of Calorifiers	Building Name	RA	Remedial Works	Monitoring Programme
64	1800's	7 storeys	Residential Development	91	4	0	Cline Road, Bounds Green, N11 2ND	No	Yes	Temperature monitoring & Sampling
65	1930's	3 storeys	Residential Development	95	1	0	Chase Court	No	Yes	Temperature monitoring & Sampling
66	1945	4 storeys	Residential Development	100	2	0	Heath Rise, Kersfield Road, London, SW15 3HF	Yes	Yes	Temperature monitoring & Sampling
67	1920-1930'	2/3 Storeys	Hospital	91	3	0	Marie Stopes, Brixton Hill	No	N/A	Temperature monitoring & Sampling
68	1800's	7 storeys	Residential Development	95	2	1	Woodlands Gate, Woodlands Way, Putney, London, SW15 2SY	10.11.11.	Yes	Temperature monitoring & Sampling
69	1800's	7 storeys	Residential Development	98	3	2	New Hurlingham Court, Ranelagh Gardens, London SW3 3UR	No	Yes	Temperature monitoring & Sampling
70	1900's	7 storeys	Residential Development	97	2	2	Arundel Mansions, Kelverdon Road, SW6 5BS	No	Yes	Temperature monitoring & Sampling
71	1900	7 storeys	Residential Development	100	2	2	Cameret Court	No	Yes	Temperature monitoring & Sampling
72	1900's	7 storeys	Residential Development	90	3	0	kenilworth Court,34A Kenilworth court Lower Richmond Road London SW15 1EN	Working on it	Yes	Temperature monitoring & Sampling
73	1994	3 Storeys	Residential Development	95	2	2	Hamilton House	No	Yes	Temperature monitoring & Sampling
74	1940	2 storeys	Residential Development	93	1	1	Rider House, 121 High Road, Loughton, IG10 4LT	01.03.13	Yes	Temperature monitoring & Sampling
75	1920	8 Storeys	Hospital	98	2	1	MS-Central London	30.11.11	Yes	Temperature monitoring & Sampling
76	1900	3 Storeys	Residential Development	95	3	1	1 Conway Street. Fitzroy Square. London. W1T 6LP.	No	Yes	Temperature monitoring & Sampling
77	1930	4 storeys	Hospital	97	2	0	MS-Reading	18.04.12	Yes	Temperature monitoring & Sampling
78	1900's	7 storeys	Residential Development	95	3	1	B R Maunder Taylor LVT Appointed Manager (Courtenay House)	No	Yes	Temperature monitoring & Sampling
79	1937	7-9 storeys	Residential Development	85	3	2	Tenby Mansions, 12-22 Nottingham Street, London.	No	Yes	Temperature monitoring & Sampling
80	1930's	2 storeys	Residential Development	80	2	2	Flat M, 49 Willington Street, London, WC2E 7BN	Yes	Yes	Temperature monitoring & Sampling
81	1940's	5 storeys	Residential Development	95	2	1	Buckingham Lodge	Yes	Yes	Temperature monitoring & Sampling
82	1945	4 storeys	Residential Development	92	8	1	Chessington Lodge, Regents Park Road, London, N3 3AA	Yes	Yes	Temperature monitoring & Sampling
83	1800's	7 storeys	Residential Development	100	3	1	Church Garth, St Johns Grove, Islington, London, N19 5RN	No	Yes	Temperature monitoring & Sampling
84	1937	7 storeys	Residential Development	91	3	1	Highgate Edge	No	Yes	Temperature monitoring & Sampling
85	1800's	7 storeys	Residential Development	98	2	0	Noblefield Heights	No	Yes	Temperature monitoring & Sampling
86	1930's	7 storeys	Residential Development	100	2	2	The Pantiles	Yes	No	Temperature monitoring & Sampling
87	1945	6 storeys	Residential Development	93	2	1	Western Beach Apartments, 36 Hanover Avenue, E16 1DZ.	No	Yes	Temperature monitoring & Sampling
88	1800's	6 storeys	Residential Development	95	2	1	Palace Court, 250 Finchley Road,NN3 6DN	No	Yes	Temperature monitoring & Sampling
89	1800's	6 storeys	Commercial	100	2	1	Redhill Chambers	No	Yes	Temperature monitoring & Sampling

	Approx Year Built	Size	Usage	Approximate Annual Occupancy rate (%)	No of CWST	No of Calorifiers	Building Name	RA	Remedial Works	Monitoring Programme
90	1900's	6 storeys	Residential Development	88	8	0	Northways Flats, CollegeCrescent ,London,NW3 5DR	No	Yes	Temperature monitoring & Sampling
91	1900	6 storeys	Residential Development	95	2	1	Collingwood Court, Queens Road, London, NW4 2HE	No	Yes	Temperature monitoring & Sampling
92	1900's	11 Storeys	Residential Development	98	4	0	Highstone Mansions	Yes	Yes	Temperature monitoring & Sampling
93	1994	5 storeys	Residential Development	100	1	2	14 Hyde Park Gardens, London W2 2LU	Yes	Yes	Temperature monitoring & Sampling
94	1940	4 storeys	Residential Development	95	2	2	79-85 Hackney Road, London, E2 8EU	Yes	Yes	Temperature monitoring & Sampling
95	1937	7 storeys	Residential Development	96	3	0	87-91,Hackney Road, London, E2 8ET	Yes	Yes	Temperature monitoring & Sampling
96	1800's	5 storeys	Residential Development	91	2	1	14-16 Long Street, London, E2 8HQ	No	No	Temperature monitoring & Sampling
97	1930's	Single storey	Industial Development (Factory)	100	3	2	Platt Industrial Estate, 1-10 Mill Place, Platt, Sevenoaks, Kent, TN15 8TB	No	No	Temperature monitoring & Sampling
98	1940's	7 storeys	Commercial	94	2	2	109 Uxbridge Road	Yes	Yes	Temperature monitoring & Sampling
99	1945	7 storeys	Residential Development	100	2	2	The Eadmund-68- 70 Brighton Road	No	No	Temperature monitoring & Sampling
100	2003	22 storeys	Residential Development	91	6	0	Proton Tower, Blackwall Way,Leamouth, E14 9GW	No	No	Temperature monitoring & Sampling
101	2003	22 storeys	Residential Development	90	2	2	Neotron Tower, Blackwall Way,Leamouth, E14 9GW	Yes	Yes	Temperature monitoring & Sampling
102	2003	22 storeys	Residential Development	95	1	1	Switch House,Blackwall Way,Leamouth, E14 9GW	Yes	Yes	Temperature monitoring & Sampling
103	1900's	2 storeys	Residential Development	93	2	0	Fallow Gate, 451 High Road, Finchley,London, N12 0AF	Yes	Yes	Temperature monitoring & Sampling
104	1994	7 storeys	Residential Development	97	3	2	Regency Apartments, Montaigne Close, Regency Street, London, SW1P 4BB	No	No	Temperature monitoring & Sampling
105	1940	9 storeys	Residential Development	98	2	2	Regency Apartments, Montaigne Close, Regency Street, London, SW1P 4BB	No	No	Temperature monitoring & Sampling
106	1920	6 storeys	Residential Development	81	3	1	Heron Court	Yes	Yes	Temperature monitoring & Sampling
107	1900	6 storeys	Residential Development	85	3	2	Dorset Court, 18-21 Dorset Street	Yes	Yes	Temperature monitoring & Sampling
108	1930	6 storeys	Residential Development	98	2	2	Rosemary Lodge Care Home	Yes	Yes	Temperature monitoring & Sampling
109	1900's	6 storeys	Residential Development	92	2	1	Trinity Court, 254 Gray's Inn Road, London, WC1X 8JX	Yes	Yes	Temperature monitoring & Sampling
110	1937	6 storeys	Residential Development	92	8	1	Priors Lodge	Yes	Yes	Temperature monitoring & Sampling
111	1800's	6 storeys	Residential Development	97	3	1	The Reach	Yes	Yes	Temperature monitoring & Sampling
112	1930's	8 storeys	Residential Development	98	3	1	1-18 Beechwood Hall, regents Park, London, N3 3AT	Yes	Yes	Temperature monitoring & Sampling
113	1945	5 storeys	Residential Development	100	2	0	Stone House	Yes	Yes	Temperature monitoring & Sampling
114	1800's	4 storeys	Residential Development	91	2	0	92 Wilton Road	No	Yes	Temperature monitoring & Sampling
115	1937	7 storeys	Residential Development	90	2	1	Ashford Court	No	Yes	Temperature monitoring & Sampling
116	1800's	7 storeys	Residential Development	93	2	1	Hillbrow(Parkgate Aspen), Richmond Hill, Richmond, Surrey, TW10 6BH	No	Yes	Temperature monitoring & Sampling
117	1940's	7 storeys	Residential Development	95	2	1	Sherwood Court, Seymour Place, W1H 5TH	Yes	Yes	Temperature monitoring & Sampling
118	1945	8 storeys	Residential Development	100	3	2	Crescent Court	No	Yes	Temperature monitoring & Sampling
119	1800's	6 storeys	Residential Development	98	2	0	Brunswick Mansions, 8 Handel Street, London, WC1N 1PE	No	Yes	Temperature monitoring & Sampling
120	1800's	6 storeys	Residential Development	100	2	0	Highpoint	Yes	Yes	Temperature monitoring & Sampling
121	1900's	4 storeys	Residential Development	100	2	1	4 Belgrave Square, London, SW1X 8PH	No	Yes	Temperature monitoring & Sampling
122	1900	3 Storeys	Residential Development	95	3	3	The Horizon, 2 Navigation Street, Leicester, LE1 3UJ	No	Yes	Temperature monitoring & Sampling

