Characterising the relationship between *Legionella longbeachae* and their amoebal hosts: Implications for Legionnaires' disease prevention

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Abstract

In New Zealand, *Legionella longbeachae* is the leading cause of Legionnaires' disease, a severe form of pneumonia that often results in hospitalisation and an intensive course of treatment. Every year, during spring and summer, cases of Legionnaires' disease caused by *L. longbeachae* infection are seen to increase. These cases are often associated with the exposure to composted plant materials, such as those found in commercial potting mix. In both natural and man-made environments, *Legionella* can parasitize free-living amoeba and subvert host cell signalling to support their replication. Whilst this interaction has been well characterised for several species of *Legionella,* very little is known about the relationship between *L. longbeachae* and its amoebal hosts and as a result, our ability to plan strategies to reduce the potential risk of human infection is limited. Using qPCR, we tested DNA from a variety of previously collected and stored environmental samples for the presence of *Acanthamoeba* spp. and *Naegleria fowleri.* Low levels of *Acanthamoeba* DNA were detected in several samples where *L. longbeachae* was present, and while there did not appear to be a relationship between *Acanthamoeba* and *L. longbeachae* in nature, *Acanthamoeba* may still be able to support the replication of *L. longbeachae in vitro*. To investigate this, a *Legionella* co-culture system was established with *Acanthamoeba polyphaga* (ATCC® 50372™). Amoebal uptake of *L. longbeachae* was observed using fluorescent microscopy, and a comparison was made using a type-strain of *Legionella pneumophila* (ATCC® 33152™). After two hours, both *L. pneumophila* and *L. longbeachae* were seen to co-localise with *A. polyphaga*. However, these findings alone were not enough to conclude whether *L. longbeachae* was being internalised or replicating within *A. polyphaga*. In an effort to help identify other potential amoebal hosts for *L. longbeachae*, culture-based techniques were used to screen a variety of environmental samples for protozoa. Viable amoeba were successfully recovered from a potting mix sample in which *L. longbeachae* had been previously isolated, and ongoing experiments have been planned to identify these species using 18S-ITS2 universal primers. In summary, this

study included a significant methodological development that will be utilised to enhance our ability to better define the relationship that *L. longbeachae* has with its amoebal hosts. Although we were unable to identify a suitable host for *L. longbeachae* in the given time frame, we have established a strong foundation for future research.

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List of abbreviations

Abbreviation	Description
ATCC	American Type Culture Collection
BCYE	Buffered charcoal yeast extract
CT	Cycle threshold
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
FLA	Free-living amoeba
GVPC	Glycine, vancomycin, polymyxin B and cycloheximide
IFA	Indirect immunofluorescence
MALDI-ToF	Matrix assisted laser desorption ionisation - Time of Flight.
$MgCl_2$	Magnesium chloride
MOI	Multiplicity of infection
NNA	Non-nutritive agar
NTC	No template control
PAS	Page's amoeba saline
PBS	Page's balanced saline
PYG	Peptone, yeast and glucose
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
RT-qPCR	Real-time quantitative polymerase chain reaction
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Chapter 1: Introduction

Legionella are gram-negative, pleomorphic bacilli that are ubiquitous in nature and are responsible for a severe form of pneumonia in humans known as Legionnaires' disease. [1, 2] *Legionella* was first recognised as a significant human pathogen following an outbreak during the 1976 American Legions Convention in Philadelphia, which resulted in 212 attendees being diagnosed with Legionnaires' disease. [2-4] Symptoms ranged from a mild flu-like illness to severe multi-system organ failure, which was fatal for many. [3] Following an intensive investigation, the aetiological agent was recognised as the environmental organism *Legionella pneumophilia* and was considered to be the primary cause of Legionnaires' disease. [2, 4]

Over the past 40 years more than 50 species of *Legionella* have been described, at least half of which have been reported to cause human disease. [5-7] Like *L. pneumophilia*, most species of *Legionella* thrive in freshwater environments, as well as man-made water systems such as spa pools, cooling towers and heating units, where they can withstand temperatures of 20-60° C. [8-10] Globally, *L. pneumophila* is reported as the cause of 90% of Legionnaires' disease cases, with *Legionella longbeachae*, *Legionella micdadei*, *Legionella bozemanii* and *Legionella dumoffii* responsible for the remaining 2-7% of cases. [5, 11, 12] However, cases of Legionnaires' disease caused by *Legionella* species other than *L. pneumophila* are often underrepresented. [13-15] This is due to many diagnostic facilities relying heavily upon urine antigen testing, a test that is only specific for *L. pneumophila* serogroup 1. [13, 14, 16] In Australia and New Zealand, *L. longbeachae* is the predominant cause of Legionnaires' disease and is routinely tested for in patients presenting with suspected pneumonia. [13, 15] Nearly two thirds of all notified cases of Legionnaires' disease in New Zealand are caused by *L. longbeachae* infection. [17] This percentage is significantly greater than those caused by *L. pneumophila*, particularly in distinct

regions where *L. longbeachae* infections can account for up to 85% of Legionnaires' disease cases each year. [13, 18-20]

1.1 Legionellosis

Legionellosis refers to any disease that is caused by *Legionella* bacteria. [5, 21] *Legionella* are obligate intracellular bacteria and legionellosis occurs when aerosolised particles containing *Legionella* bacteria are inhaled from a contaminated source. [21, 22] Once infected, the pathogen has a unique survival strategy of infecting and replicating within alveolar macrophages by avoiding fusion with the host lysosome. [7, 22] Legionellosis often manifests in two distinct forms, Legionnaires' disease and the less severe, Pontiac fever. [5, 21] Legionnaires' disease may present as a severe pneumonia that is characterised by acute respiratory, gastrointestinal and neurological symptoms with a fever that exceeds 40 °C. [3, 12] Pontiac fever is usually a milder, self-limiting illness with symptoms that often resemble those of influenza. [21, 23] However, unlike Legionnaires' disease, Pontiac fever often goes undiagnosed as it usually resolves on its own within 1-3 days. [12, 23] Elderly and immunocompromised hosts are more at risk of developing Legionnaires' disease than those who are immunocompetent. Furthermore, individuals with pre-existing conditions, such as emphysema, diabetes and a history of smoking or chronic lung disease, also have a higher risk of contracting Legionnaires' disease. [3, 24, 25] Legionnaires' disease is the cause of 2-15% of all community-acquired pneumonia cases, however, is less often associated with hospital acquired pneumonia. [26] Legionnaires' disease is clinically indistinguishable from other types of pneumonia and the general lack of awareness and implementation of routine testing specific for *Legionella* is often overlooked by clinicians. Therefore, resulting in a large number of cases going undiagnosed. [16, 21, 27] Furthermore, *Legionella* are resistant to many antibiotics, such as beta-lactam antibiotics that are commonly used to treat pneumonia. This can lead to delays in starting appropriate treatment and is often linked to a poor prognosis and the development of systemic illness that requires intensive

medical intervention. [28] This highlights the importance of implementing standardised treatment guidelines for all cases of community-acquired pneumonia to ensure any delays in treatment are avoided.

1.2 *Legionella longbeachae*

L. longbeachae is the second most predominant cause of Legionnaires' disease worldwide. [29] *L. longbeachae* was first recognised as a new species when it was isolated from the lung tissue of a patient with Legionnaires' disease in Long Beach, California in 1981. [18] Despite its original isolation, *L. longbeachae* is considered to be a rare cause of Legionnaires' disease in the United States and although it is recognised as an important emerging pathogen, the majority of research efforts worldwide still focus on *L. pneumophila*. [30, 31] Conversely, in Australasia, *L. longbeachae* is recognised as a significant human pathogen and is the primary focus of *Legionella* research. There is no significant difference between the clinical presentation of Legionnaires' disease caused by *L. pneumophila* and *L. longbeachae*. [6, 18] However, both *L. pneumophila* and *L. longbeachae* exhibit distinct ecological niches. Unlike *L. pneumophila* and other *Legionella* species, *L. longbeachae* is primarily isolated from soil environments and is rarely associated with water-borne outbreaks. [10, 13, 18, 32] Recent investigations into the genome of *L. longbeachae* have revealed many unique adaptations that reflect its soil environment. [33, 34] In 2016, a phylogenetic analysis by Joseph *et al.*, compared 19 species and 43 strains of *Legionella* and found that these species were highly diverse and shared only a conserved core genome that was made up of 1,140 genes [35] *L. longbeachae* formed a monophyletic clade with *L. dumoffi* and *L. bozemanii* based on the presence of 2,452 clade specific genes, whilst *L. pneumophila* formed a distinct clade. [35]

Comparatively, the *L. longbeachae* genome is ~500 kb larger than the *L. pneumophila* genome and has been predicted to contain 3,152 protein coding genes, as well as a 71,826 bp plasmid.

However, despite the *L. pneumophila* genome only consisting of 2,878 protein coding genes, it has a much larger plasmid (131,855 bp) than *L. longbeachae*. [34, 36] In 2010, *Cazalet* et al., published a complete genome sequence of *L. longbeachae* NSW150 and identified several genes encoding proteins that have not yet been reported in any other *Legionella* species. [34] Numerous genes were identified that indicated a plant-pathogen relationship, such as chitinases and cellulolytic enzymes that can degrade cellular material. [34] It was later hypothesised that high levels of recombination through horizontal gene transfer with various soil species has led to the acquisition of a unique accessory genome that selectively enhances the survival of *L. longbeachae* in such a diverse environment. [35]

1.2.1 Potting mix as a source for Legionnaires' disease

In 1989 an outbreak of Legionnaires' disease in South Australia caused by *L. longbeachae* led to an investigation by Steele *et al*., who found gardening and the use of commercially available potting mix was the most likely source of infection. [37] All of those affected by the outbreak were avid gardeners who acknowledged the use of commercial potting mix prior to the onset of infection. [37] Further studies investigating the presence of *Legionella* bacteria in Australian potting mix resulted in the isolation of *L. longbeachae* from 26 of the 45 (58%) locally manufactured products. [38] Several other studies have also confirmed this association, finding levels of *Legionella* as high as 1×10^5 CFU/ml in some manufactured soils. [39-42] However, in European potting mixes, *L. longbeachae* is very rarely detected and *L. bozemanii* and *L. pneumophila* are the most commonly isolated species. [38, 41] A comparative study by Steele *et al*., found several differences in the composition of commercial potting mix manufactured in Australia and Europe. In Australia and New Zealand, hammer milled bark, pine bark and sawdust are the major constituents of the product, whereas in Europe, peat moss and green waste accounts for 80% of the raw materials. [38, 39]

In a recent investigation into potting mix products in New Zealand it was shown that amongst these raw materials, pine bark had the highest rate of *L. longbeachae* contamination (unpublished data, The Infection Group, University of Otago Christchurch). Little information is available concerning the composting process of commercial potting mix, however large-scale composting facilities often manufacture large quantities of product that is maintained in piles between 40-50°C for several weeks before processing. [39] The high heat and moisture content during this process is thought to allow for the rapid multiplication of *Legionella* to detectable levels. [39] Several risk factors have since been identified concerning gardening and the handling of commercial potting mixtures. Certain behaviours such as not your washing hands immediately after gardening or vigorously spreading potting mix, particularly in enclosed areas can increase the likelihood of inhaling bio-aerosols, which is thought to be the main route of transmission for *L. longbeachae* infections. [29]

1.3 Microbiology

Legionella are nutritionally fastidious organisms that cannot be cultured on standard nutrient media. [43] *L. pneumophila* was the first species of *Legionella* to be isolated using an *in vivo* guinea pig model [4] Since its isolation, several selective media have been developed that have varied in their ability to support the growth of *Legionella in vitro*. [44] In 1978, Feeley *et al*., recognised that L-cysteine and ferric pyrophosphate were essential components for the successful laboratory culture of *Legionella.* [44] In 1979, a subsequent study by Feeley *et al*., showed that the addition of yeast extract and activated charcoal to the media improved the recovery of *L. pneumophila* by 1 x 10^2 CFU/ml. [45] Collectively, these findings led to the development of the buffered charcoal yeast extract (BCYE) medium that is routinely used for the laboratory culture of many *Legionella* species. A comparative study by Lee *et al*., investigated the growth of 28 *Legionella* species on BCYE agar and recorded the growth of several species including *L. longbeachae* to be minimal in comparison to *L. pneumophila.* [46]

However, as there have been no successful improvements made to the media for the isolation of non-pneumophila species, BCYE agar remains the basis for laboratory culture of all *Legionella* species.

