The impact of viable but non-culturable *Legionella pneumophila* on detection and disinfection: Searching for viability markers to aid in pathogen risk management

by

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Abstract

The introduction of drinking water safety plans in Alberta has resulted in increased attention towards water-based, opportunistic pathogens like *Legionella pneumophila*, which grow in premise plumbing biofilms and cannot be controlled through drinking water treatment alone. Currently, there are neither specific standards nor regulatory guidelines to control *Legionella* spp. in Canadian drinking water systems and current culture-based detection is limited by inaccuracy, 10 day incubation periods, and an inability to measure VBNC cells. Thus, it was the objective of this project to improve upon the poor reliability of *L. pneumophila* detection by examining viability assays that correlate with amoeba infectivity data, which could be coupled with a novel IMS-FCM detection system, in hopes of providing a better, timely estimate of public health risk. Experiments using the novel IMS-FCM detection system revealed sensitivity to *L. pneumophila* below the recommended action level guidelines given by Public Health Ontario (<100 CFU L^{-1}) for healthcare centres. Dose-response curves were created for *L. pneumophila* exposed to UV, heat, or monochloramine to evaluate disinfectant efficacy,

measured as 4 $\log_{10}$ reductions in culturable cells. UV dose-response curves varied significantly depending on the presence of visible light, which is thought to induce light-activated DNA damage repair machinery, resulting in significant reductions in disinfection efficacy when corrected for. The role of protozoan hosts in *L. pneumophila* disinfection and human pathogenicity was also examined, with particular focus on amoeba co-cultures of VBNC cells. Correlating *L. pneumophila* growth in amoebae with activity assays revealed CTC and ATP-based assays that may provide an appropriate measure for public health risk management action, such as when coupled to novel molecular, flow cytometry-based detection systems. The current research has demonstrated the need for an accurate, rapid *L. pneumophila* detection system for risk management that needs to address near point of use to control aerosol risks from this pathogen. In particular, the potential usefulness of ATP-CTC coupled viability assays for quantifying active, infectious cells was a novel finding of this research, which appear to be a risk to public health. For example, with point of use UV disinfection up to recommended guidelines of 16 mJ cm^{-2}, current culture-based detection would be unable to identify VBNC cells that remain infectious to *A. polyphaga* hosts and potentially human lung macrophages. The development of water safety plans through QMRA modelling must account for this uncertainty by finding more accurate detection systems that also address VBNC cells.

Preface

This thesis is an original work by Michael R. Grossi. No part of this thesis has been previously published.

Acknowledgments

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List of Abbreviations

ACES	*N-2*-aminoethanesulfonic acid
ANSI	American National Standards Institute
AOC	Assimilable organic Carbon
ASHRAE	American Society of Heating, Refrigerating and Air-Conditioning Engineers
ATCC	American Type Cell Culture
AYE	ACES yeast extract (liquid media)
BCYE	Buffered charcoal yeast extract (solid media)
BSA	Bovine serum albumin
CDC	U.S. Centers for Disease Control and Prevention
CFDA	Carboxyfluorescein diacetate
CFU	Colony forming units
CTC	*5*-cyano-*2*,*3*-di-(*p*-tolyl)tetrazolium chloride
DiBac4(3)	Bis-(*1*,*3*-dibutylbarbituric acid) trimethine oxonol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
FACS	Fluorescence activated cell sorting
FCM	Flow cytometry
FISH	Fluorescent in-situ hybridization
FITC	Fluorescein
FLA	Free-living amoeba
FSC	Forward scatter
GC	Gas chromatography
GU	Genomic units
HPC	Heterotrophic plate counts
IMS	Immunomagnetic separation
ISO	International Organization for Standardization
LD	Legionnaires' disease
LD50	Lethal dose (50%)
LED	Light emitting diode

LOB	Limit of blank
LOD	Limit of detection
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MCGL	Maximum contaminant goal level
MFI	Median fluorescent intensity
MOI	Multiplicity of infection
MS	Mass spectrometry
NDMA	*N*-nitrosodimethylamine
NSF	National Standards Foundation
OD600	Optical density (600 nm)
PAO	Phenylarsine oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEX	Crosslinked polyethylene
POU	Point-of-use
PYG	Proteose peptone, yeast extract, glucose (liquid medium)
QMRA	Quantitative microbial risk assessment
ROS	Reactive oxygen species
SCGYEM	Bovine serum, casein, glucose, yeast extract (liquid medium)
SDWA	Safe Drinking Water Act
SEM	Standard error of the mean
SG	Serogroup
SPC	Solid phase cytometry
SSC	Side scatter
SWTR	Surface Water Treatment Rules
TCC	Total cell count
THM	Trihalomethanes
UV	Ultraviolet light
VBNC	Viable but non-culturable
WHO	World Health Organization
WSP	Water safety plan

Background: An emerging threat within premise plumbing

In 2001, the World Health Organization (WHO) introduced a single framework to manage water-exposure risks that utilizes quantitative microbial risk assessment (QMRA) to ensure the safety of water systems, which for drinking water systems are referred to as water safety plans (WSP).[1] Recently, the Province of Alberta became the first jurisdiction is North America to adopt these recommendations, which encompass all aspects of drinking water supply from source to the consumer.[2] These new guidelines have prompted officials to examine the emerging threat of water-based, opportunistic pathogens like *Legionella pneumophila*, which grows in premise plumbing biofilms and cannot be controlled through drinking water treatment alone; where premise plumbing is defined as components of a drinking water distribution system within a building.[3] In 2014, WHO also issued specific guidelines for drinking water distribution systems' WSPs that addresses *L. pneumophila* control relevant to premise plumbing.[4]

L. pneumophila is a Gram-negative, opportunistic pathogen that is the main causative agent for legionellosis, a bacterial lung infection resulting in the most commonly reported waterborne disease in the United States.[5] Clinically, there are two distinct forms of legionellosis with the most understood known as Legionnaires' disease (LD), a pneumonia with a high (10-40%) case-fatality rate, and a very mild non-life threatening self-limiting Pontiac fever.[6] Exposures to *L. pneumophila* occur via inhalation of aerosols, typically from aquatic biofilms within engineered water systems, with common exposure sources including premise plumbing, cooling towers, fountains, and humidifiers.[7] Specifically, *L. pneumophila* grows to problematic concentrations within pipe-wall biofilms under warm (25 °C – 42 °C), semi-stagnant conditions, by intracellular growth within biofilm amoebae.[8] The U.S. Center for Disease Control and Prevention (CDC) has identified immune-compromised individuals, the elderly, and smokers as

groups at high risk for LD.[9] No data exists on the extent or prevalence of Pontiac fever, which results in mild symptoms including diarrhea, tiredness, and fever, possibly due to endotoxin exposure.[10] Increased surveillance and official reporting in the US has resulted in a large spike in the number of cases of LD acknowledged, as researchers and supporting healthcare staff attempt to understand and minimize risk from the main (aerosol) sources of exposure.[5] A recent CDC publication reported *L. pneumophila* to be the causative agent of two-thirds of all drinking-water disease outbreaks and all associated fatalities in the United States from 2011-2012.[11] In terms of disease burden, a compilation of Medicare and Medicaid insurance claims revealed LD cases cost an estimated $433 million USD annually.[12] In Canada, surveillance data from Public Health Ontario also shows a significant increase in legionellosis rates from 2002-2013.[13]

Microbial growth in premise plumbing presents a challenge for public health officials attempting to prevent and control LD outbreaks, since building water systems fall outside of drinking water regulations in North America.[11] Despite accounting for the majority of drinking water related disease outbreaks and health burden costs, there are currently no specific standards nor regulatory guidelines to control *Legionella* spp. in Canadian or U.S. drinking water systems.[12] Currently, the American Society of Heating, Refrigeration, and Air Conditioner Engineers (ASHRAE) and the Veterans Affairs/CDC guidance are the only North American agencies with approaches to manage *L. pneumophila* risks within building drinking water and heating/cooling systems.[14] The United States EPA Safety Drinking Water Act (SDWA) strives to achieve zero *L. pneumophila* within drinking water systems, however no regulatory monitoring requirement exist and the removal of all legionellae from water is not feasible.[3]

The dissertation is broken into four chapters that describe *L. pneumophila* detection, disinfection, the effects of free-living amoeba, and the importance of the viable but non-

culturable (VBNC) state. Each chapter consists of an abstract, introduction, materials and methods, and results and discussion section. A conclusion is present directly following the four chapters, which provides a summary of relevant findings and suggests future research directions.

1 Detection of *Legionella pneumophila* using an emerging FCM-IMS method

1.1 Abstract

This introductory chapter reviews the limitations of culture-based *L. pneumophila* detection and outlines emerging techniques that may improve diagnostic efficiency, then focuses on validating a previously reported immunomagnetic separation-flow cytometry (IMS-FCM) detection assay. The sensitivity and specificity of the IMS-FCM assay was first assessed by spiking clean water, then Edmonton tap water to evaluate method efficacy. These experiments began with calibration curves for various *L. pneumophila* serogroups and non-*pneumophila* legionellae in the presence of two commercially obtain antibodies with fluorescent labels, which allowed for measurement by flow cytometry. Unfortunately, few relevant environmental samples were obtained (i.e. healthcare building drinking water, cooling towers) in time for the project completion date (July 2017), hence this secondary aim to address a range of environmental samples was limited to spiked Edmonton tap waters.

The sensitivity experiments using flow cytometry (FCM) identified a continuous relationship between gated events and *L. pneumophila* concentration, with a limit of detection at 76 CFU over N = 10 replicate trials. This detection limit is similar to the 50 cells L^{-1} reported in the literature, and would be considered sufficient for routine monitoring of healthcare centers, since the limit falls within the lowest action level (>100 CFU legionellae L^{-1}); noting that flow cytometry may also include non-culturable cells as discussed in Chapter 4. The specificity experiments revealed that the detection system was specific to *L. pneumophila* serogroup 1, with no detection observed for *L. pneumophila* serogroup 2 or 3, or other members of the *Legionella* genus. The significance of this is debatable, as 83% of legionellosis cases are reported from serogroup 1; however, the use of detection assays biased for *L. pneumophila* may underestimate

total legionellae prevalence. Finally, IMS-FCM analyses performed with spiked Edmonton tap water samples indicated efficient *L. pneumophila* concentration from tap water by IMS. In the absence of this concentration step, the flow cytometric plot revealed no target population and non-specific staining apparent. Although promising, the results reported are limited by the lack of environmental samples to verify concentration efficacy, assay sensitivity and specificity. The effects of various matrices, high microbial loads, extracellular polysaccharides and other macromolecules associated with biofilms were not considered. This is problematic for applying the data to real-world systems, since these variables can reduce antigen-antibody binding, create false positives, and increase the background noise. In an attempt to measure the effect of biofilms, a 'first-flush' rule was placed for tap water sampling to ensure sloughed biofilm is sampled, which resulted in a pre-filtration step to be added to reduce noise.

The IMS-FCM approach has potential for automated, online detection of *L. pneumophila* and total legionellae residing within water systems. It is recommended that future research be directed to collaborations between research groups and industry for the rigourous analysis of method sensitivity and specificity and IMS purification yields within 'dirty' environmental samples through spiking experiments similar to those reported here with clean water. Attention should also be focused towards total legionellae detection, with current detection systems chronically underreporting the influence of these pathogens in legionellosis events.

1.2 Introduction

The increasing prevalence of Legionnaires' disease (LD) within developed countries has prompted public health officials to examine current practices to improve deficiencies associated with detection and management of *L. pneumophila* and total legionellae in engineered water systems. Current detection largely relies on culture-based approaches, which can be cumbersome, inaccurate, and time-consuming, especially when evaluating environmental water

samples containing other microbes and biofilm derivatives.[15] Furthermore, while LD can be induced by a multitude of *Legionella* spp. organisms, *L. pneumophila* has been the major target for clinical and environmental detection. Clinical data suggests that 95% of LD cases are the result of *L. pneumophila*; when broken up by serogroup (SG), SG1 accounts for 83% of cases, while the other SGs contribute to less than 10% of cases in Europe between 2009-2010.[16] The high proportion of cases resulting from *L. pneumophila* SG1 may be inflated by the specificity/bias of current detection methods.[6] Hence, this chapter discusses the limitations of culture-based detection and outlines emerging techniques that may improve diagnostic efficiency and accuracy for *L. pneumophila*, then provides an evaluation with an emerging immunomagnetic separation-flow cytometry (IMS-FCM) approach.[17]

1.2.1 Established and emerging detection platforms

Currently, cultured-based detection is considered the gold-standard for *Legionella* spp. detection, using the International Organization for Standardization (ISO) 11731 protocol.[17] Under this protocol, 1000 mL (drinking water systems) or 100 mL (cooling towers) environmental samples are filtered and re-suspended in 5 mL prior to plating on buffered charcoal yeast extract (BCYE) agar plates containing specific antibiotics for 7-10 days, at which point suspected colonies are re-plated on BCYE ± cysteine for 2-4 days.[18] The method is further complicated by selective acid-wash (0.2 M HCl for 5 min) or heat treatment (50 °C for 30 min) during the initial re-suspension if the sample is suspected of containing high concentrations of background microorganisms.[19] As described above, the ISO 11731 method is labour intensive, requiring a 10-15 day incubation period to yield positive results, and may underestimate cell density by 10-60%.[15, 20] Beyond these concerns, the method is unable to detect viable but non-culturable (VBNC) cells or bacteria residing within amoeba, is biased towards *L. pneumophila*

(underestimates total *Legionella* spp.), and is prone to under-reporting with samples containing high background levels of microorganisms.[16]

The major limitations of the ISO 11731 method, along with an increasing disease prevalence and aging population have prompted public health officials and researchers to examine novel detection platforms to improve both speed and accuracy of detection. Recently, a real-time quantitative polymerase chain reaction (qPCR) method, ISO/TS 12869, has been approved as a technical specification for detection of *L. pneumophila* and *Legionella* spp. in environmental water samples.[18] Concurrently, researchers reported the successful implementation of IMS-FCM for *L. pneumophila* detection, which has the potential for online automation.[17] For simplicity, emerging detection systems are classified here as nucleic acid based, immunological based, or other systems.

1.2.2 Nucleic acid based detection

The most common detection platform using nucleic acids is PCR, which employs organism/genus specific primers to amplify targeted cellular DNA. PCR-based approaches are popular due to their speed of analysis, high specificity and sensitivity, low detection limits and potential for quantification (qPCR) when DNA-binding fluorescent probes are added.[21, 22] As mentioned above, ISO/TS 12869 is the first non-culture standard for *L. pneumophila* detection, with method validation yielding a detection limit 100 GU L^{-1}, where GU are genomic unit equivalents.[20] A major review of the literature comparing culture and qPCR reported that 26/28 studies gave higher *Legionella* spp. levels with qPCR and a 72% positive sample rate, compared to 34% for culture.[23] However, the main concerns with qPCR based detection are cost, the influence/inhibition of natural organic matter, corrosion products, and other PCR inhibitors associated with environmental samples, and the overestimation of risk by not discriminating between live and dead organisms.[3, 18]

To address the concerns associated with measuring total bacterial counts, PCR assays can be modified with membrane impermeable dyes, such as ethidium monoazide or propidium monoazide.[22] The theory being that the dye can only enter cells with damaged cell membranes, where it binds DNA and can be cross-linked by light exposure, leaving it unable to participate in PCR amplification.[22] Viability PCR has been successfully used to monitor *Legionella* spp. in environmental water samples, where it was shown to have counts on average 27-fold higher than culture, with a reported detection limit of 80 GU L^{-1}.[24] Yet viability PCR is limited by the presence of environmental compounds and extracellular polymers that can inhibit PCR and interfere with dyes binding DNA, as well as concerns regarding the effectiveness of dyes to restrict amplification and enter intact cell membranes.[23]

Another nucleic acid based detection platform is fluorescent *in-situ* hybridization (FISH), which uses fluorescently labeled oligonucleotide probes (usually complementary ribosomal RNA strands) to detect specific bacterial species.[25] FISH techniques have been used to quantify airborne *L. pneumophila* levels during a shower event, where a 1 $\log_{10}$ increase in concentration was observed for FISH, compared to culture, with a detection limit of 100 cells L^{-1}.[26] The main concerns with detection by FISH are non-specific binding and the inability to discern living from dead organisms (much like qPCR).[18, 25]

1.2.3 Immunological based detection

In contrast to nucleic acid based targets, immunological detection systems use the affinity of antibodies for cell surface antigens to select for desired microorganisms with high specificity.[27] The antibody is often conjugated to a fluorescent compound to visualize and/or quantify bound complexes. A technique known as direct fluorescent antibody is frequently used for clinical analysis; however it is not suitable for environmental analysis, as the results are qualitative (presence/absence) yielding no information about concentration.[28] Quantitative

techniques have been reported, specifically solid-phase cytometry (SPC), in which environmental samples are stained with antibody, filtered down, and viewed using epifluorescence microscopy.[19] SPC has been successfully used for detection of *L. pneumophila* in domestic hot water and cooling tower samples, with a reported detection limit of 34 cells L^{-1} and consistently higher cell counts than standard culture in the 46 samples tested.[29] However, the method is limited by the overestimation of risk by combining live and dead cells, issues of cross-reactivity with non-target particulate matter, and high user input (training and validation).[19]

Antibodies can also be used to purify environmental samples, commonly through IMS columns, which use paramagnetic beads with specific affinity for antibodies or their fluorescent conjugate to selectively bind target, while eluting non-desired materials and matrix.[27] As mentioned previously, harsh acidic or heat treatments are often used to reduce background microbial levels prior to culture, potentially reducing levels of culturable *Legionella* spp.[30] IMS can be used to improve culture techniques in samples containing high background microbiota or as a labelling and purification step coupled with another detection platform such as ELISA or FCM.[18] In a comparison of 51 environmental samples, six additional samples were found to be *L. pneumophila* SG1 positive when IMS was used prior to culture.[30] An IMS-ELISA assay reported a detection limit of 93 cells per volume sampled, with an yield efficiency of 97%.[31] Additionally, the coupling of IMS and FCM (further described in §1.2.5) was reported to have a detection limit of 50 cells L^{-1}, with significantly higher levels of *L. pneumophila* in 53% of environmental samples tested, compared to culture.[17]

1.2.4 Other (emerging) detection systems

A well-known detection platform is mass spectrometry (MS), which measures the mass-to-charge (M/Z) ratio of ionized particles. Initial *L. pneumophila* detection by MS coupled to gas chromatography (GC) has been around for nearly four decades, with current coupling to matrix-

assisted laser desorption ionization – time of flight (MALDI-TOF) showing promise.[28] However the technique is not yet able to distinguish between *L. pneumophila* SGs and the results only give a qualitative measure of presence/absence based on standard spectrums.[32]

Recently, IDEXX Corporation has come out with an enzymatic culture-based detection platform specific to *L. pneumophila*, known as Legiolert®, similar to the widely used Colilert® and Enterolert® defined substrate detection systems used for detecting *E. coli* and enterococci, respectively. According to the manufacturer, the method allows for detection of one *L. pneumophila* cell in 100 mL sample within 7 days, using what is reported to be species-specific enzymatic substrate with a brown colour end-point.

Hence, to overcome culture-based biases and to consider live and dead cells often poorly resolved by molecular methods, others have been promoting FCM-based approaches, which are reviewed next.

1.2.5 Principles of flow cytometry (FCM) and potential for automation

Flow cytometers operate by passing a single layered stream of cells through a light source and measuring light scatter with fluorescent output, depending on the nature of the sample and fluorophores added.[33] Light scatter detected at low angles is reported as forward scatter (FSC) and gives an indication of particle size, while high angle detection is known as side scatter (SSC) yields information regarding internal complexity.[34] Fluorescent emissions are detected at high angles following passage through wavelength filters, using photomultiplier tubes that emit current (*I*) directly proportionally to the number of emitted photons striking the detector surface.[34] FCM data is most commonly presented as histograms and scatterplots of light scatter and fluorescence intensity. The FSC and SSC parameters are specific to the cells being analyzed, while fluorescence generally depends on the concentration of labeled probes added (Figure 1.1).[33]

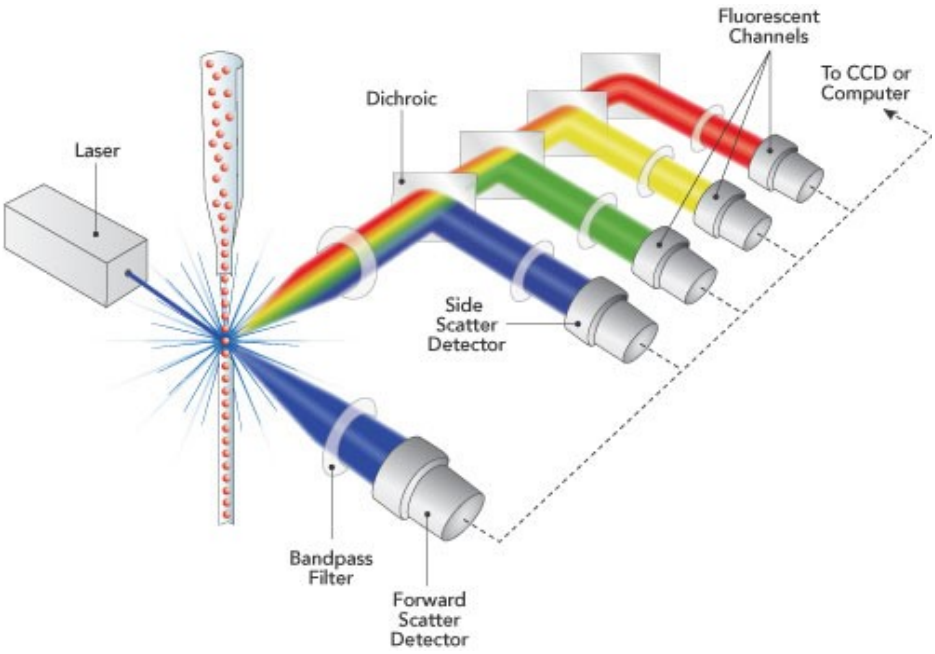

Figure 1.1 Schematic of flow cytometer operations, where a stream of cells is passed through a laser with low and high angle detectors to measure forward scatter, side scatter, and fluorescent intensity using photomultiplier tubes. Image obtained with expressed written permission from University of Alberta, Faculty of Medicine and Dentistry, Flow Cytometry Core webpage. <https://flowcytometry.med.ualberta.ca/>

The use of flow cytometry for aquatic microbiology systems has increased substantially in recent years, primarily due to technological advances that make instruments more affordable, user friendly, and easier to maintain, as well as the development of more specific stains and improved diagnostic efficiency for analyzing smaller particles.[34] Furthermore, detection by flow cytometry offers the advantage of accurate, quantitative results in a short amount of time, while also possessing the ability to examine multiple fluorophores simultaneously.[27] This allows for the detection of multiple species, or multiple cellular states (live and dead) during one sample run.[17] The main limitation of FCM based detection is the need for pre-treatment of high turbidity and biofilm derived samples, which can reduce the capture rate and potentially confounds observed signals.[34] Dilute samples can also present challenges as the low flow rates require long time intervals to detect targets, prompting researchers to develop microfluidic chambers that pre-concentrate samples.[33] Finally, flow cytometry is considered to be quasi-quantitative, as it

records a continuous response to increasing cell concentrations, yet no calibration standards are generally available (for the specific cells of interest).[35] Further complicating the quantitative nature of flow cytometry is the inability to measure volumes run, making true measures of concentration hard to calculate. Despite these concerns, carefully designed experiments can obtain meaningful quantitative results with reliable estimates of cell concentration. Perhaps the most promising aspect of flow cytometric microbial detection is the potential for automation, as commercially used for total bacterial counts in drinking water.[36] Flow cytometry based detection of total cell counting (TCC) is routinely used for monitoring by drinking water companies in Switzerland and the Netherlands, where it has been demonstrated to provide more accurate and useful data than heterotrophic plate counting.[37] An added benefit of this method was the ability to separate cells with low nucleic acid levels from those with high nucleic acid content.[37] The automated FCM detection system consisting of pre-staining apparatus, FCM, and electronics developed for TCC of drinking water is illustrated in Figure 1.2.[36]

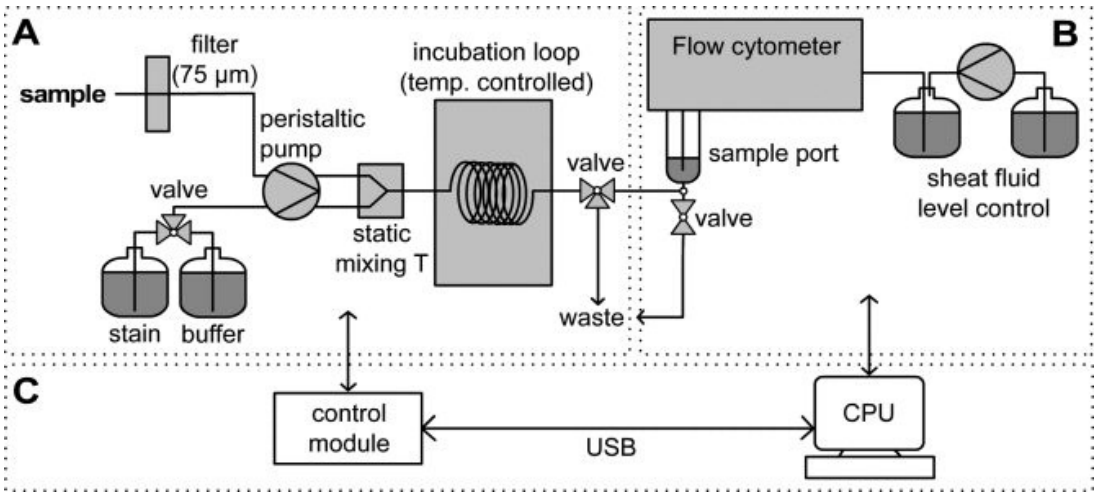

Figure 1.2 Schematic of an online FCM detection system for rapid detection of bacterial intrusions, complete with (A) staining apparatus, (B) Flow cytometer, and (C) electronics module. Image obtained from Hammes *et al*., 2012.[36]

An automated, online detection platform for monitoring *L. pneumophila* concentration in premise plumbing systems would allow public health officials to react within hours of an above

(action level) threshold. Proactive monitoring of *L. pneumophila* is recommended when dealing with a population of increased susceptibility, such as those residing within hospital and care-homes.[38] Public Health Ontario recommends specific actions after a positive detect in health-care premise plumbing based on the following *L. pneumophila* concentrations (Table 1.1).[13]

Table 1.1 Actions required following detection of *Legionella pneumophila* within a healthcare setting as outlined by Public Health Ontario "Monthly Infectious Disease Surveillance Report".

L. pneumophila Concentration	Action Required
<100 CFU L^{-1}	Focus on control measures.
>100 CFU L^{-1} and up to 1000 CFU L^{-1}	Re-assess controls and resample.
>1000 CFU L^{-1}	Immediate action required, review of control measures and risk assessment, possible disinfection of system

As mentioned above, the increasing prevalence of Legionnaires' disease within developed countries has prompted public health officials to examine current practices to improve deficiencies associated with detection of *L. pneumophila* and total *Legionella* spp. in engineered water systems. Established culture-based approaches are ineffective and often underestimate true risk, while emerging platforms, like IMS-FCM, require further application-based research and development. Hence, this chapter focused on evaluating the emerging IMS-FCM platform for detection of various *L. pneumophila* SGs.

1.3 Materials and Methods

1.3.1 Cultivation of *Legionella* species

Primary stock of *L. pneumophila* Philadelphia-1 strain (ATCC 33152) was prepared by plating onto buffered charcoal yeast extract (BCYE) agar (obtained from Alberta Provincial Laboratory, Edmonton, AB, Canada) and incubating at 37 °C for 72 h. A single colony was then inoculated into AYE medium (10 g ACES, 10 g yeast extract, 0.4 g L-cysteine, and 0.25 g iron pyrophosphate per litre) and incubated at 37 °C for 16 h, when cells reached late log phase

growth according to a predetermined bacterial growth curve calibrated to absorbance at 600 nm and colony forming units (CFU).[39] Cells were then diluted to 10^6 CFU mL^{-1} in 0.85% saline for use in experiments if not otherwise stated. Other *Legionella* species that were cultivated included: *L. pneumophila* (ATCC 33153) SG-1, *L. pneumophila* (ATCC 33154) SG-2, and *L. pneumophila* (ATCC 33155) SG-3, and non-*pneumophila* species: *L. bozemanii* (previously *Fluoribacter bozemanae*) (ATCC 33217), *L. dumoffi* (previously *Fluoribacter dumoffi*) (ATCC 33279), *L. gormanii* (previously *Fluoribacter gormanii*) (ATCC 33297), *L. longbeachae* (ATCC 33462), and *L. micdadei* (previously *Tatlocklia micdadei*) (ATCC 33218). The various species were plated onto BCYE and grown for 72 h at 37 °C, at which point pure colonies were suspended in AYE broth and incubated overnight at 37 °C. Most non-*L. pneumophila*'s were unable to grow in the AYE broth, in which case the serial dilutions were constructed directly from the BCYE plate cultures suspended in PBS with an approximate OD_{600} between 0.8-1.

1.3.2 Optimization of staining parameters (antibody titrations)

Antibody titrations were used to determine the optimal dilution factors for the commercially obtained polyclonal antibodies: FITC conjugated *L. pneumophila* polyclonal antibody (PA1-73140, Thermo Fisher Scientific, Waltham, MA, USA) and *L. pneumophila* SG 1 specific polyclonal antibody (GTX40943, Genetex, Irvine, CA, USA). Prior to the latter titration, a conjugating reaction was used to fluorescently label the antibody using the Alexa Fluor® 647 protein labeling kit following the manufacturer's enclosed instructions (Thermo Fisher Scientific). The titrations followed antibody manufacturer best practices with minor variations that are outlined below. Briefly, *L. pneumophila* (ATCC 33152) was grown to late log phase and centrifuged at 3,000 rpm for 10 min to obtain a pellet. Supernatant was discarded and the pellet was resuspended in 1 mL PBS, prior to undergoing a 2,000-fold dilution to obtain a working stock with approximate concentration of 10^6 CFU mL^{-1}. A dilution series ranging from 1:2 to

1:1000 was constructed, with 50 µL aliquots of each dilution being added to the six tubes containing 50 µL *L. pneumophila* working stock. Suspensions were vortexed and incubated for 20 min at room temperature, to which 900 µL PBS was added prior to flow cytometric analysis.

Samples were placed into a Gallios™ flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with a 488 nm, 22 mW blue solid state diode laser with five fluorescent detectors (525, 575, 620, 695, and 755 nm), and a 638 nm, 25 mW red solid state diode laser with a 660 nm detector. All runs were analyzed using Kaluza™ analysis software (Beckman Coulter, Brea, CA, USA). The optimal dilution ratio was determined by a qualitative visual inspection of the separation of FL-1 peaks (FITC λ_{EM} = 520 nm), or FL-6 peaks (Alexa Fluor® 647 λ_{EM} = 647 nm) depending on the titration being run, as well as a quantitative measure of the median fluorescent intensities (MFI) for both the signal and the noise peaks.[40] Two distinct populations, stained and unstained, were obtained in the scatterplots; with MFI values for both populations plotted to provide a visual indication of spread (Appendix Figures A1.1 and A1.2). The optimal antibody dilution range for the FITC-labeled antibody was observed between 1:50 and 1:100, with the highest separation between the two populations occurring at the 1:50 dilution. The largest MFI separation for the Alexa Fluor® 647 antibody was observed at the 1:50 dilution.

1.3.3 Method sensitivity

A dilution series was assembled for *L. pneumophila* (ATCC 33152) in order to obtain a standard concentration curve to evaluate method sensitivity. Briefly, a late-log phase suspension was serially diluted from 10^8 CFU mL^{-1} down to 10^1 CFU mL^{-1} in PBS, along with blank samples containing no *L. pneumophila*. A 50 µL aliquot of each dilution was then placed in a 1.7 mL microfuge tube, to which 50 µL of 1:50 FITC conjugated *L. pneumophila* polyclonal antibody and 50 µL of 1:50 Alexa Fluor® 647 conjugated *L. pneumophila* SG-1 specific

antibody were added. The samples were vortexed and incubated in the dark for 20 min at room temperature, to which 850 µL PBS was added prior to flow cytometric analysis.

The dilution series samples were run for 180 second intervals or 25,000 events depending on which occurred first on the Gallios™ flow cytometer. The volume uptake per 180 seconds was crudely calculated by measuring the weight of flow tubes pre and post analysis, with an average volume per 180 s run estimated at 137 µL ± 5 µL (Appendix, sample calculation A1). The number of gated events (FL-1 positive, FL-6 positive) per 180 second interval were measured and plotted as a function of CFU count to yield a standard concentration curve. The blank samples were used to determine the noise of the assay, which can be quantified as the limit of the blank (LOB); defined as the highest apparent signal measured in the absence of the analyte of interest (**Equation 1.1**).

$$LOB = \bar{x} + 1.645 * SD \qquad \text{(Equation 1.1)}$$

Where the mean and standard deviation are determined from a collection of blank samples, and the LOB is expressed in number of events per 180 s.[35]

The limit of detection (in CFUs) was determined by plotting the linear range of the standard concentration curve and calculating the expression for the line of best-fit. From this y = mx + b equation, the LOD (in number of events per 180 seconds) was added as the y-value, permitting to solve for x, the LOD (in CFU), (**Equation 1.2**).

$$LOD = LOB + 1.645 * SD \qquad \text{(Equation 1.2)}$$

Where the standard deviation is for a low positive signal and the LOD is expressed in number of events per 180 s.[35]

1.3.4 Method specificity

The affinity of the FITC and Alexa Fluor® 647 labeled antibodies for other *L. pneumophila* strains and total *Legionella* spp. (non-comprehensive list) was investigated by

running the method sensitivity protocol described above for *L. pneumophila* (ATCC 33152). Strains tested included: *L. pneumophila* (ATCC 33153) SG-1, *L. pneumophila* (ATCC 33154) SG-2, and *L. pneumophila* (ATCC 33155) SG-3, and non-*pneumophila* species: *L. bozemanii* (ATCC 33217), *L. dumoffi* (ATCC 33279), *L. gormanii* (ATCC 33297), *L. longbeachae* (ATCC 33462), and *L. micdadei* (ATCC 33218). The experiments followed the method sensitivity protocol with serial dilutions of each strain being stained and analyzed on the flow cytometer complete with blank solutions containing no bacteria.

1.3.5 IMS-FCM detection of spiked samples

To examine the noise associated with environmental sampling, several internal spiking experiments were performed using laboratory tap waters. Briefly, a 1 mL aliquot of late log-phase *L. pneumophila* was spiked into 250 mL of Edmonton tap water or distilled water, taken from the faucets in South Academic Building room 3-53 at the University of Alberta as the first 'flush' of the pipes for the respective sampling day. The samples were vacuum filtered using a 0.22 micron Isopore™ track-etched polycarbonate filter of 47 mm diameter (Sigma Aldrich, St. Louis, MO, USA). The filter was folded and placed into a 50 mL centrifuge tube containing 3 mL PBST and 10 µL of 15% BSA.[17] The filter suspension was vortexed rapidly at 15 s intervals for two minutes in order to release *L. pneumophila* adhering to the filter. The re-suspended samples were placed in a benchtop microfuge set to 5,000 rpm for 5 min to obtain a pellet, which was washed in triplicate to remove surfactant (Tween) that was found to influence antibody-antigen binding efficacy in initial trials. Following the triplicate wash steps, IMS was used to purify environmental samples containing spiked *L. pneumophila*.[27] Briefly, 50 µL of 1:50 diluted FITC-conjugated polyclonal *L. pneumophila* antibody was added (20 minute incubation) prior to addition of 10 µL anti-FITC magnetic microbeads (Miltenyi Biotech, Auburn, CA, USA)

and 50 µL of 1:50 Alexa-conjugated polyclonal *L. pneumophila* antibody. The mixture was vortexed and incubated for 20 min in the dark, while being chilled to 4 °C.[17]

IMS steps followed the manufacturer guidelines, using a MACS MS column attached to a MiniMACS™ magnetic separator (both supplied by: Miltenyi Biotech, Auburn, CA, USA). Briefly, the sample was added to the column, with a tube underneath to collect the flow-through from triplicate wash steps using 500 µL PBS (elution buffer). The MACS MS column was then removed from the magnetic separator, with a new tube being placed underneath to collect the purified *L. pneumophila* cells, following triplicate 500 µL rinses with elution buffer. Each fraction was run on the flow cytometer to provide a quantitative measure of IMS efficiency within an environmental matrix. After initial experiments revealed poor correlation between the tap water and distilled water samples, further purification methods were used to reduce the matrix effects associated with the environmental sample. Briefly, pre-filtration trials included vigourous pumping of a syringe with attached 20-G needle to disrupt potential biofilm and amoeba associated bacteria, according to literature, as well as centrifugation at 1,000 rpm for 5 min to pellet larger undesired particles and biofilm polysaccharides.[41] Another pre-filtration step used 40 µm nylon mesh sterile cell strainers (Fisher Scientific, Hampton, NH, USA), which have precedence within comparable literature, where 30 micron filters were used.[17]

1.3.6 Method modifications: *L. pneumophila* SG-6 and species specificity

The first environmental sample suspected of having *L. pneumophila* was obtained from Calgary Foothills Hospital after an apparent case of Legionnaires' disease (LD). Polymerase chain reaction (PCR) analysis confirmed the presence of *L. pneumophila* Serogroup 6. A search for suitable antibodies revealed a FITC-labeled *Legionella* direct fluorescence antibody reagent for serogroup 6 (PL209: Pro-Lab Diagnostics, Richmond Hill, ON, Canada) and non-labeled anti *L. pneumophila* serogroup 6 antibody (ab79457: Abcam, Cambridge, UK). Unfortunately, upon

antibody arrival the Calgary environmental sample was no longer available for use (either lost or used in entirety) according to relevant stakeholders. Hence, the specificity of the IMS-FCM method was only tested with the above described species and *L. pneumophila* spiked into Edmonton tap water.