	Approx Year Built	Size	Usage	Approximate Annual Occupancy rate (%)	No of CWST	No of Calorifiers	Building Name	RA	Remedial Works	Monitoring Programme
123	1900's	4 storeys	Residential Development	95	2	2	Claydon House,Holders Hill Road, London,NW4 1LS	No	Yes	Temperature monitoring & Sampling
124	2000's	7 storeys	Residential Development	100	3	1	Molasses House, Clove Hitch Quay, Wandsworth,London, SW11 3TN	No	Yes	Temperature monitoring & Sampling
125	1994	3 storeys	Residential Development	100	18	2	Conal Court	No	Yes	Temperature monitoring & Sampling
126	1920	4 storeys	Residential Development	100	5	1	Minerva Lodge	No	No	Temperature monitoring & Sampling
127	1900	7 storeys	Residential Development	100	17	2	Babington Court	No	No	Temperature monitoring & Sampling
128	1930	2/3 Storeys	Residential Development	90	6	2	Eaton House	No	No	Temperature monitoring & Sampling
129	1900's	7 storeys	Residential Development	90	4	1	Chestnuts	No	No	Temperature monitoring & Sampling
130	1937	7 storeys	Commercial	100	2	3	RMG House-EN11 0DR	No	No	Temperature monitoring & Sampling
131	1800's	7 storeys	Residential Development	100	3	3	31 Plympton Street, London, NW8 8AB	No	No	Temperature monitoring & Sampling
132	1930's	3 storeys	Residential Development	97	2	2	Sandhurst Court (Flats 1 - 57), Acre Lane, London, SW2 5TX	No	No	Temperature monitoring & Sampling
133	1940's	2 storeys	Residential Development	95	3	3	Hampton 55 (Teddington) MCL	No	No	Temperature monitoring & Sampling
134	1945	5 storeys	Residential Development	100	2	2	St. Mary Abbots Court, Warwick Gardens, London, W14 8RA	No	No	Temperature monitoring & Sampling
135	2000's	7 storeys	Residential Development	100	2	3	Antilles Bay Apartments, 3 Lawn House Close, Docklands, London E14	No	No	Temperature monitoring & Sampling
136	1994	3 storeys	Residential Development	100	2	2	9-11 Broadwick Street	Yes	No	Temperature monitoring & Sampling
137	1940	2 storeys	Residential Development	95	6	3	Bear Pit Apartments, New Globe Walk, London, SE1 9DR	No	Yes	Temperature monitoring & Sampling
138	1920	5 storeys	Residential Development	95	2	2	West End Court, Priory Road, London, NW6 3NU	No	No	Temperature monitoring & Sampling
139	1900	4 storeys	Residential Development	100	2	3	85 Robins Court, Kings Avenue, Clapham, SW4 8EE.	Yes	No	Temperature monitoring & Sampling
140	1900's	6 Storeys	Residential Development	100	8	3	Cedar Lodge-Jubilee Heights.Exeter Road, Kilburn, London, NW2 3UL	No	No	Temperature monitoring & Sampling
141	1937	4 storeys	Residential Development	100	3	2	Cocharane Close	Yes	No	Temperature monitoring & Sampling
142	1800's	7 storeys	Residential Development	100	3	1	Moorcroft	No	Yes	Temperature monitoring & Sampling
143	1930's	3 storeys	Residential Development	98	2	1	27 lexham gardens, london, W8 5JJ	No	No	Temperature monitoring & Sampling
144	1940's	5 storeys	Residential Development	100	2	1	104 Fitzjohns Avenue, London, NW3 6NT	No	No	Temperature monitoring & Sampling
145	1800's	7/8 storeys	Residential Development	100	2	1	Water Dale Manor House	No	No	Temperature monitoring & Sampling
146	2000's	2 storeys	Care Home	100	2	1	1 Friendly Street	19/09/2011	Yes	Temperature monitoring & Sampling
147	1994	Single storey	Care Home	95	6	1	10 Friendly Street	19/09/2011	Yes	Temperature monitoring & Sampling
148	1940	Single storey	Care Home	100	1	1	10 Hindmands Road	19/09/2011	Yes	Temperature monitoring & Sampling
149	1920	3 storeys	Care Home	100	2	1	114-118 Friern Road	30/07/2012	Yes	Temperature monitoring & Sampling
150	1900	2 storeys	Care Home	100	2	1	130-136 Sydenham Road	07/08/2011	Yes	Temperature monitoring & Sampling
151	1900's	3 storeys	Care Home	100	6	1	155-157 Asaph Road	19/09/2011	Yes	Temperature monitoring & Sampling
152	1937	2 storeys	Care Home	100	1	1	2-3 Townley Road	30/07/2012	Yes	Temperature monitoring & Sampling
153	1800's	5 storeys	Care Home	95	2	1	26 Liverpool Grove	19/09/2011	Yes	Temperature monitoring & Sampling
154	1940's	2 storeys	Care Home	100	8	1	27 Siddons Road	11/07/2012	Yes	Temperature monitoring & Sampling
155	1945	5 storeys	Care Home	100	3	1	272 Stanstead Road	11/07/2012	Yes	Temperature monitoring & Sampling
156	1800's	2 storeys	Care Home	100	3	1	38 Queens Road	31/07/2012	Yes	Temperature monitoring & Sampling
157	1800's	3 storeys	Care Home	100	2	1	47 Montem Road	19/09/2012	Yes	Temperature monitoring & Sampling

	Approx Year Built	Size	Usage	Approximate Annual Occupancy rate (%)	No of CWST	No of Calorifiers	Building Name	RA	Remedial Works	Monitoring Programme
158	1800's	2 storeys	Care Home	100	3	1	54 Vancouver Road	11/07/2012	No	Temperature monitoring & Sampling
159	1900's	5 storeys	Care Home	98	2	1	63 Finland Street	29/09/2011	Yes	Temperature monitoring & Sampling
160	1900	3 storeys	Care Home	100	2	1	67 Medora Road	09/10/2011	Yes	Temperature monitoring & Sampling
161	1900's	4 storeys	Care Home	95	6	1	7 Ratcliff Close	19/09/2011	Yes	Temperature monitoring & Sampling
162	2000's	6 Storeys	Care Home	100	1	1	71-73 Dunton Road	22/04/2010	Yes	Temperature monitoring & Sampling
163	1994	8 Storeys	Care Home	100	2	1	75 Woodcote Road	08/08/2012	No	Temperature monitoring & Sampling
164	1940	3 Storeys	Care Home	100	1	1	87 Friern Road	30/07/2012	Yes	Temperature monitoring & Sampling
165	1920	4 storeys	Care Home	95	2	1	Aspinden Woods	07/08/2012	Yes	Temperature monitoring & Sampling
166	1900	7 storeys	Care Home	95	4	1	Joe Richards House	19/09/2011	Yes	Temperature monitoring & Sampling
167	1930	7-9 storeys	Care Home	95	2	1	Mary Secole Court	12/04/2013	Yes	Temperature monitoring & Sampling
168	1900's	2 storeys	Care Home	95	2	1	Park Court	20/05/2013	Yes	Temperature monitoring & Sampling
169	1937	2 storeys	Care Home	100	2	1	Parkspring	14/05/2013	Yes	Temperature monitoring & Sampling
170	1800's	5 storeys	Care Home	95	2	1	Patrick Court	12/04/2013	Yes	Temperature monitoring & Sampling
171	1930's	4 storeys	Care Home	100	2	1	Priory Court	13/05/2013	Yes	Temperature monitoring & Sampling
172	1940's	3 Storeys	Care Home	100	2	1	Reader House	13/05/2013	Yes	Temperature monitoring & Sampling
173	1945	4 storeys	Care Home	95	2	1	Rosia & Dryad House	14/05/2013	Yes	Temperature monitoring & Sampling
174	1937	6 Storeys	Commercial	100	2	2	60 Gresham Street, London, EC2V 7BB	No	Yes	Temperature monitoring & Sampling
175	1800's	8 Storeys	Commercial	95	4	2	11 Berkeley Street, London, W1J 8DS	25/04/2013	Yes	Temperature monitoring & Sampling
176	1930's	3 Storeys	Commercial	100	2	2	17c Curzon Street, London, W1J 5HU	25/04/2013	Yes	Temperature monitoring & Sampling
177	1940's	4 storeys	Commercial	70	2	2	40 Gracechurch Street, London, EC3V 0BT	26/02/2013	Yes	Temperature monitoring & Sampling
178	1945	7 storeys	Residential Development	100	2	1	15 Cromwell Road, London	No	Yes	Temperature monitoring & Sampling
179	1800's	7-9 storeys	Residential Development	100	2	2	63-65 Hamilton Terrace, London	Yes	Yes	Temperature monitoring & Sampling
180	1940's	2 storeys	Residential Development	100	2	3	Hampton 50 (Teddington) MCL	No	No	Temperature monitoring & Sampling
			Colour code							
			Commercial							
			Residential Development							
			Care Home							
			Mixed use, Residential and Commercial							
			School							
			Hospital							