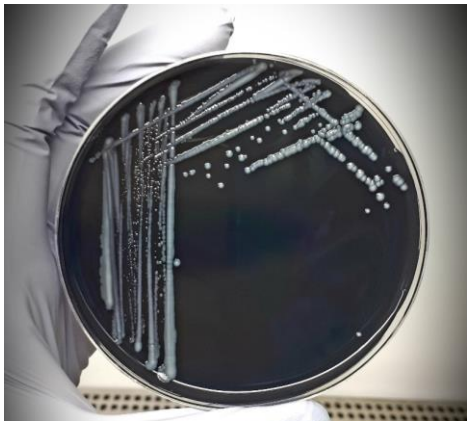

Figure 1.1 *Legionella longbeachae* serogroup 1 on BCYE agar.

L. longbeachae appear as small, grey, smooth colonies of 0.5-1.0 mm on BCYE agar approximately 72 hours after incubation at 36 °C (Fig 1.1). [47] *Legionella* colonies have a distinctive grainy appearance that is often used as an identification tool for suspected isolates. If *Legionella* is suspected, colonies should be cultured in the absence of L-cysteine and monitored for growth. [47]

Isolating *Legionella* from environmental samples can be challenging. *Legionella* growth on BCYE is often rapidly out-competed by the growth of other environmental microorganisms. [48, 49] It is estimated that the number of microbial species per gram of soil can be as high as 8.3 million in nutrient rich environments. [50] Several methods have been developed to reduce the level of microbial contamination when culturing *Legionella* species. These include, the supplementation of media with antimicrobials, and acid or heat treatment of samples prior to culture. [51-53] Through the addition of antibiotics to BCYE agar, new media have been developed to isolate *Legionella* species from complex samples. [48, 53] For example, GVPC is supplemented with glycine, vancomycin, polymyxin B and cycloheximide and has been shown to inhibit the growth of many microbial species whilst having minimal effects on *Legionella* growth. [48, 52]

1.3.1 Detection of *Legionella* in clinical samples

There are currently several different methods employed by diagnostic facilities to detect *Legionella* in clinical samples. Commonly these include urine antigen testing, culture, serology and polymerase chain reaction (PCR).[54] Urine antigen testing is a very sensitive, rapid and cost effective diagnostic technique. However, it is only specific for *L. pneumophila* serogroup 1. [55] Consequently, an over-reliance on urine antigen testing as a primary detection method for Legionnaires' disease has led to an underestimation of the number of cases that are caused by species other than *L. pneumophila* serogroup 1. [56, 57] Generally, culture based detection is considered to be the 'gold-standard' for diagnosis of Legionnaires' disease. [11, 54] However, several studies have shown that recovery rates for culture can be as low as 50%, as reviewed by Mercante *et al.,* in 2015. [58] This could be due to the inability of culture to support the growth of *Legionella* in the viable but non-culturable (VBNC) state, and the relatively high level of technical expertise required to successfully cultivate *Legionella* under laboratory conditions. [57, 58] Furthermore, only a subset of patients (~50%) can spontaneously expectorate sputum which can further decrease the sensitivity of a culture based diagnosis, when the entire population of Legionnaires' disease cases are being considered. [59] Nevertheless, many laboratories still rely upon culture as a routine diagnostic method, although it is often performed alongside a more reliable technique. Serological detection is another commonly employed diagnostic technique for Legionnaires' disease. Serology involves the detection of antibodies that are targeted against the antigen of interest. Of these methods, indirect immunofluorescence (IFA) and enzyme-linked immunosorbent assay (ELISA) are routinely used. [11] ELISA is a highly sensitive diagnostic technique; however, a reliable diagnosis of Legionnaires' disease using ELISA requires at least a four-fold increase in antibody titre. [54] Generally seroconversion can be detected within 3-4 weeks, although for a subset of cases the antibody titre can remain under the limit of detection for several weeks and is a major limiting factor of this technique. [11, 60] The introduction of nucleic acid amplification by PCR has led to

significant advances in the field of diagnostic microbiology. [61] The use of PCR to detect *Legionella* species is a highly sensitive and rapid technique, with a turnaround time of 6-8 hours. [62] Many studies have reported PCR sensitivities as high as 100% and increases of up to 30% when compared to standard culture methods. [62-64] Furthermore, unlike urine antigen testing PCR is able to detect all pathogenic species of *Legionella*, including *L. longbeachae* and is therefore an important diagnostic method for monitoring disease status. For example, in 2010 routine PCR screening for all patients presenting with suspected pneumonia was introduced in Canterbury, New Zealand. [15] A pre and post comparison study conducted by Murdoch *et al.*, found that routine testing for *Legionella* infection using PCR was associated with a 4-fold increase in the number of detected cases when compared to routine culture of patient specimens. [15, 59] PCR is therefore being recognised as a valuable diagnostic tool for Legionnaires' disease surveillance.

1.4 Ecology

In 1980 Rowbotham first demonstrated that *Legionella* are intracellular parasites of free-living amoeba (FLA). [65] FLA are protozoa that are ubiquitous in soil and water environments and play a crucial role in the life-cycle of *Legionella.* [66, 67] Since 1980 various amoebal co-culture systems have been developed using *L. pneumophila* as a model organism. These studies have led to the identification of many species of protozoa that can act as hosts for *Legionella* in the environment, as reviewed by Boamah *et al*., in 2017. [68] Of these species, *Acanthamoeba*, *Naegleria* and *Vermamoeba* are commonly found to be important host species that play a role in regulating the abundance of *Legionella* in the environment. [69] In nature, *Legionella* form and colonise established multispecies biofilms comprised of other environmental microorganisms such as *Klebsiella* and *Pseudomonas.* [70-72] As *Legionella* are fastidious organisms, complex biofilms provide sufficient nutrients for *Legionella* to survive in nutrient poor environments and offer protection from harsh environmental conditions such as,

disinfection and other antimicrobial treatments. [73, 74] In natural environments, FLA are bacterial predators that graze along the surface of biofilms, detaching and engulfing planktonic bacterial cells by phagocytosis. In soil environments, this interaction is crucial for nutrient cycling and bacterial turnover. [67, 75] However, *Legionella* species have exploited this interaction and intentionally colonise well-established biofilms to become targets of protozoan grazing. [71] *Legionella* have two distinct phases during their lifecycle, the transmissive phase and the replicative phase. [76, 77] In the absence of L-cysteine and other nutrients, *Legionella* enter the transmissive phase in which they are extremely virulent. A study by Byrne *et al*., in 1998 showed that during the transmissive phase several genes were upregulated that enhanced the infectivity potential of *L. pneumophila* in eukaryotic cells. [78] In adverse environmental conditions, *Legionella* may switch from the transmissive phase to a viable but non-culturable (VBNC) state in which metabolic activity is reduced but cellular integrity and virulence is retained. [79, 80] In this state, *Legionella* still have the potential to cause serious illness if a susceptible host is encountered. [81, 82] Conversely, when *Legionella* is phagocytosed by an amoeba it enters into the avirulent, replicative phase of its life-cycle in which it utilises host L-cysteine and other nutrients to support replication. [76, 77] Unlike many genera of bacteria, *Legionella* species have acquired several unique eukaryotic-like proteins such as, ankyrin and leucine-rich repeats as a result of this co-evolution with protozoan species in the environment. [34, 83] This interaction has led to a well-documented and very broad protozoan host-range for *Legionella* species and has enabled them to become ‘accidental’ human pathogens by infecting human lung macrophages using a similar mechanism. (Fig 1.2) [67, 72, 84]

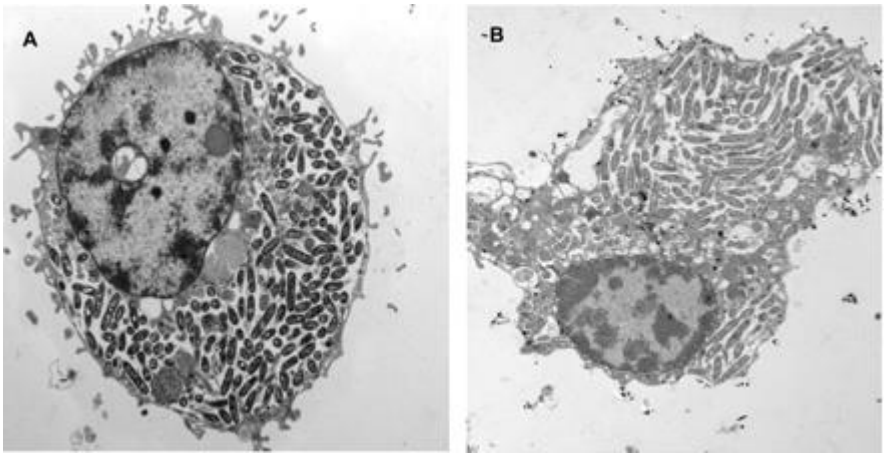

Figure 1.2 Infection of U937 macrophages (A) and *Acanthamoeba polyphaga* (B) with *Legionella pneumophila*. Source: Molmeret, 2005. [85]

1.4.1 Intracellular life-cycle of *Legionella*

Following phagocytosis, *Legionella* are thought to form a replicative niche inside the phagosome of the host known as the *Legionella* containing vacuole (LCV). [86] Within the host, *Legionella* persistence is mediated by a Dot/Icm system (T4SS) that translocates 'eukaryotic-like' effector proteins into the host cell cytosol and subverts host cell signalling. [87] This unique mechanism allows *Legionella* to persist within the LCV by blocking maturation of the phagosome to its acidic, bacteriolytic form. Several studies have investigated this interaction using *L. pneumophila* and *Acanthamoeba* species as model organisms. Using scanning electron microscopy (SEM) these studies were able to confirm that *Legionella* with defective Dot/Icm systems fail to establish replicative vacuoles and were rapidly degraded by the host following lysosomal maturation. [88-90] After infection, the LCV associates with the host cell endoplasmic reticulum and recruits ribosomal membranes that surround the LCV and provide an environmental signal that induces *Legionella* replication. [89, 91, 92] Replication continues until the hosts nutrients are depleted and bacterial growth can no longer be supported. [70] In 2002, Molmeret *et al*., showed that when the host's nutrients are depleted, *Legionella* re-enter the transmissive phase and induce host cell lysis, mediated by a cytolytic pore-forming toxin. [93] When the host's membrane lyses, hundreds of virulent legionellae are released back into the

environment where they can encounter new hosts, or persist in their VBNC state. Many genetic analyses have revealed that *L. pneumophila* cultured within amoebal host cells have upregulated expression of several virulence-related genes when compared to standard laboratory culture [94-96] These organisms were shown to be more invasive, virulent and resistant to antimicrobial therapy when introduced into murine models. This suggests that the interaction between *Legionella* and their amoebal hosts in the environment may be necessary to produce strains of *Legionella* that are capable of causing human disease. [94, 97]

1.5 *Legionella* co-culture

Co-culture has been recognised as a promising method for the recovery of fastidious intracellular microorganisms, such as *Mycobacteria*, *Listeria* and *Legionella* from complex systems. [98-100] The use of an amoebal co-culture system was first demonstrated in 1983 by Rowbotham who showed that *Acanthamoeba polyphaga* could be used to isolate *L. pneumophila* from clinical specimens. [101] Since then, numerous studies have been conducted to elucidate the diverse protozoan host range of *Legionella.* To date, 14 amoebal species, two ciliates and one slime mould have been found to support the intracellular replication of *L. pneumophila.* [68] However, for the focus of this review, only the most documented species are presented. (Table 1.1) The *Legionella* co-culture system mimics the interaction between *Legionella* and their protozoan hosts in the environment. *Legionella* are able to proliferate in the absence of competing microorganisms and can be isolated using routine culture methods following induced host cell lysis. [99] In 2013, Conza *et al.* demonstrated that the use of an *A. polyphaga* co-culture system enriched the levels of *L. pneumophila* in spiked compost by a factor of six (CFU/mL). Conversely, the same co-culture system only enriched levels of *L. longbeachae* by 1.1%, suggesting an inability for *L. longbeachae* to replicate within *A. polyphaga*. [102] Similarly, studies conducted by Neumeister *et al.*, and Wadowsky *et al.*, have shown that *L. longbeachae* is not able to replicate

within *Acanthamoeba castellanii* or *Vermamoeba vermiformis.* (Table 1.1) Interestingly, most of these studies were performed using reference strains rather than environmental or clinical isolates which may have inaccurately reflected the ability of *L. longbeachae* to proliferate in the environment. Only one study has been published that has demonstrated the ability of *L. longbeachae* to replicate within a protozoan host. In 1996, Steele and McLennan observed the rapid intracellular replication of *L. longbeachae* and other non-pneumophila species within the freshwater ciliate *Tetrahymena pyriformis* using a co-culture system. (Table 1.1) It was concluded that *T. pyriformis* may be able to serve as a host for the isolation of several species of *Legionella*, although these results are contradictory to previous investigations.