1.4 Results and Discussion

1.4.1 Method sensitivity

In order to determine the sensitivity of the FCM-based detection assay, a standard curve was created by plotting the number of dual labeled antibody-positive events against the concentration of serially diluted samples *L. pneumophila* (Figure 1.3).

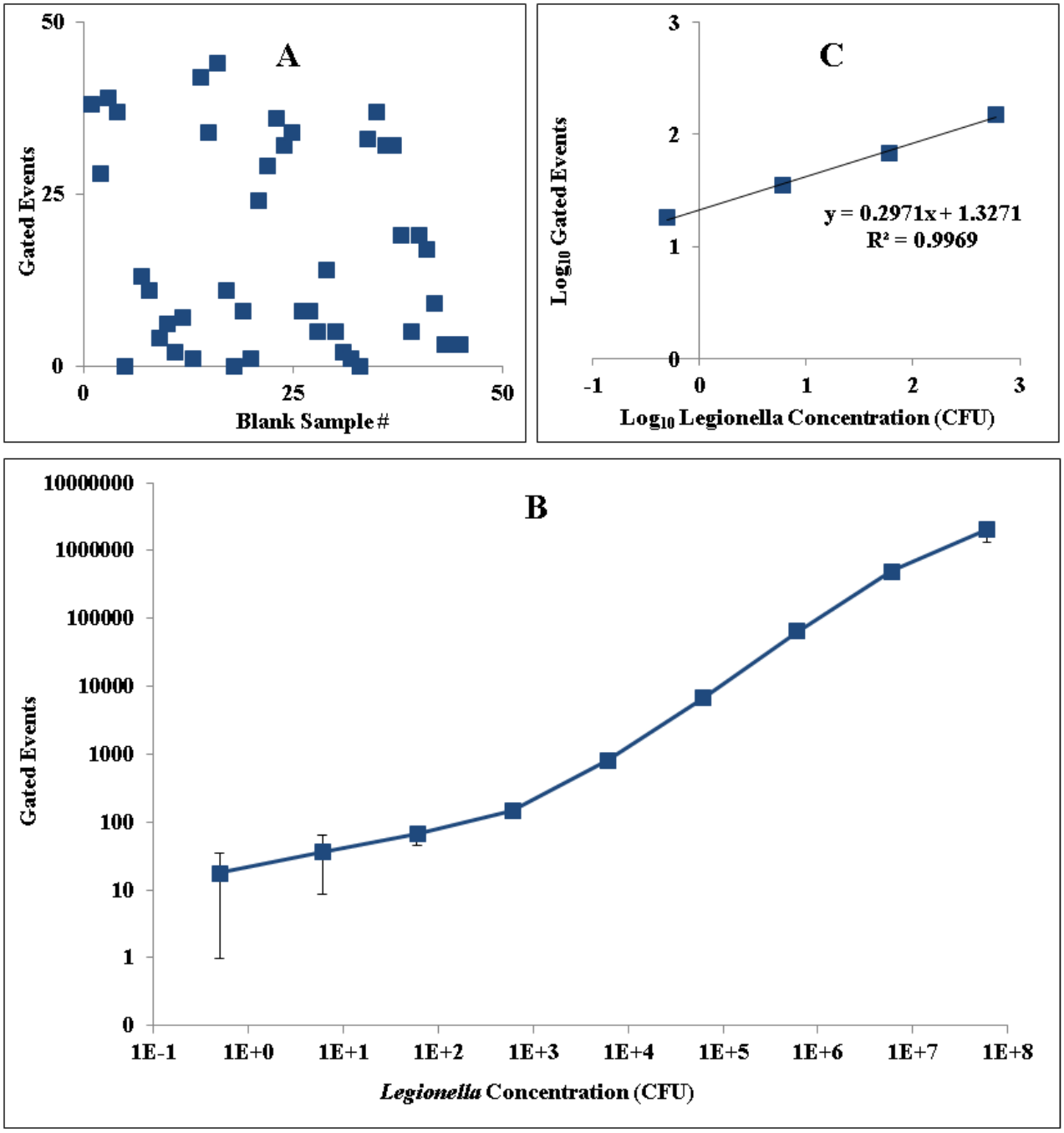

Figure 1.3 Standard curve for *L. pneumophila* (ATCC 33152) detection with gated events measured as both FL-1 and FL-6 positive regions using Gallios™ flow cytometer, with serial diluted bacterial cells and fixed concentrations of FITC and Alexa Fluor® 647 conjugated antibodies. (A) Variance in recorded events for blank samples (N = 45) containing both antibodies but no *L. pneumophila*, (B) standard curve (N = 10) with error bars ± SD, and (C) linear dynamic range with equation for linear regression and R^2 value used to estimate limit of detection for method.

From the standard curve (Figure 1.3B) it is apparent that the antibody detection system is sensitive for *L. pneumophila* detection down to low concentrations, with a linear dynamic range between 10^3 – 10^7 CFU. To quantify the lowest detectable concentration, Equations 1.1 and 1.2 were used to calculate the limit of the blank (LOB) and limit of detection (LOD), respectively, with calculations shown in the Appendix. Figure 1.3A shows the noise associated with the detection system when no *L. pneumophila* was present, with some samples having close to 50 measured events, while others had zero. The average number of events and standard deviation for blank samples tested were used to calculate the LOB = 41 events (N = 45). From this, the standard deviation for samples containing dilute *L. pneumophila* were used to calculate the LOD = 76 events (N = 10). The experimental LOD value and equation for the best-fit line (shown in Figure 1.3C) were then used to determine the lowest detectable concentration LOD = 75 CFU. This data shows that in the absence of matrix effects and microbial contaminants, the method is sensitive towards <100 CFU, which is the benchmark for action level responses in health care settings as reported in Table 1.1.

The reported detection limit of 75 CFU is comparable to values published in literature, namely the approximate LOD of 50 cells L^{-1} in Swiss samples following spikes with known *L. pneumophila* (ATCC 33152) concentrations.[17] The disparity between the literature and the current study result may arise from differences in preparing standard curves, with the published data using water samples spiked with known concentrations of bacteria, followed by a concentrating step prior to analysis via flow cytometry, while no pre-concentration step was employed in the current study. Furthermore, different antibodies, flow cytometers, and gating regimes were used. While the units appear different, the number of reported CFU and cells are equivalent, with the L^{-1} inverse volume measure indicating that the concentration step began with

1 L samples that were filtered down to 1 mL (same volume ran as reported above). Thus, the method sensitivity described here is similar to that previously reported for rapid IMS-FCM detection of *L. pneumophila*.[17]

Nonetheless, the method sensitivity experiment has several limitations that restrict the ability to accurately report the limit of detection and express real-world significance. Firstly, the standard curve (Figure 1.3B) was constructed using the expected *L. pneumophila* concentrations as determined by OD_{600} and growth curves, which rely on BCYE culture. The relative error in growth curves were carried over into the method sensitivity, since not all *L. pneumophila* are culturable and clumped cells are counted as one CFU. Secondly, in the absence of *L. pneumophila* (Figure 1.3A), there appears to be non-specific binding of the two antibodies that results in a false-positive gated event. This may arise from direct binding of the antibodies to each other, the presence of impurities in the sample (not likely), or may even involve random electronic noise within the photomultiplier tubes used to measure fluorescent signals. The variance observed for these blank samples is concerning from the point of view that true *L. pneumophila* positive samples may go undetected. The calculation of sensitivity and LOD followed International Clinical Cytometry Society working group guidelines for quantitative flow cytometry analyses. These guidelines recommend the use of equations with a 1.645X multiplier, which is commonly used for clinical applications, in comparison to a 3X multiplier common to experimental chemistry. This concern can be mitigated through inter-laboratory validation (not used here) or by comparison to reference detection platforms (i.e. BCYE culture).[35] Based on these outlined limitations, the results of the method sensitivity experiment (Figure 1.3) suggest further, more in-depth studies are required to provide an accurate measure of efficacy.

As mentioned above, the findings reported here are limited by uncertainties associated with the calibration curve, which reduce the generalizability and applicability to real-world systems. Other factors to consider are the high microbial loads and presence of interfering sugars, proteins, and other biofilm associated macromolecules within environmental samples. Furthermore, matrix effects may disrupt antibody-antigen interactions or cause false positives detections. While the method sensitivity experiment reported here gives the ideal assay response to *L. pneumophila* serogroup-1, the actual response may be confounded by a number of factors associated with the matrix and the presence of other microorganisms. Thus, environmental samples or at the very least tap water samples should be used prior to inferring real-world implications of the sensitivity reported above (further discussed in §1.4.3).

1.4.2 Method specificity

In order to determine the specificity of the FCM-based detection assay, standard curves were created for *L. pneumophila* (serogroups 1, 2, and 3), as well as non-*L. pneumophila* species. The curves were distinguished by the presence (+) or absence (−) of a continuous relationship between concentration and events detected (Table 1.2)

Table 1.2 Specificity of flow cytometry based detection assay for *L. pneumophila* (serogroups (**SG**) 1-3) and other species of *Legionella*, determined using standard curves and observing if a continuous response was detected (+) or missed (−), each assay run in triplicate.

L. pneumophila	Detected?	*Legionella* spp.	Detected?
ATCC 33152 **SG1**	+	*L. bozemanii* (ATCC 33217)	−
ATCC 33153 **SG1**	+	*L. dumoffi* (ATCC 33279)	−
ATCC 33154 **SG2**	−	*L. longbeachae* (ATCC 33462)	−
ATCC 33155 **SG3**	−	*L. micdadei* (ATCC 33218)	−

The specificity analysis (Table 1.2) revealed that the dual fluorescent antibody detection system appeared to be specific to *L. pneumophila* serogroup 1, with positive detects only being

observed for ATCC strains 33152 and 33153. The assay did not detect *L. pneumophila* serogroup 2 or 3, nor other members of the *Legionella* Genus. This result was expected as the two antibodies (commercially available) are advertised as serogroup-1 specific. Therefore, to detect other *Legionella* spp., less-specific antibodies are required. The specificity test for *L. gormanii* (ATCC 33297) was planned, yet the organism was unable to be cultured on BCYE agar plates or grown in AYE broth so no test was run.

As mentioned in the introduction, *L. pneumophila* serogroup-1 is responsible for 83% of reported legionellosis cases, with 10% being attributed to other serogroups over a 1 year period in Europe.[16] The high proportion of cases resulting from *L. pneumophila* serogroup-1 may be inflated by the specificity/bias of current detection methods.[6] Based on the reported specificity of the IMS-FCM assay (Table 1.2), a large portion of suspected cases would be detected more quickly using flow cytometric detection. However, at least 10% of cases would be missed, with the potential that this number is much higher based on the limitations and biases of our current detection systems. Therefore, it is recommended that research be focused on developing more robust detection assays for total legionellae, thus accounting for a greater proportion of cases and providing less biased reports due to the current focus on *L. pneumophila* serogroup-1.

The specificity analysis is likely limited in generalizability to real-world systems by the same factors outlined for the sensitivity experiment, namely high microbial load, matrix effects, and unspecific biofilm associated polysaccharide interference. In addition, the *Legionella* strains used here were grown in artificial media, which may lead to differences in surface protein expression compared to natural settings dominated by interactions with free-living protozoa and the biofilm environment. Therefore, as was the case for sensitivity, a true measure of detection

specificity should involve natural environmental sampling to limit potential confounding from matrices, microbial contaminants and *Legionella* host protozoa.

1.4.3 IMS-FCM detection of spiked samples

To provide a measure of immunomagnetic separation efficiency and observe minimal matrix effects associated with tap water samples, the detection assay was used on an Edmonton tap water sample spiked with 10^4 CFU *L. pneumophila* (Figure 1.4).

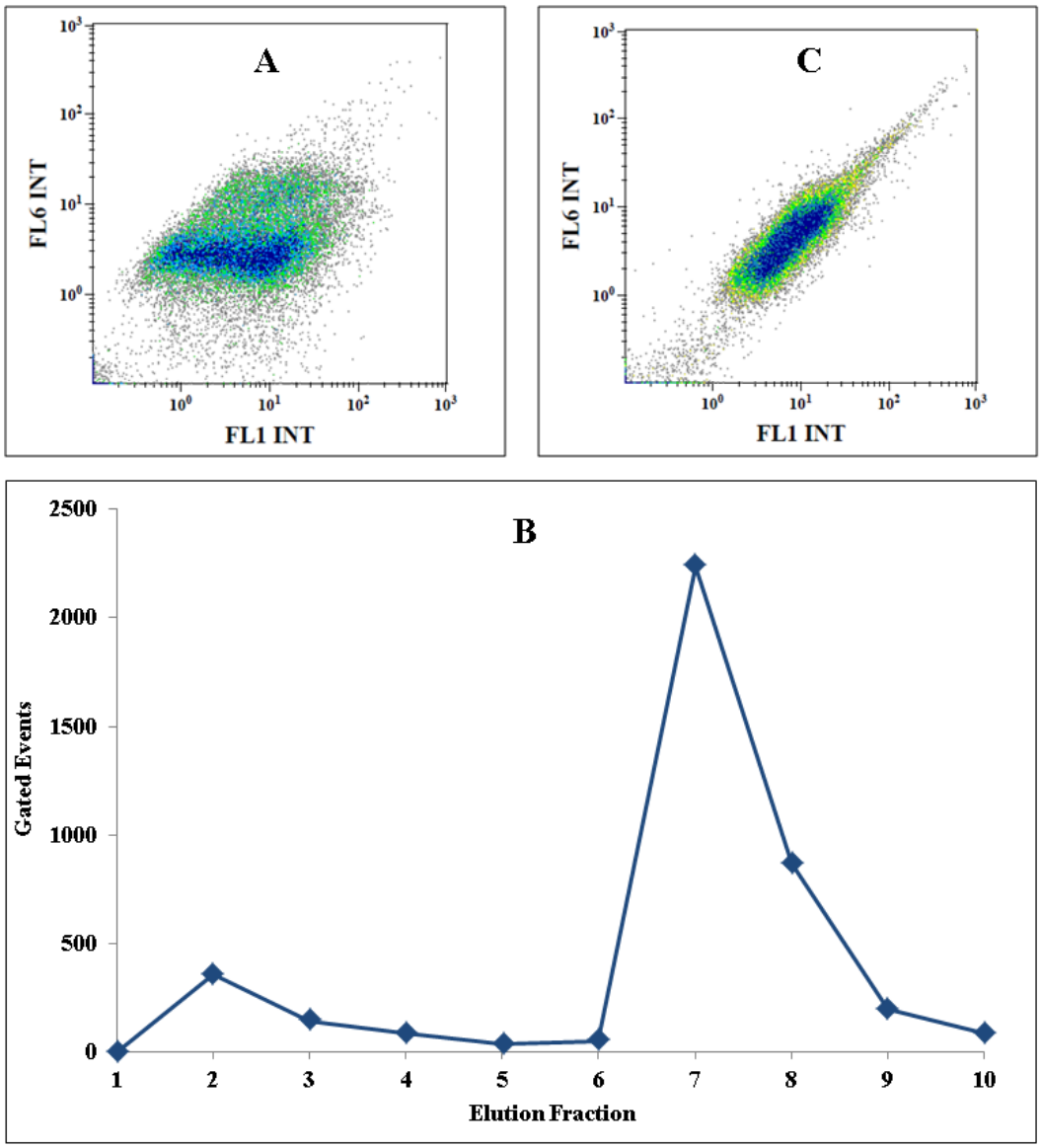

Figure 1.4 Flow cytometric plots of pre-filtered Edmonton tap water spiked with 10^4 CFU *L. pneumophila* without IMS (A) or with IMS step (C), with fractions derived from IMS column (B) showing relative efficacy of separation.

The flow cytometry and elution plots in Figure 1.4 indicate the efficacy of IMS for the purification of *L. pneumophila* from an environmental sample. In the absence of an IMS step (Figure 1.4A), the flow cytometric plot of FL-1 x FL-6 intensity is devoid of a positive population and would be best characterized by non-specific binding. However, with the IMS step (Figure 1.4C), a clear double positive population becomes apparent as the confounding matrix effects and extracellular biofilm derived molecules are washed out. The elution plot (Figure 1.4B) provides further evidence for the separation efficacy of the IMS approach, with the magnetic field removal following collection of fraction six resulting in the release of the microbead−antibody−*L. pneumophila* complex in fraction seven. The use of IMS for environmental samples has been reported for sanitary hot water and cooling tower water samples spiked with 10^3 *L. pneumophila* and *P. aeruginosa*, with culture-based detection revealing selective purification of target organism.[30] The first iteration of the IMS-FCM protocol reported purification efficacies of <95%, with the recovery rate of spiked *L. pneumophila* in tap water samples using similar approaches to those described in the current study.[27] The research also indicated that monoclonal antibodies resulted in increased separation efficacy compared with polyclonals.[27] Based on these reports and Figure 1.4, it is evident that IMS is a necessary treatment step prior to detection to minimize non-specific binding and matrix effects.

The elution plot shown in Figure 1.4B was obtained following a pre-filtration step with a 40 micron filter to reduce background noise associated with the tap water sample. As mentioned in the methods both tap and distilled water samples were used, with a pre-filter step resulting in near equivalent elution plots. In the absence of the pre-filter step, the tap water elution plot had larger reported gated events indicative of a noisy sample (not shown). It is hypothesized that the noise arises from non-specific binding to extracellular macromolecules and microbial

contaminants (microeukaryotes) present in the samples. A variety of pre-filtration or centrifugation steps were tested to determine the most effective means for reducing the background noise, with the nylon mesh filter appearing to work best for this application. However, with dirtier environmental samples, multiple purification steps may be required (including centrifugation), with care being taken to minimize loss of target cells. The number of rinses necessary for purification may also vary depending on the relative cleanliness of the sample among other variables, with six 500 µL rinses shown in the current work to be most effective for this assay.

In the methods section it was noted that the tap water samples were obtained as the 'first-flush' for the day, which is defined as the first time the taps are run over a 12 h window. The specific requirement for first-flush sampling ensures that built-up biofilm can slough off with the pressure/flow changes and is suspended in the bulk water flow, leading to more background signals. Thus, the sampling protocol results in testing of the IMS-FCM efficacy using a relevant environmental sample with contaminants. Despite this careful sampling, the implications of the assay efficacy are limited by the lack of various environmental samples, which may differ greatly from the examined tap water samples. Nonetheless, PCR-based detection is also limited by the presence of corrosion products, reaction inhibitors, and humic and other organic acids within aquatic matrices.[23] Furthermore, cooling towers, hot tubs and other engineered water systems may have high background microbial contamination due to the presence of warm, stagnant water (often untreated) with higher nutrient loads. These influences were not accounted for here, thus limiting the generalizability of the results to real-world systems.

1.4.4 Method modifications: *L. pneumophila* SG-6 and total legionellae detection

At the beginning of this project, one of the main goals was to develop a detection assay with the potential for online, automated detection that is specific for *L. pneumophila* and later on

also for total legionellae residing within drinking water samples. As other projects progressed, it became apparent that the IMS-FCM approach outlined above could meet this goal for a quick, affordable, reliable detection assay. Unfortunately, this avenue was being pursued by some of the researchers cited above, with the creation of a start-up company that produced an instrument capable of detecting *L. pneumophila* serogroups 1-14. With this knowledge, the priority for this research project moved to some of the topics addressed in other chapters of this dissertation (namely Chapters 2 and 4). However, a collaboration agreement with the start-up may allow for future examination and verification of the developed automated system. Currently, no environmental samples have been run using this new assay and a niche regarding total legionellae detection appears to remain. Future efforts should be concentrated on fostering the collaboration with the research group and company (RQmicro) behind the IMS-FCM detection platform with respect to total legionellae and viability measures (Chapter 4).

In conclusion, the IMS-FCM approach has potential for automated, online detection of *L. pneumophila* and total legionellae residing within water systems. It is recommended that future research be directed to collaborations between research groups and industry to enable rigourous analyses of method sensitivity and specificity and IMS purification yields with dirty environmental samples; possibly first via spiking experiments like those reported in the current study. Attention should also be focused towards total legionellae detection, with current detection systems chronically underreporting the influence of non-*L. pneumophila* pathogens in legionellosis cases.

2 Disinfection of *Legionella pneumophila*

2.1 Abstract

Pathogens are disinfected to sufficiently inactivate target members to meet levels considered safe-for-purpose. The main objective of this chapter was to examine the efficacy of common disinfection regimes (UV, temperature, and monochloramine) to achieve targeted 4-$\log_{10}$ reductions in culturable *L. pneumophila*. This was followed by comparisons to *E. coli* and *P. aeruginosa*, to provide relative disinfection information with respect to a common faecal indicator and saprozoic (water-based) bacterium, respectively. The experiments began with 10^6 CFU mL^{-1} *L. pneumophila* or other bacterium being exposed to UV-C irradiation (256, 268.6, or 288.6 nm), elevated temperatures (50, 55, or 60 °C), or 2.2 ppm monochloramine, with the results presented as doses required to achieve 4 $\log_{10}$ and 6 $\log_{10}$ reductions in culturable cells; which correspond with previously published secondary and primary treatment requirements, respectively, for point of use (POU) devices.

Whereas UV 253.7 nm is the most commonly used UV wavelength for disinfection, 268.6 nm was shown to be the most effective against *L. pneumophila*, requiring 7.6 mJ cm^{-2} to induce a 4 $\log_{10}$ reduction in culturable cells. Higher doses at 256 and 288.6 nm were necessary to induce similar reductions, with significant differences between 268.6 and 288.6 nm (P <0.002). The comparison with other indicator bacteria was undertaken at 256 nm, where *L. pneumophila* and *E. coli* exhibited similar responses (P = 0.235) between 10.5 mJ cm^{-2} and 12.5 mJ cm^{-2}, respectively, while *P. aeruginosa* exhibiting a much greater susceptibility, requiring only 3.9 mJ cm^{-2} (P = 0.02). Thermal survival curves revealed 60 °C to be the only temperature that effectively provided a 4 $\log_{10}$ reduction of culturable *L. pneumophila*, requiring 84 s; while 50 and 55 °C needed significantly longer durations 1940 s (P <0.001) and 780 s

(P <0.002), respectively. At 60 °C, *L. pneumophila* and *E. coli* exhibiting similar (no significant differences) in duration of exposure to achieve 4 $\log_{10}$ reduction, and again *P. aeruginosa* was more susceptible, requiring only 40 s (P = 0.017). For chemical disinfection with monochloramine at 2.2 mg L^{-1} required a Ct value of 31.2 mg min L^{-1} to achieve a 4 $\log_{10}$ reduction in culturable *L. pneumophila*; with no significant differences observed when compared to *E. coli* (P = 0.059) or *P. aeruginosa* (P = 0.504).

In comparison to previously published data, differences may be attributed to varying methodologies, starting concentrations of bacteria, and curve-fitting parameters. However, most experimental data was limited by culture-based detection, typically with a high detection limit of 10 CFU mL^{-1} and missing viable but non-culturable (VBNC) cells. Furthermore, there are concerns about the generalizability of *in-vitro* data for real-world systems, since the experiments do not account for the presence of biofilms or free-living protozoa and the growth phase of cells. Overall, 268.6 nm UV-C, 60 °C water, and 2.2 mg L^{-1} monochloramine appeared to effectively result in 4 $\log_{10}$ reduction in culturable cells, with monochloramine being more effective against *L. pneumophila*, relative to the other disinfection approaches tested against common water indicator bacteria.

2.2 Introduction

To control water-based opportunistic pathogens like *L. pneumophila*, public health officials must engage in risk assessments to understand potential hazards and mitigate risk throughout water distribution systems. Currently, a major hazard in premise plumbing with established disinfection practice is growth to problematic levels near the point of use, which is associated with outbreaks and sporadic cases.[3] While there is discussion in the literature as to the safe level of *L. pneumophila* in drinking water, based on quantitative microbial risk assessment (QMRA) modeling, there is general agreement that several $\log_{10}$ reductions (4-6) may be

necessary, depending on the exposure scenario.[42] The protocol for assessing the efficacy of an inactivation regime begins with *in-vitro* analyses to generate dose-response models that are used to predict future efficacy in real-world systems.[43] Hence, this chapter focuses on elucidating dose-response relationships for established disinfection regimes; water temperature management and monochloramination, as well as a promising point-of-use (POU) regime, ultraviolet (UV) irradiation.

2.2.1 Control and risk management

According to Health Canada, legionellosis has been a reportable disease since the 1980's, yet there is no standard for drinking water.[44] The U.S. Environmental Protection Agency (EPA) has set a maximum contaminant level goal (MCLG) of zero in its Surface Water Treatment Rule, however no regulatory requirements are associated with this goal.[45] Numerous Western European countries have enacting guidelines and regulations for *Legionella pneumophila* in drinking water distribution systems (DWDS); with Germany and France at <1,000 CFU L^{-1} and the U.K. and Netherlands at <100 CFU L^{-1}, respectively.[16] Currently, the American Society of Heating, Refrigeration, and Air Conditioner Engineers (ASHRAE) is the only North American agency with a recognized standard to manage *L. pneumophila* risks within building heating/cooling systems.[14] Despite the limited regulation in North America, industry-based researchers such as those at Ford Motor Company recognized the risks of water-based pathogens, leading to company-wide implementation of systems to prevent and manage *Legionella* spp. within their facilities over a decade ago, and since 2016 CDC has provided guidance for health-care settings.[46]

A variety of methods have been used to control legionellae within potable-water sources, including secondary disinfection with copper-silver ions, free chlorine or monochloramine, and within buildings temperature management, as well as POU applicators like filters or UV light.[43]

Restricting the formation of biofilms through reduced assimilable organic carbon (AOC) and temperatures below 20 °C have also been shown effective at managing legionellae.[16] A systematic review of potable water disinfection regimes revealed that chlorination is a widely-used, effective approach for the rapid removal of *L. pneumophila*.[46] Furthermore, a test of seven common chemical disinfectants within a series of model plumbing systems revealed that chlorine and chlorine dioxide were the most effective for disinfecting *L. pneumophila*, protozoa, and biofilms.[47] In contrast, a recent review of hospital-based disinfection systems concluded that copper-silver ionization and monochloramine appear to be the most promising in-building disinfection technologies.[43]

The lack of consensus regarding *Legionella* spp. control/management in premise plumbing systems suggests that more lab-based (*in-vitro*) research is necessary to better characterize the efficacy of common disinfection procedures, prior to their implementation in real-world systems. The aim of this chapter was to evaluate UV, water temperature, and monochloramine at inactivating *L. pneumophila*; with the end goal to provide information to support water safety plan (WSP) best-practices for *Legionella* spp. control and management within premise plumbing.

2.2.2 Ultraviolet (UV) irradiation

UV light (253.7 nm) inactivates bacterial cells by creating dimers between nucleotides (primarily adjacent thymine bases), that inhibit replication and transcription processes; while other wavelengths can induce photochemical reactions in cellular enzymes and proteins.[48, 49] UV disinfection has been described as an attractive approach for pathogen inactivation in water systems, as it does not add any chemicals nor generate significant disinfection by-products.[46] However, as UV generates no residual, it is most effective at the POU, considering the low to nil secondary disinfectant in circulating building drinking water systems that may allow for

recolonization.[43] According to the NSF International/ANSI standard document, a UV dose of 16 mJ cm^{-2} is required for Class-B POU systems, which are used for supplemental germicidal treatment of disinfected water supplies.[50] When operated as the primary inactivation treatment (Class-A POU), a dose of 40 mJ cm^{-2} is deemed sufficient for pathogen removal.[50]

Until recently, most UV disinfection studies have focused on monochromatic 253.7 nm light, being the most commonly applied wavelength for Hg-lamp UV water treatment processes.[51] The prevalence of this one wavelength is attributed to the availability of the relatively cheap, so-called low pressure Hg vapour lamps, which emit near the maximum absorbance of DNA.[48] However, the development of polychromatic UV-C medium pressure lamps and the emergence of light-emitting diode (LED) technology for UV irradiation has promoted the idea to test other UV-C wavelengths. LED systems are also suited to POU, with the added benefits of being less fragile and smaller than tradiational lamps, more energy efficient and less harmful for the environment (no Hg).[51] Recently, studies have shown that higher wavelength (lower energy) UV photons can result in more efficient inactivations compared to 253.7 nm light; suggesting that different bacterial species possess unique spectral sensitivities.[52]

Previous studies on the susceptibility of *L. pneumophila* to 253.7 nm UV-C light have reported a 4 log_{10} reduction at a dose of 5.7 mJ cm^{-2}.[53] However, within hospital water supplies, UV inactivation was found to be insufficient at controlling *Legionella* spp.; with the researchers commenting that it is best applied as a secondary disinfectant following chlorination or pasteurization based inactivation.[46] A second study within a hospital environment found a significant reduction in culturable *L. pneumophila* when irradiated directly after a primary disinfection (superchlorination or heat), however the authors make note that filters may be required to limit the impact of biofilm formation.[54] A major limitation of UV disinfection is the

potential for photoreactivation and other DNA damage repair mechanisms that can reduce the efficiency of inactivation, which will be discussed in greater detail (Chapter 4), along with having no effect in limiting or removing premise plumbing biofilm.[16]

2.2.3 Thermal disinfection

Elevated water temperatures are commonly used for disinfection of premise plumbing, as the added heat disrupts cellular membrane function and denature proteins, reducing enzyme activity within bacterial cells. The efficiency of temperature management is dependent on the ability to maintain sufficiently high temperatures and ensuring water circulates through the system to reduce periods of stagnation.[55] WHO guidelines for *Legionella* spp. control in premise plumbing recommend water temperatures should reach at least 60 °C at the heating source and at least 50 °C at the tap.[56] On the opposite end of the spectrum, *L. pneumophila* does not proliferate when water temperature is below 20 °C, with relatively slow growth observed up to 25 °C; thus it is recommended that cold water temperatures remain below 25 °C.[16]

Studies of *L. pneumophila* survival following exposure to heat have demonstrated 4 $\log_{10}$ reductions in culturable cells following exposure to 60 °C for <5 min or 70 °C for <30 s, respectively.[57] Despite the loss of culturability, viable *L. pneumophila* have been reported following a 30 min exposure to 70 °C.[58] Within hospital settings, heat shock was used to remove infectious *Legionella* in response to an outbreak event; however a low level contamination remained, that was later determined to arise from deficiencies in the hydraulics controlling hot water circulation on certain hospital floors.[55] The major limitations of temperature management are the risk of scalding and the inability to reduce biofilms and persistent FLA that harbour *L. pneumophila*.[58] Not surprisingly, it has been shown that *L. pneumophila* found within *A. polyphaga* exhibits greater resistance to thermal treatments compared to their non-bound counterparts (Chapter 3).[59]

2.2.4 Chemical disinfection

The most common type of chemical disinfectant for water treatment are oxidizing agents such as free chlorine ($HOCl/OCl^-$) and monochloramine (NH_2Cl), with non-oxidizing treatments like Cu^{2+}/Ag^+ gaining in use.[43] Oxidizing compounds increase cellular membrane permeability and react with organic components of bacterial cells, resulting in rapid disinfection. In contrast, Cu^{2+}/Ag^+ ions interfere with the enzymes of cellular respiration, affecting the ability of cells to generate energy through oxidative phosphorylation linked pathways.[46]

Aside from temperature management, free chlorine based disinfection is the most widely implemented regime for controlling problematic microbial growth in water systems.[57] Free chlorines are defined as the protonated and deprotonated forms of hypochlorite, HOCl and OCl^-, respectively.[46] Dose-response for *L. pneumophila* have revealed 2 $\log_{10}$ reduction in culturable cells following 1 h exposure at 2 mg L^{-1}, with a <6 $\log_{10}$ reduction in under 10 minutes achieved with a superchlorination dose of 20 mg L^{-1}.[59] Other survival studies have demonstrated that chlorine exposed *L. pneumophila* may remain viable, as determined through esterase activity or plating on agar containing reactive oxygen species (ROS) scavengers like pyruvate and glutamate.[60, 61] The high reactivity of free chlorine presents challenges for effective disinfection, as the molecules will interact with any available organics, producing carcinogenic trihalomethane (THM) by-products, and has been shown to have corrosive effects on pipe walls over time.[46] The long term effectiveness of free chlorine remains a concern, as recolonization and persistent low-level contamination have been reported.[46]

Chloramines are secondary disinfectants used in DWDS that have become increasingly popular in recent years as water utilities aim to reduce the amount of disinfection by-products and the corrosive effects of highly-reactive free chlorine in drinking water systems.[43] The increased efficiency of monochloramine compared to free chlorine arises from chemical stability,

with monochloramine lasting longer in water, allowing it to penetrate deeper into biofilms.[62] Chloramines are produced by reacting ammonia and free chlorine in water, forming monochloramine (NH_2Cl), and controlling these reactants to reduce production of the non-disinfecting residuals dichloramine ($NHCl_2$) and nitrogen trichloride (NCl_3).[43] Monochloramine production can be controlled by reactant ratios, but principally by pH, with the majority of studies and disinfections using a Cl_2:NH_4 ratio of 4:1 and pH 7 (Figure 2.1).

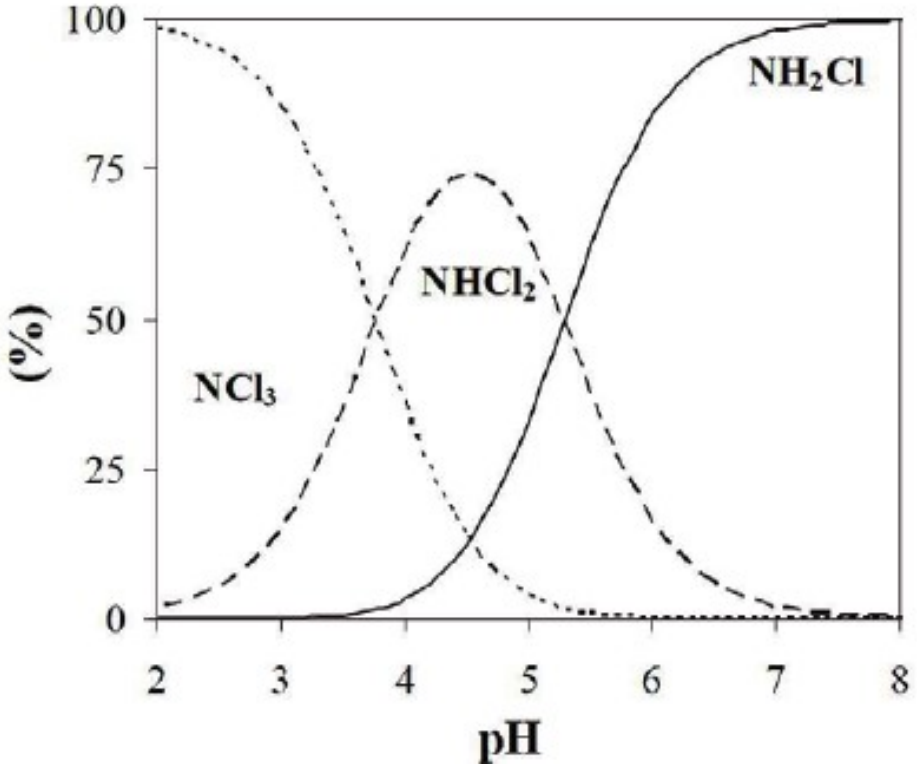

Figure 2.1 Relative abundance of specific chloramines is dependent on pH, with monochloramine being favoured at high pH, dichloramine in slightly acidic conditions, and nitrogen trichloride in acidic conditions.[63]

Experimental reports have demonstrated *L. pneumophila* susceptibility to monochloramine, with a 2 mg L^{-1} dose resulting in a complete removal of culturable cells following a 24 h exposure.[64] In real-world systems, a water utility change-over from free chlorine to monochloramine provided a 20-fold reduction in total legionellae levels in the San Francisco water supply, and yielded a 10-fold reduction in the odds of a drinking-water related

outbreak.[45] In a hospital setting, maintaining monochloramine at 2-3 mg L^{-1} was found to consistently keep *L. pneumophila* concentrations below 100 CFU L^{-1}, however concerns remain over the formation of the by-product *N*-nitrosodimethylamine (NDMA), a known carcinogen and nitrites, which have lower regulated maximum levels in Europe.[65, 66]

Non-oxidizing chemical disinfection like Cu^{2+}/Ag^{+} ionization are gaining more use due to the relatively low costs and ease of installation and maintenance.[43] Exposure to metal ions like Cu^{2+} and Ag^{+} can interrupt cellular respiration by interacting with enzyme cofactors, while also binding DNA of bacterial cells.[46] Survival curves for *L. pneumophila* have shown <6 $\log_{10}$ reduction following a 24 h exposure to 0.08 mg L^{-1} Ag^{+}, yet only a 1 $\log_{10}$ reduction after 24 h exposure to 0.4 mg L^{-1} Cu^{2+}.[67] The use of Cu^{2+}/Ag^{+} ionization as a primary disinfectant remains unclear, as outbreaks have been reported from these systems.[43, 55] Furthermore, concerns exist with regard to the ingestion of these ions, since the US EPA has set MCLG for Cu^{2+} and Ag^{+}.[46]

2.3 Materials and Methods

2.3.1 Cultivation of bacteria

Primary stock of *L. pneumophila* Philadelphia-1 strain (ATCC 33152) was prepared by plating onto buffered charcoal yeast extract (BCYE) agar (obtained from Alberta Provincial Laboratory, Edmonton, AB, Canada) and incubating at 37 °C for 96 h. A single colony was then inoculated into AYE medium (10 g N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 10 g yeast extract, 0.4 g L-cysteine, and 0.25 g iron pyrophosphate per litre), and incubated in a shaker at 37 °C for 18 h, when cells reached late log phase growth according to a predetermined bacterial growth curve calibrated to absorbance at 600 nm and colony forming units (CFU).[39] The primary stocks of *Escherichia coli* ATCC 25922, and a *Pseudomonas aeruginosa* environmental isolate (isolated from amoeba co-culture of drinking water filter, source Rafik Dey, University of Alberta) were prepared by plating onto tryptic soy agar (15 g pancreatic

digest of casein, 5 g papaic digest of soybean meal, 5 g NaCl, and 15 g agar per litre) and incubating at 37 °C for 18 h. A single colony was then inoculated into tryptic soy broth (same ingredients minus agar), and incubated at 37 °C until late log phase reached, according to a predetermined growth curve (as described above).