Temperature Monitoring Frequency	No. of Samples	No. of communal showers	Sampling Frequency	Tank(s) internal conditon at the time of out break	Thermal insulation of the tanks and pipeworks	Total Tank(s) actual approximate Storage capacity in litres	Maximum requirement of stored water @ 150 litres per person for 24 hours consumption (Residential)	Maximum requirement of stored water @ 50 litres per person for 24 hours consumption (Commercial)	No. of deadlegs in the system
Monthly	4	2	Twice Annually	Corrosion and sedimentation	Uninsulated tank	23	11,340		1
Monthly	15	1	Twice Annually	Stagnant water with bio film	Pipework not insulated	28	36,000		4
Half yearly	8	1	Twice Annually	Sedimentation	Pipework not insulated	14	21,600		1
Half yearly	5	2	Twice Annually	Scale and sedimentation	Uninsulated tank and pipework	40	14,400		4
Half yearly	3	3	Twice Annually	Corrosion	Pipework not insulated	0	17,280		1
Monthly	6	3	Twice Annually	Stagnant water with bio film	N/A	60	63,000		1
Half yearly	9	3	Twice Annually	Corrosion and sedimentation	N/A	14	114,750		2
Monthly	10	2	Twice Annually	Scale and sedimentation	Uninsulated tank and pipework	4	10,800		2
Half yearly	5	1	Twice Annually	Corrosion and sedimentation	N/A	49	4,425		1
Half yearly	12	1	Twice Annually	Sedimentation	Pipework not insulated	16	18,900		4
Half yearly	5	1	Twice Annually	Visibly clean	Satisfactory	23	67,000		0
Half yearly	8	1	Twice Annually	Corrosion	Satisfactory	13	12,000		2
Half yearly	9	1	Twice Annually	Stagnant water with bio film	N/A	15	5,400		1
Half yearly	4	1	Twice Annually	Corrosion	Uninsulated tank	27	12,930		1
Half yearly	4	1	Twice Annually	Stagnant water with bio film	Pipework not insulated	13	12,240		4
Monthly	14	1	Monthly	Stagnant water with bio film	Uninsulated tank	53	219,555		2
Quarterly	3	2	Twice Annually	Corrosion	N/A	14	126,000		2
Half yearly	31	1	Annually	Scale and sedimentation	N/A	26	9,450		1
Half yearly	4	1	Twice Annually	Sedimentation	N/A	15	17,550		4
Half yearly	26	1	Twice Annually	Scale and sedimentation	Pipework not insulated	68	12,960		2
Monthly	9	2	Twice Annually	Stagnant water with bio film	Satisfactory	66	46,000		4
Half yearly	7	1	Twice Annually	Scale, sedimentation, and corrosion with bio fi	Uninsulated tank	21	5700		4
Half yearly	5	1	Twice Annually	Scale and sedimentation	Uninsulated tank	12	11,970		2
Monthly	3	1	Quarterly	Stagnant water with bio film	Pipework not insulated	40	13,250		1
Half yearly	5	6	Twice Annually	Sedimentation	Pipework not insulated	0	30,650		1
Half yearly	9	2	Twice Annually	Stagnant water with bio film	Pipework not insulated	37	19,125		2
Half yearly	5	1	Twice Annually	Scale and sedimentation	N/A	29	7,290		2
Half yearly	5	1	Twice Annually	Corrosion and sedimentation	Uninsulated tank and pipework	18	7,650		4
Quarterly	7	2	Twice Annually	Sedimentation	Uninsulated tank and pipework	23	18,900		1
Half yearly	4	0	Twice Annually	Stagnant water with bio film	N/A	28	67,000		4
Half yearly	5	0	Monthly	Corrosion and sedimentation	Satisfactory	14	12000	2,000	1

Temperature Monitoring Frequency	No. of Samples	No. of communal showers	Sampling Frequency	Tank(s) internal conditon at the time of out break	Thermal insulation of the tanks and pipeworks	Total Tank(s) actual approximate Storage capacity in litres	Maximum requirement of stored water @ 150 litres per person for 24 hours consumption (Residential)	Maximum requirement of stored water @ 50 litres per person for 24 hours consumption (Commercial)	No. of deadlegs in the system
Quarterly	2	1	Twice Annually	Scale and sedimentation	N/A	40	5,400		4
Quarterly	2	2	Twice Annually	Sedimentation	Satisfactory	22	12,930		1
Half yearly	4	1	Twice Annually	Stagnant water with bio film	Uninsulated tank	60	12,240		1
Half yearly	4	1	Quarterly	Sedimentation	Uninsulated tank	13	219,555	4,250	2
Half yearly	7	4	Twice Annually	Scale and sedimentation	Pipework not insulated	10	126,000		2
Half yearly	7	1	Quarterly	Sedimentation	Uninsulated tank	49	17,280		1
Half yearly	5	1	Twice Annually	Sedimentation	Uninsulated tank and pipework	16	63,000		4
Monthly	4	1	Twice Annually	Visibly Clean	Uninsulated tank	23	114,750		2
Monthly	8	1	Quarterly	Visibly clean	Uninsulated tank and pipework	13	10800	1,900	2
Quarterly	7	1	Quarterly	Stagnant water with bio film	Pipework not insulated	15	4,425		1
Half yearly	3	1	Twice Annually	Corrosion	Uninsulated tank and pipework	27	18,900		1
Half yearly	5	1	Twice Annually	Stagnant water with bio film	N/A	13	67,000		4
Half yearly	3	1	Twice Annually	Stagnant water with bio film	Uninsulated tank and pipework	53	14,400		2
Quarterly	4	2	Twice Annually	Sedimentation	Pipework not insulated	14	17,280		2
Half yearly	3	1	Twice Annually	Scale and sedimentation	Pipework not insulated	26	63,000		1
Half yearly	7	1	Twice Annually	Sedimentation	Not Satisfactory	15	114,750		4
Half yearly	4	1	Twice Annually	Corrosion	Satisfactory	68	10,800		2
Half yearly	2	1	Twice Annually	Stagnant water with bio film	Uninsulated tank	66	4,425		4
Half yearly	5	1	Twice Annually	Scale, sedimentation, and corrosion with bio fi	N/A	21	18,900		4
Half yearly	4	1	Twice Annually	Scale and sedimentation	Uninsulated tank and pipework	20	67,000		2
Half yearly	4	1	Twice Annually	Stagnant water with bio film	Uninsulated tank and pipework	40	4,425		1
Half yearly	4	0	Twice Annually	Sedimentation	Uninsulated tank	20	18,900		2
Half yearly	4	0	Twice Annually	Stagnant water with bio film	Uninsulated tank	37	67,000		2
Half yearly	2	0	Twice Annually	Scale and sedimentation	Uninsulated tank and pipework	29	12,000		2
Half yearly	3	1	Twice Annually	Sedimentation	N/A	30	5,400		4
Monthly	8	1	Twice Annually	Sedimentation	Uninsulated tank and pipework	13	12930	5,000	2
Monthly	9	1	quarterly	Stagnant water with bio film	N/A	15	12,240		1
Half yearly	8	1	Twice Annually	Corrosion and sedimentation	N/A	27	10,800		1
Half yearly	6	1	Twice Annually	Stagnant water with bio film	N/A	2	36,000		4
quarterly	4	0	quarterly	Corrosion	Satisfactory	4		2,000	3
Half yearly	4	0	Twice Annually	Visibly Clean	N/A	0	4860		1
Half yearly	5	0	Twice Annually	Visibly clean	N/A	4	7,200		1