Table 1.1 Isolation of protozoan species and their interactions with *Legionella pneumophila* and *Legionella longbeachae* in the environment.

Protozoan species	**Environmental source**	**Effect on *L. pneumophila***	**Effect on *L. longbeachae***	**Reference**
Acanthamoeba polyphaga	Compost facilities Cooling towers Natural water systems	Intracellular replication	Permissive to infection	(65, 69, 102-107)
Acanthamoeba castellanii	Compost facilities	Intracellular replication	Intracellular degradation	(65, 102, 103, 108-110)
***Naegleria* spp.**	Compost facilities Natural water systems	Intracellular replication	____ _	(65, 102, 103, 111-114)
Vermamoeba vermiformis	Compost facilities Natural water systems Man-made water systems	Intracellular replication	Not permissive to infection	(102, 103, 111, 115-120)
Tetrahymena pyriformis	Natural water systems Cooling towers	Intracellular replication	Intracellular replication	(121-124)

1.6 Research rationale and objectives

It is repeatedly mentioned in the literature that *L. longbeachae* is one of the major causative agents of Legionnaires' disease. However, there is still limited information available concerning the intracellular life-cycle of *L. longbeachae*, which amoebal hosts are important mediators of this lifecycle and whether these factors can influence the ability of *L. longbeachae* to cause human disease. Reviewing previous investigations of *L. pneumophila*; which is the primarily studied species, provides a valid starting point for this study. However, due to the vast differences in the genome structure and ecology of *L. longbeachae* when compared to *L. pneumophila*, it cannot be assumed that *L. longbeachae* will utilise the same intracellular mechanisms and survival tactics. As a result of this, our ability to plan strategies and reduce the potential risk of human infection is severely limited. Therefore, the objective of this research is to gain a greater understanding of the relationship between *L. longbeachae* and its protozoan hosts by:

1. Determining the presence or absence of common amoebal species using qPCR on *L. longbeachae* positive environmental samples.
2. Developing methods to isolate and identify amoebal species that can serve as laboratory hosts to *L. longbeachae*.
3. Defining the parameters of a *L. longbeachae*/amoebal co-culture system that can subsequently be used to isolate *L. longbeachae* from complex environments.

The information gained from this study will provide insights into *L. longbeachae* survival and persistence in the environment and in turn may lead to better strategies to prevent and manage Legionnaires' disease in New Zealand, where the rates are among some of the highest in the world.

Chapter 2: Materials and Methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are summarized in Table 2.1. *L. longbeachae* F1157CHC was derived from a patient hospitalized with Legionnaires' disease in Christchurch, New Zealand. *L. pneumophila*, *Escherichia coli* and *Klebsiella pneumoniae* were laboratory type-strains obtained from the American Type Culture Collection (ATCC). All bacterial strains used in this study were provided by Canterbury Health Laboratories (CHL), Department of Microbiology. A list of all media and buffers used in this study are included in Appendix 6.1.

Table 2.1. Bacterial strains used in this study

Bacterial species	Strain	Origin
Legionella longbeachae	F1157CHC	Clinical isolate, Canterbury, NZ
Legionella longbeachae	(ATCC® 33462™)	American Type Culture Collection
Legionella pneumophila	(ATCC® 33152™)	American Type Culture Collection
Escherichia coli	(ATCC® 25922™)	American Type Culture Collection
Klebsiella pneumoniae	(ATCC® 700603™)	American Type Culture Collection

Legionella species were cultured at 36 °C on buffered charcoal yeast extract (BCYE) medium for 72 hours. Single colonies were sub-cultured between 72-96 hours for a maximum of five sub-cultures before returning to the original stock culture, as continuous sub-culturing can lead to the accumulation of undesired mutations. *E. coli* and *K. pneumoniae* were cultured on tryptic soy agar (TSA) supplemented with 5% defibrinated sheep's blood (Fort Richard, Auckland NZ) at 36 °C for 18 hours. Cultures were maintained for 72 hours before being sub-cultured onto fresh medium. Stocks of all bacterial strains were maintained in tryptic soy broth (TSB) culture medium containing 15% (v/v) glycerol and stored at -80 °C.

2.2. Amoebal strains and growth conditions

Amoebal species used in this study are summarized in Table 2.2. Amoeba were selected for this study based on their ability to support the replication of *L. pneumophila in vitro*. [103, 111, 112] *A. polyphaga* (ATCC® 50372™) was provided by the Institute of Environmental Science and Research (ESR) and was cultured axenically at 25°C in a Nunc™ T-25 cell culture flask (Thermo Fisher Scientific, MA, USA) containing 5 mL PYG media (Appendix 6.1). *A. castellanii* was derived from an eye scrapping of a patient with Acanthamoeba keratitis and was provided by CHL. *N. fowleri* (ATCC® 22758™) was purchased from ATCC by the University of Otago Christchurch, Department of Pathology and Biomedical science. *A. castellanii* and *N. fowleri* were maintained in monoxenic cultures on non-nutritive agar (NNA, appendix 6.1) plates seeded with *E. coli* or *K. pneumoniae* at 25°C.

Table 2.2. Amoebal strains used in this study

Amoebal species	Strain	Origin
[1] *Acanthamoeba polyphaga*	(ATCC® 50372™)	American Type Culture Collection
[2] *Naegleria fowleri*	(ATCC® 22758™)	American Type Culture Collection
[3] *Acanthamoeba castellanii*	-	Clinical isolate, Canterbury, NZ

[1] Institute of Environmental Science and Research (ESR), Canterbury.

[2] University of Otago Christchurch, Department of Pathology and Biomedical Science.

[3] Canterbury Health Laboratories, Department of Microbiology.

2.2.1 Culture maintenance

2.2.1.1 *Acanthamoeba polyphaga*

An ampule containing 500 µL frozen solution was thawed in a 35 °C water bath for 2-3 minutes. The solution was aseptically transferred to a sterile T-25 tissue culture flask containing 5 mL PYG medium and incubated at 25 °C for three days. After three days, the media was replaced with 5 mL fresh PYG media to remove any remaining DMSO from the culture. Flasks were examined daily for the presence of trophozoites using an Olympus CK2 Inverted Phase Tissue

Culture Microscope (Olympus, Japan). When the culture appeared to be at or near peak density the media was discarded and replaced with 1 mL fresh PYG medium. The culture was then vigorously agitated to detach adhering trophozoites from the flask surface. Aliquots of 250 µL were aseptically transferred into new T-25 culture flasks and incubated at 25 °C. This process was repeated at 8-10 day intervals to maintain axenic cultures.

2.2.1.2 *Naegleria fowleri*

An ampule containing 500 µL frozen solution was thawed in a 35 °C water bath for 2-3 minutes and aseptically transferred to a plate of ATCC medium 997 (Freshwater amoeba medium, Appendix 6.1). The solution was distributed evenly over the plate surface using an L-shaped spread bar. Plates were sealed with parafilm to prevent desiccation of the culture and incubated at 25 °C for five days. After five days, plates were examined for the presence of trophozoites using an Olympus BX53 phase-contrast microscope (Olympus, Japan). When trophozoites were evident a single scrapping was taken from the surface using a 10 µL disposable loop and transferred to the centre of a NNA plate that had been freshly inoculated with a lawn of *K. pneumoniae*. Plates were incubated at 25 °C and were observed daily for amoebal migration. When the cultures were at or near peak density a single scrapping was taken from near the migration front and transferred to a freshly bacterized plate. This process was repeated at 8-10 day intervals to maintain a monoxenic culture.

2.2.1.3 *Acanthamoeba castellanii*

An already established culture of *A. castellanii* was provided by CHL on a NNA plate seeded with *E. coli.* Plates were incubated at 25 °C and observed daily for amoebal migration. When the cultures were at or near peak density a single scrapping was taken from near the migration front and transferred to a freshly bacterized plate (as above). This process was repeated at 8-10 day intervals to maintain a monoxenic culture.

2.3 Cryopreservation of Amoeba

A. polyphaga was cultured axenically for 14 days in PYG media and examined for encystment using an Olympus CK2 Inverted Phase Tissue Culture Microscope (Olympus, Japan). Cells were harvested when 80-90% of the culture had encysted. Culture medium was discarded and replaced with 1 mL fresh PYG medium and the culture flasks were vigorously agitated to detach adherent cells. Cells were enumerated using a Kova® Glasstic slide with counting grid (CHL) and were adjusted to a final volume of 1 x 10^6 cells/mL by centrifugation at 500 x g for five minutes. The pellet was then re-suspended in a volume of fresh PYG medium necessary to yield the desired concentration. Xenically cultureable amoeba were harvested from NNA agar plates after 14 days following depletion of the bacterial food source and amoebal encystment. The plate surface was flooded with 1-2 mL Page's amoebal saline (PAS) solution (Appendix 6.1) and scraped with an L-shaped spread bar to remove adherent cells. The amoebal suspension was transferred into 1.75 mL microcentrifuge tubes and centrifuged at 500 x g for five minutes to isolate the amoeba within the suspension. The pellet was then re-suspended in 1 mL PAS (*N. fowleri*) or PYG solution (*Acanthamoeba)*. The cells were enumerated and adjusted to a final concentration of 1 x 10^6 cells/mL. A 20% solution of dimethyl sulfoxide (DMSO) was prepared with fresh PAS or PYG medium and was added in equal volumes to the amoebal suspension to produce a final solution containing 10% DMSO. Aliquots of 500 µL were transferred to 2 mL screw-capped cryovials where they were held ambient at room temperature (RT) for 15 minutes, -20 °C for 30 minutes and -80 °C for long-term storage.

2.4 Real-time (RT) quantitative polymerase chain reaction (qPCR)

DNA from 390 previously collected and stored tree bark samples were tested for the presence of *Acanthamoeba* spp. and *N. fowleri* in individual RT-qPCR assays. These samples had been previously tested for the presence of *L. longbeachae* by RT-PCR in an attempt to identify the reservoir of infection for Legionnaires' disease (Unpublished data, The Infection Group, UOC).

Bark samples were collected from *Pinus radiata*, *Pinus* spp., and an assortment of mixed species trees over the period of one year from three different sites around Christchurch, New Zealand (Table 2.3). Trees were sampled once, with the exception of *P. radiata* trees which were repeatedly sampled at three month intervals over one year to assess if there was any seasonal variation. Briefly, ~10 g of bark was collected from the north and south side of each tree at breast height (~1.5 m above ground level) and suspended in 50 mL UltraPure water (Life Technologies; CA, USA). DNA was extracted from 200 µL of the solution using a GenElute™ Bacterial Genomic DNA Kit, following the instructions provided by the manufacturer (Sigma Aldrich, MO, USA; see Appendix 6.2 for DNA extraction protocol). Each sample was processed within one week of collection and DNA was stored at -20 °C until required.

Table 2.3. Environmental sampling of tree bark

Environmental sample	Location	Month/Year sampled	No. of trees	Total
Pinus radiata	Site 1	Seasonally (Jan-Oct `18)	22	220
Pinus spp.	Site 2	October 18’	41	82
Mixed species	Site 3	November 18’	44	88

All primers and probes used in this study were synthesized by Integrated DNA Technologies (IDT, Singapore) and are summarized in Table 2.4. PCR conditions for each individual assay are described below.

Table 2.4. List of RT-qPCR primers and probes used in this study.

Target	Primer/probe	Sequence
[1] ***Acanthamoeba* spp.**	Forward primer	5'-CCCAGATCGTTTACCGTGAA-3'
	Reverse primer	5'-TAAATATTAATGCCCCCAACTATCC-3'
	TaqMan Probe	5'FAM-CTGCCACCGAATACATTAGCATGG 3'BHQ
[2] ***N. fowleri***	Forward primer	5'-GTGCTGAAACCTAGCTATTGTAACTCAGT-3'
	Reverse primer	5'-CACTAGAAAAAGCAAACCTGAAAGG-3'
	TaqMan Probe	5'FAM-ATAGCAATATATTCAGGGGAGCTGGGC-3'BHQ
[3] ***L. longbeachae***	Forward primer	5'-GTACTAATTGGCTGATTGTCTTGACC-3'
	Reverse primer	5'-CCTGGCGATGACCTACTTTCG- 3'
	MGB TaqMan Probe	5'-VIC-TATCATGCCAATAATGCGCGA-3'BHQ
[4] **ART (inhibitor control)**	Forward primer	5'-AGCGGTGACGCATGCCTTCCA-3'
	Reverse primer	5'-CAAAGGAGACATTCTCACGCTACAGTT -3'
	TaqMan Probe	5'CY5-AACACCAAGTGGCCTTTCAGGCTGCGCGACT-3'BHQ

Primers were used to amplify fragments of ~180bp (1), 153bp (2) 259bp (3) and 158bp in length (4). MGB – Minor groove binder

Legionella longbeachae

RT-PCR for the detection of *L. longbeachae* was performed on an ABI 7500 real-time PCR machine with the following thermocycling parameters; one cycle of 95°C for 2 minutes, 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. PCR conditions had been previously optimised for the ABI platform and were based upon those described by Murdoch *et al.*, 2013. [(15)] The PCR reaction mix consisted of a 2 x TaqMan™ Gene Expression Master Mix, 0.5 µM of each primer, 0.2 µM of each probe, 1 µL ART template and 5 µL of DNA in a 25µL reaction mix. Fluorescence was measured at the end of each 60 °C incubation and the results were analysed using the ABI 7500 software, version 2.0.6 (Life Technologies, CA, USA). For quantification of environmental samples, a standard curve for absolute quantification was prepared. A 0.5 McFarland standard (~1 x 10^8 CFU/mL) was prepared with *L. longbeachae* (ATCC® 33462™) using a turbidity meter (CHL). From this suspension a 10-fold series of

dilutions were prepared ranging from 1 x 10^8 to 1 x 10^0 . To validate the CFU/mL in the original suspension, 100 µL from the 1 x 10^3 and 1 x 10^2 dilutions were plated on BCYE agar (in triplicate) and incubated at 36 °C for three days, after which the number of colonies on each plate were counted and averaged and used to determine the CFU/mL of the original suspension. DNA was extracted and amplified by RT-qPCR under the conditions described above. For each sample, the Ct value was fitted to the standard curve consisting of the eight dilution points of purified DNA template and a no template control.