2.3.2 UV disinfection

The efficacy of ultraviolet (UV) light treatment on *L. pneumophila* (ATCC 33152) was examined by irradiating at emission peaks of 256, 268.6, or 288.6 nm UV-C light and measuring the log reduction of culturable cells. The experiment followed NSF International Standard guidelines for evaluating microbiological UV susceptibility, where UV dose is calculated from measured intensity, absorbance of suspended medium, and exposure time (Appendix).[21] Bacterial suspensions were irradiated with 256, 268.6, or 288.6 nm UV light using a PearlBeam™ LED collimating beam (AquiSense Technologies, Erlanger, KY, USA), with each diode emitting within a narrow band of polychromatic UV-C light (some 10 nm ½ max bandwidth; Appendix). The UV intensity was measured with an UVX-25 radiometer (UVP, Upland, CA, USA) set to the 0-200 μW cm^{-2} range, with calibration factors for the respective wavelengths tested, and UV absorbance being recorded at each wavelength tested. The UV irradiation protocol began with *L. pneumophila* (ATCC 33152) grown for 18 h to late log phase and serial diluted to a working concentration of approximately $1x10^6$ CFU mL^{-1} in 0.85% saline. An aliquot (28.3 mL) of this suspension was placed in a 55 mm x 20 mm Petri dish to a final water depth of 1 cm and mixed with a magnetic stir bar. Prior to UV exposure, a 100 μL sample of the bacterial cell suspension was serial diluted and plated onto BCYE agar to determine initial *L. pneumophila* concentrations above the detection limit of 10 CFU mL^{-1}. Following exposure, plates were incubated at 37 °C for 96 h before counting revealed the log reduction in CFU resulting from UV exposure. Upon establishing *L. pneumophila* (ATCC 33152) dose-response data, the effect of 256 nm UV light

on *E. coli* (ATCC 33152) and *P. aeruginosa* (environmental isolate) were examined. Minor deviations from the protocol outlined above involved growing the bacteria in tryptic soy broth and incubating plates at 37 °C for 18 h, prior to counting. The UV irradiance was measured at the same distance from the LEDs as the samples, with the collimating beam attached (Figure 2.2).

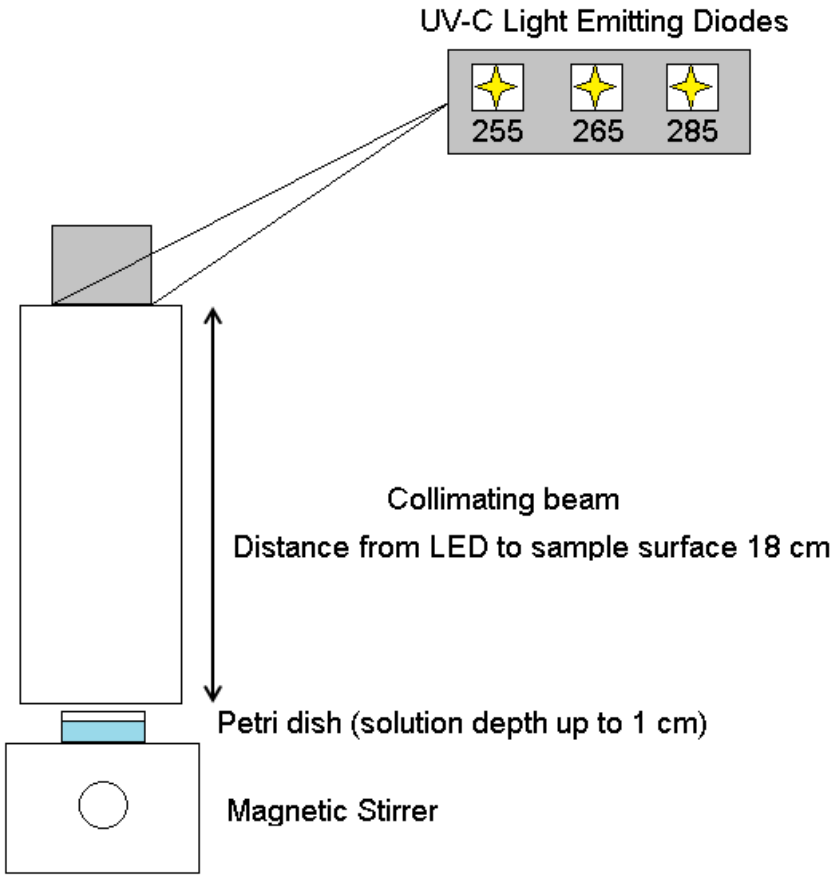

Figure 2.2 Schematic of collimating beam set-up for UV irradiation of *L. pneumophila* (ATCC 33152), *E. coli* (ATCC 25922), and *P. aeruginosa* (environmental isolate), with AquiSense PearlBeam™ LEDs emitting at 256, 268.6, and 288.6 nm maximum peak heights, respectively.

2.3.3 Thermal disinfection

The efficiency of temperature for disinfection was examined by exposing suspended cells to heat and measuring the log reduction in culturable *L. pneumophila* (ATCC 33152), *E. coli* (ATCC 25922), and *P. aeruginosa* (environmental isolate). The experiment followed an established protocol which exposed bacteria to a heated water bath using vacuum sealed bags to

obtain near instantaneous heat transfer time.[68] Briefly, 1 mL samples of $1x10^6$ CFU mL^{-1} bacteria were added to sterile plastic bags. The bags were sealed prior to being placed in a Precision GP 28 water bath (Thermo Fisher Scientific, Waltham, MA, USA) heated to 60 °C. The suspensions were exposed to heat for 0, 1, 2, 3, and 4 minutes, after which the bags were placed in a chilled water bath at 4 °C. After the heat exposure, 100 µL aliquots were serially diluted and plated on organism specific agar plates and incubated for their specified time periods. After validating the method at 60 °C, dose response curves were also constructed for *L. pneumophila* (ATCC 33152) exposed to 50 °C and 55 °C, with dose being reported as time exposed (in minutes).

2.3.4 Chemical inactivation kinetics

The efficiency of monochloramine treatment on bacteria was examined by exposing bacteria to reservoir tap water obtained from the Rossdale water treatment plant in Edmonton, Alberta courtesy of colleagues at EPCOR Utilities Inc. The chloramine levels were tested independently at the EPCOR water quality laboratory using a Series A790 amperometric titrator (Wallace and Tiernan, Warrendale, PA, USA) with acetate buffer stabilized phenylarsine oxide (PAO), as well as in house, using the CN-66 free and total chlorine color disc test kit (Hach, Loveland, CO, USA). The degradation of monochloramine was monitored over the course of 3 d to determine the expiration date, with total chlorine concentration being recorded in Appendix.

For monochloramine inactivation studies, *L. pneumophila* (ATCC 33152), *E. coli* (ATCC 25922), and *P. aeruginosa* (environmental isolate) were grown to late log phase and centrifuged at 2,000 rpm for 10 minutes to obtain a cell pellet. The supernatant was discarded and the pellet was re-suspended in 5 mL of 0.85% saline. This process was repeated in triplicate to obtain cellular suspensions free of growth medium and non-specific organics that may react with the chloramine. From here, 200 µL of the purified cell suspension was aliquoted into 19.8 mL of chloraminated water, marking time 0. Beginning at 0 minutes exposure, a 100 µL sample of the

bacterial cell suspension was serial diluted in 10% sodium thiosulfate and plated onto BCYE or TSA plates to determine initial CFUs.

The survival of *L. pneumophila* (ATCC 33152) following exposure to free chlorine was examined using a similar approach as that described for monochloramine. Briefly, household bleach (Chlorox) containing 7.4% sodium hypochlorite (74,000 mg L^{-1}) was diluted to make a 100 mg L^{-1} stock solution in sterile distilled water.[57] Immediately prior to exposure, the stock chlorine was diluted between 0.5-2 mg L^{-1}, with concentration being estimated using the colourimetric assay described above. The exposure followed a similar approach to the monochloramine treatment, with the chlorine being quenched using 10% sodium thiosulfate. The effective dose of monochloramine or chlorine residual can be calculated as the product of concentration (in mg L^{-1}) and time (in minutes).

2.3.5 Statistical analysis

The dose-response curves for each disinfection regime were analyzed for statistical significant differences between bacterial groups (*L. pneumophila, E. coli,* and *P. aeruginosa*), as well as differences within groups for *L. pneumophila* at different UV and heat doses. In order to perform this analysis, dose-response curves were created with corresponding best-fit lines for each bacterial group using a quadratic modelling system on Microsoft Excel. The null hypothesis for each of the statistical tests was no difference dose required to achieve an approximate 4 $\log_{10}$ reduction in culturable cells between bacterial species or within *L. pneumophila* groups at different doses. Microsoft Excel 2010 was used to run an unpaired, unequal variance, two-tailed Student's t-tests, with $\alpha = 0.05$, the *P*-values were reported to either accept or reject the null hypothesis.

2.4 Results and Discussion

2.4.1 UV disinfection

The effects of UV-C light exposure on *L. pneumophila* (ATCC 33152) were examined by irradiating bacterial cells using a LEDs and a collimating beam with peak maxima at 256, 268.6, and 288.6 nm (Figure 2.3).

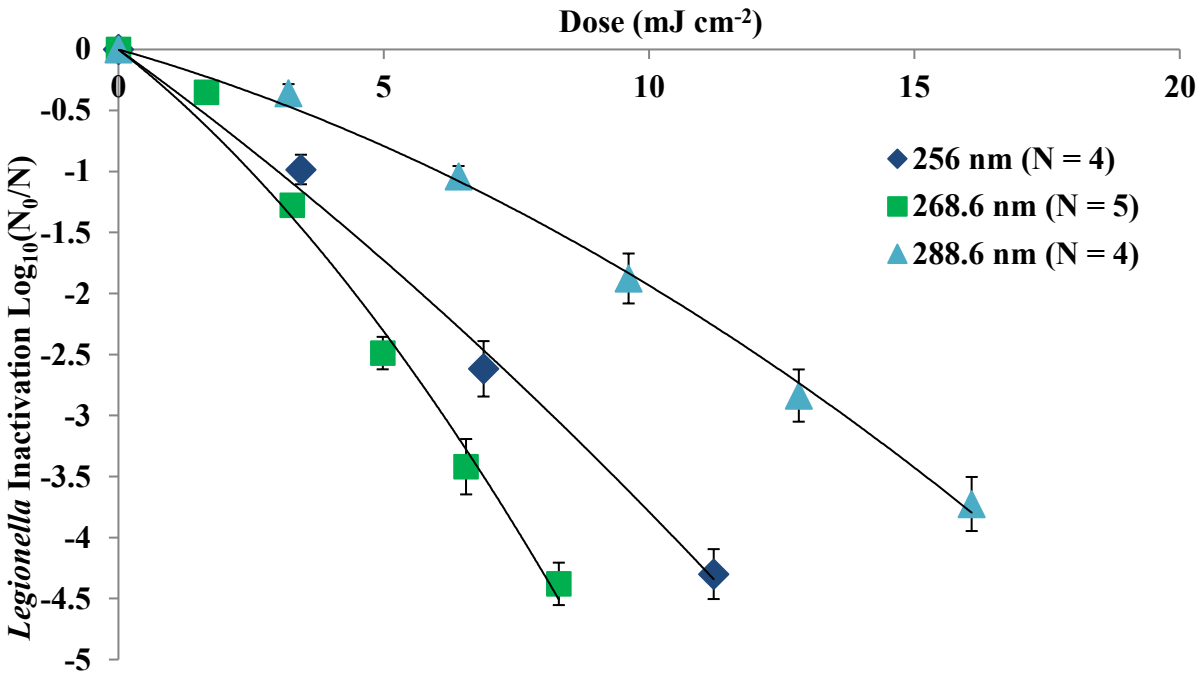

Figure 2.3 UV dose-response curve for *L. pneumophila* (ATCC 33152) exposed to LEDs emitting at peaks of 256, 268.6, or 288.6 nm UV-C light, with response reported as the reduction in culturable cells plated on BCYE agar, with a detection limit of 10 CFU mL^{-1}. The average starting concentration of culturable *L. pneumophila* was $2.3x10^6$ CFU mL^{-1}, with error bars equivalent to ± SEM for N replicate, independent trials.

From the *L. pneumophila* UV dose-response curve (Figure 2.3), equations for best-fit lines were calculated using Microsoft Excel. The equations were then used to interpolate and extrapolate the approximate UV dose required to achieve 4 log_{10} and 6 log_{10} reductions in culturable cells. A statistical test (described in methods) was then run to determine if any significant differences were observed for the three curves (Table 2.1).

Table 2.1 Equations for UV dose *L. pneumophila* response best-fit lines generated in Figure 2.3, with measure of fit (R^2) and extrapolated doses to achieve 4 or 6 $\log_{10}$ reduction for each wavelength tested.

Wavelength (nm)	Equation of Best Fit	R^2	4 $\log_{10}$ Reduction (mJ cm^{-2})	*P*-value	6 $\log_{10}$ Reduction (mJ cm^{-2})
256	$y = -0.0066x^2 - 0.3127x$	0.995	10.5	0.139	14.7
268.6	$y = -0.0245x^2 - 0.3398x$	0.9894	7.6	--	10.2
288.6	$y = -0.007x^2 - 0.1234x$	0.9971	16.7	<0.002	21.8

The *L. pneumophila* UV dose-response curve (Figure 2.3) shows greater susceptibility to light with peak emittance at 268.6 nm compared to 256 nm, requiring doses of 7.6 mJ cm^{-2} and 10.5 mJ cm^{-2} to induce 4 $\log_{10}$ reductions in culturable cells, respectively. The data demonstrates emission peaks at 268.6 nm UV-C light were statistically more effective than 288.6 nm (P = <0.002), but not for 256 nm (P = 0.139). Accordingly, the null hypothesis of no significant differences in UV dose required to achieve 4-$\log_{10}$ reductions can be rejected for the comparison of 268.6 nm and 288.6 nm, while it remains for the comparison of 256 nm and 268.6 nm. Therefore, UV disinfection with longer wavelengths should be avoided for *L. pneumophila* inactivation, as 288.6 nm has much lower efficiency compared to 268.6 nm.

As mentioned in the introduction, the majority of UV inactivation studies are run using 253.7 nm light, in part due to the widespread use of economical Hg vapour lamps.[48] As LED and medium pressure technology has developed, more research is being directed towards other wavelengths.[51] Previous studies on the susceptibility of *L. pneumophila* to 253.7 nm UV-C light have reported a 4 $\log_{10}$ reduction at a dose of 5.7 mJ cm^{-2}.[53] The apparent 5 mJ cm^{-2} deviation between the literature value and this experiment (at 256 nm) could be attributed to methodological differences. The initial concentration of bacteria of $5x10^5$ CFU mL^{-1} (literature) compared to 10^6 CFU mL^{-1} (experimental) likely resulted in less shielding and more effective

irradiation, leading to lower required doses. Furthermore, the reported suspension had a depth of 4 mm, compared to the 1 cm depth used in the current work, which would again allow for greater penetration of the photons. Thus, the deviation of UV dose required for inactivation of 4 $\log_{10}$ *L. pneumophila* is likely attributed to differences in experimental set-up.

The recommended UV doses for primary (Class-A POU) and supplemental (Class-B POU) germicidal treatments are 40 mJ cm^{-2} and 16 mJ cm^{-2}, respectively.[50] From the calculated UV doses required for 4-$\log_{10}$ reductions (Table 2.1) the Class-B POU minimum dose is almost achieved for 288.6 nm. However, one-half this dose is required to induce the same reduction in culturable cells when operating at 268.6 nm. From a risk management standpoint, doubling the dose of the more effective 268.6 nm would provide greater security for *L. pneumophila* inactivation. This highlights the importance of examining multiple wavelengths when performing dose-response inactivation curves, in order to ensure the most efficient dose is being applied. It is clear that despite the higher energy and proximity to the DNA absorption maximum associated with 256 nm photons, germicidal effects of UV light are dependent on the spectral sensitivity of each organism, possibly indicating different mechanisms such as targeting of proteins.[49, 52] A major limitation of UV disinfection is the potential for photoreactivation and other DNA damage repair mechanisms that can reduce the efficiency of inactivation, which is investigated in **Chapter 4**.

The effects of UV-C light exposure on *E. coli* (ATCC 25922) and *P. aeruginosa* (environmental isolate) were compared to *L. pneumophila* (ATCC 33152), by irradiating bacterial cells using a LEDs and a collimating beam with peak maximum of 256 nm (Figure 2.4).

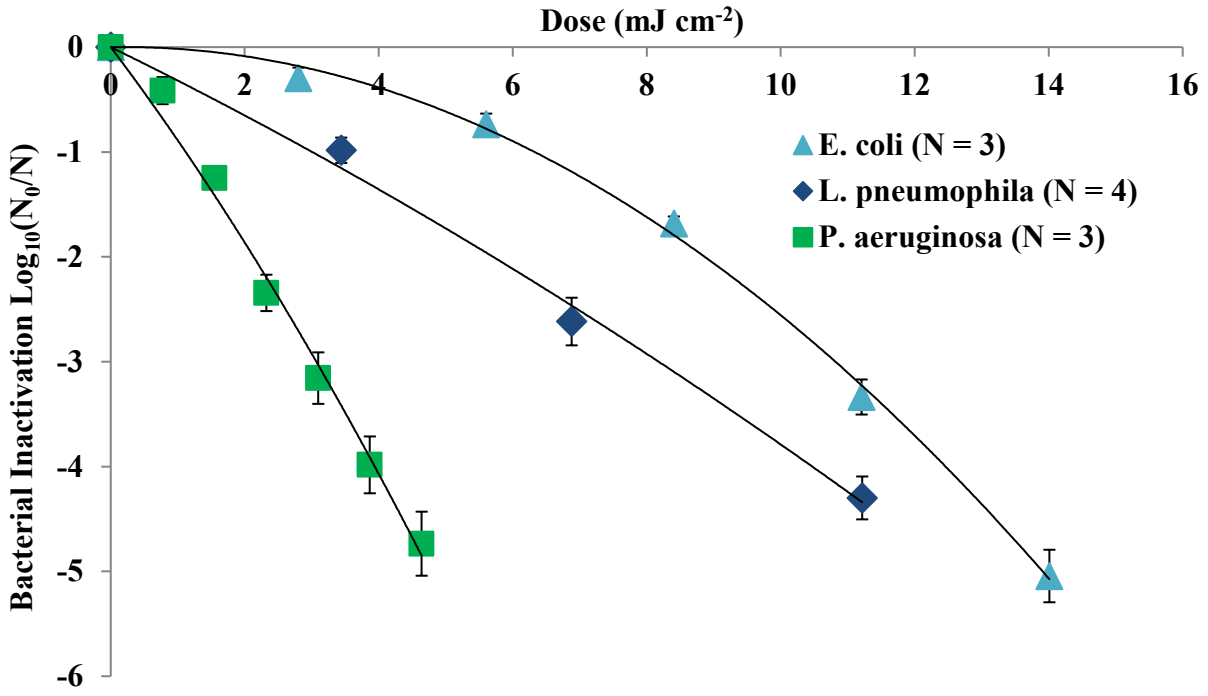

Figure 2.4 UV dose-response curve for various bacteria, including *L. pneumophila* (ATCC 33152), *E. coli* (ATCC 25922), and *P. aeruginosa* (environmental isolate) exposed to an LED emitting at maximum peak of 256 nm UV-C light, where response is reported as the reduction in culturable cells plated on BCYE agar, with a detection limit of <10 CFU mL^{-1}. The error bars are equivalent to ± SEM for N replicate, independent trials.

From the bacterial UV dose-response curve (Figure 2.4), equations for best-fit lines were calculated using Microsoft Excel. The equations were then used to interpolate and extrapolate the approximate UV dose required to achieve 4 log_{10} and 6 log_{10} reductions in culturable cells. A statistical test (described in methods) was then run to determine if any significant differences were observed for the three curves (Table 2.2).

Table 2.2 Equations for UV dose bacterial response best-fit lines generated in Figure 2.4, with measure of fit (R^2) and extrapolated log_{10} reduction for each organism tested at 256 nm.

Organism	Equation of Best Fit	R^2	4-log_{10} Reduction (mJ cm^{-2})	*P*-value	6-log_{10} Reduction (mJ cm^{-2})
L. pneumophila	$y = -0.0066x^2 - 0.3127x$	0.995	10.5	--	14.7
E. coli	$y = -0.0266x^2 + 0.0099x$	0.9979	12.5	0.235	15.2
P. aeruginosa	$y = -0.0429x^2 - 0.8461x$	0.9921	3.9	0.020	5.5

The bacterial UV dose-response curve (Figure 2.4) shows that *L. pneumophila* has an intermediate susceptibility to 256 nm UV-C irradiation, compared to *E. coli* (ATCC 25922) and *P. aeruginosa* (environmental isolate). The UV doses required to achieve 4 $\log_{10}$ reductions in culturable bacterial were similar for *L. pneumophila* and *E. coli*, with calculated values of 10.5 mJ cm^{-2} and 12.5 mJ cm^{-2}, respectively; with *P. aeruginosa* exhibiting a much greater susceptibility, requiring only 3.9 mJ cm^{-2}. The data demonstrates statistically significant differences between *L. pneumophila* and *P. aeruginosa* (P = 0.02), but not for *E. coli* (P = 0.235). Accordingly, the null hypothesis of no significant differences in 4 $\log_{10}$ reduction values between species can be rejected for *L. pneumophila* and *P. aeruginosa*, while it remains for the comparison of *L. pneumophila* with *E. coli*.

In comparing to the literature, the UV inactivation kinetics of *E. coli* K12 have been examined using 265 nm LEDs, revealing a 4 $\log_{10}$ reduction following a dose of 10.8 mJ cm^{-2}.[69] This value is relatively close to the experimentally determined value of 12.5 mJ cm^{-2}, with deviations being attributed to different LED systems and minor changes in peak wavelengths tested, as well as variations in protocols, and the use of a different *E. coli* strain. It was important to examine the UV dose-response curves for various organisms to highlight that each organism will have unique spectral sensitivities. A comprehensive review of current dose-response data concluded that germicidal effects of UV do not shadow the absorption spectrum of DNA, and that the most effective wavelength may vary across organisms.[52] Therefore, a complete scan of UV-A through UV-C dose-response should be run in order to establish the most efficient wavelength for inactivation of *L. pneumophila* or other water-based pathogens within a system.

As mentioned above, the culture-based detection system had a detection limit of 10 CFU mL^{-1}; thus it is entirely possible that a low-level of persistent *L. pneumophila* may

remain following UV irradiation. In order to reduce this concern, it is suggested that at high doses, a 1 mL sample is plated to reduce the detection limit to 1 CFU mL^{-1}. Other limits of the experiment include the calculation of UV dose, which does not account for surface reflectance or the polychromatic nature of the LEDs used, which emit a spectrum of light (half-bandwidth near 10 nm) as compared to the monochromatic emission of Hg vapour lamps. Furthermore, an issue with suspension-based irradiation involves bacterial clumping, which can result in larger apparent resistance, as cells on the top shield cells below.[48] The effect of clumping was observed when samples were above 10^6 CFU mL^{-1}, leading to erratic, non-reproducible results. This prompted all experimental samples to be diluted to approximately 10^6 CFU mL^{-1}.

The data provided here is useful for understanding the inactivation kinetics of UV disinfection within a controlled lab setting. Unfortunately, real-world premise plumbing systems are less controlled and predictable. Two major differences between lab and natural settings are the presence of biofilms and associated microbes, as well as the artificial growth conditions of plating that has been shown to yield less resistant organisms when compared to growth in tap water systems.[66] It has been shown that the efficiency of UV is reduced at least 2-fold following the introduction of *A. polyphaga*, a common free-living protozoan which may protect *L. pneumophila* from various inactivation regimes.[53] Thus, while it is important to understand the spectral sensitivity of *L. pneumophila*, other biological issues remain, such that research should also examine commonly associated host free-living protozoa to ensure that all relevant microbes are removed.

2.4.2 Thermal disinfection

The effects of elevated water temperatures on *L. pneumophila* (ATCC 33152) were examined by exposing bacterial cells to 50, 55, and 60 °C water baths (Figure 2.5).

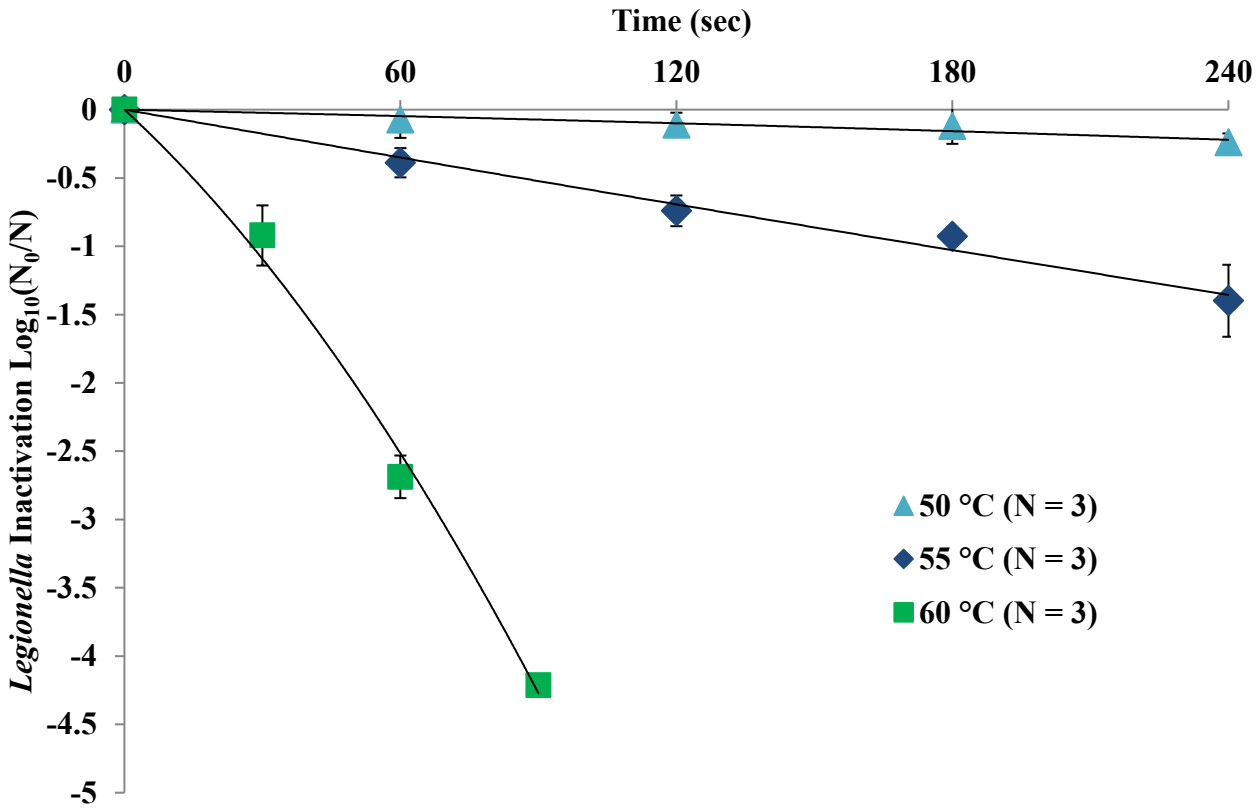

Figure 2.5 Heat dose-response curve for *L. pneumophila* (ATCC 33152) exposed to 50, 55, or 60 °C water bath, with response reported as the reduction in culturable cells plated on BCYE, with a detection limit of 10 CFU mL^{-1}. The average concentration of culturable *L. pneumophila* was $3.5x10^6$ CFU mL^{-1}, with error bars equivalent to ± SEM for N replicate, independent trials.

From the *L. pneumophila* heat survival curve (Figure 2.5), equations for best-fit lines were calculated using Microsoft Excel. The equations were then used to interpolate and extrapolate the approximate thermal dose required to achieve 4 log_{10} and 6 log_{10} reductions in culturable cells. A statistical test (described in methods) was then run to determine if any significant differences were observed for the three curves (Table 2.3).

Table 2.3 Equations for heat dose *L. pneumophila* response best-fit lines generated in Figure 2.5, using Microsoft Excel, with measure of fit (R^2) and extrapolated time to achieve 4 or 6 log_{10} reduction for each temperature tested.

Temperature (°C)	Equation of Best Fit	R^2	4-log_{10} Reduction (sec)	*P*-value	6-log_{10} Reduction (sec)
50	$y = -7x10^{-7}x^2 - 0.0007x$	0.9116	1940	--	2470
55	$y = 1x10^{-6}x^2 - 0.0059x$	0.986	780	<0.002	1305
60	$y = -0.0002x^2 - 0.0309x$	0.9941	84	<0.001	112

The *L. pneumophila* thermal inactivation curve (Figure 2.5) shows a rapid effect of exposure to 60 °C heat, requiring 84 s to induce a 4 $\log_{10}$ reduction, with 50 and 55 °C requiring greater exposure times. At 50 °C, an exposure of 1940 s (around 32.5 minutes) would be required to induce a 4 $\log_{10}$ reduction. The data demonstrates both 55 and 60 °C exposures were significantly more effective than 50 °C, (P <0.002) and (P <0.001), respectively. Accordingly, the null hypothesis of no significant differences in time required for 4 $\log_{10}$ inactivation at each temperature can be rejected. Therefore, inactivation through elevated temperature regimes should focus on >60 °C, since lower temperatures are much less effective in terms of time required to achieve the same response.

Comparing the experimental results to established literature reveals similar patterns with increased temperature trials requiring less time to achieve adequate $\log_{10}$ reductions. A recent publication determined it would take <120 minutes for a 4 $\log_{10}$ reduction at 50 °C, 10 minutes at 55 °C, and approximately 2 minutes at 60 °C, for *L. pneumophila* (ATCC 33152).[57] The only deviation between the two experiments was the 50 °C trial, which may be attributed to the curve fitting model used. In another study, a 4 $\log_{10}$ reduction was observed following a 10 minute exposure to 60 °C heat, although the starting concentration of *L. pneumophila* was in the 10^9 CFU mL^{-1} range, which may result in cell clumping and may not represent what would be expected in actual water systems.[59] Thus, the experimental results and established literature appear closely related with minor deviations being attributed to curve-fitting parameters.

As mentioned above, the WHO guidelines for *Legionella* spp. control in premise plumbing recommends water temperatures should reach at least 60 °C at the heating source and at least 50 °C at the tap.[56] From the calculated times required for 4-$\log_{10}$ reductions (Table 2.3) it is apparent that under ideal conditions, 60 °C should be kept throughout the system. However,

this may be impractical, in part due to the risk of scalding from hot water taps and poor reliability associated with maintaining these elevated temperatures.[58] Re-colonization of thermally maintained pipes has been documented; with one studying attributing a low-level contamination with poorly performing hydraulics on one floor of a hospital wing.[55] Another explanation for recurrent propagation of *L. pneumophila* following elevated temperature management is the role of free-living protozoa, some of which may be thermotolerant; providing internalized bacteria with both protection and nutrients.[59] Thus, as noted above, it is crucial to understand the inactivation kinetics of *L. pneumophila* and the major microbes that it interacts with in order to provide a true approximation of disinfection efficiency. Based on this initial data, the most efficient use of thermal inactivation is at 60 °C, with 50 and 55 °C requiring too long to attain sufficient reductions in culturable cell counts.

The effects of elevated water temperatures on *E. coli* (ATCC 25922) and *P. aeruginosa* (environmental isolate) were compared to *L. pneumophila* (ATCC 33152), by exposing the bacterial cells to a water bath at 60 °C (Figure 2.6).

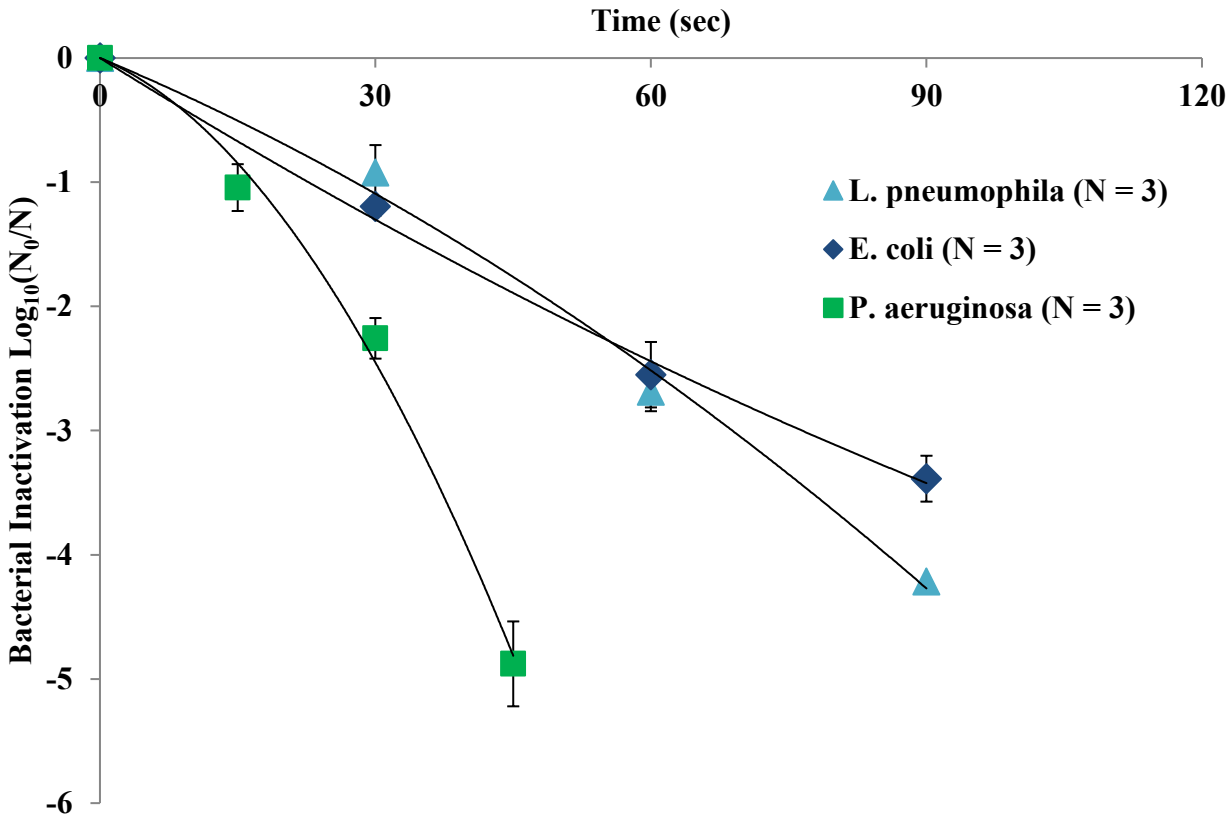

Figure 2.6 Heat dose-response curve for various bacteria, including *L. pneumophila* (ATCC 33152), *E. coli* (ATCC 25922), and *P. aeruginosa* (environmental isolate) exposed to elevated water temperature of 60 °C, where response is reported as the reduction in culturable cells plated on BCYE agar, with a detection limit of <10 CFU mL^{-1}. The error bars are equivalent to ± SEM for N replicate, independent trials.

From the bacterial temperature survival curve (Figure 2.6), equations for best-fit lines were calculated using Microsoft Excel and were used to determine time required to achieve 4 and 6 log_{10} reductions in culturable cells (Table 2.4).

Table 2.4 Equations for heat dose bacterial response best-fit lines generated in Figure 2.6, with measure of fit (R^2) and extrapolated log_{10} reduction values for each organism tested at 60 °C.

Organism	Equation of Best Fit	R^2	4-log_{10} Reduction (sec)	*P*-value	6-log_{10} Reduction (sec)
L. pneumophila	$y = -0.0002x^2 - 0.0309x$	0.9941	84	--	112
E. coli	$y = 9x10^{-5}x^2 - 0.0461x$	0.9964	110	0.807	255
P. aeruginosa	$y = -0.0017x^2 - 0.0313x$	0.9939	40	0.017	50

The bacterial thermal inactivation curve (Figure 2.6) shows that *L. pneumophila* (ATCC 33152) and *E. coli* (ATCC 25922) had similar responses to 60 °C exposure, requiring 84 s and 110 s to achieve 4 $\log_{10}$ reductions in culturable bacteria, respectively. The *P. aeruginosa* environmental isolate was much more susceptible to elevated temperatures, needing only 40 s exposure to achieve the same reduction. The data demonstrates statistically significant differences between *L. pneumophila* and *P. aeruginosa* ($P = 0.017$), but not for *E. coli* ($P = 0.807$). Accordingly, the null hypothesis of no significant differences in 4 $\log_{10}$ reduction values between species can be rejected for *L. pneumophila* and *P. aeruginosa*, while it remains for the comparison of *L. pneumophila* with *E. coli*.