Temperature Monitoring Frequency	No. of Samples	No. of communal showers	Sampling Frequency	Tank(s) internal conditon at the time of out break	Thermal insulation of the tanks and pipeworks	Total Tank(s) actual approximate Storage capacity in litres	Maximum requirement of stored water @ 150 litres per person for 24 hours consumption (Residential)	Maximum requirement of stored water @ 50 litres per person for 24 hours consumption (Commercial)	No. of deadlegs in the system
Quarterly	3	0	Twice Annually	Visibly clean	N/A	0	12,600		2
Half yearly	3	0	Twice Annually	Corrosion	N/A	14	5,400		1
Half yearly	5	0	Twice Annually	Visibly clean	N/A	6	14,400		1
Monthly	9	0	Twice Annually	Visibly Clean	N/A	0		2,500	1
Half yearly	3	0	Twice Annually	Corrosion	N/A	0	18,900		1
Half yearly	3	0	Twice Annually	Visibly Clean	N/A	2	37,800		2
Half yearly	4	0	Twice Annually	Visibly Clean	N/A	0	18,900		2
Half yearly	9	0	Twice Annually	Visibly Clean	N/A	2	44,100		1
Half yearly	7	0	Twice Annually	Visibly Clean	Satisfactory	14	18,900		2
Half yearly	4	0	Twice Annually	Visibly Clean	N/A	12	5,400		1
Half yearly	4	0	Twice Annually	Visibly Clean	N/A	0	3,600		1
quarterly	8	0	quarterly	Visibly clean	N/A	11		3,000	1
Half yearly	4	0	Twice Annually	Visibly clean	N/A	3		1,500	2
Monthly	5	0	Twice Annually	Visibly clean	Satisfactory	10		2,500	1
Half yearly	10	0	Twice Annually	Corrosion	N/A	0	25,200		1
Half yearly	4	0	Twice Annually	Corrosion	N/A	0	29,300		1
Half yearly	6	0	Twice Annually	Visibly Clean	Satisfactory	0	3,600		2
Quarterly	11	0	Annually	Visibly Clean	Satisfactory	0	9,000		2
Half yearly	4	0	Twice Annually	Visibly clean	N/A	0	23,040		1
Half yearly	6	0	Twice Annually	Visibly clean	N/A	0	12,600		1
Half yearly	5	0	Annually	Scale and sedimentation	N/A	0	12,600		1
Half yearly	6	0	Twice Annually	Visibly clean	N/A	0	25,200		1
Monthly	5	0	Twice Annually	Visibly clean	Satisfactory	0	9,450		1
Half yearly	7	0	Twice Annually	Corrosion	N/A	0	10,800		1
Half yearly	3	0	Twice Annually	Visibly clean	N/A	0	16,200		1
Half yearly	4	0	Annually	0	Satisfactory	4		4500	3

Temperature Monitoring Frequency	No. of Samples	No. of communal showers	Sampling Frequency	Tank(s) internal conditon at the time of out break	Thermal insulation of the tanks and pipeworks	Total Tank(s) actual approximate Storage capacity in litres	Maximum requirement of stored water @ 150 litres per person for 24 hours consumption (Residential)	Maximum requirement of stored water @ 50 litres per person for 24 hours consumption (Commercial)	No. of deadlegs in the system
Half yearly	6	0	Twice Annually	Visibly clean	N/A	0	15,120		1
Half yearly	6	0	Twice Annually	Visibly Clean	N/A	4	16,200		1
Half yearly	5	0	Twice Annually	Visibly clean	N/A	0	19,800		2
Half yearly	4	0	Twice Annually	Corrosion	N/A	4	9,000		1
Half yearly	3	0	Annually	Visibly Clean	N/A	6	6,840		1
Half yearly	4	0	Annually	Visibly Clean	N/A	0	9,400		1
Half yearly	6	0	Annually	Corrosion	N/A	0	9,000		1
Half yearly	3	0	Annually	Visibly clean	N/A	2	N/A		2
Monthly	4	0	quarterly	Visibly Clean	N/A	0		3,500	2
Half yearly	4	0	Twice Annually	Visibly Clean	N/A	2	12,600		1
Half yearly	4	0	Twice Annually	Visibly Clean	Satisfactory	9	39,600		2
Half yearly	4	0	Twice Annually	Visibly Clean	N/A	0	39,600		1
Half yearly	3	0	Twice Annually	Visibly Clean	N/A	0	39,600		1
Half yearly	3	0	Twice Annually	Visibly clean	N/A	5	3,420		1
Monthly	7	0	monthly	Visibly clean	N/A	3	12,600		2
Monthly	4	0	Twice Annually	Visibly clean	Satisfactory	5	32,400		1
Half yearly	4	0	Twice Annually	Corrosion	N/A	0	16,200		1
Half yearly	4	0	Twice Annually	Corrossion	N/A	0	10,800		1
Monthly	3	0	Twice Annually	Visibly clean	Satisfactory	0	10,800		2
Monthly	3	0	Twice Annually	Visibly Clean	Satisfactory	0	10,800		2
Monthly	4	0	Twice Annually	Visibly Clean	N/A	0	10,200		1
Half yearly	6	0	Twice Annually	Visibly Clean	N/A	0	10,800		1
Half yearly	6	0	Twice Annually	Visibly clean	N/A	0	11,600		1
Half yearly	3	0	Twice Annually	Visibly clean	N/A	0	9,000		1
Half yearly	3	0	Twice Annually	Visibly Clean	Satisfactory	0	7,200		1
Half yearly	5	0	Twice Annually	Corrosion	N/A	0	12,600		1
Half yearly	3	0	Twice Annually	Visibly clean	N/A	0	12,600		1
Half yearly	10	0	Twice Annually	Corrosion	N/A	0	12,600		1
Half yearly	3	0	Twice Annually	Visibly Clean	N/A	4	12,800		2
Half yearly	5	0	Twice Annually	Visibly Clean	N/A	0	10,600		2
Quarterly	5	0	Twice Annually	Visibly Clean	N/A	2	10,800		1
Half yearly	4	0	Twice Annually	Visibly clean	Pipework not insulated	5,000	7,200		
Half yearly	6	0	Twice Annually	Visibly clean	Pipework not insulated	4,000	5,100		1

Temperature Monitoring Frequency	No. of Samples	No. of communal showers	Sampling Frequency	Tank(s) internal conditon at the time of out break	Thermal insulation of the tanks and pipeworks	Total Tank(s) actual approximate Storage capacity in litres	Maximum requirement of stored water @ 150 litres per person for 24 hours consumption (Residential)	Maximum requirement of stored water @ 50 litres per person for 24 hours consumption (Commercial)	No. of deadlegs in the system
Half yearly	4	0	Annually	Visibly clean	Pipework not insulated	6,000	6,800		
Half yearly	5	0	Twice Annually	Visibly clean	Satisfactory	12,000	12,600		
Half yearly	20	0	Twice Annually	Visibly clean	Satisfactory	5,600	8,100		
Half yearly	7	0	Twice Annually	Visibly clean	Satisfactory	4,000	6,800		
Half yearly	19	0	Twice Annually	Visibly clean	Satisfactory	18,000	14,400		
Half yearly	7	0	Twice Annually	Visibly Clean	Pipework not insulated	15,000	9,720		
Half yearly	7	1	Twice Annually	Corroded	Pipework not insulated	32,000	22,680		
Monthly	5	0	Twice Annually	Sedimentation	Satisfactory	6,000		5,500	1
Half yearly	5	0	Twice Annually	Visibly clean	Satisfactory	10,000	12,600		
Half yearly	5	1	Twice Annually	Visibly clean	Satisfactory	8,000	7,800		
Half yearly	6	1	Twice Annually	Visibly Clean	Pipework not insulated	6,000	6,840		2
Monthly	4	0	Twice Annually	Corroded	Satisfactory	8,000	9,000		
Half yearly	4	0	Twice Annually	Sedimentation	Satisfactory	12,000	12,600		
Half yearly	4	0	Twice Annually	Visibly clean	Pipework not insulated	6,000	5,400		
Half yearly	9	0	Twice Annually		Pipework not insulated	12,000	10,260		
Half yearly	3	0	Twice Annually		Satisfactory	8,000	8,500		
Half yearly	5	0	Twice Annually		Satisfactory	8,000	7,200		
Half yearly	11	0	Twice Annually		Satisfactory	18,000	21,600		
Half yearly	5	0	Twice Annually		Pipework not insulated	6,000	7,200		
Half yearly	5	0	Twice Annually		Pipework not insulated	20,000	25,200		
Half yearly	4	0	Twice Annually		Satisfactory	4,000	5,300		2
Half yearly	4	0	Twice Annually		Satisfactory	16,000	18,000		
Half yearly	5	1	Twice Annually		Satisfactory	20,000	25,200		
Half yearly	6	2	Twice Annually		Satisfactory	1000	1500		
Half yearly	10	2	Twice Annually	Stagnant water with oily film	Pipework not insulated	3,600	2,850		1
Half yearly	5	2	Twice Annually		Satisfactory	1500	1500		
Half yearly	5	2	Twice Annually		Satisfactory	2000	3000		
Half yearly	5	2	Twice Annually		Satisfactory	2000	2250		
Half yearly	9	2	Twice Annually		Satisfactory	1800	2700		
Half yearly	5	3	Twice Annually		Satisfactory	1000	1800		
Half yearly	6	3	Twice Annually		Pipework not insulated	5000	7800		1
Half yearly	13	3	Twice Annually		Satisfactory	2	3000		
Half yearly	7	2	Twice Annually		Satisfactory	3,000	3,750		
Half yearly	7	2	Twice Annually		Satisfactory	3,000	3,000		
Half yearly	7	3	Twice Annually		Satisfactory	4,000	4500		