Acanthamoeba and Naegleria fowleri

RT-qPCR for the detection of *Acanthamoeba* spp. and *N. fowleri* was performed on an Applied Biosystems (ABI) 7500 real-time PCR machine with the following thermocycling parameters; one cycle of 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 63°C for 60 seconds. Fluorescence was measured at the end of each 63 °C incubation step and the results were analysed as above. Initial PCR conditions were based upon those previously described by Qvarnstrom *et al.*, 2006. [125] The initial PCR reaction mix consisted of 2 x TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific, MA, USA), 0.2 µM of each primer, 0.1 µM of each probe and 5 µL of DNA in a 25 µL reaction mix.

2.4.1 PCR optimisation

DNA from laboratory cultures of *A. castellanii* and *N. fowleri* were used to test the efficiency of each PCR. DNA was extracted using a GenElute™ Bacterial Genomic DNA Kit following the instructions provided by the manufacturer. Initially, PCR was performed under the conditions mentioned above. Once the target DNA was successfully amplified, the PCR conditions were modified to achieve maximum efficiency on the ABI 7500 platform. As the TaqMan gene expression master mix contains $MgCl_2$ and dNTP concentrations that have all

been validated on the ABI system, further optimisation of these components were not required. However, primer and probe concentrations were optimised in a series of assays. Primer pairs were tested in triplicate at concentrations ranging from 0.2 µM to 0.5 µM in increments of 0.1 µM and probe concentrations were tested in triplicate at 0.1 µM and 0.2 µM. Thermocycling conditions were kept constant throughout the optimisation process. Optimal annealing temperatures were calculated using the Tm calculator (Thermo Fisher Scientific, MA, USA) and were not altered from those previously described. All primer pairs were tested in singleplex PCRs at the estimated optimal conditions before multiplexing, with the addition of an artificial construct (ART) as a PCR inhibition control. ART is constructed from a reference sequence (GenBank accession number: U17140) and was inserted into a plasmid carried by *E. coli* (CHL). ART template DNA was obtained from CHL and was diluted (10-fold) and amplified under the selected conditions until the CT (cycle threshold where fluorescence is detected) was consistent and between 28-34 cycles in each assay.

To verify that the selected conditions were suitable for use, a standard curve analysis was conducted. Standard curves were established using 6-day cultures of *A. castellanii* and *N. fowleri.* Cells were harvested by flooding plates with 1 mL Page's balanced saline (PBS) solution and the surface was scrapped with an L-shaped spread bar. Cells were enumerated using a Fuchs-Rosenthal counting chamber (Thomas Scientific, NJ, USA) and a 10-fold series of dilutions were prepared ranging from 10^0 to 10^{-6} from the original suspension. DNA was extracted and amplified by RT-qPCR under the selected conditions. For each sample, the Ct value was fitted to a standard curve consisting of the six dilution points of purified DNA template and a NTC. Once the standard curve was linear and reproducible, the conditions were not altered any further. A 1×10^{-2} dilution of *N. fowleri* and *A. castellanii* were used as positive controls for each PCR assay. This DNA also served as an internal calibrator to monitor the efficiency of the individual assays (Appendix 6.3). Following optimisation, the final PCR

conditions were as follows; 2 x TaqMan™ Gene Expression Master Mix, 0.5 µM of each primer, 0.2 µM of each probe, 1 µL of ART template and 5 µL of DNA in a 25 µL reaction mix.

2.4.2 Analytical sensitivity

The minimum detectable value for each individual RT-qPCR was estimated from analysis of the standard curve. The point in the standard curve where linearity was lost or no product was amplified was considered to be the analytical sensitivity of that particular assay.

2.4.3 Validation of primer specificity

The RT-qPCR primers used in this study were designed for the amplification of the *N. fowleri* and *Acanthamoeba* spp. 18S ribosomal RNA (rRNA) gene sequences. [125] To validate the specificity of these primers, partial 18S rRNA gene sequences for *A. polyphaga* (GenBank accession number: AY237735.1) and *N. fowleri* (GenBank accession number: KY062165.1) were retrieved from the National Centre for Biotechnology Information Nucleotide Database (NCBI, MA, USA). Sequences were downloaded into Geneious (version 10.2.6, Biomatters Ltd, Auckland NZ) and the primer and TaqMan probe sequences were mapped to the 18S rRNA gene. The PCR product was then excised and input into the Basic Local Alignment Search Tool (BLAST) by NCBI and the top 100 matches were viewed to ensure those with >90% sequence identity were specific to the genus of interest.

2.5 Amoebal enrichment and co-culture

A. polyphaga (ATCC® 50372™) was cultured in PYG medium at 25 °C in a T-25 surface cell culture flask for six days until it was near peak density. The cultures were harvested by vigorously agitating the flask to detach the adherent cells. Cells were enumerated using a Kova®

Glasstic slide with counting grid and were then diluted in a volume of fresh PYG medium necessary to yield a total concentration of 1 x 10^6 cells/mL. Amoeba were seeded in 12-well culture plates (Sigma Aldrich, MO, USA) at 1 x 10^6 cells/mL and were incubated at 30°C for one hour. Whilst the cells were incubating, a 0.5 McFarland standard of *L. longbeachae* and *L. pneumophila* (positive control) were prepared in saline (as previously described) and stained with a Vybrant® DiO cell-labelling solution (see 2.5.1). Following incubation, each well was washed once with PAS solution and replaced with a 1 mL 1:10 dilution of fresh pre-warmed PYG:PAS. Each well was inoculated with 100 µL of Dio-labelled *Legionella* (MOI 10:1) and the plates were centrifuged at 500 x g for five minutes to allow the *Legionella* and *A. polyphaga* to interact. Following centrifugation, the plates were incubated at 30 °C for two hours. Each plate of *L. longbeachae* and *L. pneumophila* was cultured with *A. polyphaga* in duplicate wells with the inclusion of an amoeba only control for visual comparison.

2.5.1 Dio-labelling *Legionella*

A 1 mL suspension of a 0.5 McFarland standard of *Legionella* was transferred to a 1.75 mL microcentrifuge tube and centrifuged at 13, 800 x g for five minutes to concentrate the bacteria. The supernatant was discarded and the pellet was re-suspended in 200 µL of PBS. The *Legionella* was stained with 1 µL of Vybrant® DiO cell-labeling solution (1mM stock; Thermo Fisher Scientific, MA, USA) and incubated at 36°C for 20 minutes in a light-proof container. Following incubation, 800 µL of PBS was added to the suspension to make up a final volume of 1 mL. The suspension was washed twice with PBS by centrifugation at 13, 800 x g for five minutes to remove any unbound dye from the solution. An aliquot of the supernatant from the final wash step was kept as a control to show that all the unbound dye had been removed.

2.5.2 Slide preparation and fluorescence microscopy

Culture wells were washed twice with PYG:PAS (1:10) solution to remove any extracellular bacteria from the suspension. Each well was aspirated several times to disturb the monolayer and a 500 µL aliquot from each well was transferred to a 1.75 mL microcentrifuge tube. The suspension was then washed again by centrifugation at 1000 x g for five minutes. The pellet was re-suspended and fixed in 500 µL of 4% paraformaldehyde for 30 minutes at RT. The suspension was centrifuged at 1000 x g for five minutes and the pellet was resuspended in 200 µL of PBS. 100 µL of each suspension was placed in the centre of a pre-cleaned microscope slide (in duplicate) and was left to air-dry for approximately one hour. The actin cytoskeleton of *A. polyphaga* was counterstained with Texas Red-conjugated phalloidin (5U/mL in PBS; Invitrogen, CA, USA) overnight at 4°C. Coverslips were mounted with ProLong1 gold antifade reagent (Invitrogen; CA, USA) and the slides were examined using a Zeiss Axioimager Z1 microscope with a 40x EC Plan Neofluar NA 1.4 objective and ApotomeTM structure illumination system (Zeiss, Germany).

2.6 Isolation of *Legionella longbeachae* from environmental samples

A five litre bag of randomly selected, commercially available potting mix was purchased from a local hardware store in Christchurch. This potting mix was primarily composed of composted *P. radiata*, pumice and organic fertilisers (>90%). Samples of ~5 g were repeatedly taken from the top layer of the soil and placed into individual Petri dishes over a three month period. Each sample was dampened with 5 mL of UltraPure water (Life Technologies, CA, USA). Each Petri dish was sealed with parafilm and incubated at 25 °C for at least 14 days to encourage the growth of any native bacteria. After 14 days, ~0.5 g samples of the pre-incubated potting mix were suspended in 1 mL of UltraPure water and centrifuged at 500 x g for five minutes to separate the bacterial cells from larger soil particles. The supernatant was transferred to sterile

microcentrifuge tubes and diluted to 1/100. Aliquots of 100 µL of the diluted sample were plated on GVPC agar (in triplicate) and incubated at 36°C for 72 hours. After 72 hours, plates were examined daily for suspected *Legionella* colonies for a period of seven days before the cultures were discarded. Suspected colonies were selected on the basis of colony morphology. Colonies were purified and sub-cultured onto fresh BCYE agar and incubated at 36 °C for a further 72 hours. Cultures were maintained by routinely sub-culturing single colonies onto fresh BCYE agar as previously described. A single colony from the purified culture was sub-cultured onto TSA with 5% defibrinated sheep blood (Fort Richard, Auckland NZ) and incubated at 36°C for 72 hours. Colonies of isolates that failed to grow on the above medium were examined under an Olympus SZX10 Binocular Zoom Stereo-Microscope (Olympus, Japan) for the distinctive grainy appearance that is characteristic of *Legionella*. Finally, isolates were identified by MALDI-ToF mass spectrometry. Briefly, an inoculum of purified bacteria was transferred to a 96-well MALDI steel plate and a 1 µL suspension of 70% formic acid was added to each sample and allowed to air-dry. Following this, 1 µL of α-Cyano-4-hydroxycinnamic acid (CHCA matrix) was added to each sample. The samples were processed using the MicroFlex LT (CHL) and the results were analysed by the MALDI Biotyper version 3.1 (Bruker Daltonics, Germany).

2.7 Isolation of amoeba from environmental samples

Isolation of amoeba from environmental samples was based on the methods previously described by Amaro and Shuman (2019). [(126)] These methods were a modification of the amoeba "walk-out" method originally described by Neff (1958). [(127)] A 1.5 g sample of pre-incubated potting mix was placed in the centre of a grade 1, 11 µm filter paper disk (Whatman; Thermo Fisher Scientific, MA, USA). Filter disks were placed in the centre of a NNA plate seeded with a lawn of live *E. coli* (Fig 2.1). Plates were sealed with parafilm and incubated at 25 °C for five

days. From day five, the agar surface was examined daily for amoebal migration using an Olympus CH-2 Binocular Microscope (Olympus, Japan). Areas of interest were marked with a fine tip marker and 1 cm^2 agar blocks were excised and placed surface side down onto the centre of a freshly bacterised NNA plate. Plates were incubated at 25 °C for seven days and observed for amoebal migration as previously described. After several days, an agar square containing a single trophozoite was excised and sub-cultured onto a freshly bacterised NNA plate as previously described. This process was repeated several times to ensure clonality of each isolate.

Figure 2.1. Isolation of protozoa from environmental samples using the amoeba "walk-out" method, modified from Amaro and Shuman (2019).