Interestingly, the environmental isolate (*P. aeruginosa*) was less resistant to temperature stress than two lab culture strains (*L. pneumophila* and *E. coli*). Previous studies have shown that environmental isolates are more resistant to chemical intrusion than similar lab strains.[64] This may be attributed to previous exposures or changes in gene expression. However, a comparison of lab and environmental *L. pneumophila* found that the environmental strain exhibited much lower resistance to thermal stress compared to the lab strain.[58] Furthermore, continual sub-culturing of lab-based strains can lead to lower resistance over time.[64] Therefore, it is important that experimental studies account for the types of strains (lab, clinical, environmental) and how many sub-cultures are occurring, since both of these factors will affect inactivation kinetics.

The majority of literature examining thermal inactivation of *E. coli* is directed towards food safety, with reference strains (K12 and ATCC 25922) having D_{60} values between 0.1 – 0.5 minutes following steam treatment; where the D-value represents the time interval required to achieve a 1 $\log_{10}$ reduction.[68] In a study of microbes commonly found following rainwater harvesting, *P. aeruginosa* was reported to be less resistant than other microbes, with $D_{60} = 49$ s;

although the starting concentration of bacteria was reported to be $2x10^{10}$ CFU mL^{-1}.[70] As discussed above, bacteria exhibit added resistance above 10^6 CFU mL^{-1}, which should be considered an artifact of the experimental design. These artifacts are due to shielding, clumping, and other interactions, which affect reproducibility and limit its generalizability to real-world systems. Thus, the inactivation study has similar results to the established literature, demonstrating rigour in the experimental methods employed here.

Initial heat-shock trials involved a hot plate to which cuvettes were added; however the results were inconsistent as the cuvettes required certain time intervals to heat to desired temperatures. These concerns were removed by using sealable bags and a water bath set-up, which allowed for near-instantaneous heat transfer and reproducible trials. The effects of cell clumping were minimized by serial dilutions of samples to 10^6 CFU mL^{-1} (as discussed above). Immediately following heat exposure, the sealed bags were submerged in 10 °C water to rapidly cool the bacterial samples, ensuring that the exposure times were not underestimated.

2.4.3 Chemical disinfection

The effects of monochloramine on *E. coli* (ATCC 25922) and *P. aeruginosa* (environmental isolate) were compared to *L. pneumophila* (ATCC 33152), by exposing the bacterial cells to 2.2 mg L^{-1} monochloraminated tap water (Figure 2.7).

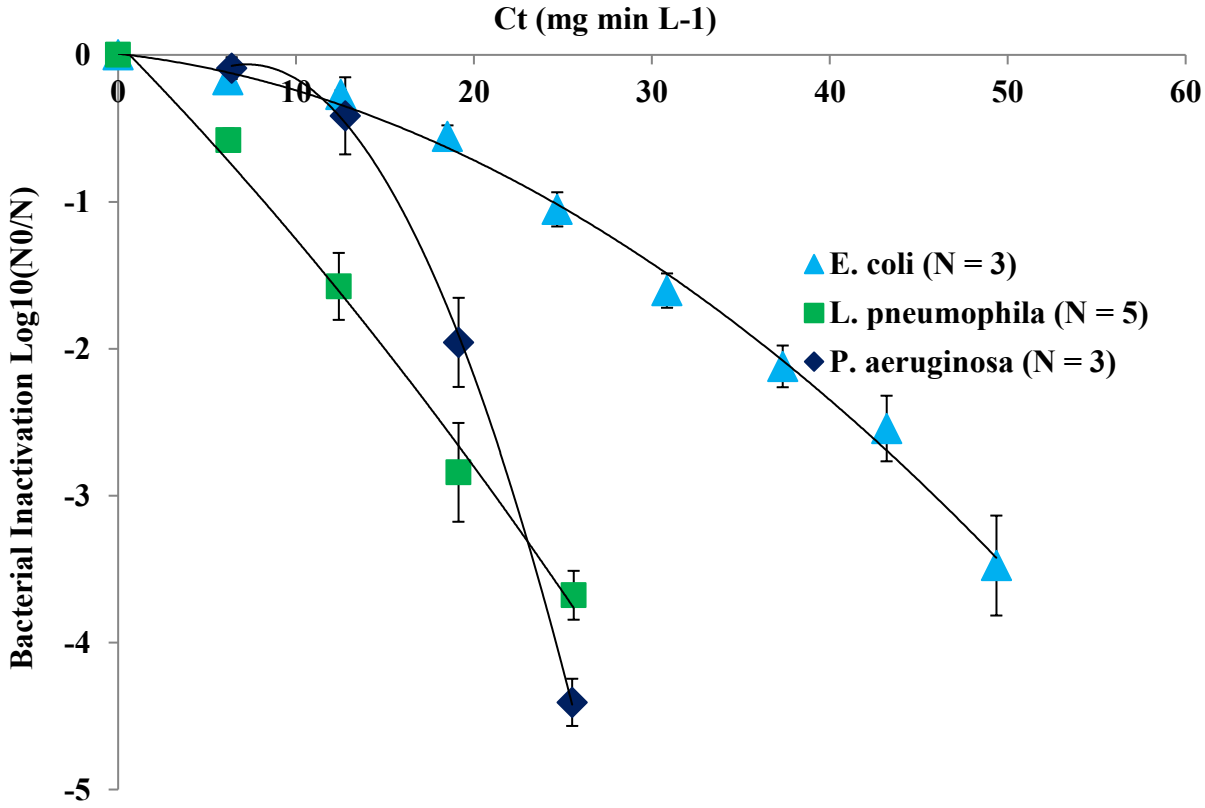

Figure 2.7 Monochloramine dose-response curve for various bacteria, including *L. pneumophila* (ATCC 33152), *E. coli* (ATCC 25922), and *P. aeruginosa* (environmental isolate) exposed to 2.2 mg L^{-1} monochloraminated tap water obtained from EPCOR Rossdale water treatment plant in Edmonton, AB; where response is reported as the reduction in culturable cells plated on BCYE agar, with a detection limit of 10 CFU mL^{-1}. The error bars are equivalent to ± SEM for N replicate, independent trials.

From the bacterial monochloramine survival curve (Figure 2.7), as described above, equations for best-fit lines were used to interpolate and extrapolate the approximate chloramine doses required to achieve 4 log_{10} and 6 log_{10} reductions in culturable cells (Table 2.5).

Table 2.5 Equations for monochloramine dose bacterial response best-fit lines generated in Figure 2.7, with measure of fit (R^2) and doses required for 4 or 6 log_{10} reduction tested at 2.2 mg L^{-1} monochloramine in tap water.

Organism	Equation of Best Fit	R^2	4-log_{10} Reduction (mg min L^{-1})	*P*-value	6-log_{10} Reduction (mg min L^{-1})
L. pneumophila	$y = -0.0014x^2 - 0.1119x$	0.9919	31.2	--	39.1
E. coli	$y = -0.0011x^2 - 0.013x$	0.9954	54.0	0.059	67.4
P. aeruginosa	$y = -0.0101x^2 + 0.0868x$	0.9945	24.7	0.504	29.0

The bacterial survival curves for exposure to 2.2 mg L^{-1} exposure (Figure 2.7) show that *L. pneumophila* (ATCC 33152) and *P. aeruginosa* (environmental isolate) had similar responses, requiring 31.2 and 24.7 mg min L^{-1} to achieve 4 $\log_{10}$ reductions in culturable bacteria, respectively. Surprisingly, *E. coli* (ATCC 25922) had greater resistance to monochloramine, requiring a dose of 54 mg min L^{-1} to achieve the same reduction. The data reveals no statistically significant differences between *L. pneumophila* and the other species tested, with *E. coli* approaching significance ($P = 0.059$), although random chance cannot be ruled out. Accordingly, the null hypothesis of no significant differences in 4-$\log_{10}$ reduction values between species can be rejected for *L. pneumophila* and *P. aeruginosa*, as well as *L. pneumophila* and *E. coli*.

Comparing the experimental results to established literature reveals deviations in the doses required for reductions in culturable cells in some studies, while others show similar results. It has been shown that *L. pneumophila* (ATCC 33152) can remain culturable for 24 h in the presence of 1.5 mg L^{-1} monochloramine; however no culturable cells were observed at 2 mg L^{-1} over the same time period.[64] Another study revealed that *L. pneumophila* quickly enters a viable but non-culturable (VBNC) state in response to monochloramine exposure.[71] These results cannot be directly compared to the experimental survival curve, as there is no inactivation kinetic data (response curves), only initial and final concentrations, or biofilm associated bacteria, respectively.[64, 71] Finally, a 2 $\log_{10}$ reduction in culturable *L. pneumophila* was observed following a 5 minute exposure to 2 mg L^{-1} monochloramine, with 15 minutes required to achieve the same reduction at 1 mg L^{-1}.[66] This result is nearly identical to the experimental data presented, supporting sound methodologies and scientific rigour in the protocol used in the current study.

The deviations between experimental and published data may arise from residual organic components in the literature samples, as well as unmeasured by-product formation during the reactions used to create monochloramine in lab. This experiment used monochloramine taken directly from the taps at the EPCOR Rossdale water treatment plant that serves the city of Edmonton, Alberta; thus there were accurate measures of true chloramine levels. Furthermore, care was taken to remove any non-bacterial organic components that could interfere with monochloramine.

The susceptibility of *L. pneumophila* to monochloramine compared to other microorganisms was surprising, in part due to the fact that *L. pneumophila* is comparatively more resistant to free chlorine and can withstand doses of 50 mg L^{-1}.[9] It has been argued that less reactive monochloramines are more effective against *L. pneumophila* than free chlorine, since they can penetrate into inhabited biofilms.[62] Despite this claim, there is evidence that *Legionella* can increase in relative abundance following chloramine treatment, through resistance selection processes.[72] A widely cited paper on the efficacy of chloramine disinfection of *L. pneumophila* used a case-control epidemiological study; which was limited by potential for underreporting of outbreak events, failure to disclose secondary disinfection regimes, confounding, and bias.[73] Furthermore, a review of hospital disinfection methods suggests that prolonged studies are required in order to determine if monochloramine is a suitable inactivation approach.[43] The ambiguity around the inactivation kinetics of chloramines suggest that more *in-vitro* analyses are required before definitive statements on efficacy compared to chlorines can be made.

The title of this section reads “chemical disinfection”, suggesting that more than just monochloraminated water would be tested for disinfection efficacy. Initially, the goal of the project was to examine both free chlorine (derived from sodium hypochlorite), and Ag^{+}/Cu^{2+}

ions against *L. pneumophila*. Unfortunately, both of these inactivation regimes were abandoned over the course of this project due to reasons explained below. For the inactivation by Ag^{+}/Cu^{2+} ions, personal correspondence with Emilie Bedard at Ecole Polytechnique de Montreal during a conference in Ottawa, ON, revealed that their lab has been studying the inactivation kinetics of the metal ions on *L. pneumophila*. The collaborators readily shared their findings and suggested future research links related to VBNC infectivity following Ag^{+}/Cu^{2+} exposure (undertaken and presented in Chapter 4).

The free chlorine trials were attempted over the course of three months, with each trial yielding unique, non-reproducible results. Initially, the trials were run using household bleach diluted in sterile milliQ water. The poor reproducibility was thought to arise from the hypotonic suspension, causing bacteria to lyse upon introduction into the sample. To combat this, solutions were then diluted in 0.85% saline with similar precision issues arising. A new source of hypochlorite was attempted, with samples being obtained from EPCOR; however this did not change the results. Other sources of error in the experiment that were examined included: plastics vs. acid-washed glassware, pH sensitivities (hypochlorite added to PBS) and colourimetric assay accuracy (tested against EPCOR lab and another lab-based assay); each revealing the characteristic poor precision across multiple trials. With this series of failed trials, it was decided to abandon the free chlorine inactivation kinetics for the foreseeable future.

2.4.4 Explanation of statistical analyses and method limitations

Initially, the dose-response curves were constructed using linear modelling without forcing the curves through the origin (0, 0). Upon submission of a paper for review, multiple reviewers noted that the data should have a parabolic fit with the lines being forced through the origin. The justification for using a parabolic fit as opposed to a linear model is the presence of shouldering and tailing regions within dose-response curves that restrict the linear response to

the middle of the curves. The shoulder occurs at low doses, while tailing is prevalent at high doses, usually in response to clumping effects.[48] Shouldering leads to a perceived "threshold dose" at which inactivation (log_{10} reduction) begins. This is most prominent in Figure 2.7, where the best-fit model for a *P. aeruginosa* environmental isolate has a shoulder above the x-axis, suggesting that low monochloramine doses may actually be beneficial to the organism. The positive region of this curve shows that the parabolic fit is not perfect, however it appears to have better goodness-of-fit (approximated by R^2) than the linear models used. As for fitting the data through the origin, the point (0, 0) must be considered, since the remainder of the doses are calculated as a factor of the initial concentration prior to exposure. It should be noted that for the monochloramine trial, a time zero (no exposure) sample was not possible based on the protocol used. Instead, the bacterial samples were added to the chloraminated water with a magnetic stir bar, and a time-adjusted "control" was taken within 10 s of exposure.

The results for each dose-response curve were reported as the dose required to achieve either a 4-log_{10} or 6-log_{10} reduction in culturable cell counts. A 4-log_{10} reduction corresponds to a 99.99% removal of microorganisms, and was obtained from the US EPA Safe Drinking Water Act (SDWA) Surface Water Treatment Rules (SWTR), which also legislates residual disinfection above 0.2 mg L^{-1} and continuous monitoring of disinfectant residual entering distribution systems serving >3000 people.[45] The 6 log_{10} reduction value was chosen specifically from QMRA modelling of *L. pneumophila* within premise plumbing systems, where a concentration of $3.5x10^6$ CFU mL^{-1} is required to cause infection.[74] Thus, the estimated doses required to achieve 4 log_{10} and 6 log_{10} inactivation provide a measure of efficacy as would be necessary to reduce risk within drinking water systems under current SDWA regulations and

available QMRA models. Sample calculations of the doses required to achieve appropriate reductions are included in the Appendix section.

Some of the limitations of the experimental approach have been discussed above, with most falling into issues of culture-based detection or generalizability of data in real-world systems. The use of BCYE agar plates to detect culturable *L. pneumophila* is limited by a relatively high detection limit (10 CFU mL^{-1}) and the potential to underrepresent viable but non-culturable (VBNC) cells. Starting with the detection limit, it is possible that at high doses having removed 3 or 4 log_{10} cells, a small number of persistent cells may remain. Under this circumstance, the inactivation regime would select for resistance strains that could then propagate when provided nutrients or growth factors. The occurrence of VBNC cells is the focus of Chapter 4, however it can be mentioned that this physiological state is induced in response to environmental stress/challenges. Therefore, VBNC cells can lose their culturability following exposure to disinfection, yet can remain viable, possessing the potential to reproduce and propagate under appropriate conditions. These are the main concerns with regard to culture-based detection of inactivated *L. pneumophila* cells.

In the introductory paragraphs of this chapter it was noted that the *in-vitro* analysis is the initial phase in examining the efficacy of a potential inactivation regimes.[43] These studies are important for evaluating effective doses and comparing between strains and species of microorganisms that may be encountered in real-world systems. However, as discussed above *in-vitro* experiments are limited in their generalizability for real-world systems. An important factor that is difficult to replicate in lab settings is the interaction of *L. pneumophila* with free-living protozoa and the biofilm environment. Furthermore, chemical disinfection *in-vitro* and premise plumbing differ in the presence of organic molecules that can consume residual disinfectants.[66]

Finally, the conditions with which *L. pneumophila* are grown will play a role in the resistance to disinfectants. It has been shown that *L. pneumophila* grown in broth is more susceptible to inactivation compared to the same strain in tap water.[66]

The initial stage in evaluating the efficacy of disinfectants on *L. pneumophila* is to perform *in-vitro* analyses to establish dose-response models. The inactivation kinetics revealed within this stage of experiment is important for QMRA and risk modelling, as it provides a simple system free from biofilms and free-living protozoa that are common in premise plumbing. It is the goal of this research to help provide more effective measures for reducing risk of legionellosis outbreaks by finding an effective inactivation regime. The main points of this chapter should be that 268.6 nm UV-C, 60 °C water, and 2.2 mg L^{-1} monochloramine can all effectively achieve a 4 $\log_{10}$ reduction in culturable cells, with monochloramine being more effective against *L. pneumophila*, relative to the other regimes tested on multiple microorganisms. Ensuring that the loss of culturability equals loss of viability is the focus of Chapter 4. Future goals could involve the addition of a free chlorine dose-response model that could then be compared to monochloramine.

3 Role of free-living amoebae in supporting pathogenic *Legionella pneumophila*

3.1 Abstract

The complex interactions between free-living amoeba (FLA) and *L. pneumophila* are examined in this chapter; as they have important impacts on pathogen disinfection, detection, and opportunistic pathogen human infectivity. Using co-culture experiments with *Acanthamoeba polyphaga* and *Willaertia magna* as FLA hosts, conditions were optimized for *L. pneumophila* infection and to ensure that measured bacteria were intracellular derived. Finally, *W. magna* vesicles were analyzed using FCM to resolve between trophozoites, cysts, and extracellular bacteria.

The co-culture experiments revealed differential interactions with *L. pneumophila* and the two FLA tested, causing cytotoxicity and growth inhibition in *A. polyphaga*, whereas no apparent effect on *W. magna*. This finding is significant, as it suggests a pathway through which *W. magna* escapes the lethal effects of *L. pneumophila* infection. The effects of FLA on *L. pneumophila* growth were also studied, with a temporary lag phase appearing for *W. magna* co-cultures, whereas no growth lag was observed with *A. polyphaga*. This observation may explain the reduced pathogenicity of *L. pneumophila* to *W. magna*, compared with many other FLA. In particular, pathogenic legionellae were isolated within vacuoles and expelled as membrane enclosed vesicles from *W. magna*. Intracellular bacterial growth was verified by fluorescent microscopy, with co-culture methods being optimized to limit extracellular growth in PYG medium. An isolation protocol for *W. magna* vesicles containing GFP *L. pneumophila* was tested using FCM, however no distinct sub-population was observed, potentially due to rupturing of membranes.

Water-based opportunistic pathogens like *L. pneumophila* rely on complex interactions with protozoa and aquatic biofilms for protection, feeding, and growth. This chapter illustrates the differential response of two FLA hosts to *L. pneumophila* infection, highlighting the potential impacts on subsequent human pathogenicity. It has been shown that within a few days of co-culture, *L. pneumophila* can grow above critical action levels and may exist in densely packed vesicles expelled from FLA hosts, increasing the risk of opportunistic infections with respect to human lung macrophages. Thus, it is recommended that routine scanning for FLA be considered, based on the likely prerequisite for amoeba-host growth amplification to critical action levels and heightened virulence prior to aerosolization and human exposures.

3.2 Introduction

Water-based opportunistic pathogens like *L. pneumophila* rely on complex interactions with free-living amoeba (FLA), as well as other protozoa and aquatic biofilms for protection, feeding, and growth. These relationships can become problematic when residual disinfectant levels drop within drinking water systems, usually near the tap or outlet, as infectious *L. pneumophila* reach critical concentrations through propagation in these favourable growth environments.[57] There is evidence to suggest that the evolutionary interactions between protozoa and *L. pneumophila* have resulted in opportunistic human pathogenicity with respect to human macrophages as accidental end hosts.[6] Hence, this chapter examines important aspects related to where *L. pneumophila* grow in engineered water systems, and how these relationships influence the detection, disinfection, and potential human pathogencity of *L. pneumophila*.

3.2.1 Ecology of *Legionella pneumophila* and FLA hosts

L. pneumophila is present within aquatic biofilms, both in natural settings and engineered water systems.[7] Major exposure sources include premise plumbing, cooling towers, and hot tubs, where warm water can stagnate, allowing *L. pneumophila* to amplify within biofilms and free-

living protozoa.[75, 76] The preferred temperature range for *L. pneumophila* falls between 25 − 42 °C, with optimal growth, motility, and virulence assumed at 37 °C.[77] Incidence rates of LD correlate with environmental temperature, humidity, and precipitation, with peak observations between July and September in the U.S. and Western Europe.[16] Colonization of hot water systems with legionellae correlates with increased Mn concentration and heterotrophic plate counts (HPC), with Cu concentration and high temperatures (>55 °C) suppressing growth.[76] Plumbing material has been shown to differentially influence *L. pneumophila* growth, thought to involve favourability of biofilm formation, with PEX supporting more biofilm growth than copper or stainless steel.[78] However, evidence is mounting for Cu-based biofilms supporting a microbiome more supportive of *L. pneumophila* growth than PEX or PVC.[79] Furthermore, the biofilm-amoeba environment provides legionellae a competitive edge, with respect to accessible nutrients, reduced disinfectant residuals and low risk of predation.[3, 5] Recent evidence has suggested that *L. pneumophila* must be phagocytized by free-living protozoa in order to proliferate within drinking water distribution systems, with most evidence for FLA serving as a predictor of *Legionella* spp. colonization.[45]

Like *L. pneumophila*, FLA occupy diverse settings in nature and have been found to colonize various engineered water systems, including water treatment plants, cooling towers, air conditioners, and drinking water distribution systems.[80] Examination of exposure sources following legionellosis cases often reveal the presence of both *L. pneumophila* and FLA, with the latter being a known supporter of intracellular replication of the former.[77] In general, this has led to FLA being regarded as Trojan horses of the microbial world, due to their ability to form endosymbiotic to parasitic relationships with a variety of intracellular viruses, fungi, and

bacteria, some of which are human pathogens.[81] In this chapter the focus is on the interactions of *L. pneumophila* with two known FLA hosts *Acanthamoeba polyphaga* and *Willaertia magna*.

Members of the *Acanthamoeba* genus are ubiquitous within aquatic biofilms and have been associated with the transmission of human diseases, including legionellosis and viral pneumonia.[3] In addition, *A. polyphaga* has been shown to be an opportunistic water-based pathogen itself, causing keratitis among contact lens users as well as some cases of granulomatous encephalitis.[82] The life cycle of *A. polyphaga*, like most FLA, consists of a trophozoite stage characterized by feeding and replication, as well as a cyst stage, in which the organism becomes dormant with low metabolic activity, yet can remain viable for years.[83] The morphological differences between a FLA trophozoite and cyst are shown in Figure 3.1.

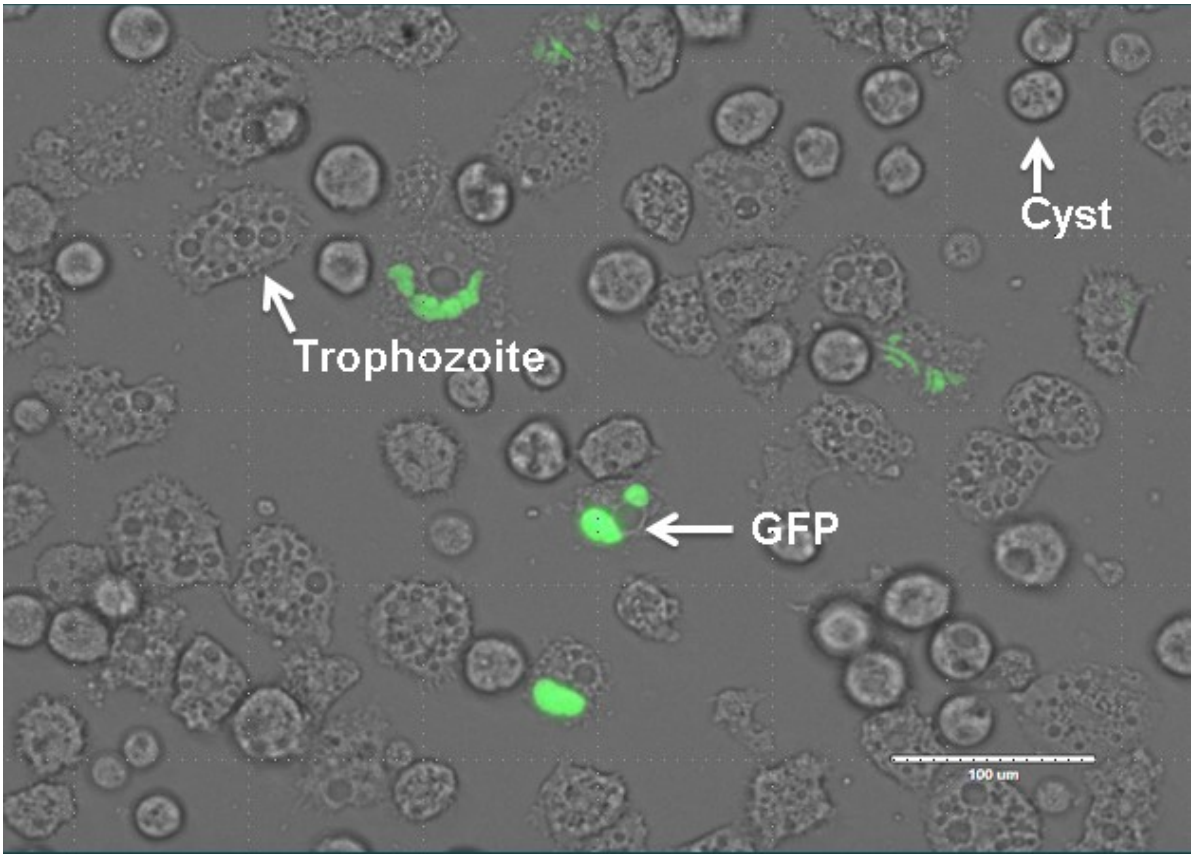

Figure 3.1 Trophozoite and cyst stages of *A. polyphaga* life cycle taken 2 d post infection with GFP labeled *L. pneumophila*. Note the thick outer wall and circular shape of the cyst compared to the free moving, metabolically active trophozoite.

The majority of studies examining the interaction between *L. pneumophila* and their FLA hosts have relied on *Acanthamoeba* spp. and *Vermamoeba vermiformis* (previously *Hartmanella vermiformis*), which show cytotoxic/pathogenic effects on the amoebae within a few days of infection. Recently, a novel response has been reported, in which *W. magna* was found to resist the growth inhibition and cytotoxicity commonly observed during internalization of *L. pneumophila* for up to 7 d post infection.[84] There are few studies on *W. magna* - *L. pneumophila* interactions, with one report indicating the prevalence of both organisms within aquatic floating biofilms.[85] Currently, it is thought that its resistance to *L. pneumophila* infection involves the blocking of bacterial-induced lysis as well as a reduction of intracellular replication, possibly through the expulsion of undigested *L. pneumophila* rich vesicles.[84] Hence, the interactions of legionellae and *W. magna* vesicles was also examined to fill this knowledge gap.

3.2.2 Implications of FLA – *L. pneumophila* relationship in pathogen detection

The close relationship between *L. pneumophila* and their FLA hosts, particularly *A. polyphaga*, make both organisms key targets for detection platforms aimed at monitoring and controlling water-based pathogens. The relationship can be modified for the purpose of isolating and enriching viable *L. pneumophila* within FLA, initially derived from clinical samples.[86] In addition, FLA can be used to resuscitate viable but non-culturable (VBNC) *L. pneumophila* to prove the bacteria remains alive and infectious (Chapter 4).[87] Furthermore, calculations of *L. pneumophila* density within *A. polyphaga* have approximated average value of <300 bacteria, with a maximum of <1,300.[88] These studies illustrate a major concern with current detection of *L. pneumophila* which may not account for FLA internalized bacteria, leading to underestimation of VBNC cell counts and their associated public health risks.

The dual threat of *A. polyphaga* as a facilitator of bacterial and viral infections, as well as its own human pathogenicity has prompted research into potential detection platforms for both

clinical and environmental assays, with the focus on IMS-FCM compatible procedures (as used for *L. pneumophila,* Chapter 1). To date, there are few reports of successful *Acanthamoeba* spp. detection systems, with most research focused on PCR, and the development of specific antibodies or other surface protein/carbohydrate related markers. A promising study reported the generation and isolation of eight monoclonal antibodies following mouse infections with *A. castellani*, with two of the antibodies binding unfixed *A. polyphaga* cysts reliably.[89] Unfortunately, the antibody panel did not effectively bind to trophozoites or environmental samples, making them useful for clinical detection of cysts only.[89] Another study evaluated the specificity of various lectins, which are cell-surface carbohydrate binding proteins, for *A. polyphaga*, based on the knowledge that the amoeba binds corneal mannose glycoproteins prior to infection.[90] This lectin study reported similar carbohydrate exposures in trophozoites and cysts, with *N*-acetylglucosamine being the most abundant; however this type of detection would be prone to non-specific binding associated with environmental matrices non-target microorganisms.[90] Thus, there is a gap in current IMS-FCM compatible detection systems for *A. polyphaga* and *Acanthamoeba* spp.

3.2.3 Implications of FLA – *L. pneumophila* relationship in disinfection

The interactions between FLA, *L. pneumophila*, and aquatic biofilms have major consequences on the efficacy of various disinfection regimes. FLA hosts and the biofilm environment offer protection from disinfectants, reduce the effective concentrations of oxidizing agents, and shield bacteria from lethal doses. The importance of these interactions is highlighted by the need to restrict biofilm formation in order to control legionellae within potable water sources, usually through reduced AOC and low temperatures.[16] As discussed in Chapter 2, the efficacy of disinfection can be overestimated when the role of biofilms and FLA hosts are not

considered. Thus, an appropriate disinfection protocol should result in reduced *L. pneumophila*, FLA, and their biofilm niche, in order to reduce the risk of future re-colonization.

The removal of *L. pneumophila* from premise plumbing is complicated by FLA internalization, which may offer protection from treatment processes and residual disinfectants.[7] *L. pneumophila* found within *A. polyphaga* have also been shown to exhibit greater resistance to physical treatments, such as thermal and UV irradiation.[59, 91] When comparing chlorine efficacy, FLA internalized *L. pneumophila* was only reduced 3 $\log_{10}$ following a 30 min exposure to 0.5 mg L^{-1} free chlorine, whereas an axenic culture exhibited a 5 $\log_{10}$ reduction following a 6 min exposure.[57] Another study on monochloramine treatments revealed that in the presence of *V. vermiformis* and biofilm, disinfection efficacy was reduced from 2 $\log_{10}$ to 0.7 $\log_{10}$ for 180 min exposures at 0.5 mg L^{-1}, compared with freely suspended bacteria.[92] These examples highlight the protective nature of FLA hosts at limiting the effects of common disinfection treatments on *L. pneumophila*.

In addition to the reduced disinfection efficacy when *L. pneumophila* resides within FLA, public health officials must also concern themselves with inactivating the hosts such that re-colonization risk is lowered, (i.e. also reducing FLA cyst entry into DWDS and growth of trophozoites). Following various disinfection regimes including free chlorine, monochloramine, or copper-silver ionization, FLA were shown to resist treatment and serve as reservoirs for *L. pneumophila* protection and propagation.[93] *A. polyphaga* cysts are resistant to many disinfectants and high temperatures, allowing for internalized bacteria to persist in treated water systems.[81] A study reported viable *A. polyphaga* cysts were recovered after treatment with 100 mg L^{-1} chlorine for 10 minutes, suggesting current measures are inadequate for controlling amoebae bound legionellae.[59]

3.2.4 Implications of FLA – *L. pneumophila* relationship to public health

While FLA are the natural hosts for *L. pneumophila*, the bacteria exhibits opportunistic pathogenicity (accidently, as a dead-end host) with respect to human macrophages, ultimately leading to incidence of LD.[6] Of note, the intracellular infection pathways for protozoa and human cells are strikingly similar, with *L. pneumophila* evading lysosomal fusion, propagating, and eventually overwhelming the host.[94] The similarity is thought to arise as a consequence of the evolutionary relationship between *L. pneumophila* and its FLA hosts, which has led to the acquisition and expression of genes conferring resistance to mammalian phagocytes, such as lung macrophages (Figure 3.2).[6]

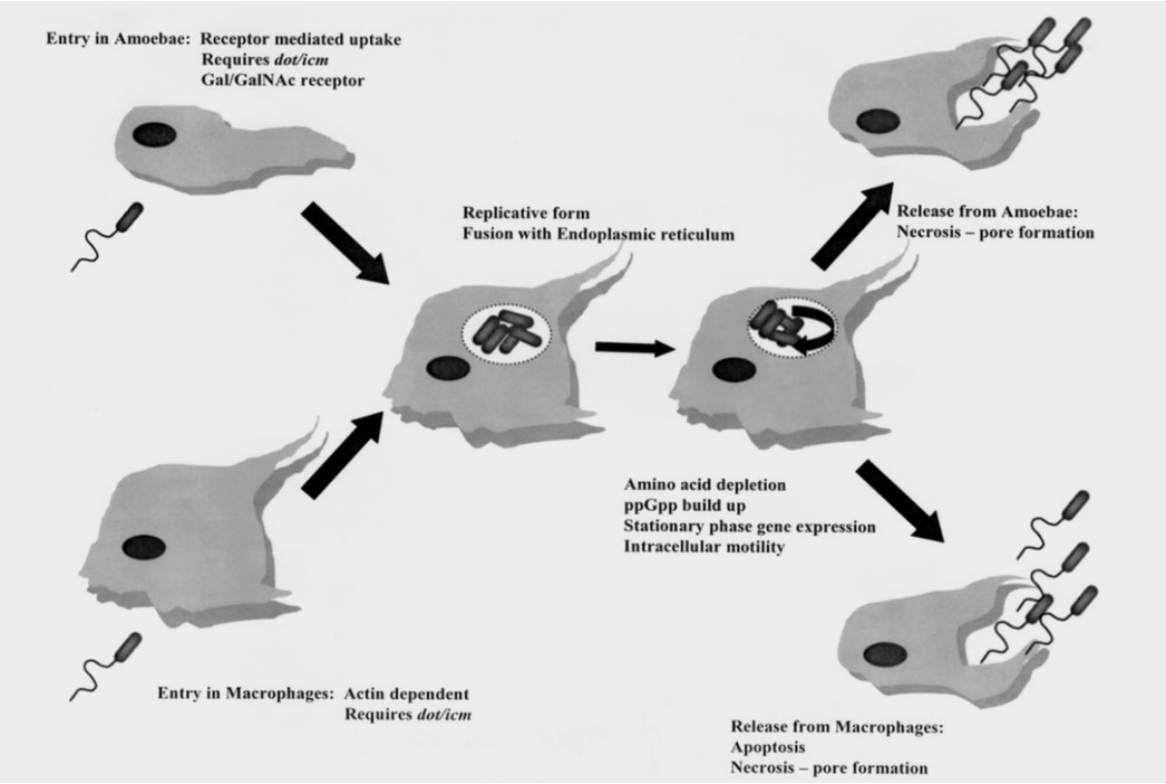

Figure 3.2 The uptake of *Legionella pneumophila* by free-living amoeba (top) by coiling phagocytosis, compared to the interaction of the bacteria with human lung macrophages (bottom). It is hypothesized that the strong relationship between *L. pneumophila* and FLA may have selected for genes conferring resistance to human lung macrophages (Fields *et al*,. 2002).[6]

This theory is supported by the lack of reports of disease transmission from infected individuals, with all confirmed cases occurring via primary exposure to source (one possible

exception to date). Furthermore, the relationship of *L. pneumophila* with its amoeba hosts has unique features compared to a similar group of saprozoic pathogens, the non-tuberculous mycobacteria, such as its ability to survive encystation of *Acanthamoeba* spp., residing within the core of the cysts.[95] In contrast, most ingested organisms adhere to the outer wall of the double-walled cysts, effectively killing these pathogens.[83] Additionally, there is evidence that suggests cysts can become airborne, creating a situation in which *Acanthamoeba* spp. acts as both the biological host and the vector for transmission of *L. pneumophila*.[81]

Thus, the interactions of FLA and *L. pneumophila* have resulted in the bacterium becoming an opportunistic human pathogen, with the greatest risk of exposure occurring within shared environments (often engineered water systems) that offer disinfection protection, relatively rich nutrient niches, reduced competition, and aerosol generation (as the primary exposure route). The ubiquity of FLA within engineered water systems and their associations with human pathogens like *L. pneumophila*, requires that any research project examining water-based bacterial pathogens include considerations of the role of FLA. Therefore, this chapter addresses the interactions between two FLA hosts and *L. pneumophila*, to fill current knowledge gaps and enable improved public health risk communication resulting a better understand of these associations.

3.3 Materials and Methods

3.3.1 Cultivation and counting free-living amoebae

Acanthamoeba polyphaga (ATCC 30461) was grown in 712 PYG medium (20 g proteose peptone, 1 g yeast extract, 0.985 g magnesium sulphate heptahydrate, 0.0588 g calcium chloride, 1.0 g sodium citrate, 0.0196 g ammonium iron(II) sulphate hexahydrate, 0.67 g sodium phosphate dibasic heptahydrate, 0.34 g potassium phosphate monobasic, and 18 g of glucose per litre).[57] The amoebae cultures were grown for 4 d at 25 °C before sub-culturing by adding 200

µL of the culture into 5 mL of fresh PYG. *Willaertia magna* strain Z503 (ATCC 50035) was grown in SCGYEM medium (10 g isoelectric casein, 2.5 g glucose, 5 g yeast extract, 1.325 g sodium phosphate monobasic, 0.8 g potassium phosphate dibasic, and 100 mL heat inactivated fetal bovine serum per litre).[84] The amoebae cultures were grown for 4 d at 25 °C before sub-culturing by adding 200 µL of the culture into 5 mL of fresh SCGYEM. Amoebae were harvested after 2 d of growth and counted on a Bright-Line metallized hemacytometer (Hausser Scientific, Horsham, PA, USA) with the assistance of a Primovert inverted microscope (Zeiss, Oberkochen, Germany) at 10x magnification. A positive identification for amoeba was counted for both the trophozoite and cyst morphologies. The cultures were then diluted to 10^5 amoeba mL^{-1} in PYG medium prior to use in experiments.