Temperature Monitoring Frequency	No. of Samples	No. of communal showers	Sampling Frequency	Tank(s) internal conditon at the time of out break	Thermal insulation of the tanks and pipeworks	Total Tank(s) actual approximate Storage capacity in litres	Maximum requirement of stored water @ 150 litres per person for 24 hours consumption (Residential)	Maximum requirement of stored water @ 50 litres per person for 24 hours consumption (Commercial)	No. of deadlegs in the system
Half yearly	8	3	Twice Annually		Satisfactory	3000	3,600		
Half yearly	7	3	Twice Annually		Satisfactory	4,000	5,880		1
Half yearly	7	4	Twice Annually		Satisfactory	3,000	3,600		
Half yearly	12	4	Twice Annually	Stagnant water with oily film	Tank not insulated properly	9,000	5,400		
Half yearly	7	3	Twice Annually		Satisfactory	6,000	7,200		
Half yearly	8	4	Twice Annually		Pipework not insulated	8,000	9,600		
Half yearly	6	3	Annually		Satisfactory	4,000	3,600		
Half yearly	7	3	Twice Annually		Satisfactory	4,000	4,560		
Half yearly	9	3	Twice Annually		Pipework not insulated	8,000	7,980		
Half yearly	4	0	Twice Annually		Satisfactory	8,000	11,400		1
Half yearly	9	5	Twice Annually		Satisfactory	3,000	2,700		
Half yearly	10	6	Twice Annually		Satisfactory	2,000	2,400		
Half yearly	9	5	Twice Annually	Stagnant water with oily film	Tank not insulated properly	8,000	7,125		
Half yearly	9	5	Twice Annually		Satisfactory	6,000	6,000		
Half yearly	9	5	Twice Annually		Tank not insulated properly	4,000	3,600		
Half yearly	8	4	Twice Annually		Satisfactory	4,000	4,560		
Half yearly	7	3	Twice Annually		Satisfactory	2,000		1,500	
Half yearly	8	2	Twice Annually		Satisfactory	7,000		5,700	
Half yearly	6	2	Twice Annually		Tank not insulated properly	3,000		2,250	
Half yearly	6	1	Twice Annually	Stagnant water with oily film	Satisfactory	4,000		1,400	
Half yearly	4	0	Twice Annually		Tank not insulated properly	18,000	25,200		
Half yearly	4	0	Twice Annually		Pipework not insulated	14,000	30,000		1
Half yearly	6	1	Twice Annually	Visibly Clean	Pipework not insulated	6000	6840		2

Number of Legionella Outbreak(s)	Legionella detected samples	Sample temperature in Degree Celcious H - Hot, C - Cold	Season of outbreaks	High TVC Count	Remediation Method Used	Resampling Result
Yes - Legionella (SG1) -1	Hot tap -2 and CWST -1	H - 49, C - 20	June		Chlorination	Legionella Not Detected
Yes - Legionella (SG2-14) - 0	CWST -3	H- 60 C - 19	May		Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0	CWST - 2 and Hot tap -1	C - 20 and H - 60	November	Yes - TVC EST1458	Chlorination of cold water tank feeding calorifiers	Legionella and TVC Not Detected
Yes - Legionella (SG2-14) - 0	Hot tap - 4	H - 60 C-23	November			
Yes - Legionella (SG2-14) - 1	Cold -1	H-53 C - 23	0		Chlorination	Not Detected
Yes - Legionella (SG2-14) - 2	Hot tap - 2	H - 52 C -22	May - November		Chlorination of cold water tank feeding calorifiers	Legionella and TVC Not Detected
Yes - Legionella (SG2-14) - 1	Hot tap - 2	H - 48 C- 15	0		Pasteurisation	Legionella Not Detected
Yes - Legionella (SG2-14) - 2	Hot tap - 2	H - 48 C-14	0		Chlorination	Not Detected
Yes - Legionella (SG2-14) -1	Hot tap - 2	H - 49 C- 20	March		Chlorination	Not Detected
Yes - Legionella (SG2-14) - 1	Hot tap -2	H - 46 C- 23	May		Chlorination	Not Detected
Yes - Legionella (SG2-14) - 1	CWST-1	H-48 C-15	March		Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0	Shower	H-49 C- 17	0		Chlorination of cold water tank feeding calorifiers	Legionella and TVC Not Detected
Yes - Legionella (SG2-14) - 0	Hot tap -2 and Showers -2	H - 60 C-19	June			
Yes - Legionella (SG2-14) - 1	Hot tap -3 and CWST -3	H - 54, C 17	0		Chlorination of Hot Water System	Legionella Not Detected
Yes - Legionella (SG2-14) -0	Hot tap - 1	H -53 C-22	November		Chlorination	Not Detected
Yes - Legionella (SG2-14) -0	Hot -1 and Cold -1	H - 48 C - 19	May	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) -0	CWST -2	H-49 C-20	0			
Yes - Legionella (SG2-14) -0	CWST -2	H-52 C- 23	May		Chlorination	Not Detected
Yes - Legionella (SG2-14) -0	Hot tap -2 and Cold tap -1	H - 53 and C - 20	March		Chlorination	Not Detected
Yes Legionella (SG2-14) - 1	Hot tap -1 and CWST -1	H -50, C - 11	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes Legionella (SG2-14) - 1	Hot tap -1 and CWST -1	H - 60 and C - 18	April		Pasteurisation	Legionella Not Detected
Yes Legionella (SG2-14) - 1	Showers -2 and CWST -1	H- 48 C- 10	May	Yes - TVC >10000	Chlorination	TVC Not Detected
Yes Legionella (SG2-14) - 1	Hot tap -3, Cold tap -1 and CWST -2	H-53 C-15	0			
Yes Legionella (SG2-14) - 1	Hot tap -5 and CWST- 1	H - 54 - 57 and C - 21	November		Chlorine Dioxide	Legionella Not Detected
Yes Legionella (SG2-14) - 6	Showerhead -6	H - 55 C- 8	January		Chlorination	Not Detected
Yes Legionella (SG2-14) - 2	Hot tap -6	H - 60 C-23	November	Yes - TVC Tanks >10000	Chlorination	
Yes Legionella (SG2-14) -1	Hot tap -1 and CWST -1	H - 60 and C - 24	May		Chlorination	Not Detected
Yes Legionella (SG2-14) -1	CWST -2	H-56 C- 12	0		Chlorination	Not Detected
Yes - Legionella (SG2-14) - 2	Cold -1	H- 49 C- 23	June			
Yes - Legionella (SG2-14) - 0	Hot tap - 2	H - 60 C- 19	May			
Yes - Legionella (SG2-14) - 0	Hot tap - 2	H - 59 C- 20	November			

Number of Legionella Outbreak(s)	Legionella detected samples	Sample temperature in Degree Celcious H - Hot, C - Cold	Season of outbreaks	High TVC Count	Remediation Method Used	Resampling Result
Yes - Legionella (SG2-14) - 1	Hot tap - 2	H - 59 C-22	May			
Yes - Legionella (SG2-14) - 2	Hot tap - 2	H - 45 C -9	0			
Yes - Legionella (SG2-14) - 2	Hot tap -2	H - 49 C-20	May			
Yes - Legionella (SG2-14) - 2	Cold tap -1 and CWST-1	H-48 C-13	January	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 2	Shower	H-52 C-12	0			
Yes - Legionella (SG2-14) -1	Hot tap -2 and Showers -2	H - 52 C- 20	March			
Yes - Legionella (SG2-14) -1	Hot tap -3 and CWST -3	H - 46 C- 20	May			
Yes - Legionella (SG2-14) -1	Hot tap - 1	H -49 C- 15	April			
Yes - Legionella (SG2-14) -1	Hot -1 and Cold -1	H - 48 C - 5	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) -0	CWST -2	H-52 C-15	October	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 1	CWST -2	H- 53 C-5	0			
Yes - Legionella (SG2-14) - 1	Showers -2 and CWST -1	H-48 C-22	May			
Yes - Legionella (SG2-14) - 0	Hot tap -3, Cold tap -1 and CWST -2	H -49 C-18	November			
Yes - Legionella (SG2-14) - 1	Hot tap -5 and CWST- 1	H-48 C- 6	0			
Yes - Legionella (SG2-14) - 2	Hot tap -6	H -52 C- 22	May- November			
Yes - Legionella (SG2-14) - 1	Hot tap -6	H - 53 C- 20	March			
Yes - Legionella (SG2-14) - 1	Hot tap -1 and CWST -1	H - 48 C-10	0			
Yes - Legionella (SG2-14) - 0	CWST -2	H-60 C-19	April	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 1	Cold -1	H-48 C- 15	May			
Yes - Legionella (SG2-14) - 0	Hot tap - 2	H - 52 C-8	0			
Yes - Legionella (SG2-14) - 1	Hot tap - 2	H - 53 C-23	November			
Yes - Legionella (SG2-14) - 3	Hot tap - 2	H - 52 C-13	December, January, Feb	Yes - TVC EST7542	Chlorination	TVC Not Detected
Yes - Legionella (SG2-14) - 0	Hot tap - 2	H - 52 C-23	May			
Yes - Legionella (SG2-14) - 3	Hot tap -2	H - 48 C- 20	May- November			
Yes - Legionella (SG2-14) -1	Cold tap -1 and CWST-1	H-49 C- 5	0			
Yes - Legionella (SG2-14) -1	Hot tap - 2	H -46 C-8	0			
Yes - Legionella (SG2-14) -2	Hot tap - 2	H - 49 C-15	June. October			
Yes - Legionella (SG2-14) -2	Hot tap - 2	H - 48 C-12	0			
Yes - Legionella (SG2-14) - 1	Hot tap -2	H -49 C-23	May			
0		H-60 C-11	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0		H-60 C-10	0			