2.8 Statistical analysis

Statistical analysis to compare the levels of *L. longbeachae* (CFU/mL), *N. fowleri* (cells/mL) and *Acanthamoeba* spp. (cells/mL) was performed using R studio® version 1.2.1335 (Rstudio Inc, MA, USA, 2019). A Pearson's Chi-squared test with Yate's continuity correction was used to determine whether there was a significant difference between the levels of *L. longbeachae*, *N. fowleri* and *Acanthamoeba* spp. when compared to two variables, tree type and season. The

Chi-squared test is based on the null hypothesis that the above species are independent of each variable. Secondly, the strength and direction of association between *L. longbeachae* and *Acanthamoeba* spp. or *N. fowleri* was measured using the Pearson product-moment correlation coefficient. For all analyses, a probability value (P-value) of 0.05 was used as the threshold for significance. Data was provided by and used with the permission of The Infection Group, UOC.

Chapter 3: Results

3.1 Optimisation of PCR

For this study, 0.5 µM of forward and reverse primers and 0.2 µM of TaqMan probe were chosen as optimal concentrations for the amplification of both *N. fowleri* and *Acanthamoeba* spp. on the ABI 7500 system. These concentrations represented the lowest primer concentration that reproducibly yielded the lowest Ct values for each assay (Fig 3.1).

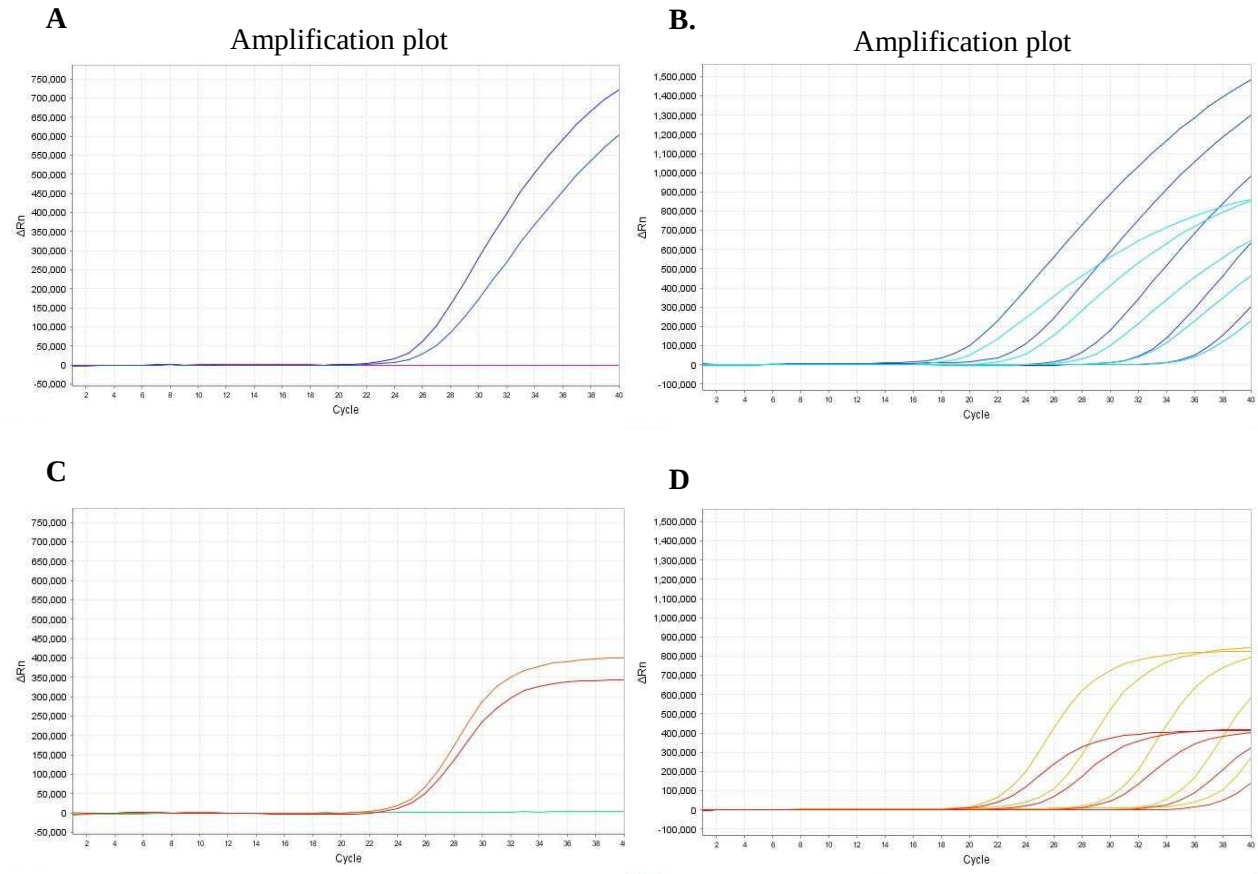

Figure 3.1 Optimisation of PCR conditions. **(A)** Comparison of 0.2 µM and 0.5 µM of *Acanthamoeba* forward and reverse primers. **(B)** Comparison of 0.1 µM and 0.2 µM of *Acanthamoeba* probe in a 10-fold dilution series **(C)** Comparison of 0.2 µM and 0.5 µM of *N. fowleri* forward and reverse primers **(D)** Comparison of 0.1 µM and 0.2 µM *N. fowleri* probe in a 10-fold dilution series.

For both *N. fowleri* and *Acanthamoeba* spp., increasing the concentration of forward and reverse primers from 0.2 µM (as previously cited) to 0.5 µM resulted in an increased ΔRn value (emitted fluorescence – background fluorescence) and therefore increased the magnitude of the

fluorescent signal generated. This signal was further increased by increasing the concentration of the TaqMan probe for both *N. fowleri* and *A. castellanii* to 0.2 µM, from the previously described 0.1 µM. *N. fowleri* retained a sigmoidal curve throughout the optimisation process; however, the same was not observed for *Acanthamoeba* despite optimisation of the conditions. This indicates that there may have been inefficiencies in the binding of the primers or probe to the target sequence. Due to this, only a reduced efficiency (70%) was possible for this assay. However, as this study relied upon the use of pre-designed primers that had been cited in multiple publications, this could not be avoided.

3.2 Standard curve analysis

Triplicate plate counts for *L. longbeachae* were averaged and used to calculate the CFU/mL of the original suspension, as 1.365×10^7. The total cell counts for *A. castellanii* and *N. fowleri* were 1.98×10^5 (cells/mL) and 1.97×10^5 (cells/mL), respectively. These values were used to establish a standard curve based on the absolute quantification method (Fig 3.2-3.4). Standard curve analysis of all three individual targets showed a linear relationship between the emitted fluorescence and DNA concentration over five or more log scales. This relationship demonstrates that the sample DNA was amplifying exponentially per PCR cycle. The R^2 values were used to evaluate the ability of the standard curve to accurately predict the value of 'x' or the unknown in the environmental samples. All R^2 values for the three individual targets were >0.99. Thus, providing confidence that we could correlate the two values (x and y) for quantification.

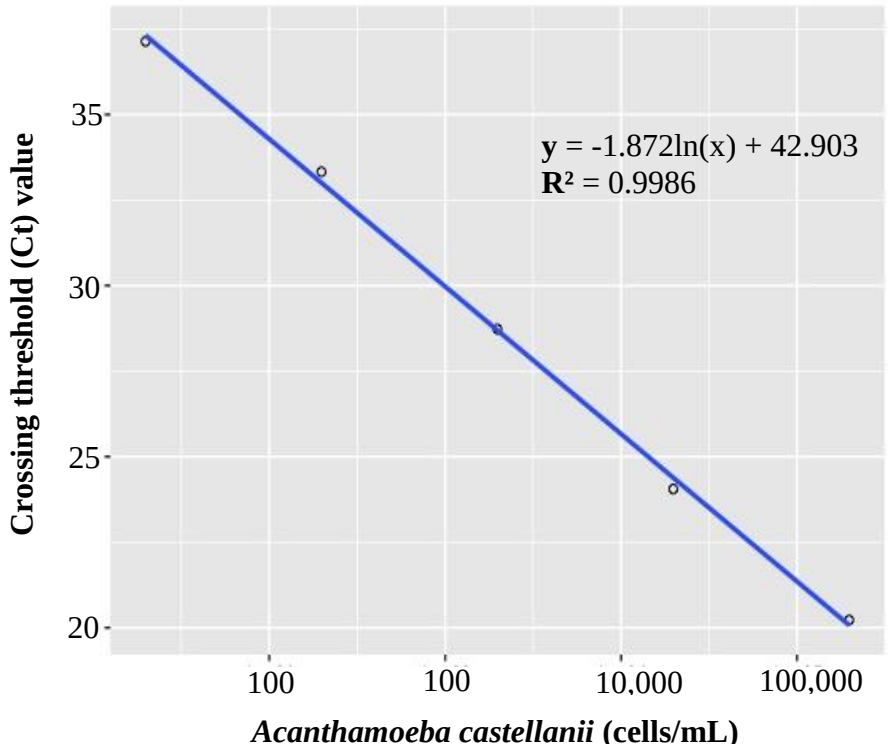

Figure 3.2 Standard curve consisting of five serial dilution points of *A. castellanii.* The Ct values for the five dilutions were plotted against the log of the concentration (cells/mL) to produce the standard curve. Data depicted corresponds to one of the standard curves obtained. Efficiency = 70.5%. Figure produced in R studio® version 1.2.1335.

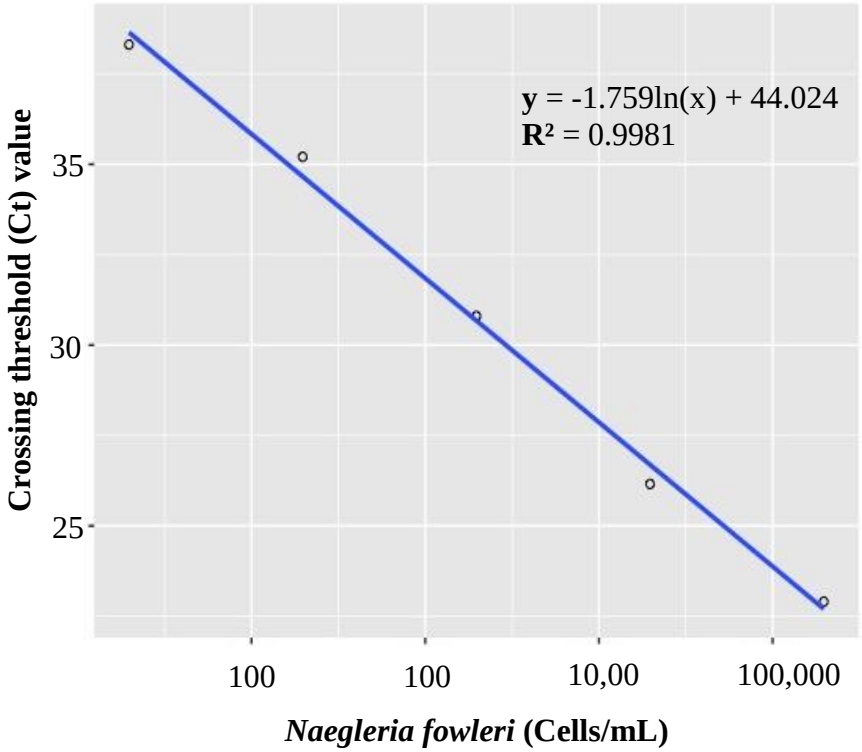

Figure 3.3. Standard curve consisting of five serial dilution points of *N. fowleri.* The Ct values for the five dilutions were plotted against the log of the concentration (cells/mL) to produce the standard curve. Data depicted corresponds to one of the standard curves obtained. Efficiency = 78%. Figure produced in R studio® version 1.2.1335.