3.3.2 Amoeba co-culture

Axenic cultures of *A. polyphaga* and *W. magna* were counted and diluted down to 10^5 amoebae mL^{-1} in 5 mL PYG, separately. At the same time, approximately 10^9 CFU mL^{-1} *L. pneumophila* was prepared in 0.85% saline solution by measuring OD_{600}, against a previously calibrated CFU count. A 25 µL aliquot of the *L. pneumophila* suspension was added to the centrifuge tubes containing FLA, establishing a 50:1 multiplicity of infectivity (MOI) ratio. The mixture was then centrifuged at 1000 rpm for 8 min to ensure sufficient contact between bacteria and amoebae. The infected amoebae were vortexed and 1 mL suspension were added into a 24 well, which was placed into an incubator at 37 °C, with dark conditions, for the duration of the experiment. Interactions were examined by recording the total number of amoebae visible, as well as the number of culturable *L. pneumophila* for 0, 1, 2, 3, and 4 d post-infection. The morphological status of the amoebae (trophozoite or cyst) were examined using an EVOS® FL Auto Cell Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) at 20x magnification. In order to determine the number of culturable *L. pneumophila,* the contents of a

well were vigorously agitated using a 3 mL syringe with a 20-G needle to lyse amoeba, releasing intracellular bacteria, in accordance with standard protocols.[41] The well contents were then serially diluted, plated on BCYE agar plates and incubated for 72 h at 37 °C.

3.3.3 Verifying and quantifying intracellular growth

Microscopy and a series of control experiments were used to verify that *L. pneumophila* was internalized within FLA hosts, and that measured concentrations were accurate. The lab was fortunate to obtain two GFP-labeled *L. pneumophila* (ATCC 33152) strains, one with GFP carrying plasmid, and one with chromosomally-expressed GFP *Legionella pneumophila* obtained from Dr. Karen Brassinga's lab (University of Manitoba, Winnipeg, Canada). Over the course of the 4 d co-culture experiments, the internalization of GFP *L. pneumophila* was monitored using the EVOS fluorescent microscope described above, with 20x magnification with MOIs of 100. In order to determine the amount of bacteria residing within a FLA, gentamicin wash steps were used to remove free-floating *L. pneumophila*. At 2 h post infection, a stock solution of 50 mg mL^{-1} gentamicin sulphate was diluted to a final concentration of 100 µg mL^{-1} in 5 mL of the co-culture. After a brief vortex the suspension was incubated at 37 °C for 2 h, at which point the suspension was centrifuged at 1000 rpm for 8 min to pellet amoebae and internalized *L. pneumophila*. Three wash steps were used to remove gentamicin, with the pellet being resuspended in 5 mL PYG.[96] Co-cultures were grown for 4 d at 37 °C, with samples being plated onto BCYE agar every 24 hours, to compare against non-gentamicin treated cells. To ensure optimal growth conditions, various control parameters were tested, including temperature (25 or 37 °C) and media (PYG, SCGYEM, AYE), with no major differences observed for temperature and reduced *L. pneumophila* growth in SCGYEM and AYE broths (data not shown).

3.3.4 *W. magna* vesicle detection

As part of a colleague's research project (PhD student, Md. Shaheen), it was discovered that *W. magna* can release vesicles containing high concentrations of *L. pneumophila* (visible with GFP labels) when co-cultured in nutrient limiting conditions (dH_2O) with an MOI of 100. Based on the relative size and high fluorescent character of the vesicles, it was hypothesized that they could be detected by FCM and separated by fluorescent-activated cell sorting (FACS). The first attempt at analyzing the vesicles used the Gallios™ flow cytometer to measure FL-1 GFP fluorescence and forward scatted (FSC) parameters. The reasoning for this distinct gating region was the size differences of *W. magna* trophozoites, cysts, and vesicles, along with the high proportion of GFP-expressing cells expected within the vesicles. The samples were also run on the Fortessa X-20 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) at the Medical Microbiology and Immunology flow core at the University of Alberta.

3.4 Results and Discussion

3.4.1 Interactions between FLA and *L. pneumophila*

The complex associations of *A. polyphaga* and *W. magna* with *L. pneumophila* were examined through co-culture experiments, with total amoebae and bacteria concentrations being recorded daily. The morphological changes in amoeba over the experiments are shown in the Appendix (Figures A3.1 and A3.2), with examples of the effects illustrated for *L. pneumophila* on *A. polyphaga* (Figure 3.3) and *W. magna* (Figure 3.4).

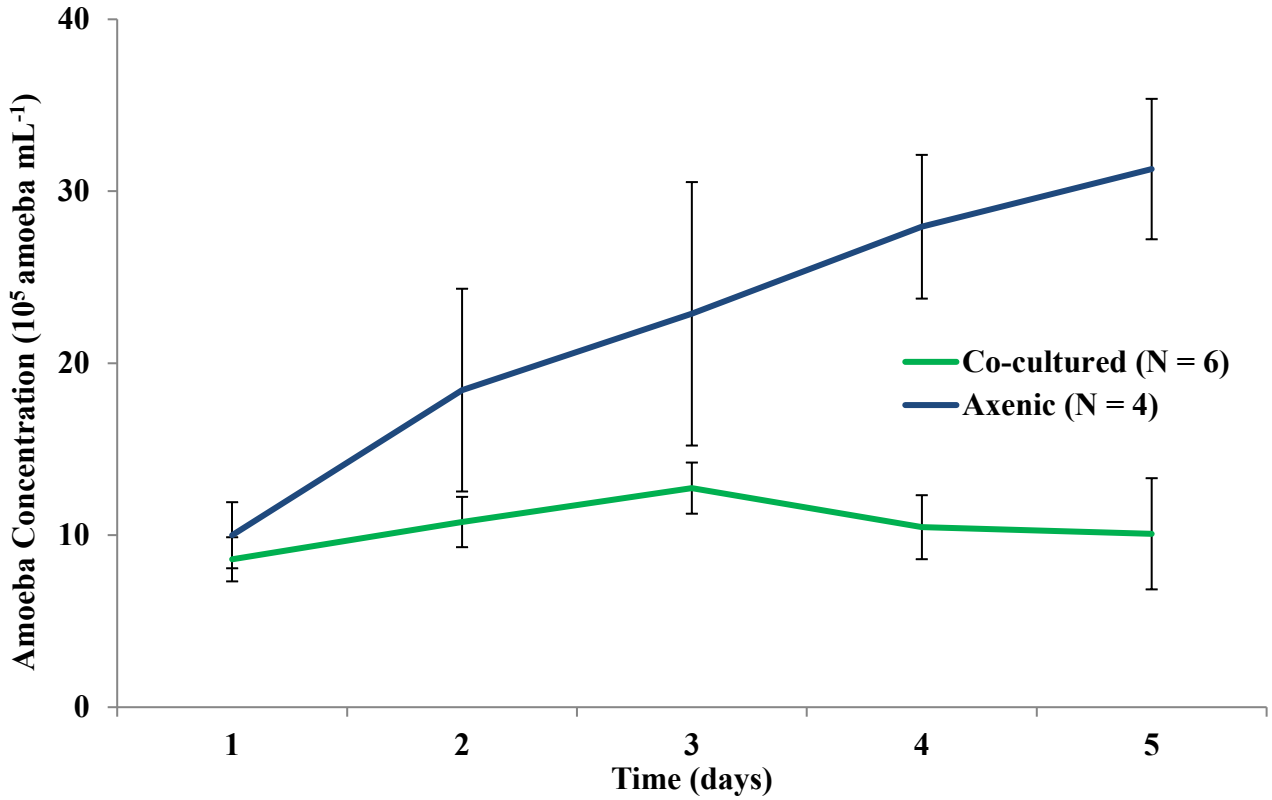

Figure 3.3 Change in *A. polyphaga* growth over 4 d co-culture with *L. pneumophila* compared with an axenic culture, measured by counting total amoeba (trophozoites and cysts) using a hemacytometer. Error bars equivalent to ± SEM for N replicate, independent trials.

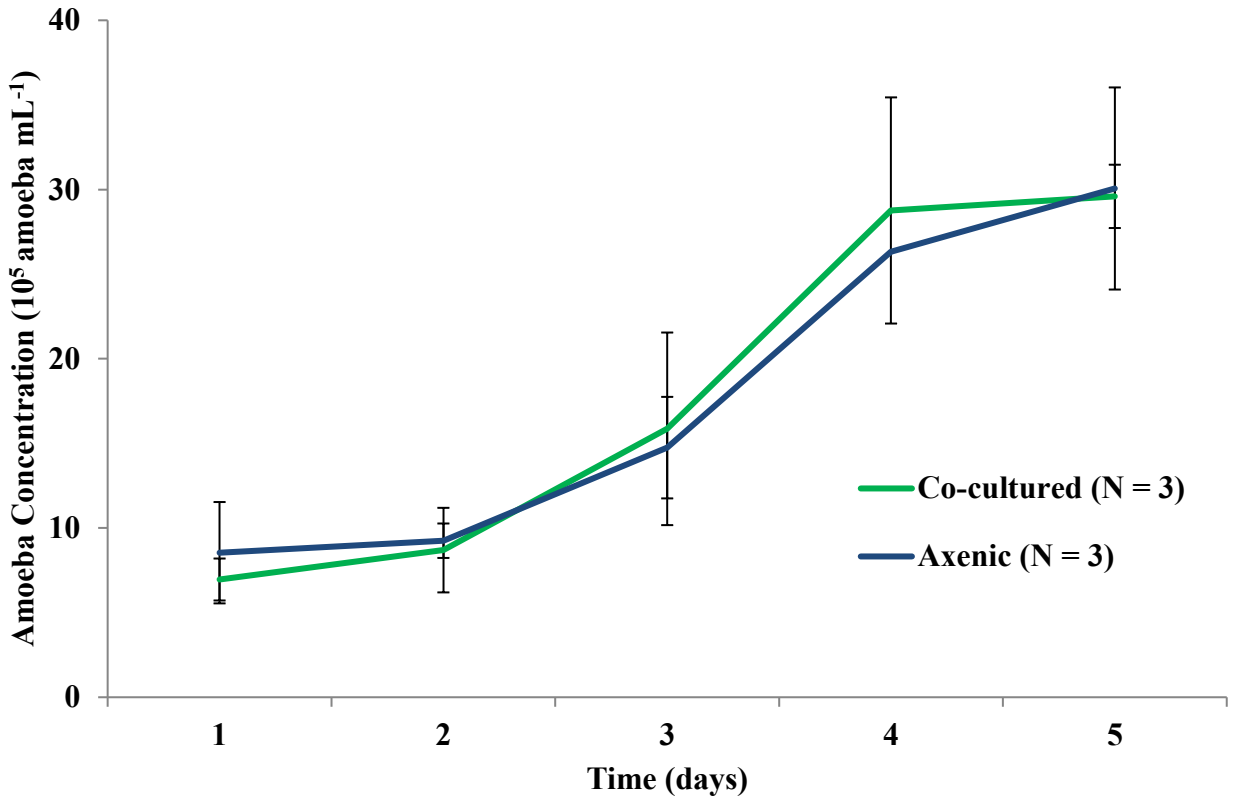

Figure 3.4 Change in *W. magna* growth over 4 d co-culture with *L. pneumophila* compared with an axenic culture, measured by counting total amoeba (trophozoites and cysts) using a hemacytometer. Error bars equivalent to ± SEM for N replicate, independent trials.

The two plots showing the effects of *L. pneumophila* on *A. polyphaga* and *W. magna* illustrate differential impacts on growth between the two FLA tested. When *A. polyphaga* is exposed to *L. pneumophila* (Figure 3.3), growth was limited in comparison to an axenic culture, which increased 3-fold over the 4 d co-culture. In contrast, the *W. magna* plot (Figure 3.4) shows negligible differences between axenic and co-culture samples, with both increasing 3-fold over the 4 d experiment. From these observations, it is clear that *L. pneumophila* affects the growth of *A. polyphaga*, while *W. magna* growth does not appear to change with the addition of the opportunistic bacterial pathogen. The results reported in the current study are consistent with previous reports, where co-culture experiments with a MOI of 50 resulted in a slight reduction in *A. polyphaga* levels over 4 d and a 4-fold increase in *W. magna* concentration within the same

period.[84] As mentioned above, *W. magna* is different in that this host has been shown to resist growth inhibition and cytotoxicity commonly observed during internalization of *L. pneumophila* for up to 7 d post infection.[84] This is contrasted by the apparent growth inhibition observed for *A. polyphaga* (Figure 3.3) and the relative change in morphology from feeding trophozoites to cysts (shown in Appendix). Hence, further examination of the differential interactions of the two FLA (*A. polyphaga* and *W. magna*) with *L. pneumophila* may provide evidence towards a specific mechanism through which the latter can avoid the pathogenic effects of an infection. Evidence from a colleague's research project suggests that the evasion may involve the expulsion of *L. pneumophila* in the form of vesicles (discussed in § 3.4.3).

In addition to the study of *L. pneumophila* infection on FLA growth, the co-cultures were also plated on BCYE agar each day to provide an indication of *L. pneumophila* growth preference among the two FLA tested (Figure 3.5).

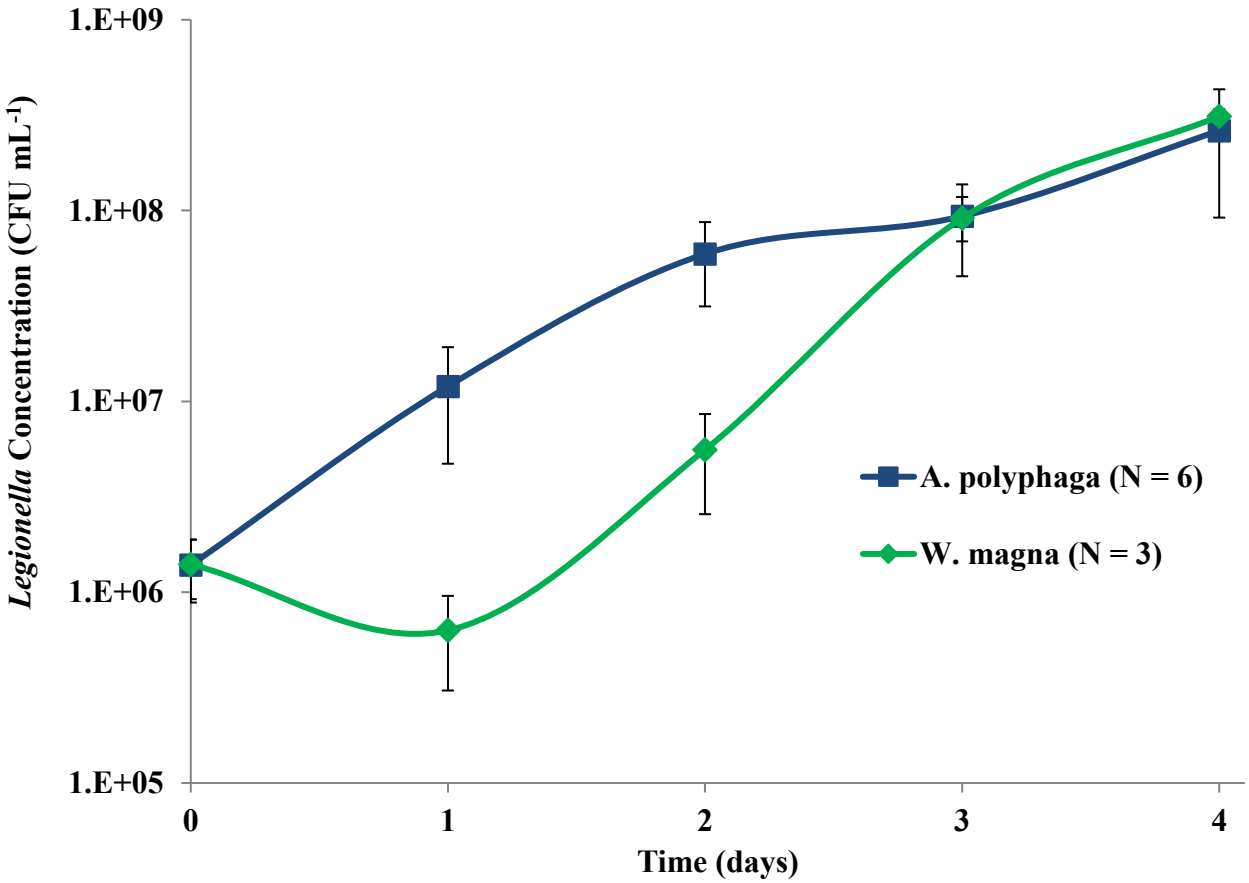

Figure 3.5 Change in *L. pneumophila* growth over 4 d co-culture with either *A. polyphaga* or *W. magna*, measured by plating on BCYE agar following brief dispersion with syringe and 20G needle. Error bars equivalent to ± SEM for N replicate, independent trials.

From the interaction graph (Figure 3.5) there is an apparent lag in *L. pneumophila* growth when co-cultured with *W. magna*, compared with *A. polyphaga*. The lag appears to be temporary, with both co-cultures reaching >10^8 CFU *L. pneumophila* within 4 d of infection. The deviation between the two experiments may arise from selective packaging of internalized bacteria within *W. magna* within the first 24 h post-infection. This observation may explain the reduced cytotoxicity of *L. pneumophila* to *W. magna*, compared with other FLA described, in which bacteria may be partitioned within the amoeba such that when a vacuole is filled, it can be expelled.[97] This theory is supported by literature, where a similar experiment revealed few internalized bacteria at the 36 h mark, suggesting that *L. pneumophila* is efficiently removed

from *W. magna* following ingestion.[84] However, the result of the current experiment differ with literature with respect to the duration of the lag phase, with literature reporting a 2-3 d lag, versus 1 d lag in the current study. This may be attributed to different bacterial strains or the use of SCGYEM in the literature study, which is better for supporting *W. magna* growth and may limit the ingestion of *L. pneumophila* compared to a less favourable medium like PYG. Finally, the current experiment highlights the risk posed by the interactions of *L. pneumophila* with FLA hosts and the aquatic biofilm, as the 4 d incubation resulted in rapid propagation of bacteria above risk assessment action levels.[74] Thus a main takeaway from this data set is the 3 $\log_{10}$ increase in *L. pneumophila* that occurs within a short period of time resulting from interactions with FLA hosts.

3.4.2 Intracellular *L. pneumophila* detection

To verify that the amoeba co-culture experiments were successfully measuring intracellular bacterial replication, GFP labels were used to observe *L. pneumophila* growth within FLA using fluorescent microscopy (Figure 3.6).

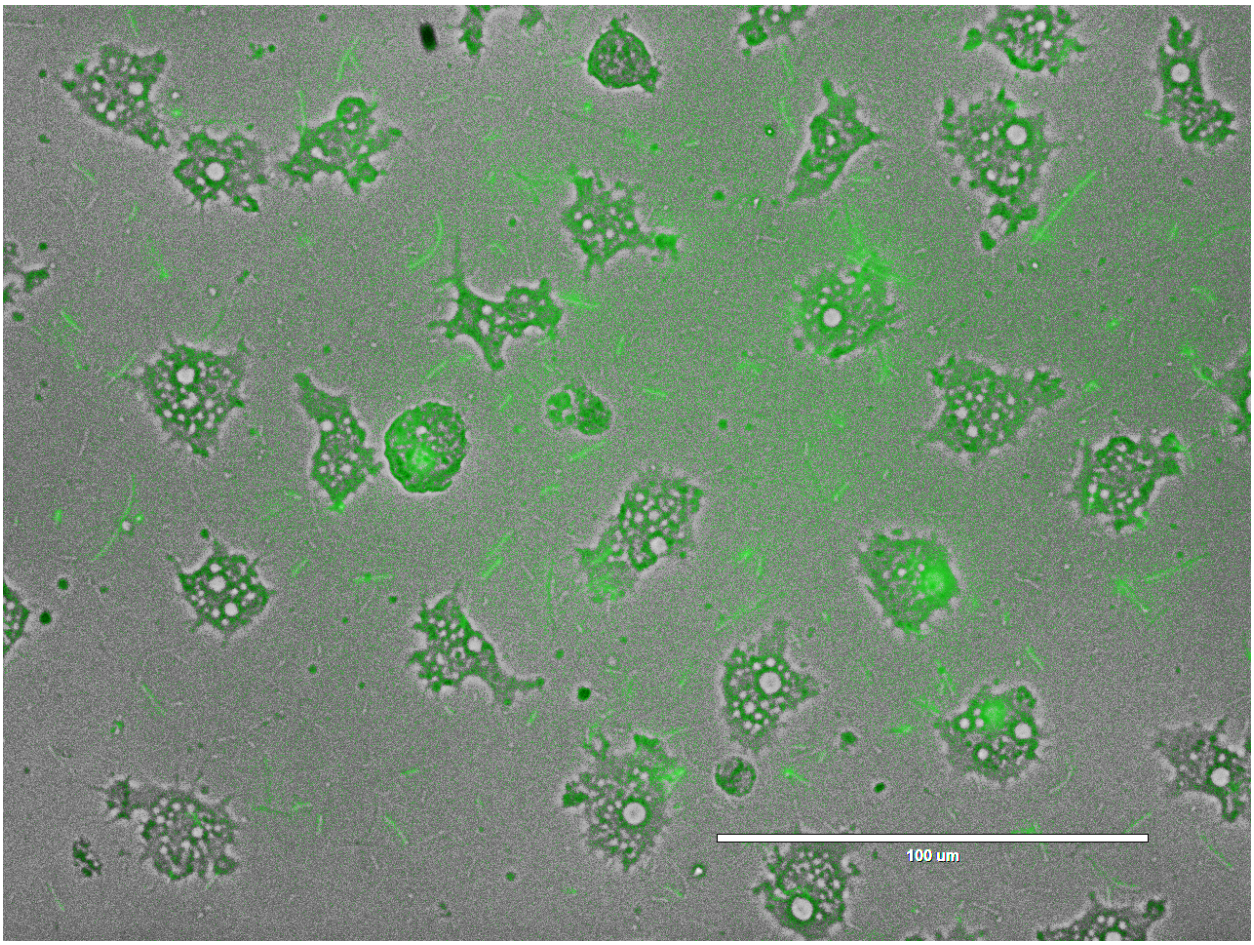

Figure 3.6 Verification of *L. pneumophila* internalization in *A. polyphaga* at 24 h after infection with an MOI of 100, image obtained using fluorescent microscopy with the chromosomally labeled GFP courtesy of the Karen Brassinga laboratory. Note that there are non-internalized GFP *L. pneumophila*, as no gentamicin washes were employed.

The amoeba co-culture image (Figure 3.6) clearly shows pockets of bright green, internalized *L. pneumophila* within *A. polyphaga*, within 24 h of mixing. The packaging of bacteria into specific regions of the amoeba involves phagosomes, which may recruit other cytoplasmic organelles including lysosomes, mitochondria, and the endoplasmic reticulum shortly after infection.[94] The verification of intracellular growth image (Figure 3.6) also shows a large population of extracellular *L. pneumophila* residing within the liquid medium. There are two main explanations for this, with the first being a higher than usual MOI being used for the purposes of more rapid visualization of phagosomes. Secondly, no gentamicin washes were used to rid the sample of extracellular bacteria within 2 h of infection. The MOI used for this image

was 2-fold greater than that used for the interaction studies and 10-fold greater than that used for VBNC analyses (Chapter 4). The differences between an MOI of 100 versus an MOI of 10 can be visualized in Figures 3.6 and 3.1, respectively. With a lower MOI, there are less extracellular bacteria present at the 2 d mark. In addition, the two images differ in the type of GFP *L. pneumophila* used, with Figure 3.6 using the chromosomal-label, which through anecdotal evidence generates more rigid cell chains compared with the plasmid-GFP *L. pneumophila* (shown in Figure 3.1), potentially making it harder to ingest by the amoeba.

A series of control experiments were run to ensure optimal co-culture performance and limit extracellular bacterial growth. The main parameters tested for optimization were temperature, medium, and presence/absence of gentamicin. The two temperatures tested, 25 and 37 °C are reported as optimal for *A. polyphaga* and *L. pneumophila*, respectively; with some research suggesting that bacterial growth should be measured under ideal amoebae conditions to provide a true measure of pathogenicity.[58] The control at 25 °C revealed negligible differences in both bacterial performance and amoebae growth inhibition (not shown). Similarly, the addition of gentamicin washes had minimal impact on *L. pneumophila* replication, as the PYG medium selected for the co-culture experiments conferred negligible bacterial growth compared to AYE and SCGYEM (not shown). The optimal medium was chosen based on ability to support amoeba and the internalization of bacteria, while limiting extracellular proliferation. Hence, the AYE did not fit the criteria, as it is the preferred artificial growth medium of *L. pneumophila*, while the SCGYEM also supported some extracellular growth.

3.4.3 Isolation of *W. magna* vesicles containing *L. pneumophila*

The release of vesicles from *W. magna* containing *L. pneumophila* was monitored using chromosomally expressed GFP in the bacteria. The quick method for vesicle release involved an MOI of 100 and dH_2O to stimulate feeding (Figure 3.7).

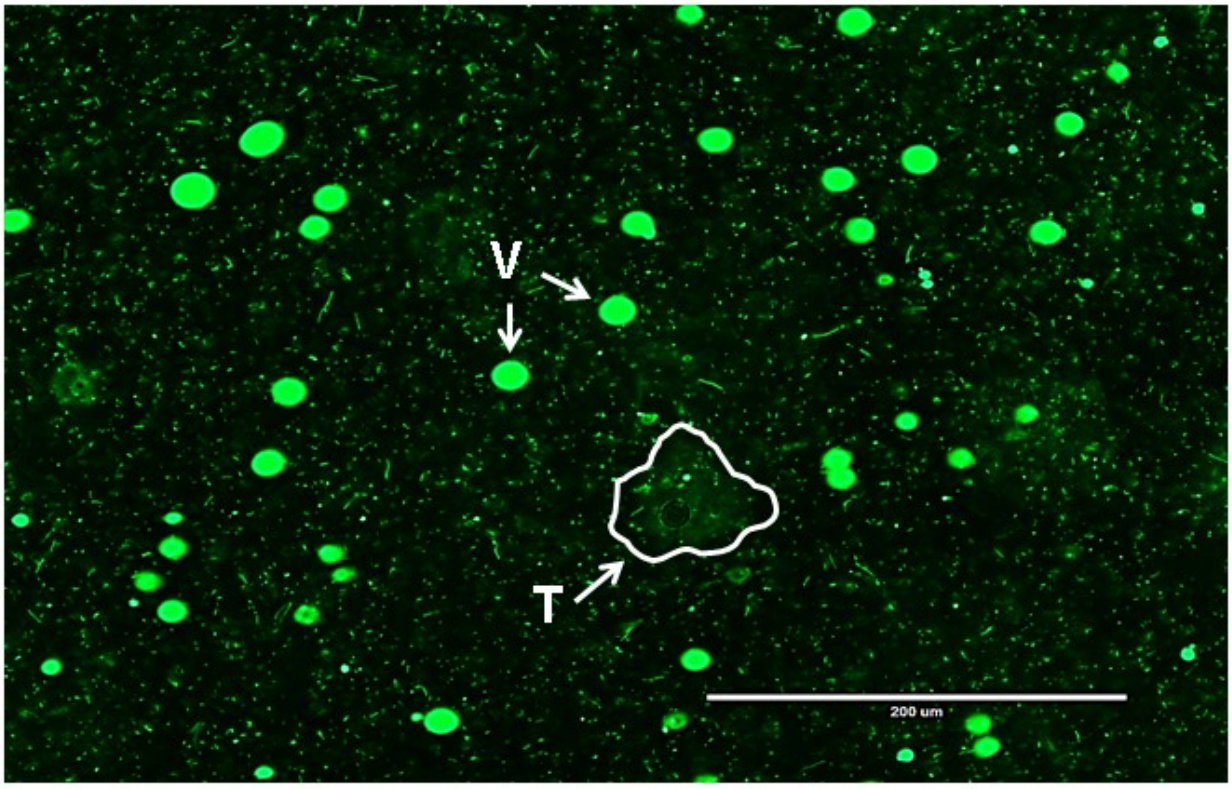

Figure 3.7 Vesicle release from *W. magna* within 24 h of ingestion of *L. pneumophila* with MOI 100 in dH_2O, visualized using fluorescent microscopy and chromosomal-labeled GFP bacteria courtesy of Dr. Karen Brassinga. Note the size difference between vesicles denoted as (V) and *W. magna* trophozoites (T).

Following detection of *W. magna* vesicles containing *L. pneumophila*, attempts were made to separate them from the amoebae and free floating bacteria. Based on the size differences between vesicles, trophozoites, cysts, and bacteria, it was hypothesized that flow cytometry may be used to gate the desired population using FSC and FL-1 scatterplots. The FCM data is included in the Appendix (Figure A3.3); however no distinct sub-populations were observed when the samples were run on either the Gallios or Fortessa instruments available. In addition to running the vesicles on multiple FCMs, the flow cytometry specialist from the University of Alberta, Faculty of Medicine and Dentistry, Flow core manipulated various voltage and gain parameters in an attempt to visualize the population. The inability to detect the vesicles may involve rupturing of the delicate membranes upon entering the FCM sheath fluid and orifice

adjacent to laser light interrogation. It should be noted that previous attempts to visualize stained FLA trophozoites and cysts also failed to resolve between sub-populations, which is surprising considering FCM was designed for measuring blood cells of similar size ranges. In deliberations over the failure to resolve vesicles by FCM, other techniques were considered, specifically density gradient centrifugation using Ficoll, however due to time constraints, no separation tests were run. Concern remains over the impact of centrifugal forces on the thinly enclosed vesicles. Thus, it is a major focus of this lab group to find a method for isolation of intact vesicles to better understand the *W. magna* response to *L. pneumophila*.

As mentioned above, the interactions between FLA and *L. pneumophila* have direct impacts on bacterial disinfection and detection, both key aspects of the current MSc study. The disinfection studies reported in Chapter 2 involved UV, heat, and monochloramine challenges that led to reduced *L. pneumophila* culturability. A scan of relevant literature revealed accounts of each disinfection regime being used to compare axenic *L. pneumophila* with FLA internalized cells (discussed in § 3.2.3). However, there is no data on the protection offered by *W. magna* trophozoites, cysts or vesicles (see Conclusions). For detection systems, an *A. polyphaga* specific monoclonal antibody array was requested from another research institution; but unfortunately the communications did not materialize into tangible materials to test. As well, a lectin-based detection system was considered but never attempted due to concerns about specificity and selectivity of carbohydrate binding domains when dealing with environmental samples containing biofilm derivatives and high microbial loads. With no good options for FLA specific antibodies or lectins for FCM detection, the project was abandoned. Despite the lack of progress, it is recommended that routine scanning for FLA be considered, based on the tight interactions between protozoans and *L. pneumophila* that allow for rapid growth above critical action levels.

In summary, water-based opportunistic pathogens like *L. pneumophila* rely on complex interactions with protozoa and aquatic biofilms for protection, feeding, and growth. This chapter illustrates the differential response of two FLA hosts to *L. pneumophila* infection, highlighting the potential impacts on human pathogenicity. It has been shown that within a few days of co-culture, *L. pneumophila* can grow above critical action levels and may exist in densely packed vesicles expelled from FLA hosts, increasing the risk of opportunistic infections with respect to human lung macrophages. Thus, it is recommended that routine scanning for FLA be considered, based on the tight interactions between protozoans and *L. pneumophila* that allow for rapid growth above critical action levels.

4 Are we managing pathogen risk through disinfection?

4.1 Abstract

The main objective of this chapter is to answer the question posed above in the title regarding the efficacy of disinfection techniques at reducing the risk of inhaling infectious *L. pneumophila* associated with premise plumbing systems. This task is complicated by the induction of the viable but non-culturable (VBNC) state *L. pneumophila* enters in response to environmental stressors (e.g. disinfection processes) and as part of its intracellular life-cycle, making it undetectable by current BCYE culture used in most surveillance systems. Despite restricted metabolic rates with VBNC cells, it has been shown to remain infectious. Hence, experiments were run to evaluate the infectivity of *L. pneumophila* disinfected by UV, heat, and monochloramine treatments, using an amoeba co-culture approach with *Acanthamoeba polyphaga* serving as a susceptible host cell. In addition, a previously reported light-activated DNA repair mechanism was evaluated to provide corrected dose-response data for *L. pneumophila* following exposure to UV-C light. This was followed by an exploratory data analysis with common viability assays to correlate with amoeba infectivity.

The photoreactivation experiment revealed that 268.6 nm UV-C was the most effective wavelength for achieving up to 6 $\log_{10}$ reduction in culturable *L. pneumophila* after accounting for DNA repair mechanisms. The difference in efficacy was observable at higher UV doses, where a 40 mJ cm^{-2} exposure resulted in a complete loss of 10^6 mL^{-1} culturable cells at 268.6 nm, with approximately 10^2 and 10^5 *L. pneumophila* remaining culturable at 256 and 288.6 nm. The amoeba co-culture experiment represents the first evidence for UV-C induced VBNC *L. pneumophila* remaining infectious to a free-living host, *A. polyphaga*. Within 4 d of co-culture, the bacteria reached QMRA action levels, 10^6 CFU mL^{-1}, through amplification in free-living amoeba. The failure to resuscitate *L. pneumophila* exposed to 40 mJ cm^{-2} at 256 nm UV-

C, 60 °C water (2 min), or 2.2 mg L^{-1} monochloramine (15 min) was attributed to low concentrations of viable cells, varying bacterial loads and differing MOIs compared to previous studies reported.

Exploratory data analyses were performed for *L. pneumophila* viability following exposure to UV, heat, or monochloramine disinfection using live-dead, esterase, CTC, and ATP-luminescence based 'viability' assays. For UV disinfection, a small decrease in esterase activity, −6%, at 40 mJ cm^{-2} and a +26% increase in ATP production were observed. For heat disinfection, large reductions were observed in esterase activity (−87%), electron transport function (−55%), and ATP production (−78%), with an increase in membrane permeability. For monochloramine disinfection, reductions in electron transport chain function (−17%,) and ATP production (−23%) were observed. The analyses indicated that culture does not provide an accurate measure of viability, since cells lost ability to be cultured prior to major changes in the activity assays. A collection of viability markers may provide a more representative measure of risk compared to current culture-based detection, since UV-C irradiated *L. pneumophila* lose culturability, yet retain activity, increased ATP production, and the ability to be resuscitated by amoeba co-culture.

While it has been shown that UV (40 mJ cm^{-2}), heat, and monochloramine disinfection can reduce *L. pneumophila* infection risk, as measured by amoeba co-culture infectivity; the results may overstate the efficacy of these treatments. The exploratory data analysis revealed that there was not a complete loss of viability markers for the harshest of disinfections (60 °C for 5 min), suggesting that *L. pneumophila* may possess necessary growth elements and enzyme activity that rule out cell death. Furthermore, *in-vitro* analysis does not account for the protecting/shielding effects of biofilms or free-living protozoan hosts. Thus, it is recommended

that further studies are conducted with biofilm and amoeba present to provide a more generalizable (real-world applicable) indicator of disinfection efficacy and reductions in pathogen risk; since the available information would indicate that current disinfection protocols may not adequately reduce pathogen risk

4.2 Introduction

When *Legionella pneumophila* concentrations reach an action level within premise plumbing, the response often involves a thermal treatment or superchlorination event to reduce viable bacteria numbers and cases of legionnaires' disease (LD). Despite these disinfection regimes, re-colonization of the premise plumbing has been observed, in part due to a stress-induced, resilient physiological state that goes unreported as the cells lose culturability upon adaptation.[98] Many uncertainties remain about these non-culturable bacterial pathogens, such as what risk if any do these cells pose to public health, and what activity/viability markers may be used to detect non-culturable cells that may remain a risk to public health.