Number of Legionella Outbreak(s)	Legionella detected samples	Sample temperature in Degree Celcious H - Hot, C - Cold	Season of outbreaks	High TVC Count	Remediation Method Used	Resampling Result
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-8	0			
Yes - Legionella (SG2-14) - 0		H-60 C-7	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12	0			
Yes - Legionella (SG2-14) - 0		H-60 C-8	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0		H-60 C-6	0			
Yes - Legionella (SG2-14) - 0		H-60 C-10	0			
Yes - Legionella (SG2-14) - 0		H-60 C-6	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-9	0			
Yes - Legionella (SG2-14) - 0		H-60 C-11	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-4	0			
Yes - Legionella (SG2-14) - 0		H-60 C-2	0			
Yes - Legionella (SG2-14) - 0		H-60 C-6	0			
Yes - Legionella (SG2-14) - 0		H-60 C-8	0			
Yes - Legionella (SG2-14) - 0		H-60 C-10	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0		H-60 C-12	0			
Yes - Legionella (SG2-14) - 0		H-59 C-10	0			
Yes - Legionella (SG2-14) - 1		H-60 C-8	0			
Yes - Legionella (SG2-14) - 0		H-60 C-9	0			
Yes - Legionella (SG2-14) - 0		H-60 C-7	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-11	0			

Number of Legionella Outbreak(s)	Legionella detected samples	Sample temperature in Degree Celcious H - Hot, C - Cold	Season of outbreaks	High TVC Count	Remediation Method Used	Resampling Result
Yes - Legionella (SG2-14) - 0		H-60 C-12	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-8	0			
Yes - Legionella (SG2-14) - 0		H-60 C-10	0			
Yes - Legionella (SG2-14) - 0		H-60 C-7	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-59 C-8	0			
Yes - Legionella (SG2-14) - 0		H60- C -5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-8	0			
Yes - Legionella (SG2-14) - 0		H-60 C-6	0			
Yes - Legionella (SG2-14) - 0		H-60 C-9	0			
Yes - Legionella (SG2-14) - 0		H-60 C-11	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12	0			
Yes - Legionella (SG2-14) - 0		H-60 C-4	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 4		H-60 C-8	0	Yes - TVC Tanks >10000	Chlorination	Not Detected
Yes - Legionella (SG2-14) - 0		H-60 C-10	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12	0			
Yes - Legionella (SG2-14) - 0		H-60 C-6	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-8	0			
Yes - Legionella (SG2-14) - 0		H-60 C-9	0			
Yes - Legionella (SG2-14) - 2		H-60 C-11	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-6	0			
Yes - Legionella (SG2-14) - 0		H-60 C-9	0			
Yes - Legionella (SG2-14) - 0		H-60 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-7	0			
Yes - Legionella (SG2-14) - 1		H-58 C-5	0			
Yes - Legionella (SG2-14) - 0		H-60 C-12				

Appendix-1

Number of Legionella Outbreak(s)	Legionella detected samples	Sample temperature in Degree Celcious H - Hot, C - Cold	Season of outbreaks	High TVC Count	Remediation Method Used	Resampling Result
				Yes - TVC Tanks >10000	Chlorination	
				Yes - TVC Tanks >10000	Chlorination	
				Yes - TVC Tanks >10000	Chlorination	Not Detected

Number of Legionella Outbreak(s)	Legionella detected samples	Sample temperature in Degree Celcious H - Hot, C - Cold	Season of outbreaks	High TVC Count	Remediation Method Used	Resampling Result
				Yes - TVC Tanks >10000	Chlorination	Not Detected
				Yes - TVC Tanks >10000	Chlorination	Not Detected
				Yes - TVC Tanks >10000	Chlorination	Not Detected

Bldg No.	Occupancy rate	No. of water tanks	No. of calorifiers	Tank's internal conditions	Thermal insulation	Excess water storage (%)
1	4	8	5	4	4	23
2	3	7	3	6	3	28
3	3	6	3	3	3	14
4	4	4	4	5	5	40
5	3	12	0	2	3	0
6	3	8	1	6	7	60
7	3	6	3	4	6	14
8	3	8	3	5	5	4
9	4	9	4	4	8	49
10	4	4	2	3	3	16
11	2	11	2	1	1	23
12	2	16	2	2	1	13
13	3	14	2	6	0	15
14	3	12	2	2	4	27
15	3	10	3	6	3	13
16	3	4	2	6	4	53
17	4	7	2	2	7	14
18	4	6	3	5	6	26
19	5	3	4	3	6	15
20	4	3	4	5	3	68
21	3	30	0	6	1	66
22	3	6	4	7	4	21
23	3	5	2	5	4	12
24	4	2	2	6	3	40
25	0	3	3	3	3	0
26	4	5	3	6	3	37
27	5	5	3	5	6	29
28	5	4	2	4	5	18
29	5	3	5	3	5	23
30	3	9	1	6	9	28
31	2	13	2	4	1	14
32	3	14	2	5	0	40

Bldg No.	Occupancy rate	No. of water tanks	No. of calorifiers	Tank's internal conditions	Thermal insulation	Excess water storage (%)
33	3	21	1	4	1	22
34	3	16	0	6	4	60
35	3	11	3	3	4	13
36	4	4	4	5	3	10
37	4	12	5	3	4	49
38	5	6	2	3	5	16
39	4	7	2	1	4	23
40	5	10	1	1	5	13
41	3	5	0	6	3	15
42	3	4	2	2	5	27
43	4	5	3	6	6	13
44	3	24	6	6	5	53
45	4	7	4	3	3	14
46	4	6	3	5	3	26
47	3	13	3	3	2	15
48	3	8	4	2	1	68
49	3	30	0	6	4	66
50	5	6	4	7	6	21
51	3	3	2	5	5	20
52	3	7	2	6	5	40
53	3	8	2	3	4	20
54	2	4	2	6	4	37
55	3	4	2	5	5	29
56	3	6	2	3	6	30
57	3	10	2	3	5	13
58	4	3	2	6	8	15
59	4	7	2	4	9	27
60	3	5	3	6	11	2
61	1	2	2	2	1	4
62	1	3	1	1	0	0
63	1	2	0	1	0	4
64	1	4	0	1	0	0

Bldg No.	Occupancy rate	No. of water tanks	No. of calorifiers	Tank's internal conditions	Thermal insulation	Excess water storage (%)
65	1	1	0	2	0	14
66	1	2	0	1	0	6
67	1	3	0	1	0	0
68	1	2	1	2	0	0
69	1	3	2	1	0	2
70	1	2	2	1	0	0
71	1	2	2	1	0	2
72	1	3	0	1	1	14
73	1	2	2	1	0	12
74	1	1	1	1	0	0
75	1	2	1	1	0	11
76	1	3	1	1	0	3
77	1	2	0	1	1	10
78	1	3	1	2	0	0
79	2	3	2	2	0	0
80	2	2	2	1	1	0
81	1	2	1	1	1	0
82	1	8	1	1	0	0
83	1	3	1	1	0	0
84	1	3	1	5	0	0
85	1	2	0	1	0	0
86	1	2	2	1	1	0
87	1	2	1	2	0	0
88	1	2	1	1	0	0
89	1	2	1	0	1	4
90	2	8	0	1	0	0
91	1	2	1	1	0	4
92	1	4	0	1	0	0
93	1	1	2	2	0	4
94	1	2	2	1	0	6
95	1	3	0	1	0	0
96	1	2	1	2	0	0