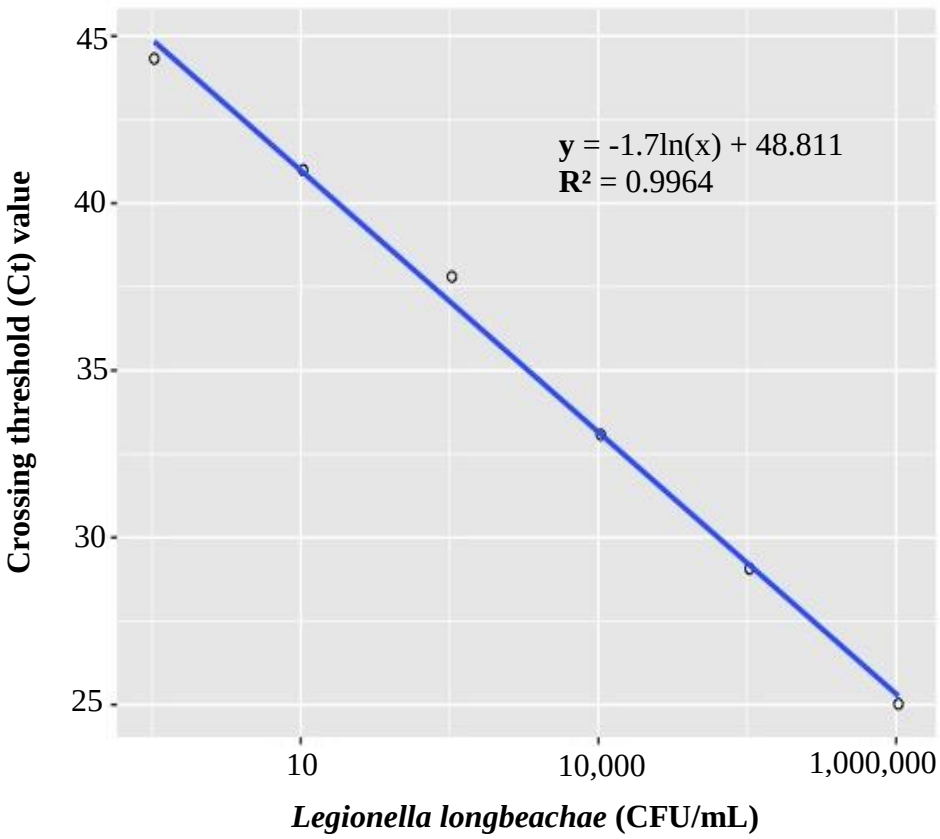

Figure 3.4 Standard curve consisting of six serial dilution points of *L. longbeachae*. The Ct values for the six dilutions were plotted against the log of the concentration (CFU/mL) to produce the standard curve. Data depicted corresponds to one of the standard curves obtained. Efficiency = 96.9%. Figure produced in R studio® version 1.2.1335.

3.2.1 Analytical sensitivity

The analytical sensitivity for *L. longbeachae, N. fowleri* and *A. castellanii* was determined by standard curve analysis and is summarised in Table 3.1. *N. fowleri* and *A. castellanii* had similar sensitivities, with both assays able to detect <20 cells/mL in a purified sample. No product was amplified at a dilution of 1 x 10^{-6}. Therefore, the analytical sensitivity for both *N. fowleri* and *A. castellanii* was between 2 and 20 cells/mL. *L. longbeachae* was detected at 10.365 CFU/mL, but linearity was lost at 1.036 CFU/mL. This value was considered to be outside the range of linear regression and was excluded from the standard curve analysis. Therefore, the analytical sensitivity for *L. longbeachae* was between 1 and 10 CFU/mL on the ABI 7500 platform.

Table 3.1 Analytical sensitivity of individual RT-qPCR assays

Target	Minimum detectable concentration	Ct value
A. castellanii	19.8 (cells/mL)	37.13783
N. fowleri	19.7 (cells/mL)	38.6169
L. longbeachae	10.365 (CFU/mL)	44.32436

3.3 Detection of *L. longbeachae, Acanthamoeba* spp. and *N. fowleri* in environmental samples using RT-qPCR

An analysis of the presence of *L. longbeachae* from 390 previously collected and tested DNA samples was evaluated by assigning samples to one of two groups, pine trees (*Pinus* spp.) and 'other' or mixed species trees. *P. radiata* trees (a sub-group of pine trees), which were repeatedly sampled over one year, were then further categorised by sub-grouping them according to the season in which they were sampled; autumn, winter, spring and summer.

L. longbeachae was detected in 47% of the samples tested. Of these samples, 73% of the *L. longbeachae* positive samples were detected on pine trees (*Pinus* spp.) and the remaining 27% on the mixed species trees. *N. fowleri* was not detected in any of the 390 samples tested; however, *Acanthamoeba* spp. was detected in 11.7% of the samples tested. Of these samples, 80% of the *Acanthamoeba* positive samples were detected on pine trees and the remaining 20% on the mixed species trees. Furthermore, the levels of both *L. longbeachae* (CFU/mL) and *Acanthamoeba* spp. (cells/mL) were significantly higher ($P < 0.05$) on these pine trees when compared to the mixed species trees (Fig 3.5-3.6). However, the distribution of these boxplots was heavily skewed, and in both instances, the upper quartile range was considerably larger. This suggests that the levels of *L. longbeachae* and *Acanthamoeba* spp. were highly variable between individual pine trees, but in general, most of the positive samples exhibited relatively low organism levels.

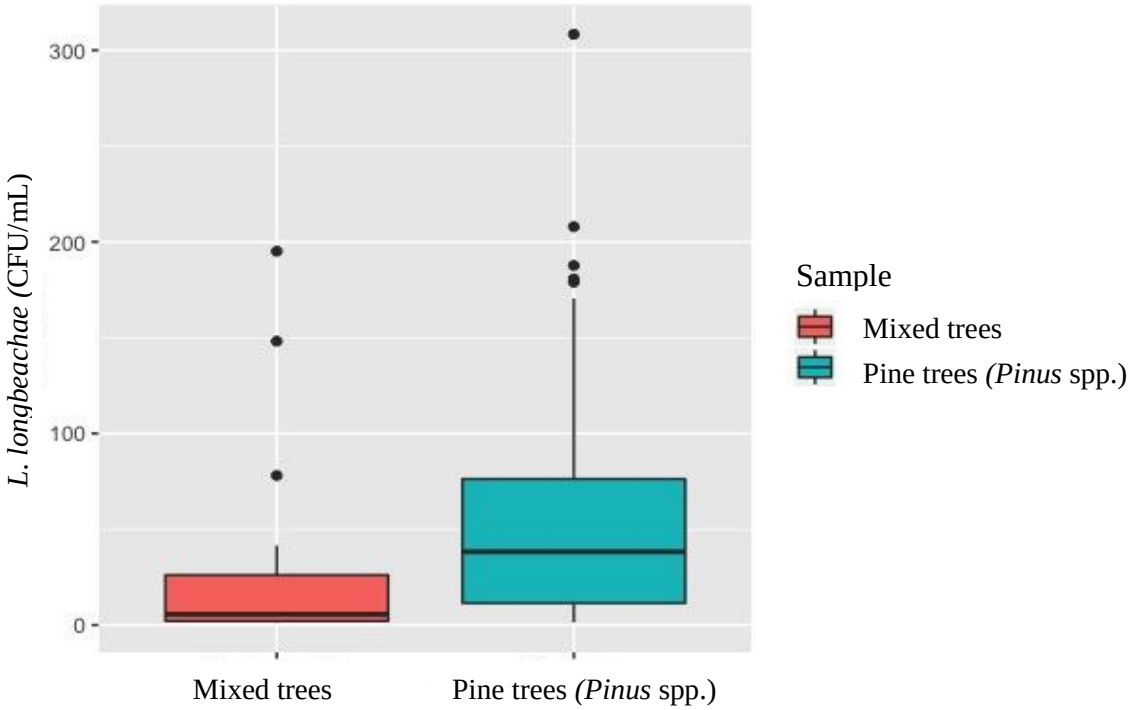

Figure 3.5 Levels of *L. longbeachae* (CFU/mL) quantified by standard curve analysis on mixed species trees and pine trees. Trees were sampled in spring of 2018 and analysed by qPCR. $P < 0.0005$. Vertical lines represent the interquartile range and the solid bars represent the median. Figure produced in R studio® version 1.2.1335.

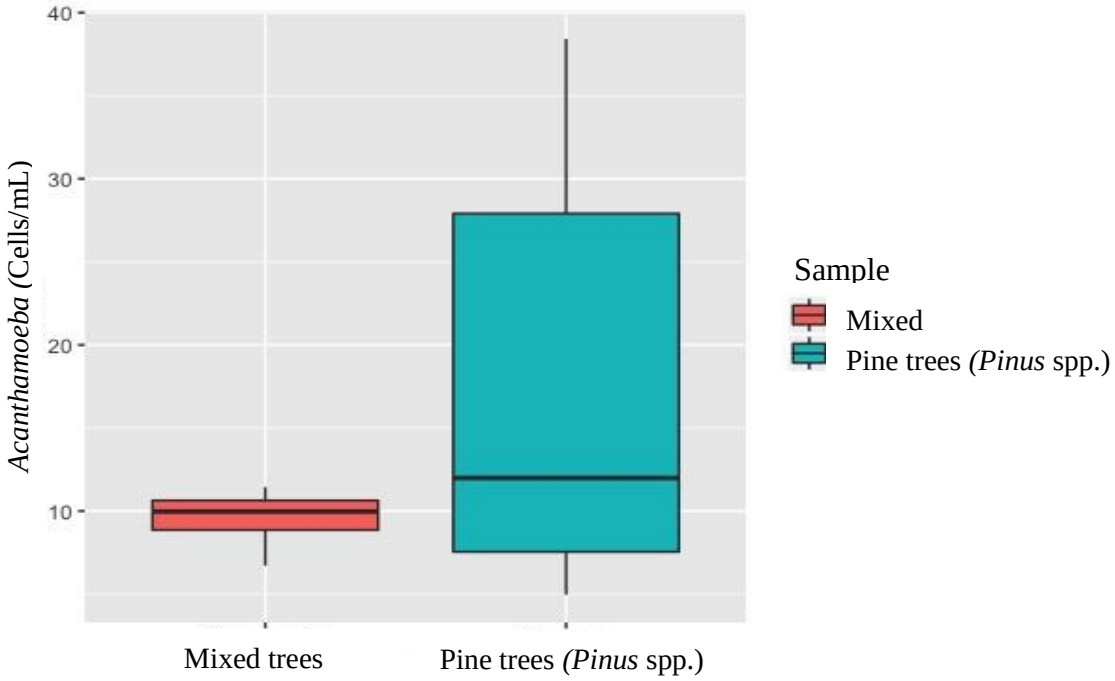

Figure 3.6 Levels of *Acanthamoeba* (cells/mL) quantified by standard curve analysis on mixed species trees and pine trees. Trees were sampled in spring of 2018 and analysed by qPCR. $P < 0.05$. Vertical lines represent the interquartile range and the solid bars represent the median. Figure produced in R studio® version 1.2.1335.

The presence of *L. longbeachae* on *P. radiata* trees varied over the seasons. We observed a 4.5x increase in positivity rates in spring (59%) when compared to any other season. There was also a significant increase (P <0.0005) in the levels of *L. longbeachae* (CFU/mL) on *P. radiata* trees in spring (Fig 3.7). These findings correlate to an increase in the clinical cases of Legionnaires' disease observed in spring that have been reported in previous studies. [6, 18] However, as previously observed, this distribution was heavily skewed and the majority of the samples exhibited relatively low organism levels. Interestingly, *L. longbeachae* was not detected on any of the *P. radiata* trees sampled in summer, which is contradictory to the findings in the literature. [128] Unlike *L. longbeachae*, no seasonal difference was observed between the positivity rates or levels of *Acanthamoeba* (cells/mL), and these levels appeared to be randomly distributed throughout the year (Fig 3.8). However, the small sample size made it difficult to exclude random variation as a contributing factor of this observation.

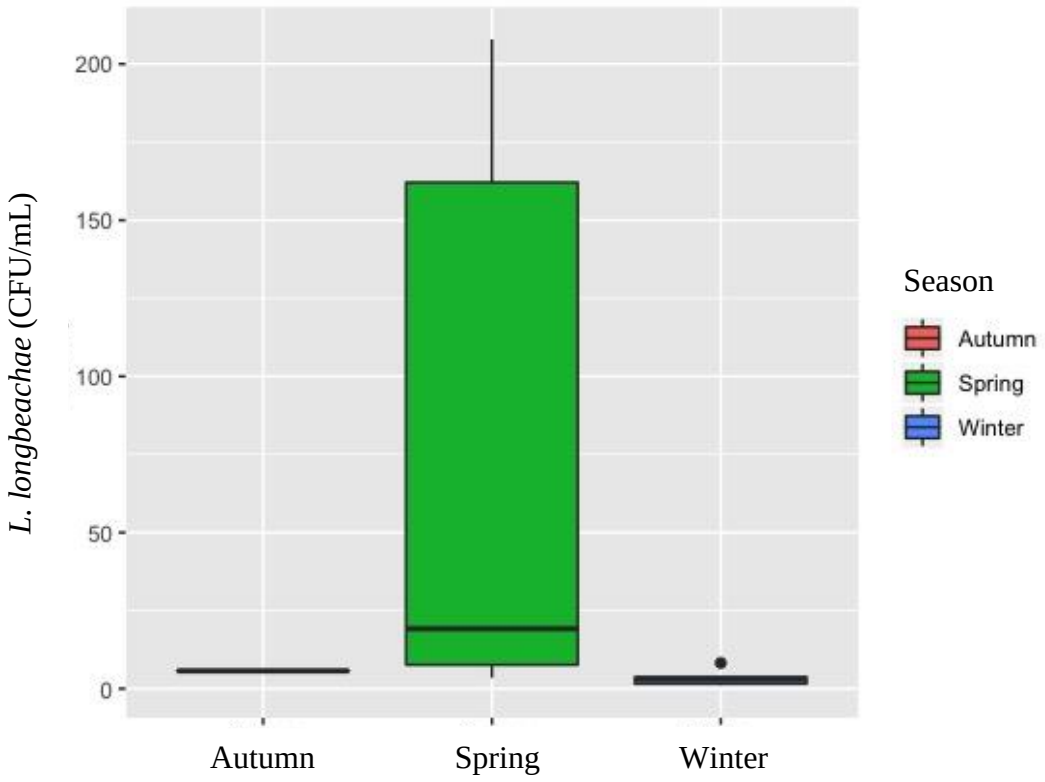

Figure 3.7 Levels of *L. longbeachae* (CFU/mL) quantified by standard curve analysis on *Pinus radiata* trees. Trees were sampled repeatedly in spring, summer, autumn and winter of 2018 and analysed by qPCR. P < 0.0005. Vertical lines represent the interquartile range and the solid bars represent the median. Figure produced in R studio® version 1.2.1335.