4.2.1 Quantitative microbial risk assessment (QMRA)

QMRA is a step-wise protocol used to estimate potential human health risk, and specifically aid in setting target levels to manage pathogen risks.[18] The protocol is used in the development of water safety plans (WSPs) and aims to identify hazards, sources of exposure, and applying dose-response equations to characterize risk associated with specific pathogens.[1, 3] The power of QMRA is reduced by uncertainty, which may arise from the absence of information, the generalizability of experimental work, or model assumption uncertainty.[99] QMRA for legionellae is generally limited by uncertainties associated with quantifying total legionellae as opposed to infectious cells, use of conservative exposure estimates, and the efficacy of pathogen reduction within their environments.[3]

To date, there is only an animal-based dose-response model for *L. pneumophila* that used guinea pigs exposed to bacterial aerosols, concluding an infectious dose required <129 organisms, with a 50% lethal dose (LD_{50}) in the range of 10^5 organisms.[100] Despite the lack of a human dose-response model, several risk assessments have been carried out to estimate the concentration of *L. pneumophila* required to cause an infection in humans. The guinea pig model has been used to estimate exposure and calculate risk for three real-world spa outbreaks, with the analysis yielding similar results to the number of reported LD cases.[101] Furthermore, an exposure model for *L. pneumophila* entering through inhalation during a 15-minute shower event estimated a critical concentration of $3.5x10^6$ CFU mL^{-1} is necessary within the pipe-wall biofilms for possible legionellosis (Figure 4.1).[74]

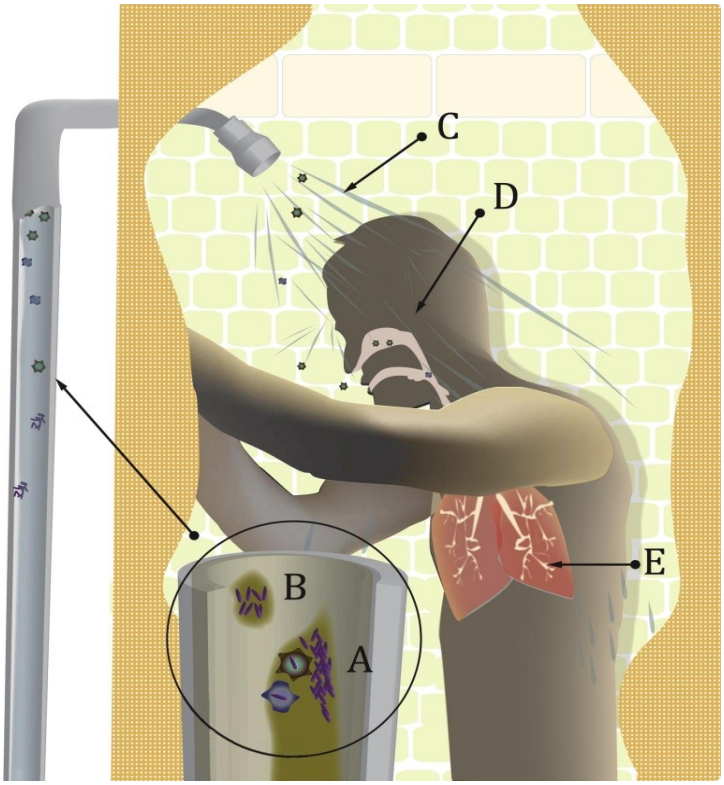

Figure 4.1 QMRA modeling for *L. pneumophila* exposure during 15 minute shower event using back calculations assuming one bacterium delivered to the alveoli can cause infection. Model predicts roughly $3.5x10^6$ CFU.mL^{-1} *L. pneumophila* required in biofilm necessary for one cells to gain entry to lungs.[74]

In order to better manage risk and mitigate legionellosis cases, further research is required with respect to establishing effective disinfection regimes and quantifying *L. pneumophila* that may remain infectious following treatment.

4.2.2 The viable but non-culturable (VBNC) physiological state

VBNC bacterial are defined as cells that have lost their ability to grow on artificial media, generally formed in response to unfavourable environmental/chemical conditions, including nutrient starvation, changes in temperature or pH, chlorination, and UV irradiation.[102] The VBNC state is thought to be an evolutionary adaptation among Gram-negative bacteria for long-term survival within a stress-filled environment, similar to spore formation in Gram-positive bacteria; while others hypothesize the state is merely a stage along the pathway to cell death.[18, 103] VBNC bacteria exhibit unique cell morphologies, metabolic rates, stress resiliencies, and virulence that help distinguish them from dead, dormant, and non-stressed living cells.[102, 103] To date, a total of 85 bacterial species have been reported to exhibit a VBNC state, with 51 of these being known human pathogens, including *L. pneumophila*.[102]

The VBNC state is part of the natural life-cycle of legionellae and can be induced by starvation, chemical disinfectants, temperature changes, and UV light.[18] Changes in nutrient availability have been shown to result in morphological and physiological changes in *L. pneumophila*, where a decrease in available nutrients lead to the expression of virulence factors, triggering a conversion from non-motile, thin-walled replicative cells to motile, thick-walled persistent cells.[61] VBNC *L. pneumophila* can be classified as (a) damaged and near death, (b) injured but may be repairable, and (c) physiologically adapted cells.[18, 61] The distinction between these VBNC cell states has important consequences for public health officials, as (b) and (c) may remain infectious and underreported by traditional culture-based detection platforms.[61]

4.2.3 Are VBNC *L. pneumophila* a risk to public health?

A major limitation of culture-based detection of *L. pneumophila* is the underestimation of potential risk arising from the inability to detect VBNC cells. Despite restricted metabolic rates, VBNC cells have been shown to retain pathogenic properties.[104] In 2012, Alberta Health Services reported a legionellosis outbreak affecting six individuals in the Calgary region, however no exposure source was isolated possibly as a result of legionellae residing within the VBNC state.[105]

To assess the potential public health risk associated with VBNC *L. pneumophila* residing within premise plumbing, the infectivity of the bacterial cells can be examined using preferred free-living protozoan hosts. The ability of FLA hosts to 'resuscitate' VBNC *L. pneumophila* was first observed after non-culturable bacterial cells subjected to nutrient scarce sterile tap water for 180 days quickly regained culturability following co-culture with *Acanthamoeba castellanii*.[87] Since this initial study, researchers have reported the resuscitation of VBNC *L. pneumophila* following inactivation by synthetic drinking water, free chlorine, monochloramine, and heat.[58, 60, 61, 71, 106, 107] Details of the reported resuscitations can be seen in Table 4.1.

Table 4.1 Reported use of free-living amoeba host co-cultures for resuscitation of viable but non-culturable *Legionella pneumophila* after inactivation stress, references listed in order of publication date.

Legionella Strain	*Acanthamoeba* Strain	Disinfection Agent	Result	Reference
L. pneumophila (Philadelphia-1 JR32)	*A. castellanii* (ATCC 30234)	Sterile tap water exposure for 180 d (nutrient scarcity)	VBNC bacteria approached 10^7 CFU.mL^{-1} within 3 days of co-culture following exposure	Steinert *et al*., 1997
L. pneumophila (ATCC 33152)	*A. polyphaga* (ATCC 30461)	Synthetic drinking water exposure for 190 d (nutrient scarcity)	Viable cell count varied 0.5 $\log_{10}$ over 190 day period, exponential growth of VBNC bacteria following co-culture to 10^6 CFU mL^{-1} within 7 d	Hwang *et al*., 2006
L. pneumophila (ATCC 33152) and 6 other strains	*A. polyphaga* (ATCC 50998)	1024 ppm sodium hypochlorite exposure for 22 and 46 h	*L. pneumophila* (ATCC 33152) resuscitated 6 $\log_{10}$ within 2 d of co-culture following exposure	Garcia *et al*., 2007
L. pneumophila (CIP 105349)	*A. castellanii* (ATCC 50739)	1 mg mL^{-1} monochloramine	Biofilm associated VBNC cells regained culturability after amoeba co-culture, cells remained VBNC in absence of amoeba	Alleron *et al*., 2008
L. pneumophila (CIP 107692)	*A. polyphaga* (Linc AP-1)	0.5 ppm free chlorine exposure for 24 h (derived from bleach)	VBNC bacteria regained culturability within 5 d of co-culture, approaching 10^6 CFU mL^{-1}, results not observed when amoeba absent	Dusserre *et al*., 2008
L. pneumophila (CIP 103854)	*A. castellanii* (ATCC 30234)	0 – 0.27 mM sodium hypochlorite exposure for 1 h	Co-culture data may be for 3 or 10 d, definite resuscitation observed, focus of paper directed towards plating with ROS scavengers	Ducret *et al*., 2014
L. pneumophila (Philadelphia, LP1-008)	*A. polyphaga*	70 °C heat exposure for 30 min	VBNC bacteria regained culturability within 4 d of co-culture, unable to resuscitate with macrophage-like cells	Epalle *et al*., 2015

From the data presented in Table 4.1, there appears to be knowledge gaps with respect to silver-copper ionization and ultraviolet (UV) irradiation induced VBNC states in *L. pneumophila* and their potential for FLA host infectivity. A literature search revealed the presence of light-dependent DNA damage repair mechanisms in non-culturable *L. pneumophila* that changed a 3 $\log_{10}$ reduction of culturable cells into a 0.5 $\log_{10}$ reduction after photoreactivation.[108] Thus it appears UV induces a VBNC state in *L. pneumophila*, however there was no report regarding the potential of VBNC cell infectivity and subsequent risk to public health.

4.2.4 Activity/viability assays with potential to detect VBNC cells

Amoeba co-culture experiments are some of the most biologically relevant assays for assessing the risk to public health associated with VBNC *L. pneumophila*. Despite this, amoeba co-culture is time consuming and can be vulnerable to contamination. Due to these concerns, researchers have turned to other markers of activity/viability that can be coupled to molecular-based detection platforms (i.e. not limited to artificial media culture-based detection), being quicker, and well correlated with amoeba co-culture results. A variety of markers may be useful for the detection of viable *L. pneumophila*, including membrane permeable and impermeable dyes, metabolic activity indicators, and measures of growth potential. The coupling of immunomagnetic separation purification with flow cytometry (IMS-FCM) and an activity marker may represent the most feasible platform for the rapid detection of total legionellae and viable *L. pneumophila* within environmental water samples.[17] A non-comprehensive list of up and coming viability markers that may or may not be coupled to FCM are listed in Table 4.2, which has been modified from literature reviews on detecting VBNC bacteria.[18, 102, 103]

Table 4.2 Viability/activity based assays for detection of viable *L. pneumophila* and other microorganisms complete with details on mechanism of action and potential modification for analysis by FCM.[18, 102, 103]

Viability Assay	Mechanism: What does it measure?	Potential for coupling to FCM (Yes/No)	Reference(s)
Amoebae co-culture[a]	Ability of bacteria to infect host organism	No	See Table 5.1
BacLight™ live/dead staining[b]	Syto9 dye binds to all cells while PI dye only enters damaged cells	Yes	Allegra *et al*., 2011, Keserue *et al*., 2012
$DiBac_4(3)$[b]	Stain can enter cells following loss of membrane potential	Yes	Wang *et al*., 2010
Viability PCR[b]	EMA/PMA dye enters damaged cells and intercalates with DNA to reduce amplifiable nucleic acids	No	Yanez *et al*., 2011, Ditommaso *et al*., 2014
CTC[c]	Non-fluorescent reagent enters cell to compete with O_2 as final electron acceptor and fluoresces upon reduction	Yes	Rahman *et al*., 1994 Creach *et al*., 2003
CFDA or ChemChrome V6[c]	Non-fluorescent reagent enters cell and is cleaved by esterase enzyme to produce a fluorescent product	Yes	Yamamoto *et al*., 1996 Wang *et al*., 2010
Luminescence[c]	Luciferase enzyme catalyzes reaction with ATP producing light, provides measure of cellular ATP content (short-lived)	Maybe	Lindback *et al*., 2010 Reyneke *et al*., 2016
RT-PCR[c]	Presence of short-lived mRNA, translation potential	No	Alleron *et al*., 2013
Direct viable counts[d]	VBNC cells in growth medium with antibiotics can elongate without division, provides measure of growth potential	Maybe	Garcia-Hernandez *et al*., 2011

Provides measure of [a] infectivity, [b] membrane integrity, [c] metabolic activity, and [d] growth potential

From the data present in Table 4.2, the various activity assays can be divided into four main categories, including infectivity measures (discussed above), tests of membrane integrity, metabolic activity, and growth potential. The operational theory and literature examples of these assays are explained below.

4.2.5 Activity assays measuring membrane integrity

Maintaining an intact, selectively permeable plasma membrane is critical for bacterial viability, since the membrane protects the cell from external elements, houses important proteins for oxidative phosphorylation, and retains valuable nutrients within the cytoplasm.[18] Therefore, multiple viability assays have been developed to test the plasma membrane permeability and/or ability to maintain an electronic potential gradient. Table 4.2 lists three activity markers that examine membrane integrity, including live/dead staining, $DiBac_4$, and viability PCR.

A widely used assay is Live/Dead® BacLight™ bacterial viability staining, which uses two dyes with different polarities; Syto9 (λ_{em} = 503 nm, green) is a membrane permeable dye that binds DNA, while propidium iodide (λ_{em} = 617 nm, red) is a membrane impermeable dye that can only enter cells with damaged cell membranes.[17, 102] The operational theory behind this assay is that viable cells will have intact cell membranes and stain green (Syto9 label), while injured/damaged cells will be stained by both dyes and appear largely red.[102] The live-dead stain was developed for fluorescent microscopy but can be modified for flow cytometric analyses of *L. pneumophila*.[17, 109] While the BacLight™ kit is the most commonly used live-dead stain, others involving SYBR green or acrimide orange have also been reported.[18, 107] The accuracy of the assay was analyzed by sorting live and dead sub-populations using an BD FACSAria III fluorescence activated cell sorter (FACS) (Becton Dickinson, Franklin Lakes, NJ, USA), which were then plated on BCYE to determine if dead cells remained culturable.

Other viability assays relying on an intact cellular membrane include viability PCR and indicators of membrane potential, like bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBac_4(3)$). Viability PCR (described in Chapter 1) uses membrane impermeable compounds like ethidium monoazide and propidium monoazide that enter damaged/injured cells and intercalate to cellular DNA.[22] Upon a light-induced cross-linking reaction, the DNA of damaged cells is unable to be amplified by PCR, thus providing a measure of viable bacteria, nut cannot be used for UV-irradiated cells.[110] Viability PCR has been successfully used to monitor total *Legionella* spp. in environmental samples and *L. pneumophila* within aerosols.[24, 111] $DiBac_4(3)$ provides a measure of membrane integrity, since this molecule can only enter bacterial cells that have lost membrane potential.[18] This assay has been used to examine the effects of chlorine disinfection on viable cell counts for *Legionella beliardensis* and *E. coli* using flow cytometry.[112]

4.2.6 Activity assays measuring metabolic rate

While VBNC cells are less metabolically active than growing cells, they can continue to carry out cellular respiration, produce adenosine triphosphate (ATP), perform protein synthesis, and ingest nutrients.[102] These metabolic functions distinguish viable cells from dead or severally damaged cells, allowing for multiple activity assays based on measuring metabolic activity. These include measures of cellular respiration and enzyme activity, as well as assays of short-lived substrates like ATP and mRNA.

Active bacterial cells typically require functional electron transport chains to maintain the proton gradients across the cell membrane required for the synthesis of high-energy ATP molecules. An assay that tests for electron transport chain function involves 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a non-fluorescent substrate that replaces O_2 as the terminal electron acceptor and reduces to a fluorescent red precipitate, CTC-formazan (λ_{em} = 630 nm, red), distinguishing actively respiring cells from dead cells.[113] This assay has taken the place of *p*-

iodonitrotetrazolium violet, another formazan producing reagent, which has been used to detect other VBNC cells, such as *Shigella dysenteria*.[114]

Another marker for metabolic activity is enzyme function, since damaged or non-viable cells may have denatured proteins upon exposure to the extracellular environment or disinfection stressors. Esterases are a common enzyme target for viability assays, with carboxyfluorescein diacetate (CFDA) and ChemChrome V6 being the most used products.[18] The non-fluorescent reagents of these assays can enter bacterial cells and are cleaved by active esterase enzymes, producing fluorescein (λ_{em} = 525 nm, green).[115] It has been shown that esterase activity is a good indicator for actual bacterial activity in *L. beliardensis* and *E. coli*.[112] Esterase activity has been used to quantify viable *L. pneumophila* in natural hot water sources, with the assay showing higher or equivalent counts compared to plating.[29]

Metabolically active bacterial cells contain an abundance of ATP, but lose this high-energy molecule shortly after cell death.[116] Thus, an activity assay measuring the concentration of ATP present within the intracellular environment can be used to estimate viable cell counts. Briefly, the assay uses luciferase enzyme coupled to other reagents, which in the presence of ATP, will create a luminescent product.[117] The approach has been successfully implemented for the detection of viable *L. pneumophila* within harvested rainwater tanks and to evaluate ATP levels in VBNC *Listeria monocytogenes* cells.[116, 117] While auto-fluorescence occurs quite frequently in biological samples, luminescence is much less common. The difference between fluorescence and luminescence can be seen in Figure 4.2.

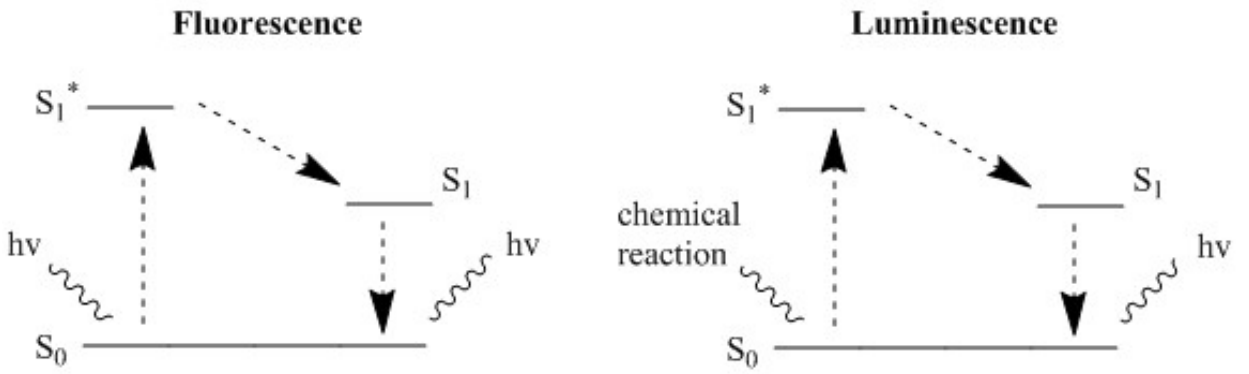

Figure 4.2 Energy diagrams for fluorescence and luminescence, with the main difference between the two processes being the excitation source, which is either photons (hν) or a chemical reaction, respectively. Thus luminescence is more sensitive than fluorescence considering there is no excitation wavelength that can interfere with detection of the emission wavelength.

It has been shown that VBNC cells can continue to synthesize proteins, prompting researchers to examine transcription and translation related assays. Reverse transcriptase PCR (RT-PCR) can be designed to detect short-lived cellular mRNA, which can then be reverse transcribed to form complementary DNA (cDNA) for amplification.[102] RT-PCR relies on the quick turnover of mRNA within cells, which may last for seconds to hours.[18] It has been shown that VBNC *L. pneumophila* express virulence proteins, that may allow for detection based on specific primers.[118] Despite their promising results, RT-PCR and viability PCR were not examined as this project is focused on viability assays adaptable to FCM.

4.2.7 Activity assays measuring growth potential

To date, the most useful approach to assess the public health risk associated with VBNC *L. pneumophila* remains resuscitation by susceptible protozoan hosts to infection. In order to remain infectious, VBNC cells must be able to replicate and express virulence factors. Thus, an assay that examines the growth of VBNC cells, such as direct viable counting, may correlate well with resuscitation data, since these assays subject VBNC cells to nutrient rich media in the presence of various inhibitors (of cell division), resulting in cellular elongation without division.[102] Viable cells will continue to grow while non-viable cells remain the same length,

leading to distinct populations that can be distinguished microscopically.[119] This approach has been successfully implemented for the detection of viable *Lactobacillus delbrueckii*, with novobiocin producing the longest filaments, while pipemidic acid had minimal effect on cell length.[120]

4.3 Materials and Methods

4.3.1 Photoreactivation of *L. pneumophila*

In order to study the presence of previously reported light-activated DNA damage repair mechanism, 10^6 CFU mL^{-1} *L. pneumophila* (ATCC 33152) was exposed to UV-C light (as described in Chapter 2) in order to achieve up to a 6 log_{10} reduction in culturable cells.[108] Briefly, *L. pneumophila* was exposed to 10, 20, 30 and 40 mJ cm^{-2} doses of 256, 266.8, or 288.6 nm UV light. Following inactivation, 1 mL samples of the suspensions were subjected into ambient light or dark conditions at room temperature for 24 h. The samples were then plated onto BCYE agar to determine net photoreactivation (defined here as the change from culturability at 0 h post-UV to 24 h post-UV in the presence on ambient light). A secondary control with non-UV irradiated *L. pneumophila* in 0.85% saline was run to determine if cells were able to replicate within the osmotically neutral, minimal medium. All experiments were run in triplicate.

4.3.2 Resuscitation of disinfected, non-culturable cells through amoeba co-culture

Acanthamoeba polyphaga (ATCC 30461) was used to assess the resuscitation capabilities of disinfected *L. pneumophila* (ATCC 33152) within a free-living amoeba host. Briefly, an axenic culture of *A. polyphaga* was diluted to 10^5 amoeba mL^{-1} in 5 mL PYG 712 medium (formulation described in Chapter 3), to which approximately 10^6 CFU mL^{-1} of non-stressed or UV, heat, or monochloramine disinfected *L. pneumophila* was added to a desired multiplicity of infection (MOI) of 10 bacteria-to-amoeba. The bacteria-amoeba mixture was centrifuged at 1000 rpm for 5 min to induce contact between cells. The co-culture was then vortexed briefly to re-

suspend cells before 1 mL suspensions were pipetted into 24 well plates. The concentration of viable (resuscitated) *L. pneumophila* was determined by plating samples on BCYE agar at 0, 24, 48, 72, and 96 h post infection. A wide gauge syringe was used to vigorously agitate the mixtures, to optimize dispersion of *A. polyphaga* allowing access to internalized *L. pneumophila* as previously described.[41] A set of control samples containing disinfectant stressed and non-stressed *L. pneumophila* without *A. polyphaga* were run to examine the effects of amoeba growth medium on bacterial replication.

4.3.3 Examining correlations of activity assays with amoeba resuscitation

Three fluorescent assays measuring membrane permeability, electron transport chain function, and enzyme activity were examined for potential correlation with amoeba resuscitation data analyzed by flow cytometry (Gallios™ flow cytometer, Beckman Coulter, Brea, CA, USA). Each assay was standardized according to the manufacturer protocols or best-practices from available literature, with characteristic graphs being shown in the Appendix (Figures A4.3 – A4.5). Upon calibration, each assay was used to determine the percent change in viable cell population following disinfection at increasing doses up to 2.5 times dose the required to achieve 6 $\log_{10}$ reductions in culturable cells. For each activity assay, 10^6 CFU mL^{-1} *L. pneumophila* were exposed to 10, 20, 30, or 40 mJ cm^{-2} 256 nm UV-C, 1, 2, 3, 4, or 5 min at 60 °C, and 15, 30, 45, or 60 ppm min monochloramine. Prior to stress events, an aliquot of *L. pneumophila* was taken to serve as the non-stressed (control) proportions, to which each disinfected sample would be compared against. Fluorescent dyes and colourless reagents were added prior to flow cytometric analysis and incubated in accordance with recommended protocols. All fluorescent data was recorded on the Gallios™ flow cytometer, analyzed using Kaluza™ software (Beckman Coulter, Brea, CA, USA). The same protocol was used for the luminescence based activity assay

with measurements using a FLUOstar Omega filter-based multi-mode microplate reader (BMG Labtech, Ortenburg, Germany).

The Live/Dead® BacLight™ bacterial viability kit (Molecular Probes, Eugene, OR, USA) was used to determine the proportion of bacteria with damaged cell membranes. The assay followed the manufacturer's instructions, with 3 µL of 20 mM PI and 3 µL of 3.34 mM Syto-9, being added to a 2 mL sample of bacteria, followed by 15 minute incubation at room temperature. Gating used a collection of FL-1 and FL-4 histograms and an FL-1 X FL-4 scatterplot to distinguish the population of viable cells from membrane damaged bacteria. CTC (Sigma Aldrich, St. Louis, MO, USA) was used to determine the proportion of bacteria with active electron transport chains, using a previously published assay; in short, with 100 µL of a 50 mM CTC solution being added to 1 mL samples, followed by 1 hour incubation at 37 °C.[113] The FCM gating regime used a FL-4 histogram and an FL-1 X FL-4 scatterplot to distinguish the population of metabolically active cells from total cell count. The colourless molecule, 6-carboxyfluorescein diacetate (CFDA) (Sigma Aldrich, St. Louis, MO, USA), was used to determine the proportion of bacteria with esterase enzyme activity. Method standardization followed established literature, with 2.5 µL of 10 mM stock 6-CFDA being added to 500 µL samples with 50 µL of 10 mM EDTA, followed by a 30 min incubation at 35 °C.[112] The FCM gating regime used a FL-2 histogram and an FL-2 X FL-4 scatterplot to distinguish the population of metabolically active cells from total cell count.

In addition to the three fluorescent activity assays, the luminescent BacTiter-Glo™ Microbial Cell Viability Assay kit (Promega, Madison, WI, USA) was used to quantify intracellular ATP concentrations. The protocol followed the manufacturers' instructions, with 100 µL of the luminescence reagent being added to 100 µL cell suspensions in an opaque 96 well

plate. The relative ATP concentration was determined by comparing the cellular luminescence values to a standard curve with known ATP concentrations. As mentioned above, top luminescence of each well was recorded using a multi-mode microplate reader in contrast to the fluorescent assay detection by flow cytometry.

4.3.4 Statistical analysis

The photoreactivation plot was analyzed for statistically significant differences between 256, 268.8 and 288.6 nm (±10 nm) wavelengths at each dose tested. The null hypothesis for this test was equivalent means for each wavelength at a respective UV dose. Microsoft Excel 2010 was used to run an unpaired, unequal variance, two-tailed Student's t-tests, with $\alpha = 0.05$, the *P*-values were reported to either accept or reject the null hypothesis. The correlation of fluorescent and luminescent activity assays with amoeba co-culture results was considered exploratory data analysis, in which the main objective was to look for patterns among the data. No statistical testing was run.

4.4 Results and Discussion

4.4.1 Photoreactivation of *L. pneumophila*

Many bacterial species exhibit light and dark-specific DNA damage repair mechanisms, with the former being referred to as photoreactivation. The effectiveness of UV-C light on *L. pneumophila* has been shown to be dependent on the absence of visible light post irradiation.[108] Hence the ability of *L. pneumophila* (ATCC 33152) to repair DNA damage following exposure to UV light was examined, as seen in Figure 4.3

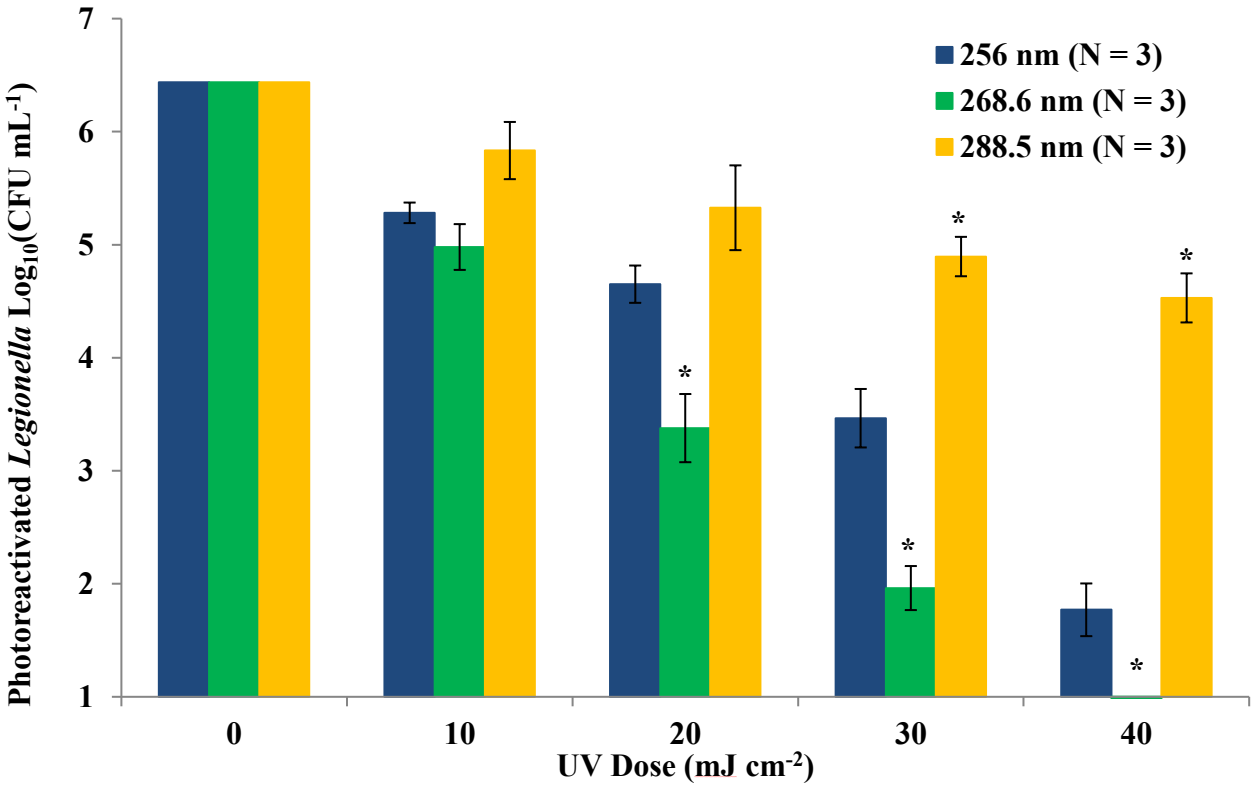

Figure 4.3 Photoreactivation of *Legionella pneumophila* (ATCC 33152) after exposure to 256, 268.6, or 288.6 nm UV-C light at 10, 20, 30, and 40 mJ cm^{-2}. Average initial *L. pneumophila* concentration pre-UV exposure $3x10^6$ CFU mL^{-1} shown as 0 mJ cm^{-2} dose. Not shown are controls ran in the dark and non-exposed cells in 0.85% saline, error bars ± SEM with statistically significant differences between mean values denoted as (*) compared to 256 nm.

From the *L. pneumophila* photoreactivation plot (Figure 4.3), a statistical test (described in methods) was run to determine if any significant differences were observed for the three wavelengths tested as each respective dose. Figure 4.3 shows that 268.6 nm UV-C was the most effective wavelength for achieving up to 6 log_{10} reduction in culturable *L. pneumophila* after accounting for DNA repair mechanisms. The difference in efficacy was observable at higher UV doses, where a 40 mJ cm^{-2} exposure resulted in an apparent complete loss of culturable cells at 268.6 nm, with approximately 10^2 and 10^5 *L. pneumophila* remaining culturable at 256 and 288.6 nm, respectively. Statistical analysis revealed significant differences in mean photoreactivation values for 266.8 nm compared to 256 nm at 20 mJ cm^{-2} (P = 0.034), 30 mJ cm^{-2} (P >0.01), and

40 mJ cm^{-2} (P = 0.017) and 288.6 nm compared to 256 nm at 30 mJ cm^{-2} (P = 0.01) and 40 mJ cm^{-2} (P >0.001), respectively.

The photoreactivation data presented above were compared to the UV dose-response curve presented in Chapter 2, which estimated UV doses of 14.7, 10.2, and 21.8 mJ cm^{-2} would result in 6 $\log_{10}$ reductions in culturable cells at 256, 268.6, and 288.6 nm, respectively. From Figure 4.3 it is apparent that these doses are insufficient to yield a true 6 $\log_{10}$ reduction after accounting for DNA damage repair mechanisms, considering culturable cells were observed at each wavelength following exposure to 30 mJ cm^{-2}. Furthermore, the main conclusion from the initial dose response curve (Chapter 2) was a significant difference between 256 and 288.6 nm, with no rejection of the null hypothesis for 256 and 268.6 nm. As shown above, the corrected dose-response (accounting for photoreactivation) shows significant differences for both 268.6 and 288.6 nm, when compared to 256 nm. Therefore, UV disinfection with 268.6 nm displays a statistically significant greater efficacy compared to 256 nm and 288.6 nm, respectively. In addition, the differences in culturable cell counts directly following exposure (Chapter 2) and after 24 h incubation in ambient light (Figure 4.3) clearly indicate that UV-C exposure induces a VBNC state in *L. pneumophila*. The bacteria is non-culturable directly after exposure, however light-activated DNA damage repair mechanisms promote re-culturability, demonstrating that the stressed *L. pneumophila* remain viable during this time. Thus, UV-C light exposure induces a VBNC state in *L. pneumophila*, which can regain culturability through incubation in ambient light conditions.

Photoreactivation of UV irradiated *L. pneumophila* and other legionellae was first reported in 1985, where a 4 $\log_{10}$ reactivation of culturable cells was reported.[121] Another study demonstrated an apparent 3 $\log_{10}$ reduction in *L. pneumophila* exposed to low-pressure 253.7 nm

UV light became 0.5 $\log_{10}$ after accounting for photoreactivity.[108] Interestingly, the ability of *L. pneumophila* to photoreactivate is more dependent on the time exposed to visible light than the time irradiated by UV light.[108] The current experimental results are similar to those described in literature, where $<10^6$ *L. pneumophila* were irradiated and photoreactivation was performed under similar protocols. It should be noted that at least two controls were run during each experiment, with one sample being incubated under dark conditions (no growth observed) and a non-exposed *L. pneumophila* sample being incubated in ambient light for 24 h to demonstrate cells do not replicate or grow in the osmotically neutral, nutrient poor medium. These controls strongly indicate that the photoreactivation reported above is a result of light-activated DNA damage repair mechanisms.

The corrected *L. pneumophila* dose-response curve for UV-C exposure suggests that the NSF guidelines for effective POU UV treatment should be revisited. Recall that the NSF International/ANSI standard document mandates a UV dose of 16 mJ cm^{-2} for Class-B POU systems, which are used for supplemental germicidal treatment of disinfected water supplies; while a dose of 40 mJ cm^{-2} is deemed sufficient for pathogen removal when operated as the primary inactivation treatment (Class-A POU).[50] Based on the results reported above, neither of these treatments would be sufficient to achieve up to a 6 $\log_{10}$ inactivation of *L. pneumophila* at 256 or 288.6 nm, with only the 268.6 nm exposure causing complete loss of culturable cells at 40 mJ cm^{-2}. Operating a POU applicator at these low efficacy wavelengths may lead to poor inactivation performance and increased risk of legionellosis cases, particularly in biofilm rich environments such as shower heads where light may reach, and amoeba may also grow. Accordingly, further research is required to determine the true population of VBNC and potentially infectious *L. pneumophila* following UV inactivation. Once this is achieved, QMRA

modeling could provide an improved measure of risk, allow for better monitoring of hazards, and ideally mitigate cases.

A plausible explanation for the photoreactivation phenomenon of legionellae must account for their ecological preference to inhabit freshwater environments where exposure to UV and visible light are more common than in engineered water systems.[122] *Legionella* spp. have been shown to express a melanin-like pigment when grown in certain conditions, although there appears to be no relationship with UV light sensitivity.[121] The ecological significance of *L. pneumophila* photoreactivation remains unclear, however VBNC cells that can be re-cultured by visible light suggests the organism may remain infectious to preferred hosts.

4.4.2 Amoeba co-culture

The VBNC state of *L. pneumophila* is of concern to public health as cells remain infectious to free-living amoeba hosts and potentially human macrophages after various disinfection processes, as described in Table 4.1. Therefore, *L. pneumophila* exposed to UV, heat, or monochloramine to achieve up to a 6 $\log_{10}$ reduction in culturable cells were incubated with *A. polyphaga* to determine if inactivated cells remained infectious (Table 4.3).

Table 4.3 Resuscitation potential for disinfected *L. pneumophila* (ATCC 33152) from an initial concentration of $<10^6$ CFU mL^{-1} when grown in PYG 712 medium with *A. polyphaga* (ATCC 30461) for 4 d at 37 °C.

Disinfection	Dose	Resuscitation by co-culture
UV-C (256 nm)	16 mJ cm^{-2}	+
UV-C (256 nm)	40 mJ cm^{-2}	−
Heat (60 °C)	2 min	−
Monochloramine (2.2 ppm)	30 ppm min	−

From Table 4.3 it is apparent that *L. pneumophila* remained infectious to *A. polyphaga* following a 16 mJ cm^{-2} exposure to 256 nm UV-C light, while a 40 mJ cm^{-2} UV dose did not allow for resuscitation above the detection limit of 10 CFU mL^{-1}. Similarly, *L. pneumophila*

exposed to 60 °C water for 2 min or 2 mg L^{-1} monochloramine for 15 min were not resuscitated through amoeba co-culture. The amplification of *L. pneumophila* within amoeba following a 16 mJ cm^{-2} exposure to 256 nm UV-C can be seen in Figure 4.4.