Bldg No.	Occupancy rate	No. of water tanks	No. of calorifiers	Tank's internal conditions	Thermal insulation	Excess water storage (%)
97	1	3	2	1	0	2
98	1	2	2	1	0	0
99	1	2	2	1	0	2
100	1	6	0	1	1	9
101	1	2	2	1	0	0
102	1	1	1	1	0	0
103	1	2	0	1	0	5
104	1	3	2	1	0	3
105	1	2	2	1	1	5
106	2	3	1	2	0	0
107	2	3	2	2	0	0
108	1	2	2	1	1	0
109	1	2	1	1	1	0
110	1	8	1	1	0	0
111	1	3	1	1	0	0
112	1	3	1	1	0	0
113	1	2	0	1	0	0
114	1	2	0	1	1	0
115	1	2	1	2	0	0
116	1	2	1	1	0	0
117	1	2	1	2	0	0
118	1	3	2	1	0	4
119	1	2	0	1	0	0
120	1	2	0	1	0	2

Number of deadlegs	Hot water temperature	Cold water temperature	Seasonality	No. of showerheads	No. of *Legionella* outbreaks
1	4	4	4	2	1
4	0	4	3	1	0
1	0	4	3	1	0
4	0	5	3	2	0
1	3	5	0	3	1
1	3	5	3	3	2
2	4	2	0	3	1
2	4	2	0	2	2
1	4	4	2	1	1
4	5	5	3	1	1
0	4	2	2	1	1
2	4	3	0	1	0
1	0	4	4	1	0
1	3	3	0	1	1
4	3	5	3	1	0
2	4	4	3	1	0
2	4	4	0	2	0
1	3	5	3	1	0
4	3	4	2	1	0
2	3	0	0	1	1
4	0	3	2	2	1
4	4	0	3	1	1
2	3	2	0	1	0
1	3	5	3	1	0
1	2	0	1	6	6
2	0	5	3	2	0
2	0	5	3	1	3
4	3	1	0	1	1
1	4	5	4	2	1
4	0	4	3	0	1
1	1	4	3	0	1
4	1	5	3	1	1

Number of deadlegs	Hot water temperature	Cold water temperature	Seasonality	No. of showerheads	No. of *Legionella* outbreaks
1	5	0	0	2	2
1	4	4	3	1	2
2	4	1	1	1	2
2	3	1	0	4	1
1	3	4	2	1	1
4	5	4	3	1	1
2	4	2	2	1	1
2	4	0	0	1	1
1	3	2	4	1	0
1	3	0	0	1	1
4	4	5	3	1	1
2	4	3	3	1	0
2	4	0	0	2	1
1	3	5	3	1	2
4	3	4	2	1	1
2	4	0	0	1	1
4	0	4	2	1	0
4	4	2	3	1	1
2	3	0	0	1	0
1	3	5	3	1	1
2	3	1	1	0	3
2	3	5	3	0	0
2	4	4	3	0	3
4	4	0	0	1	1
2	5	0	0	1	1
1	4	2	4	1	2
1	4	1	0	1	2
4	4	5	3	1	1
3	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0

Number of deadlegs	Hot water temperature	Cold water temperature	Seasonality	No. of showerheads	No. of *Legionella* outbreaks
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	1	0	0	0	0
1	0	0	0	0	1
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
3	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	1
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0

Number of deadlegs	Hot water temperature	Cold water temperature	Seasonality	No. of showerheads	No. of *Legionella* outbreaks
2	1	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	4
1	0	0	0	0	0
2	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	2
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
2	1	0	0	0	1
1	0	0	0	0	0

Appendix 3

Table: 1

Legionella outbreak: Possible contributed factors to the outbreak

Bldg No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	1937	Res	70	23	2	H,C	51, 18	OCT-APR	2	Sediment	S
2	1945	Res	100	28	0	WT	17, 19	FEB-AUG	2	Water Stagnation	NS
3	1900	Res	85	14	1	C Tap	18	JUN	1	Clean	S
4	1930	Res	70	40	0	WT	18-22	FEB-AUG	3	Sediment & scale	NS
5	1900	Mix	75	N A	2	WT	16	APR	2	Water Stagnation	NS
6	1930	Mix	60	60	0	WT, H-tap	16,18, 52	FEB-AUG	3	Corrosion & oily film	NS
7	2000	Care Home	100	NA	1	Shower	NA	JUN-DEC	3	Clean	NS
8	1900	Res	100	NA	1	H-2	47, 51	MAY-NOV	2	Clean	S
9	1937	Res	70	49	0	H-2, WT	55, 20	JAN- JUL	3	Water stagnation & oily film	S
10	1940	Res	75	16	1	H-2	56, 54	FEB-AUG	2	Clean	NS
11	1940	Res	80	23	2	H-2	55	MAR-SEP	2	Sediment & scale	NS
12	1940	Res	65	13	1	H-2, C	53,20	NOV-MAY	3	Clean	S
13	1800	Res	65	15	0	H-3,C-1, WT-2	47-50, 20, 19,18	DEC-JUN	6	Corrosion	NS
14	2000	Mix	75	27	0	H-2	57, 55	JAN- JUL	2	Sediment & scale	NS
15	1800	Res	85	13	0	H	53	JAN	1	Clean	NS
16	1940	Mix	35	53	0	H-5, WT-1	54-57, 21	NOV-AUG	6	Water stagnation & oily film	S

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Bldg No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
17	2000	Res	70	14	0	H-2	51,53	JAN-JUL	2	Clean	NS
18	2003	Res	85	26	1	C-1, WT -1	16,11	SEP-MAR	2	Water stagnation & oily film	S
19	2000	Res	90	15	1	H-6	55-58	JAN-JUL	6	Clean	S
20	1940	Res	25	68	0	H-3, WT-3	54-55, 17-22	DEC-JUN	6	Water stagnation & oily film	NS
21	1930	Res	60	66	3	H-1,WT-1	50, 18	FEB-AUG	2	Water stagnation & oily film	N S
22	1940	Res	85	21	0	H-6	52-56	APR-OCT	6	Clean	NS
23	1800	Res	80	NA	0	H-1, WT - 1	56, 22	JUN-DEC	2	Sediment & scale	S
24	1930	Res	90	40	2	H-1, WT-1	56, 18	MAY-NOV	2	Corrosion	NS
25	1945	Res	70	20	1	H-4	54-58	NOV-MAY	4	Clean	NS
26	1930	Care Home	85	37	0	WT-2	22,14	JUN-DEC	2	Water stagnation & oily film	NS
27	1920	Care Home	95	29	0	Shower-2, WT-1	20	JUN-DEC	3	Sediment & scale	NS
28	1800	Care Home	85	NA	1	H-2, Shower -2	54, 56	NOV-MAY	4	Clean	NS

Appendix 3

Table: 2

Outbreak in buildings with 100% Occupancy

Bldg. No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	1945	Res	100	28	0	WT	17, 19	FEB-AUG	2	Water Stagnation	NS
2	2000	Care Home	100	NA	1	Shower	NA	JUN-DEC	3	Clean	NS
3	1900	Res	100	NA	1	H-2	47, 51	MAY-NOV	2	Clean	S

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Table: 3

Outbreak in buildings with occupancy rate 50< and 100>

Bldg. No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	1937	Res	70	23	2	H,C	51, 18	OCT-APR	2	Sediment	S
2	1900	Res	85	14	1	C Tap	18	JUN	1	Clean	S
3	1930	Res	70	40	0	WT	18-22	FEB-AUG	3	Sediment & scale	NS
4	1900	Mix	75	N A	2	WT	16	APR	2	Water Stagnation	NS
5	1930	Mix	60	60	0	WT, H-tap	16,18, 52	FEB-AUG	3	Corrosion & oily film	NS
6	1937	Res	70	49	0	H-2, WT	55, 20	JAN- JUL	3	Water stagnation & oily film	S
7	1940	Res	75	16	1	H-2	56, 54	FEB-AUG	2	Clean	NS
8	1940	Res	80	23	2	H-2	55	MAR-SEP	2	Sediment & scale	NS
9	1940	Res	65	13	1	H-2, C	53,20	NOV-MAY	3	Clean	S
10	1800	Res	65	15	0	H-3,C-1, WT-2	47-50, 20, 19,18	DEC-JUN	6	Corrosion	NS
11	2000	Mix	75	27	0	H-2	57, 55	JAN- JUL	2	Sediment & scale	NS
12	1800	Res	85	13	0	H	53	JAN	1	Clean	NS
13	2000	Res	70	14	0	H-2	51,53	JAN-JUL	2	Clean	NS