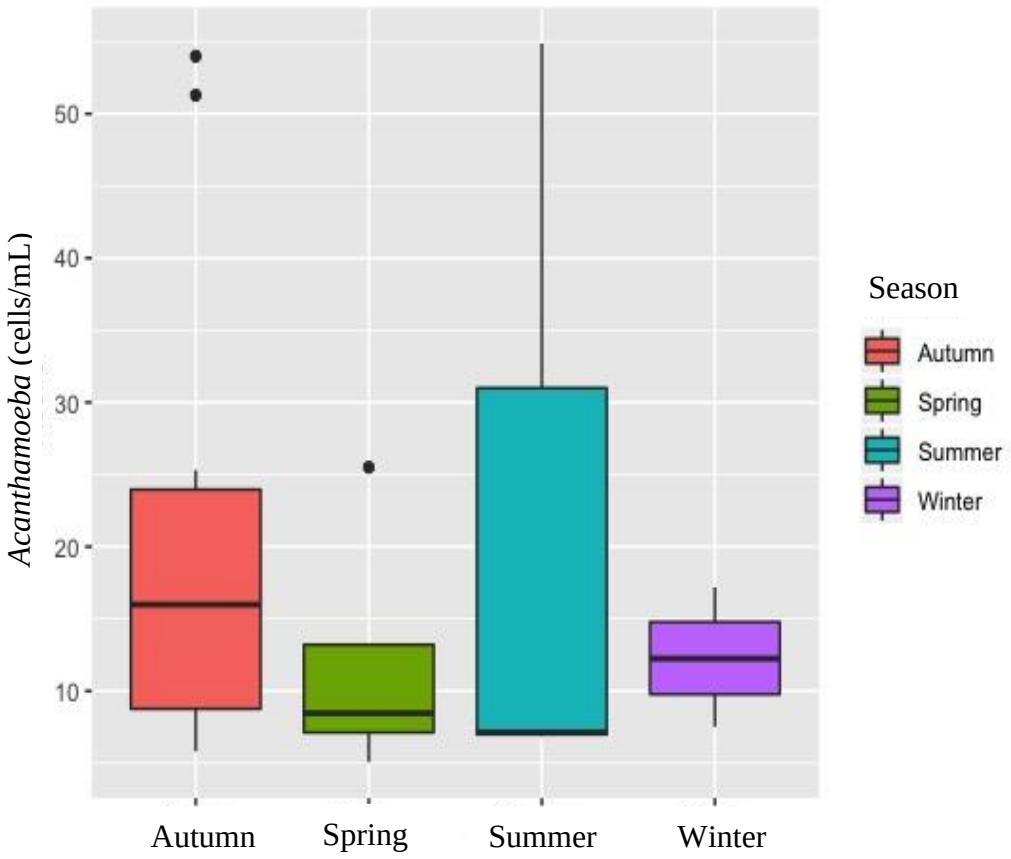

Figure 3.8 Levels of *Acanthamoeba* (cells/mL) quantified by standard curve analysis on *Pinus radiata* trees. Trees were sampled repeatedly in spring, summer, autumn and winter of 2018 and analysed by qPCR. P = 0.08. Vertical lines represent the interquartile range and the solid bars represent the median. Figure produced in R studio® version 1.2.1335.

To evaluate the relationship between *L. longbeachae* and *Acanthamoeba* in nature, trees that were positive for *L. longbeachae* and *Acanthamoeba* spp., were plotted against each other and evaluated using a Pearson's product-moment correlation. Only 5% of the total trees sampled were positive for both organisms and interestingly, all of these positives occurred on *Pinus* spp. trees. However, the relationship between the two was non-linear, indicating that there was no correlation between species (Fig 3.9). Thereby suggesting that the presence of *Acanthamoeba* does not influence the presence of *L. longbeachae* in nature.

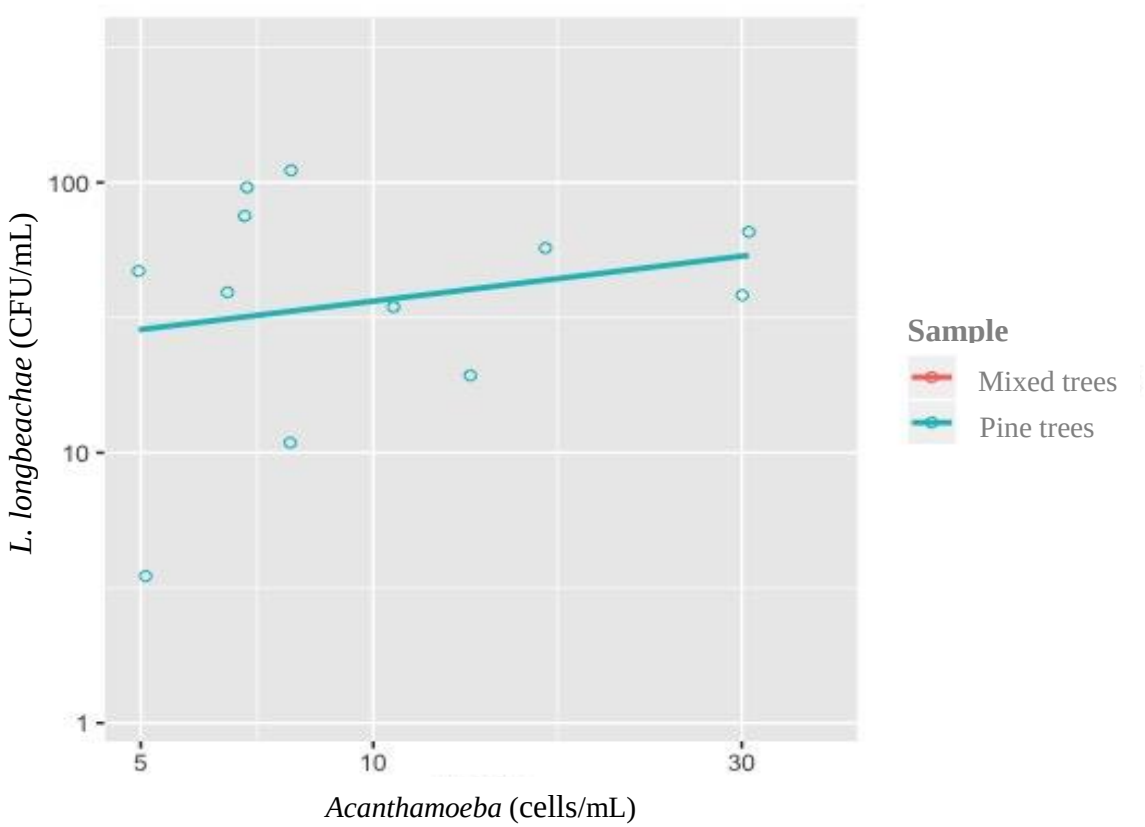

Figure 3.9 Relationship between *L. longbeachae* positive samples and *Acanthamoeba* positive samples. N=12. P = 0.97, calculated using a Pearson product-moment correlation efficient. Figure produced in R studio® version 1.2.1335.

3.4 Validation of primer specificity

The top 100 matches for the *Acanthamoeba* spp. 18s rRNA PCR product (Fig 3.10A) all showed 100% sequence similarity to the *Acanthamoeba* genus. Sequences with 100% sequence similarity to the *N. fowleri* 18s rRNA PCR product (Fig 3.10B) were all specific to *N. fowleri*. However, several other *Naegleria* species including, *Naegleria lovaniensis*, *Naegleria minor* and *Naegleria neopolaris* all shared more than 90% sequence similarity with the excised PCR product. This could have resulted in the amplification of non-specific products; however, as *N. fowleri* was not detected in any of the samples tested, this did not interfere with the results of this study.

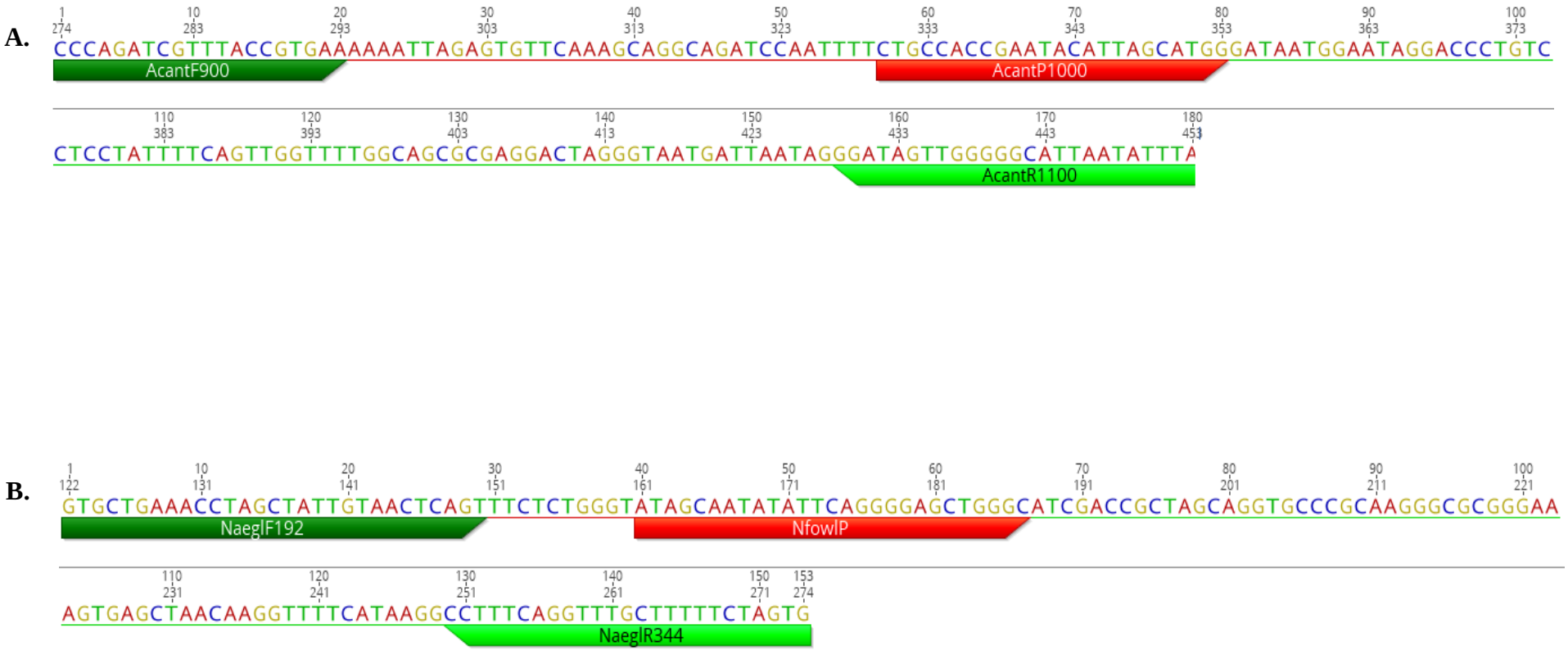

Figure 3.10 Representation of the amplified regions of the *Acanthamoeba* **(A)** and *N. fowleri* **(B)** 18S rRNA gene by RT-qPCR. Forward and reverse primers are shown in green and TaqMan probes are shown in red. Expected PCR product sizes for each oligonucleotide primer set are approximately 180 bp, varying by a few base pairs dependent on species **(A)** and 157 bp **(B).** Images produced in Geneious (version 10.2.6).