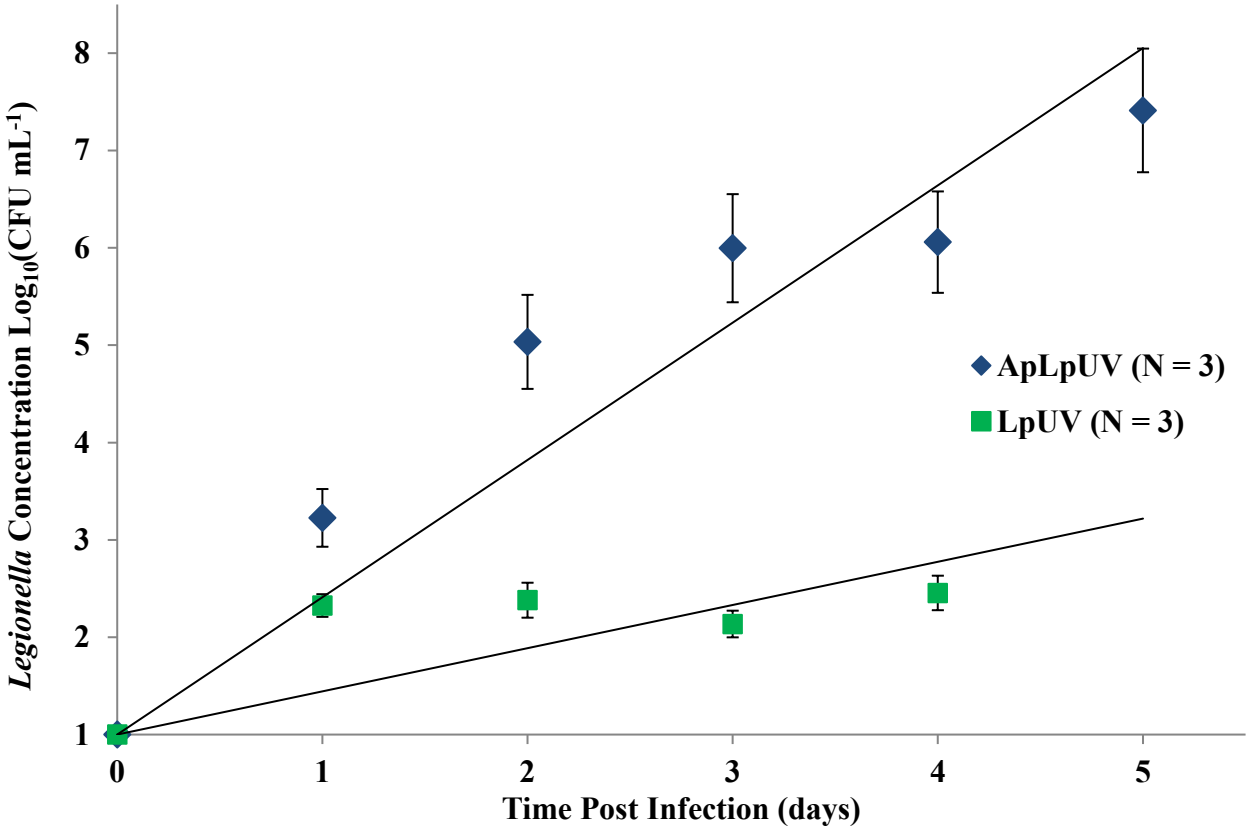

Figure 4.4 *Legionella pneumophila* (ATCC 33152) resuscitation in PYG 712 medium containing *Acanthamoeba polyphaga* (ATCC 30461) following exposure to 16 mJ cm^{-2} 256 nm UV-C light over a 5 d incubation period at 37 °C (diamonds). Also shown is UV stressed *L. pneumophila* grown in absence of *A. polyphaga* (squares). The average concentration of culturable bacteria was $1.4x10^6$ CFU mL^{-1}, with error bars equivalent to ± SEM for N replicate, independent trials and a detection limit of 10 CFU mL^{-1}.

The *L. pneumophila* resuscitation plot (Figure 4.4) clearly shows an amplification of UV stressed bacteria following co-culture with *A. polyphaga*. The *L. pneumophila* was exposed to a 16 mJ cm^{-2} dose of 256 nm UV-C light prior to co-culture, resulting in a complete loss of culturable cells. The co-culture resuscitation of the non-culturable *L. pneumophila* indicates that they remain infectious to amoeba hosts following UV disinfection. Within 4 days of co-culture,

the bacteria reached QMRA estimated like infection levels, $<10^6$ CFU mL^{-1}, through amplification in free-living amoeba. This finding is significant in that it demonstrates the NSF guidelines for Class-B POU secondary disinfection systems would be inadequate for removal of pathogenic *L. pneumophila*. Furthermore, culture-based detection would find no risk, as the bacteria are non-culturable directly following UV treatment, yet remain infectious and can regain culturability over time (potentially also with lung macrophages).[123]

To ensure that the resuscitation involved amplification within the free-living amoeba hosts, a variety of controls were used. Firstly, UV stressed *L. pneumophila* were added to the PYG 712 medium in the absence of *A. polyphaga*. For this control, minor resuscitation was observed (Figure 4.4), with UV stressed *L. pneumophila* regaining culturability within 1 d of co-culture, peaking in the 10^2 CFU mL^{-1} range over the course of the experiment. This suggests that the nutrient rich PYG 712 may allow for VBNC *L. pneumophila* to regain culturability through a novel dark DNA repair mechanism. Secondary controls involving non-stressed *L. pneumophila* (see Appendix Figure A4.6) revealed that the PYG 712 medium did not serve as a growth medium in the absence of *A. polyphaga*. In addition, a GFP *L. pneumophila* control was used to visualize amoeba infections over the duration of the experiment, confirming that the host organism was the main driver of bacterial amplification (Chapter 3). Finally, care was taken to ensure that the experiment was performed under dark conditions to prevent photoreactivation.

While UV disinfection was insufficient at a dose of 16 mJ cm^{-2}, resuscitation was not observed for *L. pneumophila* exposed to 40 mJ cm^{-2} 256 nm UV-C light. A possible explanation for this observation involves the photoreactivation plot (Figure 4.3), which shows the corrected UV-C dose-response curve, accounting for light-activated DNA damage repair. At a UV dose of 16 mJ cm^{-2}, the corrected dose-response curve suggests 10^5 CFU mL^{-1} *L. pneumophila* remain

viable, despite being non-culturable. In contrast, at 40 mJ cm^{-2} there was $>10^2$ CFU mL^{-1} *L. pneumophila* that may remain viable that could be re-cultured via photoreactivation pathways. Therefore, the observed resuscitation of UV stressed *L. pneumophila* following 16 mJ cm^{-2} exposure but not at 40 mJ cm^{-2} exposure may be attributed to the number of viable bacteria present in the samples. While in theory, only one viable bacterium is necessary to infect and amplify within free-living amoeba, the high detection limit (10 CFU mL^{-1}) and strict timelines of the experiment (only 4 d) make it unlikely to observe resuscitation with low viable cell counts. Furthermore, relatively high prey to host amoebae cells are needed for infection. Therefore, to provide greater evidence of 40 mJ cm^{-2} efficacy against *L. pneumophila*, it is recommended that co-culture lasts upwards of 7-10 d and that 1 mL samples (or cell concentrates) are plated to lower the detection limit to 1 CFU mL^{-1}. Figure 4.4 illustrates the first reported instance of UV-C induced VBNC *L. pneumophila* infecting a free-living amoeba host, hence further research is recommended to evaluate the true efficacy of UV disinfection on other water-based pathogens.

The infectivity of VBNC *L. pneumophila* was evaluated following various disinfection processes (Table 4.1), including heat treated and monochloramine exposed *L. pneumophila*.[58, 71] Despite these published accounts, the co-culture experiments described in Table 4.3 indicate that no resuscitation was observed for either 60 °C heat treated nor 2.2 mg L^{-1} monochloramine exposed *L. pneumophila* (ATCC 33152) with *A. polyphaga* (ATCC 30461) as the free-living protozoan host. The conflicting experimental data can be attributed solely to differing methodologies and organisms used. It has been reported that VBNC *L. pneumophila* (Philadelphia-1) were able to infect *A. polyphaga* following a 30 min heat treatment at 70 °C.[58] The literature methods varied from experimental approaches in that 10^8 CFU mL^{-1} (compared with 10^6) were inactivated, the MOI was 100 (compared with 10), and a different strain of

L. pneumophila was used.[124] Furthermore, the resuscitation data reported in literature are inconsistent, with culturable cell counts peaking at $<10^2$ CFU mL^{-1} and no correlation between number of *L. pneumophila* added and resuscitation capacity. Similarly, the reported resuscitation of monochloramine inactivated *L. pneumophila* varied from most co-culture experiments in that exposed bacteria were part of a biofilm environment (as opposed to free suspensions), cells were treated with 1 ppm monochloramine, and different bacterial strains and *Acanthamoeba* spp. species were used.[71] The role of the biofilm environment in shielding/protecting *L. pneumophila* from inactivation restricts the ability to compare the published data with the experimental findings (Table 4.3).[59] Thus, the apparent contradictory evidence with the current experimental data compared to previous reports can be attributed to methodological differences, such as higher bacterial loads, differing MOIs, and the addition of the biofilm environment for protection.

The VBNC state induced in response to 256 nm UV-C irradiation (the major germicidal wavelength used in commercial systems) could have major implications for public health risk. The current culture based approach for detecting *L. pneumophila* would have concluded that no risk existed within a tap water sample that had been irradiated with UV-C light at a point-of-use filter. While no culturable cells were detected, the *L. pneumophila* that resides within the water sample are still infectious in amoeba, which are their main proliferation route in water, and possibly also via lung macrophage growth within the human host. Should biofilm develop post UV treatment in a POU device, it is possible that within 5 d of initial UV exposure, the VBNC *L. pneumophila* could amplify within biofilm FLA to levels approaching the critical concentration estimate to likely cause infection via aerosol exposures; hence an infectious dose could develop if inappropriate legionellae control and management systems are in place.[74] Overall, it is extremely important that we continue to examine other means for detecting

L. pneumophila within premise plumbing, as current culture methods do not generally account for VBNC cells that remain infectious and pose a potential risk to public health.

4.4.3 Correlating viability assays with infectivity

While amoeba co-culture should be considered the gold-standard for assessing public health risk associated with VBNC *L. pneumophila*, the method is time-consuming, prone to contamination, and relies on culture-based detection, which has a series of limitations (**Chapter 1**) that have prompted the search for novel molecular-based approaches, including flow cytometry. Therefore, a variety of fluorescent or luminescent based activity assays (Figure 4.5) were examined to find correlations with experimental resuscitation data reported above.

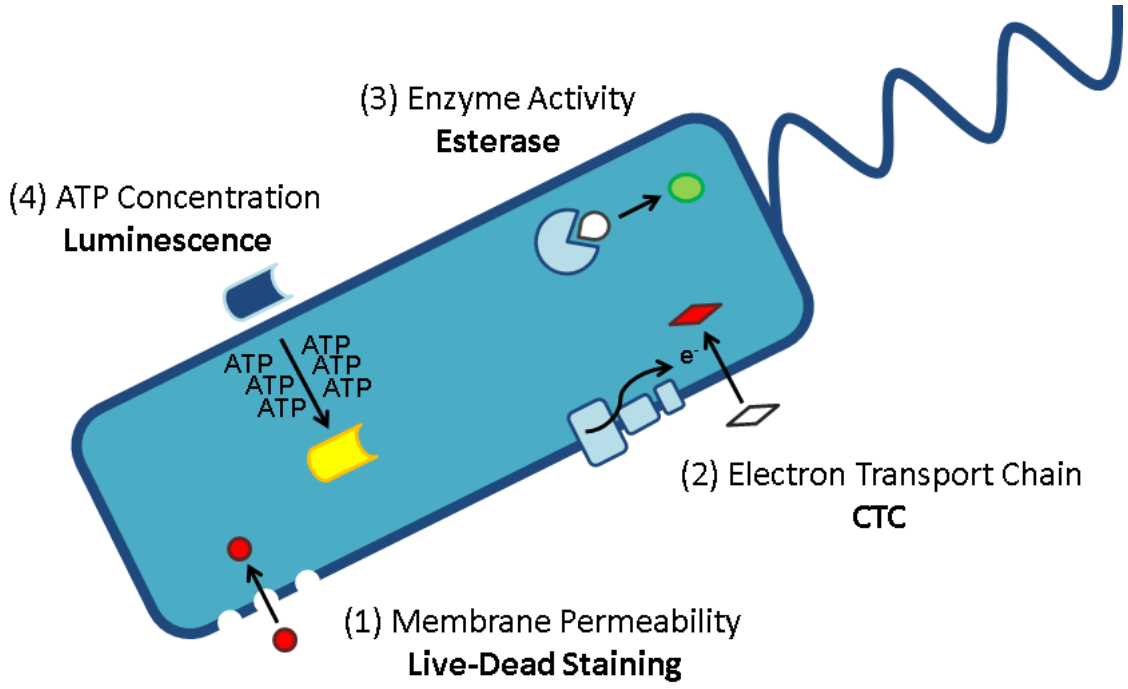

Figure 4.5 Fluorescent and luminescent viability assays tested for correlation with experimental amoeba co-culture data (Table 4.3), created using Microsoft Powerpoint, adapted from similar figure in sourced literature.[37]

The comparison of activity-based assays with BCYE culture performance for 256 nm UV-C, 60 °C heat, and 2.2 ppm monochloramine are shown in Figures 4.6 – 4.8, respectively.

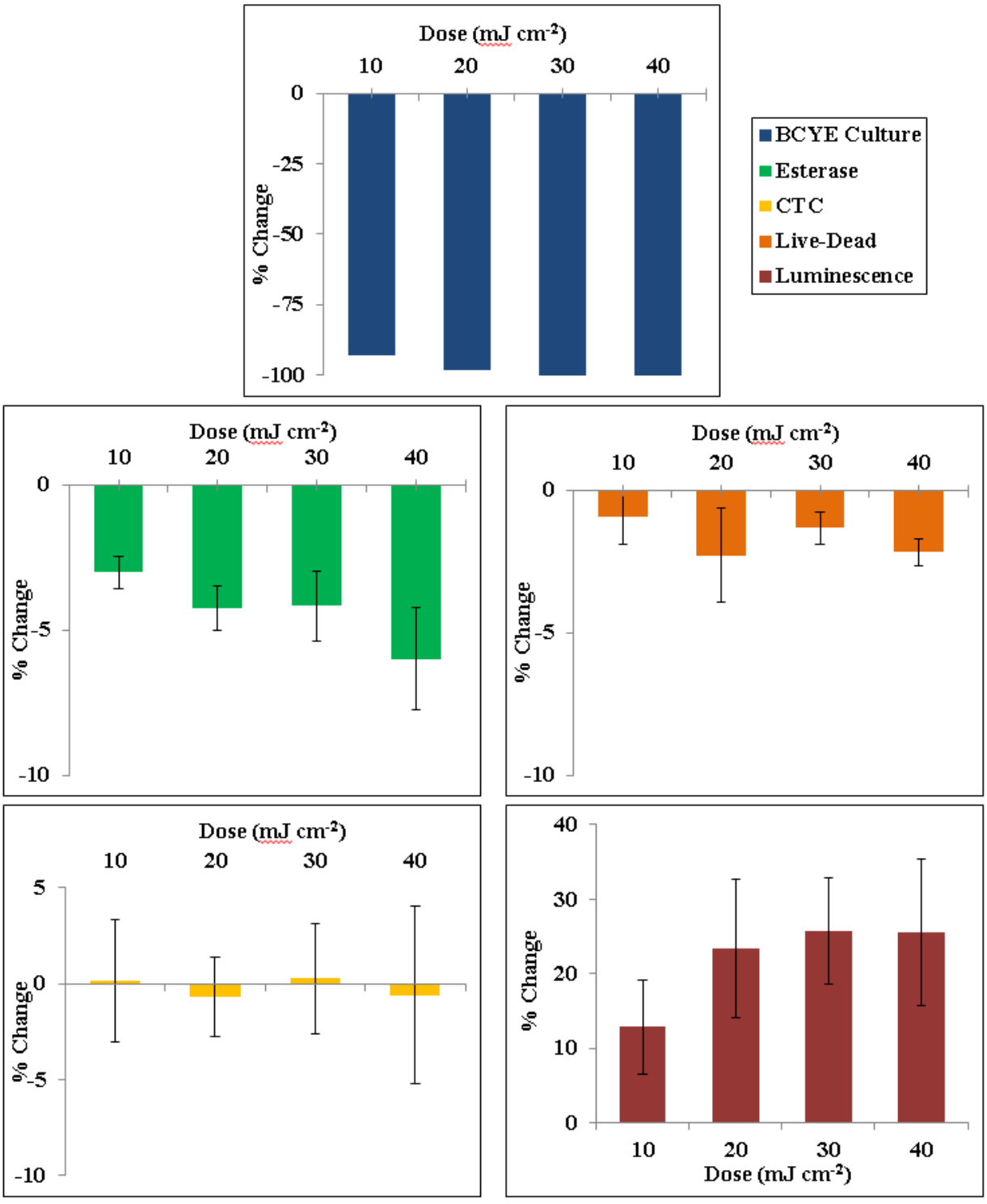

Figure 4.6 Activity assays following UV exposure including three fluorescent (esterase, CTC, and Live-Dead) and one luminescent (ATP) activity assays with BCYE culture for $<10^6$ *L. pneumophila* (ATCC 33152) irradiated with increasing doses of 10, 20, 30, and 40 mJ cm^{-2} UV-C at 256 nm. Data is reported as percent change in activity compared to non-stressed (0 mJ cm^{-2}) *L. pneumophila*, with error bars equivalent to ± SEM for N = 4 replicate, independent trials.

The exploratory data analysis of the percent change in various viability assays following UV disinfection (Figure 4.6) shows trends for esterase activity and ATP concentration, while membrane permeability and electron transport function remain unchanged. The small decrease in esterase activity, −6%, at 40 mJ cm^{-2} suggests that the 256 nm UV-C may be causing photochemical reactions that reduce enzyme function through protein folding.[49] Surprisingly, cellular ATP production appears to increase substantially, +26%, at 30 mJ cm^{-2}, where it plateaus, in comparison to non-stressed *L. pneumophila*. The increase in ATP production following UV inactivation stress may arise from increased exonuclease activity and substrate level phosphorylation, as cellular enzymes repair damaged DNA.[125] Thus, the activity trends presented here confirm that UV-C inactivation affects proteins as well as DNA, and that the increased ATP concentration following stress may provide insight into the true risk associated with these VBNC *L. pneumophila*.

The increase in ATP production following UV irradiation may explain why UV was the only inactivation regime yielding *L. pneumophila* that could be resuscitated by amoeba co-culture. As is apparent from Figures 4.7 and 4.8, the increasing ATP trend following inactivation stress was unique to 256 nm UV-C, whereas sharp decreases were observed for both heat and monochloramine inactivation. Thus, luminescent viability assays of intracellular ATP concentration in *L. pneumophila* may provide a measure of public health risk following inactivation. This may be the first comprehensive analysis of *L. pneumophila* activity following UV stress, with most literature focused on *E. coli* to date. However, the available publications on *E. coli* did not analyze ATP activity, yet found minimal change in esterase or electron transport function (CTC) for 50 mJ cm^{-2} UV.[126]

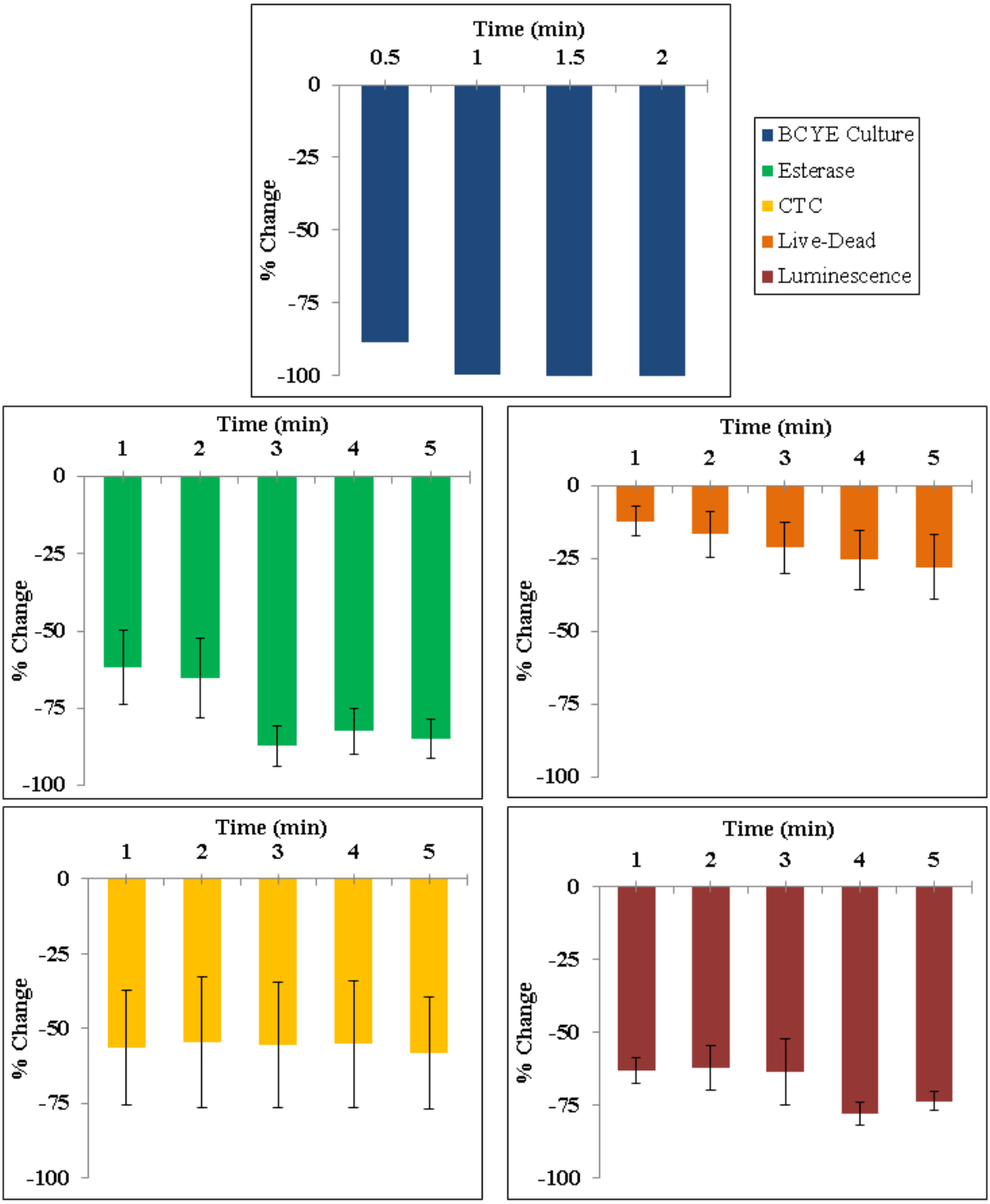

Figure 4.7 Activity assays following heat exposure including three fluorescent (esterase, CTC, and Live-Dead) and one luminescent (ATP) activity assays with BCYE culture for $<10^6$ *L. pneumophila* (ATCC 33152) exposed to 60 °C for durations of 1, 2, 3, 4, and 5 min. Data is reported as percent change in activity compared to non-stressed (0 min) *L. pneumophila*, with error bars equivalent to ± SEM for N = 3 replicate, independent trials.

The exploratory data analysis of the percent change in various viability assays following thermal disinfection (Figure 4.7) shows large reductions in esterase activity, electron transport function, and ATP production, with an increase in membrane permeability. The large decrease in esterase activity, −87%, at 3 min exposure time confirms that high temperatures inactivate bacteria by denaturing proteins, causing reduced enzyme function. Similarly, the electron transport chain and ATP production rely heavily on proteins to establish the proton gradient necessary for oxidative phosphorylation and to shuttle various redox cofactors, thus explaining the large reductions, -55% and -78% at 3 min or 4 min exposure, respectively. Finally, the live-dead staining assay revealed an increase in membrane permeability (shown as percent change in live population in Figure 4.7) as exposure time increased, which may be explained by increased fluidity of the phospholipid bilayer or the inactivation of membrane proteins. The similar trends across the four activity assays indicate that heating water to 60 °C readily inactivates *L. pneumophila* through protein denaturation and increased membrane permeability.

The trends observed in the current work appear to agree with published literature for heat inactivation of *L. pneumophila*. In one study, live-dead staining was used to determine the relative percentage of live, viable, and dead cells following a 5 min exposure to 60 °C heat; with >60% of a laboratory reference strain of *L. pneumophila* retaining an impermeable membrane, compared to the experimentally determine value of >75%. Another study reported that following a 30 min treatment at 70 °C, approximately 25% of *L. pneumophila* retained membrane integrity, suggesting that they may remain viable.[109] The large reductions in the activity assays and the increase in membrane permeability following heat inactivation may explain why *L. pneumophila* was not resuscitated by amoeba co-culture. Lower enzyme activity following heat inactivation would be expected to greatly reduce the infectivity of *L. pneumophila*.

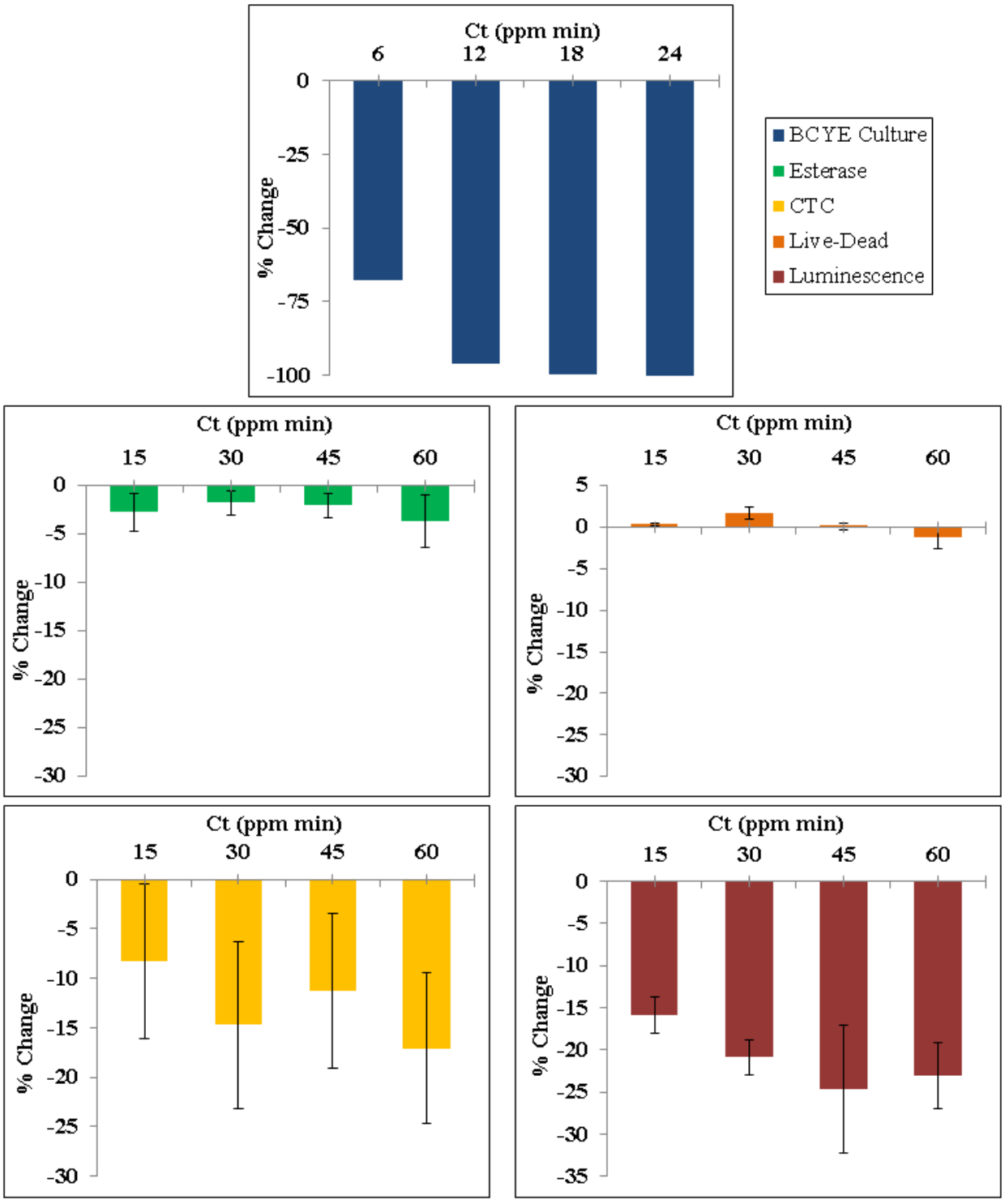

Figure 4.8 Activity assays following monochloramine exposure, including of three fluorescent (esterase, CTC, and Live-Dead) and one luminescent (ATP) activity assays with BCYE culture for $<10^6$ *L. pneumophila* (ATCC 33152) exposed to 2 mg L^{-1} monochloramine for durations of 7.5, 15, 22.5, and 30 min. Data is reported as percent change in activity compared to non-stressed (0 min) *L. pneumophila*, with error bars equivalent to ± SEM for N = 4 replicate, independent trials.

The exploratory data analysis of the percent change in various viability assays following monochloramine disinfection (Figure 4.8) shows large reductions in electron transport chain function and ATP production, with no trends in membrane permeability or esterase activity. The decreases in electron transport, −17%, and ATP production, −23%, suggest that monochloramine disinfection causes disruption of cellular respiration and oxidative phosphorylation, which are linked through the establishment and use of a proton gradient. Monochloramine is an oxidizing agent and may act directly on various proteins, including those associated with electron transport or replace O_2 as an electron receptor, or may function indirectly by disrupting the establish gradients necessary to drive oxidative phosphorylation. ATP production and electron transport chain function appear tightly linked for all three disinfection regimes, with UV having an increase in ATP associated with no change in CTC levels, while heat and monochloramine disinfection result in large reductions of activity for both assays, respectively. Therefore, a combination of ATP and CTC based viability assays may provide a good measure of disinfection efficacy with the treatment types examined.

To date, there is no comprehensive evaluation of activity assay performance following *L. pneumophila* disinfection. The majority of published reports describe an activity assay used in conjunction with culture-based detection or novel molecular-based approaches. For monochloramine, 29% of *L. pneumophila* were reported to have intact membranes 15 d after exposure, while a separate experiment indicated 10% of cells maintained esterase activity after 145 d.[71] Another study reported that when *L. pneumophila* (ATCC 33152) was exposed to 1.5 mg L^{-1} monochloramine from 24 h, $<10^3$ CFU mL^{-1} retained electron transport function, with no activity observed when the dose was increased to 2 mg L^{-1}.[64] While not directly comparable, there is considerable data for *L. pneumophila* activity following free chlorine disinfection. A

viability assay measuring esterase activity was used to quantify total viable counts of *L. pneumophila* following free chlorine treatment reported VBNC cells remained after 48 h exposure to 0.5 mg L^{-1} HOCl.[60] The extracellular ATP concentration was shown to increase following *E. coli* hypochlorite exposure (given a stable matrix) as the cellular membrane is lysed.[127]

As mentioned above, the main objective of the exploratory data analysis for the three disinfection regimes was to determine if any activity assay correlated with the observed resuscitation of VBNC *L. pneumophila* following amoeba co-culture. The analysis for each disinfection regime (Figures 4.6 – 4.8) suggest a mechanism through which *L. pneumophila* remains infectious following UV-C exposure, yet cannot be resuscitated after exposure to heat or monochloramine. The increased ATP production following UV-C treatment indicates that *L. pneumophila* may ramp up exonuclease activity in response to DNA damage. While the ATP concentration was found to increase, there was no change in CTC, live-dead, or esterase activity, which indicate cells still possess all the markers of being active. This was not observed for monochloramine or heat exposure, where ATP and electron transport chain function were disrupted or all major markers of activity were reduced, respectively. However, the knockout of one 'viability' marker, cells may remain a risk if they can repair damage over time, or purposefully reduce metabolic activity upon induction of a VBNC state in response to stress. Some have recommended that dead cells be reclassified as those that have irreversibly lost all ability to reproduce, elongate, and synthesize proteins.[126] Thus, it is apparent that a true marker of resuscitation potential must account for different components possessed by viable cells, including enzyme function, membrane permeability, electron transport function, and ATP

production. A further cautionary note here relates to Pontiac fever, a mild form of legionellosis, which may related more to endotoxin level, that may be associated with dead cells.[10]

Another observation from the exploratory data analysis (Figures 4.6 – 4.8) involves the relative change in culturable *L. pneumophila* measured on BCYE agar in response to stress. A clear trend is present, in which *L. pneumophila* exhibits major reductions in culturable cells prior to observable changes in activity assays. This can be seen in Figure 4.6, where a 10 mJ cm^{-2} exposure results in some 90% loss of culturable cells, with only a minor decrease in esterase activity and increased ATP production. Likewise, the monochloramine analysis (Figure 4.8) has lower doses for BCYE culture compared to the activity assays, as reduced culturability is observed prior to major changes in electron transport chain function or ATP production. This indicates that culture does not provide an accurate measure of activity (and potential infectivity), since cells lost ability to be cultured prior to major changes in the 'activity' assays. Furthermore, this demonstrates the problematic nature of the VBNC state, which can be switched on under moderate environmental stress to allow for survival. Given that current detection relies on culture, the VBNC state represents a major concern for evaluating true public health risk and the efficacy of legionellae control and management. Thus, a collection of viability markers may provide a more representative measure of risk compared to current culture-based detection, since UV-C irradiated *L. pneumophila* lost culturability, yet showed no major loss of activity, increased ATP production, and the ability to be resuscitated by amoeba co-culture.

4.4.4 Explanation of method limitations

Initially, the photoreactivation study was run following a complete loss of culturability, however upon manuscript submission, external reviewers were critical that these doses were not biologically relevant and may overstate the ineffectiveness of UV-C inactivation. To address these concerns, research was conducted to find the NSF International/ANSI standard, which

recommends primary and secondary (Class A and B) POU doses of 40 and 16 mJ cm^{-2}, respectively.[50] These doses were then used as benchmarks for the photoreactivation and amoeba co-culture experiments to ensure that the stated results would be as relevant as possible.

The amoeba co-culture experiments followed the protocols for similar resuscitation trials which are outlined in Table 4.1. Two variables that may limit the generalizability of the experimental data are the presence of external (non-infectious) *L. pneumophila* and the MOI used. The possibility of extracellular growth with amoeba-derived nutrients was not explicitly examined, yet can be with the introduction of gentamicin to the media to remove non-internalized *L. pneumophila* provided concerns remain regarding this issue. When comparing similar studies that used gentamicin compared to those that did not, the authors were able to verify the same results for similar inactivation by free chlorine.[60, 61, 106] The co-culture experiment was designed to match previous VBNC studies, none of which employed antibiotics during the co-culture experiments.[58, 60, 61, 71] The MOI used for the current experiment was standard for resuscitation studies, with average MOIs ranging between 10 to 100.[58, 60, 106] Therefore, while concerns may remain regarding the measurement of *L. pneumophila* not residing within free-living amoeba hosts and the likelihood of ratios used, the current co-culture study followed the same set of protocols as other commonly cited papers.

It was emphasized when introducing the activation assay section that the results were meant to explore potential trends with respect to amoeba co-culture. No statistical significance testing was employed beyond the use of SEM to show the relative variability within each data set. This is particularly important for small trends, which are most readily visualized for UV (see live-dead, CTC, and esterase), as well as monochloramine (see live-dead and esterase), where the percent change is often less than ± 5%. Even with larger changes in response to varying

stressors, change of 90% or 99% would be equivalent of 1 or 2 $\log_{10}$, respectively. The initial concentration for each activity assay was about 10^6 CFU mL^{-1}, which would require detection of active/viable cells down to the 0.0001% to ensure sufficient removal of risk. At the current populations reported above, the activity assays indicate that following maximum exposures, $<10^5$ *L. pneumophila* may remain viable and potentially infectious. Even for temperature disinfection, which showed visible reductions in activity for each assay, a large non-zero proportion of potentially infectious bacteria would remain. Thus, the data is meant to be interpreted as population trends as opposed to definitive inactivation of total bacteria present within a sample.

Furthermore, the activity assays used in this chapter do not discriminant viable from non-viable cells with pinpoint accuracy. When standardizing the live-dead staining protocol using ethanol killed *L. pneumophila*, the viable and dead sub-populations were sorted using FACS and then plated on BCYE. The experiment showed that a small proportion of PI (+) "dead" cells remained culturable, proving that these assays should be interpreted with caution (data not shown); with similar findings being confirmed in literature.[22] Live-dead assays have drawn criticism for apparent shifts from green to red, caused by intercalation of DNA to effectively kill viable cells and variations between expected viability and observed populations for complex samples containing biofilms.[128] Since CTC acts as a terminal electron acceptor, it effectively inhibits the electron transport chain, resulting in cell death shortly after incubation.[126] As reported above, ATP based assays do not discriminate between external and internal ATP (without additional pre-treatment steps that were not used here), which may result in observed increases in ATP production when cells are being lysed.[127]

Despite the limitations listed above, activity assays can provide a wealth of knowledge regarding the mechanisms through which bacteria are disinfected. In moving from exploratory

data analysis to statistically significant findings, it is recommended that FACS be used to ensure that cells counted as dead are not culturable; although this may not be practical if cells are VBNC. The concern regarding externalized ATP being measured in response to cell lysis did not occur in this experiment, since the live-dead staining revealed only minor changes in membrane permeability and the thermal disinfection reduced ATP levels. The activity assay experiments were used to evaluate trends across several disinfection processes. All viability assay results must be considered with caution, since there are limitations and are not 100% accurate.

The main objective of this chapter was to answer the question presented in the title, "are we effectively managing *L. pneumophila* exposure risk through disinfection?". Based on the results and discussion provided above, the answer to this question is "potentially… but probably not", since it was shown that *L. pneumophila* can remain infectious despite being VBNC and that following heat or monochloramine treatments, cells possessed some activity markers that are attributed to living/potentially infectious cells. The "potentially" part of the answer stems from the observation that three of the four disinfection regimes tested resulted in no resuscitation by amoeba co-culture, while the "but probably not" part of the answer comes from an understanding of the inherent limitations of the current work. The experiments run here were simplistic in that a lab strain of *L. pneumophila* was exposed to UV, heat, or monochloramine *in-vitro*, and then incubated with *A. polyphaga* under ideal conditions to determine resuscitation potential.

In Chapter 2 it was noted that *in-vitro* analysis is the initial phase in examining the efficacy of potential inactivation regimes. These studies are important for evaluating effective doses and comparing between strains and species of microorganisms that may be encountered; yet often lack generalizability to real-world systems. An important factor that is difficult to replicate in lab settings is the interaction of *L. pneumophila* with free-living protozoa and the biofilm

environment. This is exemplified with the comparison of VBNC *L. pneumophila* resuscitation following monochloramine exposure in literature, while growth was not observed in this experiment.[118] The main difference between the two experiments was that the literature example exposed a biofilm containing *L. pneumophila*, while the current experiment used free suspensions of cells. This highlights the limitations of *in-vitro* analyses, which do not account for the protecting/shielding effects of biofilms or free-living protozoan hosts. Thus, it is apparent that current disinfection protocols may remove pathogen risk, however it is more likely that we are overestimating the efficacy of these treatments and that further research should be focused on removing the favourable biofilm environments that can lead to rapid proliferation of infectious *L. pneumophila*.