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14	**2003**	**Res**	**85**	**26**	**1**	**C-1, WT - 1**	**16,11**	**SEP-MAR**	**2**	**Water stagnation & oily film**	**S**
15	**2000**	**Res**	**90**	**15**	**1**	**H-6**	**55-58**	**JAN-JUL**	**6**	**Clean**	**S**
16	**1930**	**Res**	**60**	**66**	**3**	**H-1,WT-1**	**50, 18**	**FEB-AUG**	**2**	**Water stagnation & oily film**	**N S**
17	**1940**	**Res**	**85**	**21**	**0**	**H-6**	**52-56**	**APR-OCT**	**6**	**Clean**	**NS**
18	**1800**	**Res**	**80**	**NA**	**0**	**H-1, WT - 1**	**56, 22**	**JUN-DEC**	**2**	**Sediment & scale**	**S**
19	**1930**	**Res**	**90**	**40**	**2**	**H-1, WT-1**	**56, 18**	**MAY-NOV**	**2**	**Corrosion**	**NS**
20	**1945**	**Res**	**70**	**20**	**1**	**H-4**	**54-58**	**NOV-MAY**	**4**	**Clean**	**NS**
21	**1930**	**Care Home**	**85**	**37**	**0**	**WT-2**	**22,14**	**JUN-DEC**	**2**	**Water stagnation & oily film**	**NS**
22	**1920**	**Care Home**	**95**	**29**	**0**	**Shower-2, WT-1**	**20**	**JUN-DEC**	**3**	**Sediment & scale**	**NS**
23	**1800**	**Care Home**	**85**	**NA**	**1**	**H-2, Shower - 2**	**54, 56**	**NOV-MAY**	**4**	**Clean**	**NS**

Appendix 3

Table: 4

Outbreak in buildings with occupancy rate 50>

Bldg. No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	1940	Res	25	68	0	H-3, WT-3	54-55, 17-22	DEC-JUN	6	Water stagnation & oily film	NS
2	1940	Mix	35	53	0	H-5, WT-1	54-57, 21	NOV-AUG	6	Water stagnation & oily film	S

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Table: 5

Outbreak on the basis of building usage - Mix Use (Residential and commercial)

Bldg. No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	1900	Mix	75	N A	2	WT	16	APR	2	Water Stagnation	NS
2	1930	Mix	60	60	0	WT, H-tap	16,18, 52	FEB-AUG	3	Corrosion & oily film	NS
3	2000	Mix	75	27	0	H-2	57, 55	JAN- JUL	2	Sediment & scale	NS

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Table: 6

Outbreak on the basis of building usage – Care Home

Bldg. No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	2000	Care Home	100	NA	1	Shower	NA	JUN-DEC	3	Clean	NS
2	1930	Care Home	85	37	0	WT-2	22,14	JUN-DEC	2	Water stagnation & oily film	NS
3	1920	Care Home	95	29	0	Shower-2, WT-1	20	JUN-DEC	3	Sediment & scale	NS
4	1800	Care Home	85	NA	1	H-2, Shower - 2	54, 56	NOV-MAY	4	Clean	NS

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Table: 7

Legionella outbreak: Sites with up to 25% Excess water storage

Bldg. No.	Age	Usage	Occup-ancy Rate (%)	Excess Water Storage (%)	No. of dead legs	Sample Detected (H/C - tap/WT/ Shower)	Sample Tempe-rature (s)	Sampling Month	Total number of outbreaks in 6 years	Tank's Internal condition	Thermal Insulation
1	1937	Res	70	23	2	H,C	51, 18	OCT-APR	2	Sediment	S
2	1900	Res	85	14	1	C Tap	18	JUN	1	Clean	S
3	1940	Res	75	16	1	H-2	56, 54	FEB-AUG	2	Clean	NS
4	1940	Res	80	23	2	H-2	55	MAR-SEP	2	Sediment & scale	NS
5	1940	Res	65	13	1	H-2, C	53,20	NOV-MAY	3	Clean	S
6	1800	Res	65	15	0	H-3,C-1, WT-2	47-50, 20, 19,18	DEC-JUN	6	Corrosion	NS
7	1800	Res	85	13	0	H	53	JAN	1	Clean	NS
8	2000	Res	70	14	0	H-2	51,53	JAN-JUL	2	Clean	NS
9	2000	Res	90	15	1	H-6	55-58	JAN-JUL	6	Clean	S
10	1940	Res	85	21	0	H-6	52-56	APR-OCT	6	Clean	NS
11	1945	Res	70	20	1	H-4	54-58	NOV-MAY	4	Clean	NS

Appendix- 4

Bldg No.	Occupancy rate	No. of water tanks	No. of calorifiers	Tank's internal conditions	Thermal insulation	Excess water storage (%)
1	4	8	5	2	4	31
2	2	4	4	6	2	18
3	3	3	2	6	6	24
4	4	14	2	0	0	50
5	4	5	3	1	5	10
6	4	5	2	6	1	40
7	5	6	3	4	6	5
8	3	8	2	5	7	17
9	3	4	2	6	9	19
10	5	5	3	4	4	44
11	4	4	4	5	0	43
12	4	4	5	3	3	30
13	5	3	0	4	1	18
14	4	2	2	6	8	23
15	4	6	3	5	3	11
16	5	24	2	5	3	18
17	5	7	2	5	1	22
18	4	6	3	5	0	45
19	4	3	3	5	2	10
20	3	5	1	5	4	42
21	5	30	0	4	2	5
22	4	6	4	2	0	17
23	4	3	2	3	6	19
24	5	4	2	2	2	44
25	5	3	3	2	3	20
26	4	13	4	1	6	31
27	4	9	1	2	1	29
28	4	3	3	2	1	0
29	4	2	2	3	1	4
30	5	8	3	3	0	0
31	3	9	2	1	7	14
32	2	4	0	4	4	10
33	0	1	2	2	0	14

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34	1	2	2	1	0	6
35	1	3	0	1	0	0
36	1	2	1	2	0	0
37	1	3	2	1	0	12
38	1	2	2	1	0	0
39	1	2	2	1	0	2
40	2	2	3	1	1	14
41	1	2	2	1	0	0
42	1	1	1	1	0	0
43	1	2	3	1	0	11
44	1	3	2	1	0	3
45	1	2	2	1	1	10
46	2	3	1	2	0	0
47	1	3	2	2	0	0
48	1	2	2	1	1	0
49	2	2	1	1	1	0
50	1	8	1	1	0	0
51	1	3	1	1	0	0
52	1	3	1	0	0	0
53	1	2	0	0	0	0
54	1	2	3	0	1	0
55	2	2	1	2	0	0
56	1	2	1	1	0	0
57	1	2	2	0	1	0
58	2	2	2	0	1	0
59	1	2	0	1	1	0
60	1	1	2	1	0	0
61	1	1	1	1	0	0
62	1	1	0	0	2	0
63	1	2	0	1	0	0
64	2	2	1	0	0	0
65	1	3	2	0	0	0
66	1	4	0	1	0	0

Number of deadlegs	Hot water temperature	Cold water temperature	Seasonality	No. of showerheads	No. of Legionella outbreaks
4	2	5	4	5	3
4	2	0	4	4	2
3	1	1	5	4	2
4	3	1	5	5	2
4	2	5	0	5	1
5	3	1	4	3	1
2	2	4	0	4	1
2	2	4	0	5	3
1	3	0	5	4	3
4	6	0	5	4	3
2	1	3	4	0	4
2	2	4	0	0	2
1	2	0	4	4	1
1	6	5	1	0	2
4	6	3	5	3	1
2	2	4	4	3	0
2	6	3	5	0	0
1	2	0	5	3	1
4	2	1	5	2	1
3	4	5	4	2	3
4	2	4	5	2	3
4	3	3	5	3	2
2	2	5	0	0	0
5	6	1	3	3	2
2	3	5	0	1	3
4	2	5	2	3	0
2	3	4	3	3	3
5	4	4	3	0	2
5	0	2	0	1	4
5	0	2	5	2	2
4	0	3	4	5	2
5	0	0	3	5	3
1	0	0	0	0	0

1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	1	0	0	1
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
2	0	0	0	0	1
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	2
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	1
1	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	2
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0
1	0	1	0	0	0
0	0	0	0	0	0

Bldg No.	Occupancy rate	No. of water tanks	No. of calorifiers	Tank's internal conditions	Thermal insulation	Excess water storage (%)
1	2	3	4	2	6	9
2	2	5	5	3	4	4
3	4	4	4	4	5	0
4	4	4	4	5	3	3
5	5	4	14	2	0	0
6	1	2	2	1	1	0
7	1	1	1	1	1	0
8	1	1	1	1	0	0
9	1	2	1	1	0	0

Number of deadlegs	Hot water temperature	Cold water temperature	Seasonality	No. of showerheads	No. of Legionella outbreaks
12	1	3	0	5	4
21	4	6	0	5	4
6	2	1	3	4	0
5	2	2	4	0	0
13	4	3	1	5	5
4	0	0	0	0	0
2	0	0	0	0	0
1	0	0	0	0	0
1	0	0	0	0	0