This experiment represents the first evidence for UV-C induced VBNC *L. pneumophila* remaining infectious to a free-living host, *A. polyphaga*. This finding is significant as it fills in a knowledge gap relating to VBNC infectivity, while also prompting further research into the efficacy of UV inactivation as a POU application for *L. pneumophila* control and management. While it has been shown that UV (40 mJ cm^{-2}), heat, and monochloramine disinfection can remove *L. pneumophila* pathogen risk, as measured by amoeba co-culture infectivity; the results may overstate the efficacy of these treatments. The exploratory data analysis revealed that there was not a complete loss of viability markers for the harshest of disinfections (60 °C for 5 min), suggesting that *L. pneumophila* may possess necessary growth elements and enzyme activity that rule out cell death. Thus, it is recommended that further studies are conducted with biofilm and amoeba present to provide a more generalizable (real-world applicable) indicator of disinfection efficacy and reductions in pathogen risk; since the available information would indicate that current disinfection protocols may not adequately remove pathogen risk.

Conclusions and Future Directions

The introduction of drinking water safety plans in Alberta has resulted in increased attention towards underreported water-based, opportunistic pathogens like *L. pneumophila*, which grow in premise plumbing biofilms and cannot be controlled through drinking water treatment alone. The development of these plans that address hazards from source to customers' taps relies on QMRA to address enteric pathogen reduction needs, but has yet to address water-based pathogens that are limited by uncertainties associated with the speed and accuracy of current culture-based detection, as presented in this dissertation for measuring infectious *L. pneumophila* (including VBNC). Thus, a major objective was to improve upon the poor reliability of culture-based *L. pneumophila* detection by examining viability assays that correlate with amoeba infectivity data, which could be coupled with a novel IMS-FCM detection system, in hopes of providing a better, timely repose system for public health risk management.

An evaluation of established and emerging *L. pneumophila* detection platforms listed multiple concerns regarding current detection using BCYE agar plates, and suggested the need to move towards more accurate nucleic-acid and immunology-based molecular systems. In addition, a recently reported IMS-FCM detection system was shown to detect *L. pneumophila* below the recommended action level guidelines given by Public Health Ontario (<100 CFU L^{-1}). Dose-response curves were created for *L. pneumophila* exposed to UV, heat and monochloramine to evaluate the efficacy of each disinfectant, with 268.6 nm UV-C, 60 °C water, and 2.2 mg L^{-1} monochloramine achieving relatively rapid 4 $\log_{10}$ reductions in culturable cells. UV dose-response curves varied significantly depending on the presence/absence on visible light, which is thought to induce light-activated DNA damage repair machinery, resulting in significantly lower disinfection efficacy when corrected for. The role of FLA in *L. pneumophila* detection, disinfection and potential human pathogenicity was examined, with particular focus on

amoeba co-cultures of VBNC cells. These experiments demonstrated poor disinfection efficacy by UV light, while no bacterial growth was reported following heat or monochloramine treatments. Correlating *L. pneumophila* growth in amoeba with fluorescent and luminescent activity assays revealed CTC and ATP-based approaches may provide an appropriate measure of cells of public health risk, with potential for coupling to novel molecular, flow cytometry-based detection systems.

Some future areas of study related to this project include collaborations with industry to evaluate sensitivity of novel detection platforms, including IMS-FCM, with the goal of establishing an automated, online system for continuous monitoring; of particular interest is RQmicro (Rapid Quantitative Microbiology, Zurich, SUI), which will produce automated units to measure *L. pneumophila*. A comprehensive analysis of disinfection regimes is desired, with free chlorine and Ag^{+}/Cu^{2+} ionization providing useful comparisons to monochloramine, with respect to dose-response and amoeba co-culture findings. The protective nature of *W. magna* trophozoites, cysts, and vesicles against bacterial disinfection is a current knowledge gap, as is the quantification of *L. pneumophila* residing within these amoebal forms. It is also recommended that novel detection platforms for FLA hosts be identified, such that risk managers can monitor precursor conditions that favour *L. pneumophila* propagation. Finally, statistical significance testing of the activity assays should be performed to report important trends associated with disinfection efficacy.

Based on the thesis objectives and results summarized above, the current research has demonstrated the need for an accurate, rapid *L. pneumophila* detection system, and the potential usefulness of ATP-CTC coupled viability assays for quantifying active, infectious cells that could be a risk to public health. Following UV disinfection up to recommended guidelines of 16

mJ cm^{-2}, current culture-based detection would be unable to identify VBNC cells that remain infectious to *A. polyphaga* hosts and potentially human lung macrophages. Thus, current detection may underestimate infectious *L. pneumophila*, since VBNC cells are not recorded yet may still cause infection. The development of WSPs through QMRA modelling must account for this uncertainty by finding more inclusive detection systems for all forms of infectious legionellae; otherwise legionellosis rates will continue to be underreported and vulnerable high-risk groups will continue to be put at risk.

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Appendix A

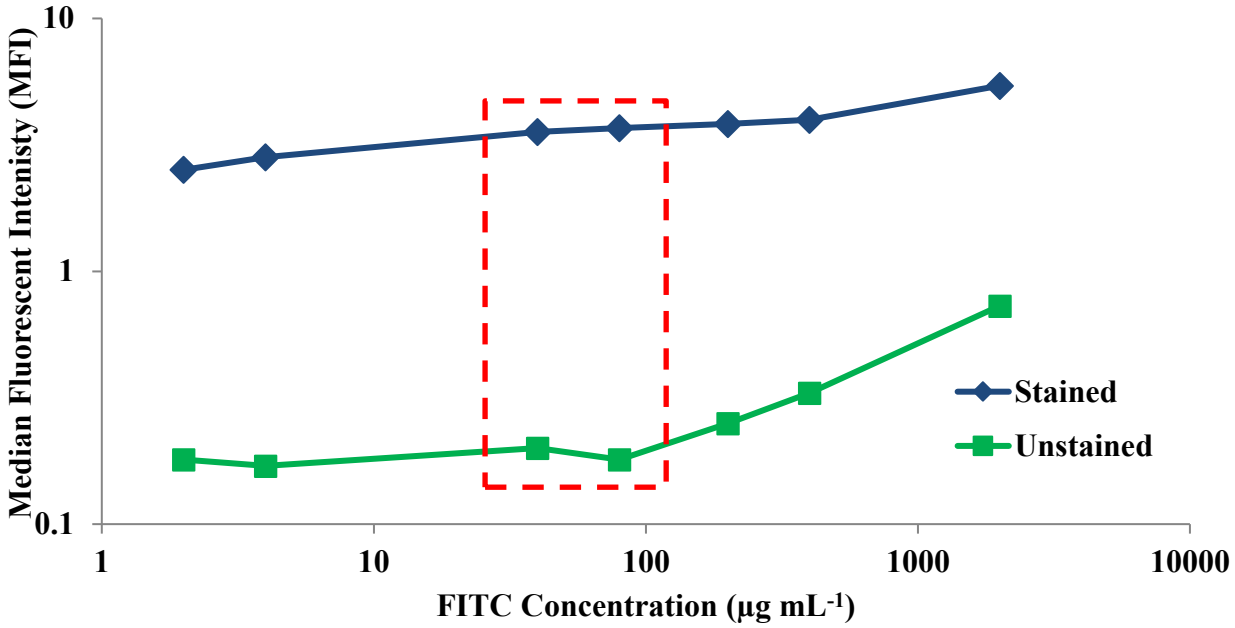

Figure A1.1 FITC titration measured as median fluorescent intensity (MFI) values for stained and unstained populations at different concentrations of FITC conjugated *L. pneumophila* specific antibody (PA1-73140) diluted from stock of 4-5 mg mL^{-1}, with red box denoting maximum separation efficiency.

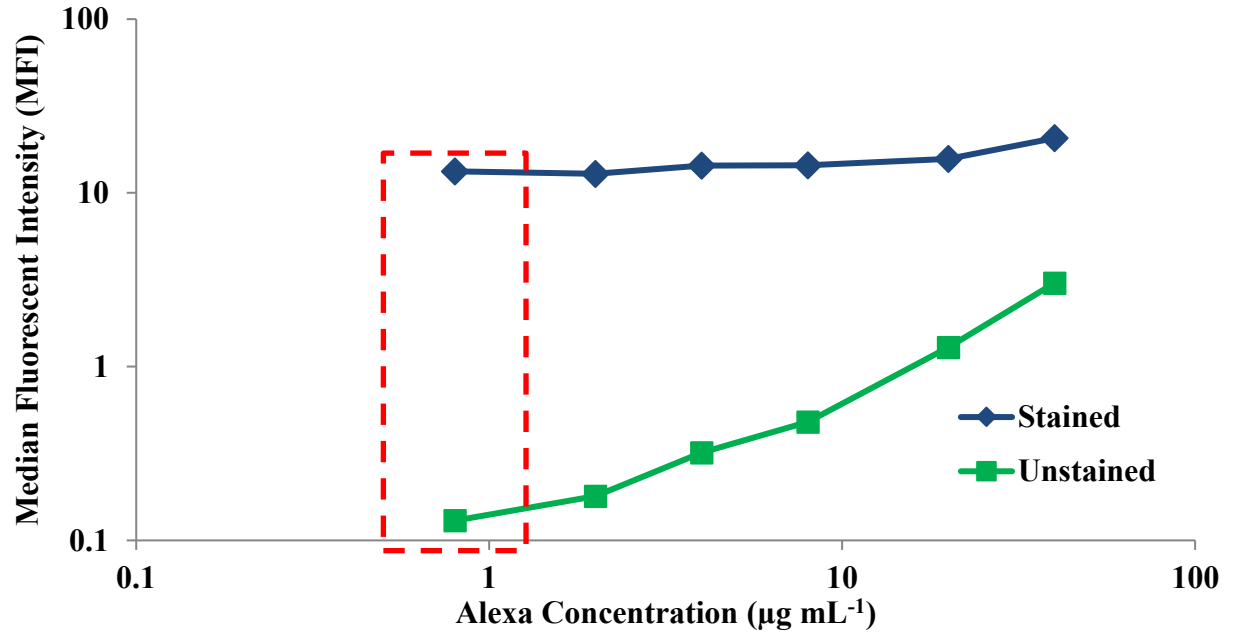

Figure A1.2 Median fluorescent intensity (MFI) values for stained and unstained populations at different concentrations of Alexa Fluor® 647 conjugated *L. pneumophila* specific antibody (GTX40943) diluted from stock of 4 mg mL^{-1}, with red box denoting maximum separation efficiency.

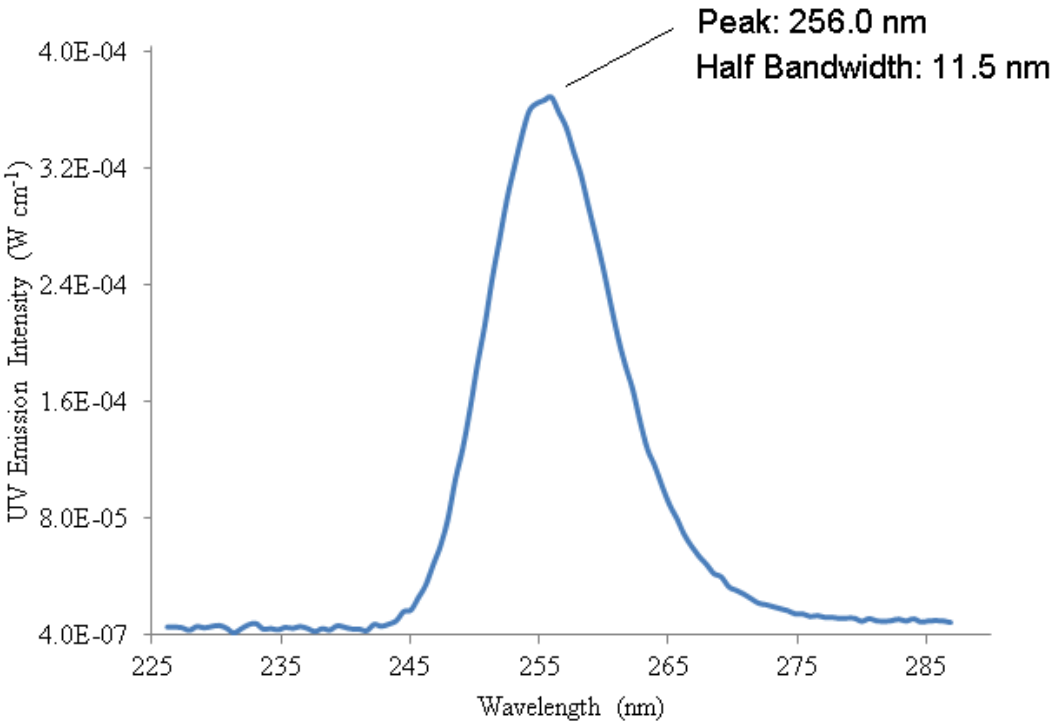

Figure A2.1 UV emission spectrum for 255 nm LED, with peak maximum reached at 256 nm and a half bandwidth of 11.5 nm.

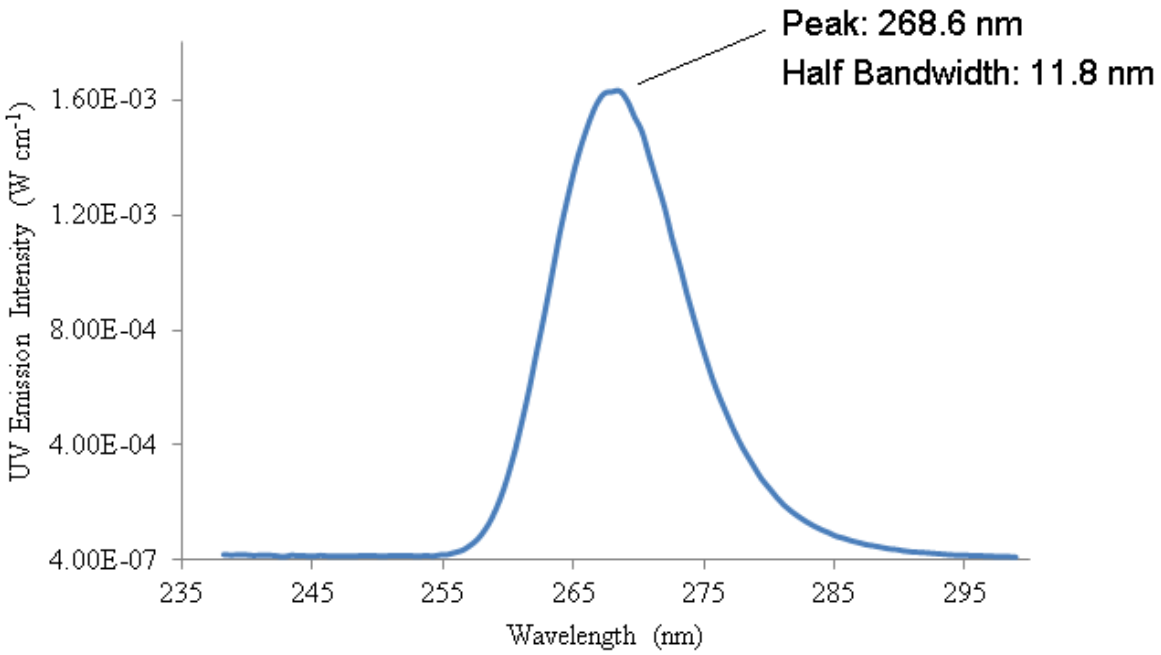

Figure A2.2 UV emission spectrum for 265 nm LED, with peak maximum reached at 268.6 nm and a half bandwidth of 11.8 nm.

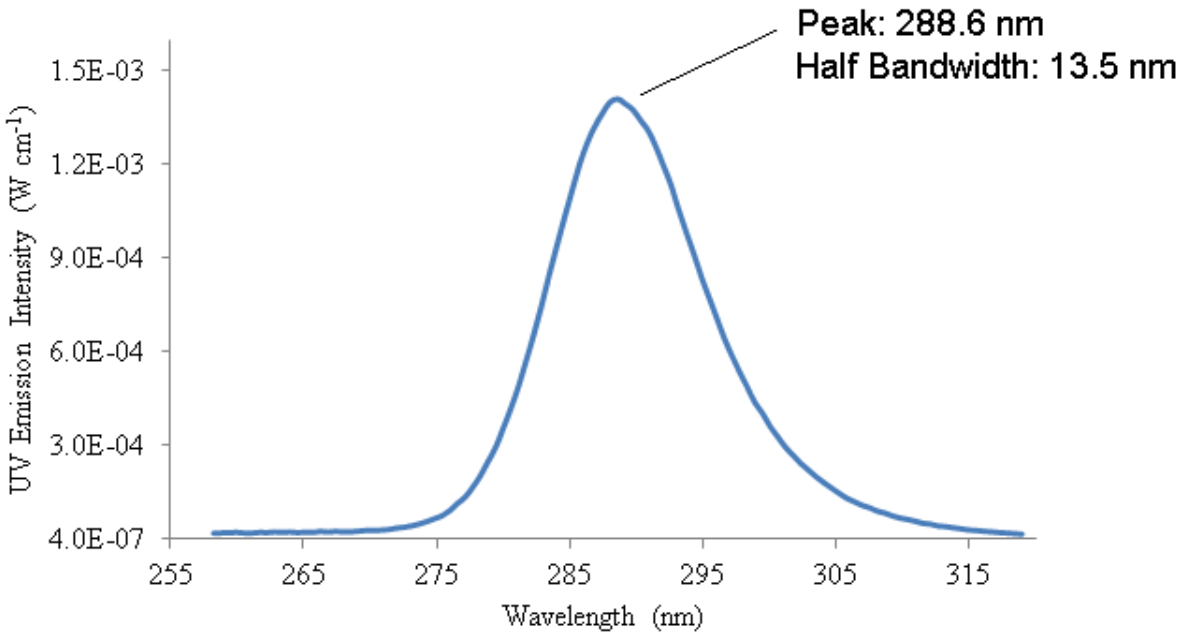

Figure A2.3 UV emission spectrum for 285 nm LED, with peak maximum reached at 288.6 nm and a half bandwidth of 13.5 nm.

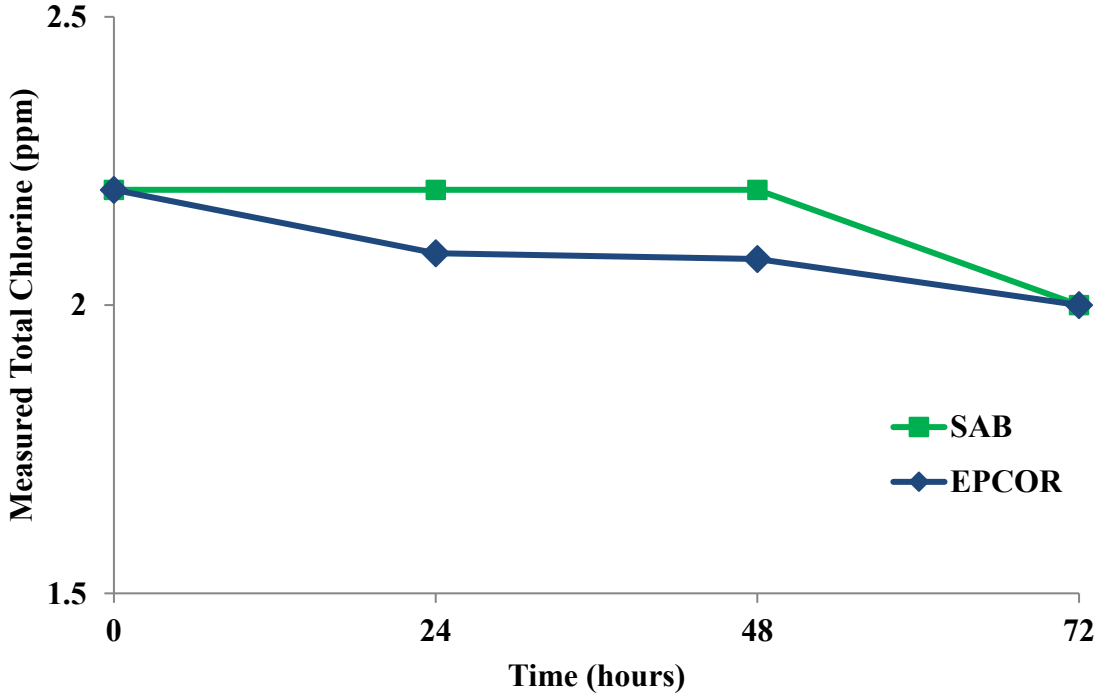

Figure A2.4 Monitoring of monochloramine degradation over 3 day period using EPCOR laboratory based PAO test and in-laboratory (SAB) colourimetric assay.

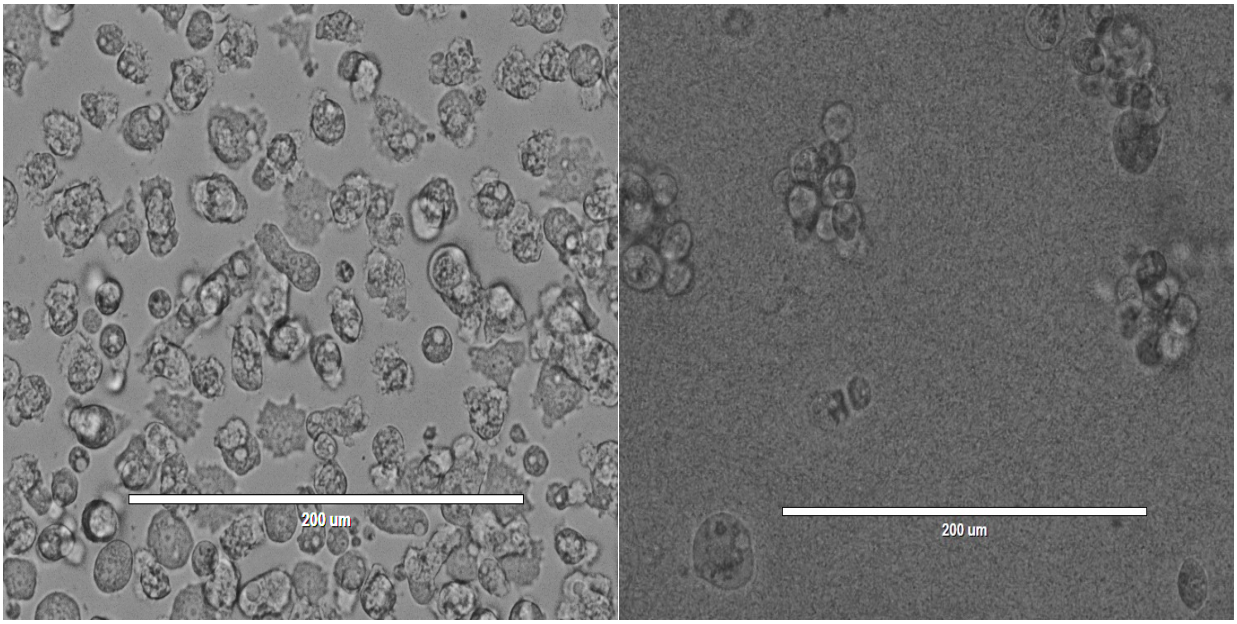

Figure A3.1 Morphological differences between healthy *A. polyphaga* (ATCC 30461) [left] and *A. polyphaga* infected with *L. pneumophila* (ATCC 33152) [right], captured after 72 h growth in SCGYEM medium at 37 °C under 5% CO_2 using EVOS® FL auto cell imagining system at 20x magnification.

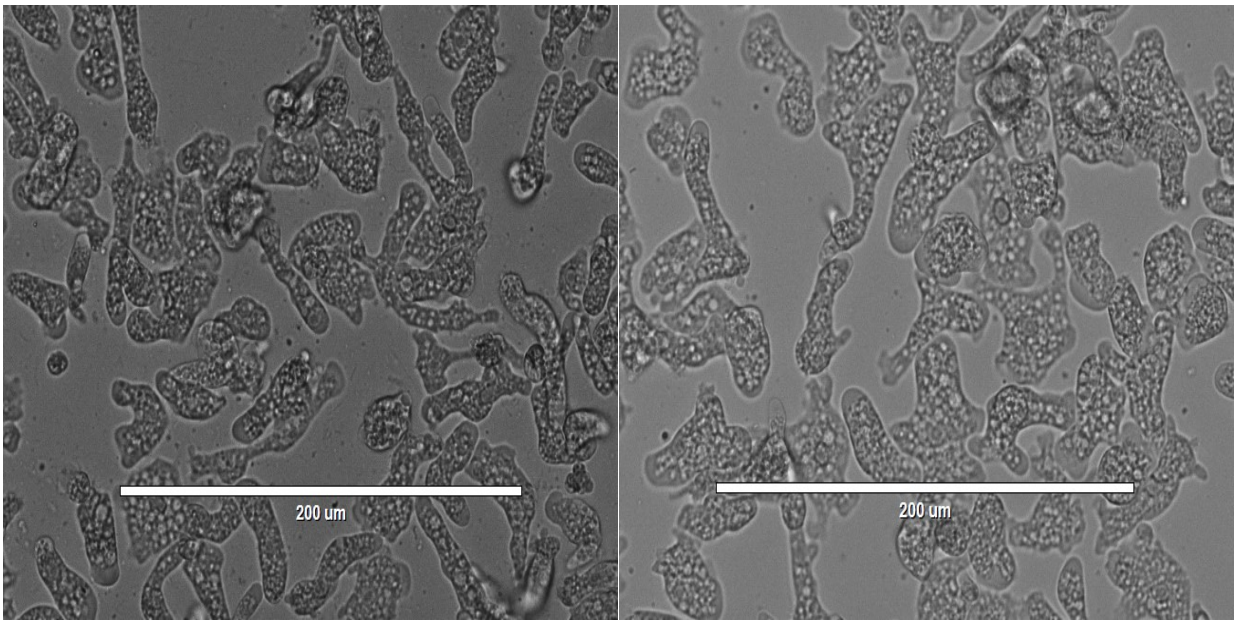

Figure A3.2 Morphological differences between healthy *W. magna* (ATCC 50035) [right] and *W. magna* infected with *L. pneumophila* (ATCC 33152) [left], captured after 48 h growth in SCGYEM medium at 37 °C under 5% CO_2 using EVOS® FL auto cell imagining system at 20x magnification.

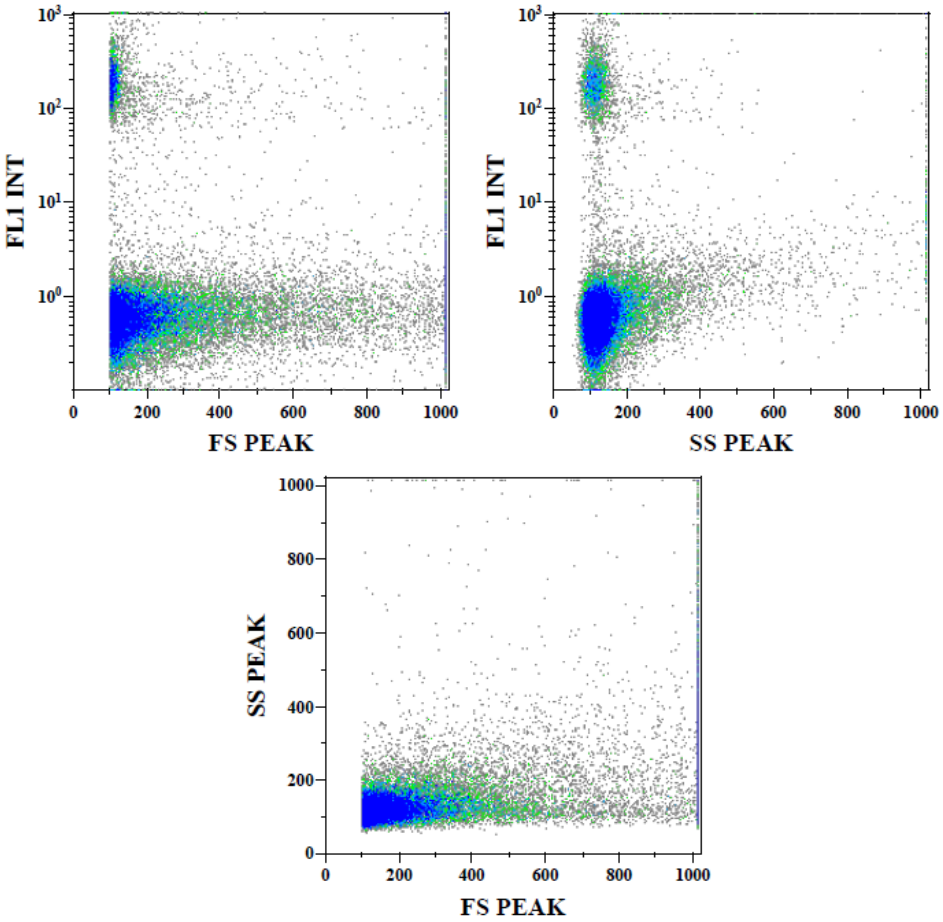

Figure A3.3 FCM plot of *W. magna* vesicles suspended with free-floating GFP *L. pneumophila* and amoebae trophozoites and cysts.

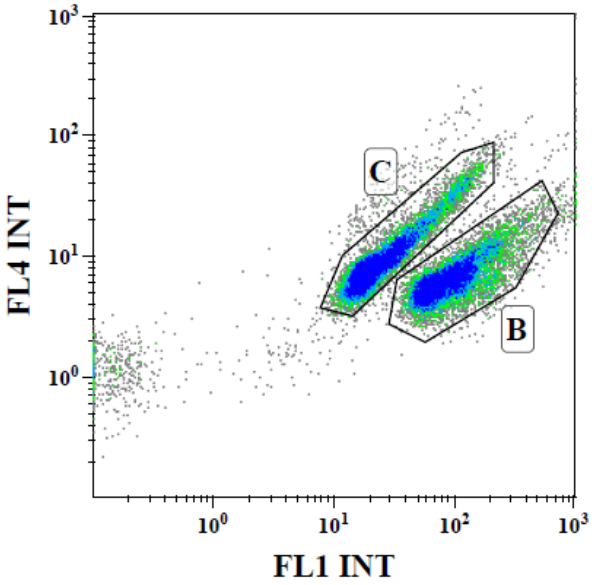

Figure A4.1 FCM scatterplot of live-dead staining with (C) dead population and (B) viable cell count based on relative FL-4 and FL-1 intensities.

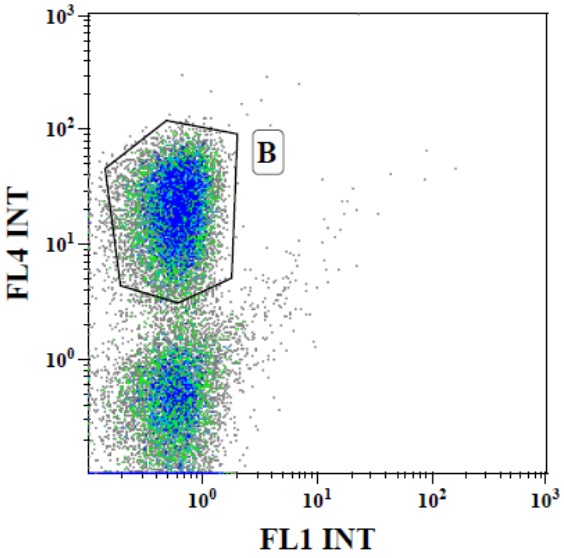

Figure A4.2 FCM scatterplot of CTC assay with (B) metabolically active cells that have reduced CTC to CTC-formazan.

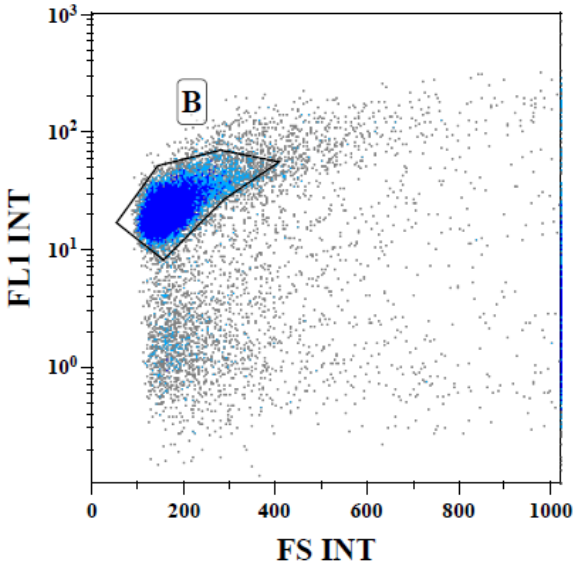

Figure A4.3 FCM scatterplot of esterase assay with (B) metabolically active cells that have cleaved CFDA to FITC.

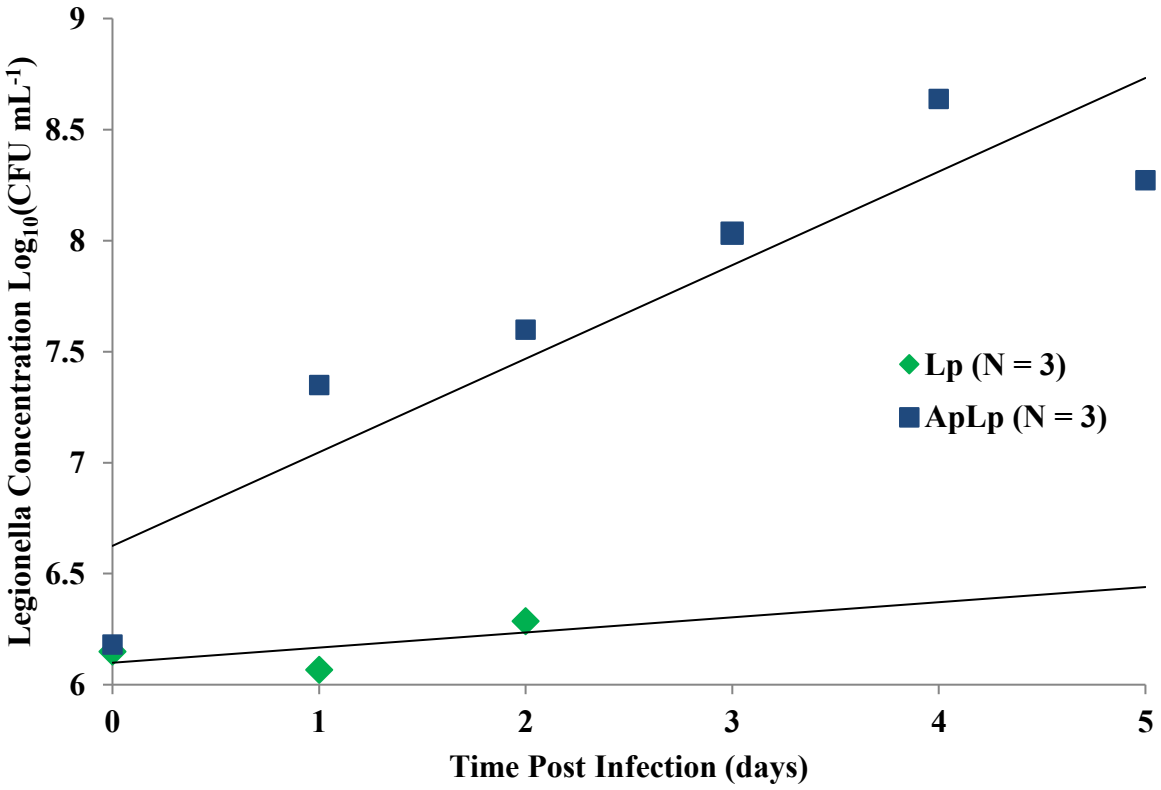

Figure A4.4 Co-culture control showing *L. pneumophila* growth in PYG medium in the presence and absence of *A. polyphaga* hosts.

Appendix B

Sample Calculation B1.1 Estimating FCM volume per run

Average mass uptake = 0.1366 g

Density = 1.0 g mL^{-1}

Volume uptake = (0.1366 g)/(1.0 g mL^{-1})

Volume uptake = 137 µL

Sample Calculation B1.2 Limit of the blank for N = 45 samples

$$LOB = \bar{x} + 1.645 * SD$$

$$\bar{x} = 17.1$$

SD = 15.1

LOB = 17.1 + 1.645*15.1

LOB = 41 events

Sample Calculation B1.3 Concentration limit of detection for FCM method

Equation for best-fit line: y = 0.2971x + 1.3271

y = 76 events

$$x = 10^{\frac{\log_{10}(76)-1.327}{0.2971}}$$

LOD = 75 CFU

Sample Calculation B2.1 Effective UV dose (in mJ cm^{-2})

$$E_{AVG} = 0.98\left[\frac{E_o}{L}\left(\frac{(T)^L - 1}{\ln(T)}\right)\right]$$

E_o = 32.3 µW cm^{-2}

L = 1 cm

T = (1 – A) = 0.892

E_{AVG} = 29.9 µW cm^{-2}

$$Dose = \left(\frac{exposure\ time\ \times\ E_{AVG}}{1000}\right)$$

Dose = (29.9 µW cm^{-2} * 60 s)/1000

Dose = 1.8 mJ cm^{-2}

Sample Calculation B2.2 Effective dose for 4 $\log_{10}$ reduction

Equation for best-fit line: $y = -0.0066x^2 - 0.3127x$

$$y = -4.0$$

$$x = -b \pm \sqrt{\frac{b^2 - 4ac}{2a}}$$

$$x = -(-0.3127) \pm \sqrt{\frac{(-0.3127)^2 - 4(-0.0066) * (-4)}{2 * (-0.0066)}}$$

x = 10.5 mJ cm^{-2}