3.5 Amoebal enrichment and co-culture

A co-culture system was designed in an attempt to mimic the interactions between *L. longbeachae* and *Acanthamoeba* in nature. After two hours, both *L. pneumophila* and *L. longbeachae* were seen to co-localise with *A. polyphaga*; and in some instances, inside what appeared to be distinct vacuoles (Fig 3.11). The similarities observed between *L. pneumophila* and *L. longbeachae* suggests that *A. polyphaga* may to be able to support the replication of *L. longbeachae* under laboratory conditions. However, the use of this method alone did not provide sufficient contrast for us to determine whether *A. polyphaga* had internalised the *Legionella* or whether it was only associated with the cell surface, as there was evidence of non-internalised bacilli in some samples. This method also required extensive optimisation, and due to the time constraints of this project, we were unable to increase the incubation time beyond two hours. Therefore, we were unable to conclude whether *L. longbeachae* was replicating or being degraded by *A. polyphaga* following amoebal uptake, and further investigations into this using the methods developed in this study are required.

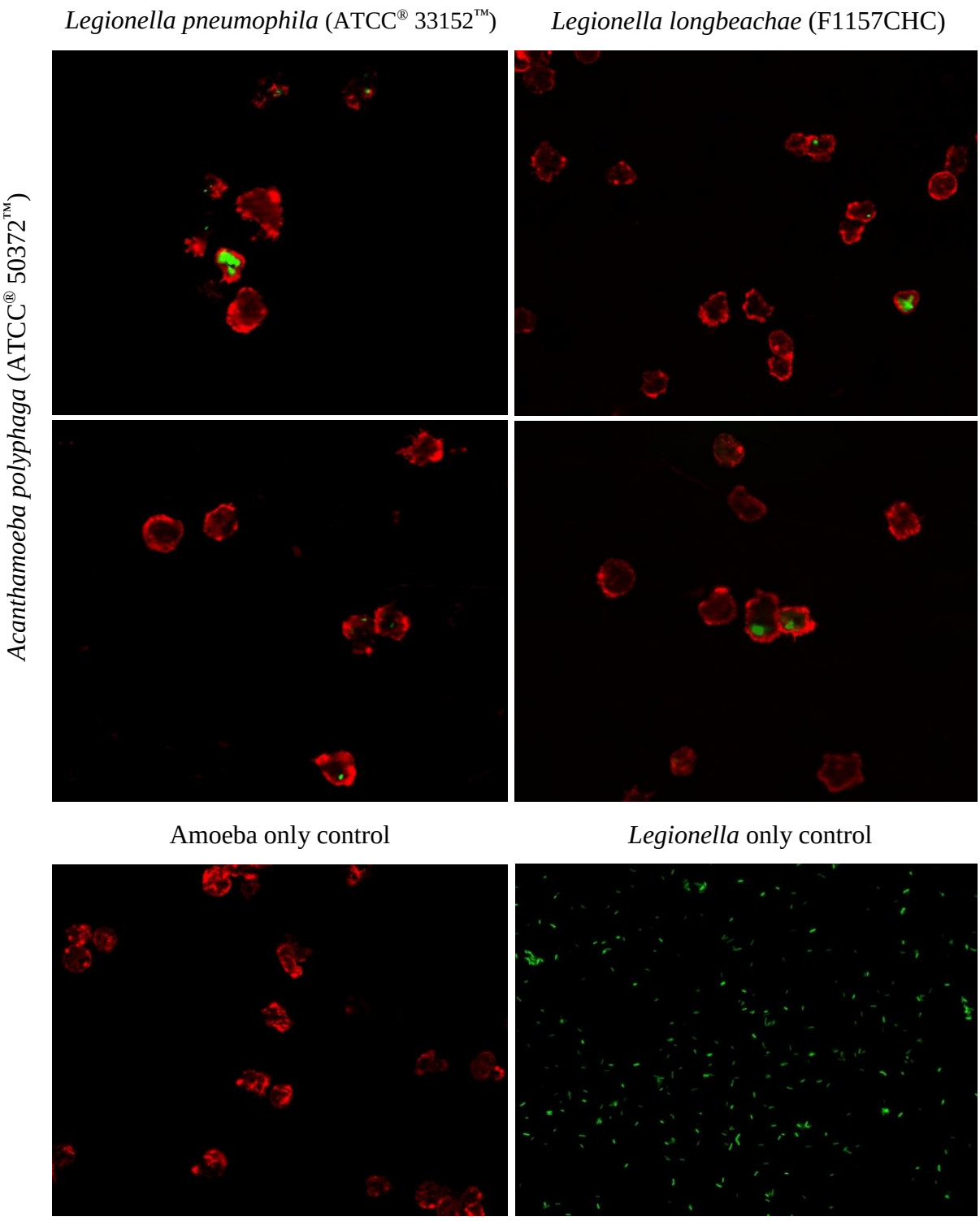

Figure 3.11 Preliminary co-culture of *A. polyphaga* and *Legionella* at an MOI of 10:1. Wells were incubated at 30°C for 2 hours before trophozoites were harvested. Texas Red-conjugated phalloidin-stained the actin cytoskeleton of *A. polyphaga* (red) and Vybrant® DiO cell-labelling solution-stained the *Legionella* plasma membrane (green). Images were taken on the Zeiss Axioimager Z1 microscope with a 40x EC Plan Neofluar NA 1.4 objective and are representative of three replicates.

3.6 Isolation of *Legionella longbeachae* from potting mix

Two isolates of *L. longbeachae* were successfully recovered from a single bag of locally acquired potting mix using the developed methods (Fig 3.12). The identification of these isolates were confirmed by MALDI-ToF mass spectrometry, with score values >2.300 indicating a highly probable species identification (Fig 3.13). However, the recovery was low, and only two isolates were obtained over a three-month sampling period. This low recovery could be linked to low organism burden within the sample or an uneven distribution of *L. longbeachae* throughout the product. During the development of this isolation protocol, it seemed that both the dilution and centrifugation steps were important, as *L. longbeachae* could not be isolated from samples in which it had been previously recovered when either step was removed. However, the overgrowth of competing microorganisms within the sample likely led to some isolates being overlooked.

Figure 3.12 Representative image of a *L. longbeachae* environmental isolate on GVPC agar.

Analyte Name	Analyte ID	Organism (best match)	Score Value
D5 (+++) (A)	#2	Legionella longbeachae	2.403
D6 (++) (A)	#2	Legionella longbeachae	2.272
D7 (+++) (A)	#4	Legionella longbeachae	2.368
D8 (+++) (A)	#4	Legionella longbeachae	2.544

Figure 3.13. A screenshot showing the output from the MicroFlex LT. Samples were processed in duplicate (#2 and #4) and the results were analysed by the MALDI Biotyper version 3.1 (Bruker Daltonics, Germany).

3.7 Isolation of free-living amoeba from potting mix

Using the developed methods, amoeba could be isolated from all of the samples of potting mix in which *L. longbeachae* was previously recovered. However, no amoeba could be isolated from the stored *P. radiata* samples, most likely due to degradation and loss of viability of the samples over time. After five days, cultures were rapidly overcome by fungal contamination, which appeared as a filamentous growth from the edge of the filter disk. The addition of GVPC (containing two fungicides, polymyxin B and cycloheximide) to the media inhibited the growth of amoeba and also failed to control the spread of fungal contaminants. Due to this, the repetitive sub-culture method, as described by Neff was employed. [127] After several sub-cultures, contamination was significantly reduced. However, this was a prolonged process and as a result, we were unable to identify any of the isolates using the 18S-ITS2 universal primers as planned. When observed under the phase-contrast microscope, all of the isolated amoebae appeared morphologically similar (Fig 3.14.), suggesting that all isolates belonged to the same genus.

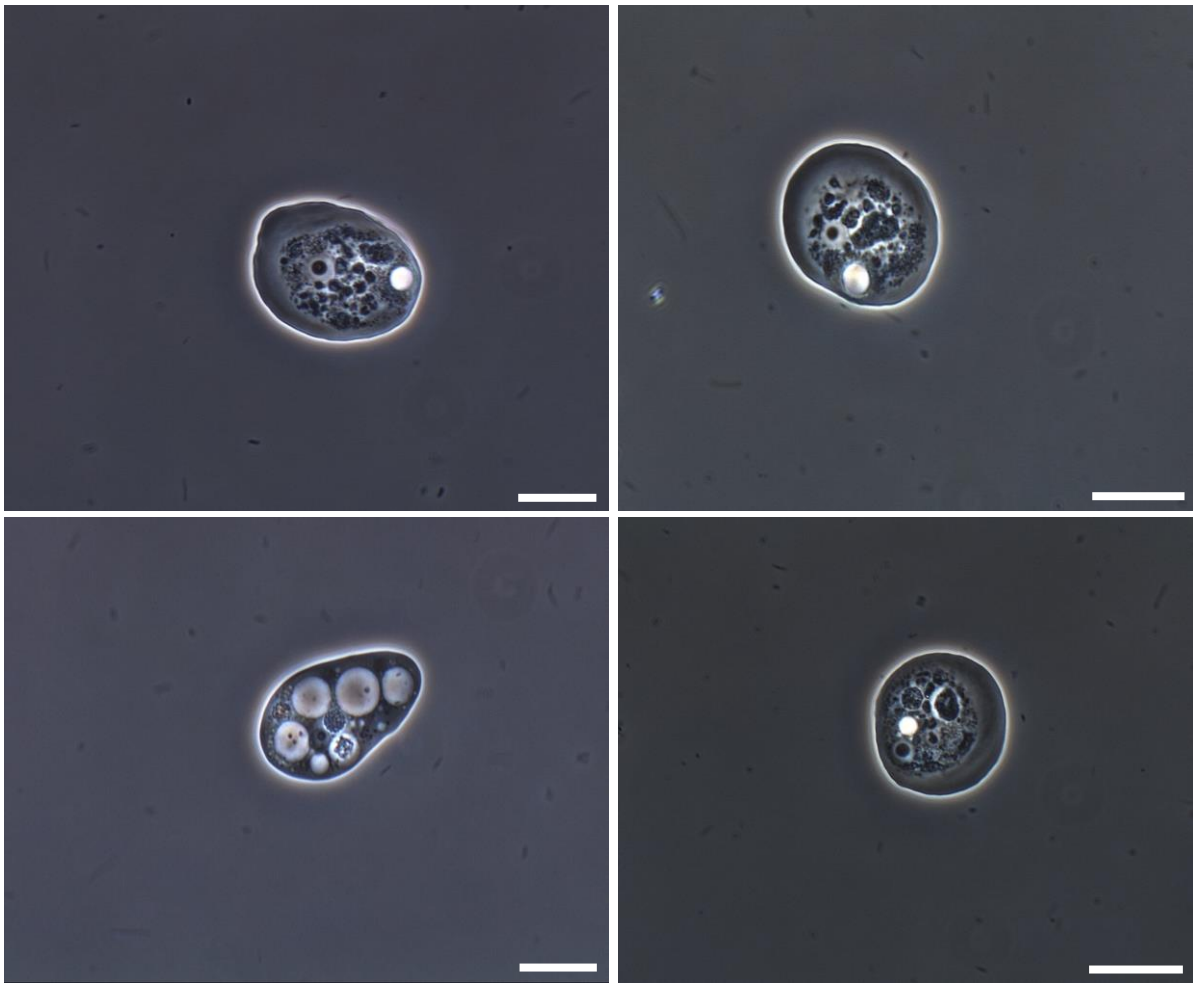

Figure 3.14. Representative images of environmental free-living amoeba isolated from *L. longbeachae* positive potting mix samples. Amoeba were isolated using methods modified from Amaro and Shuman (2019). Images taken using the OLYMPUS cellSens Entry 1.15. Bars represent 10 µm.

Chapter 4: Discussion

In New Zealand, *L. longbeachae* is recognised as a significant human pathogen and is the major causative agent of Legionnaires' disease. [15, 17] However, there are still many unresolved questions regarding the interactions between *Legionella* species and their amoebal hosts, and how these interactions can contribute to human disease. Therefore, the primary aim of this research was to gain a better understanding of the relationship between *L. longbeachae* and their amoebal hosts, which in turn, could be used to develop new strategies to assess and reduce the risk of human infection.

4.1 Discussion of study findings

The results from this study suggest that neither *Acanthamoeba* nor *N. fowleri* has the ability to serve as a natural host for *L. longbeachae*. *Acanthamoeba* was only detected at low levels in a small percentage of samples where *L. longbeachae* was also present and no relationship was observed between the samples that tested positive for *Acanthamoeba* and those that tested positive for *L. longbeachae*. These findings were unexpected as *Acanthamoeba* is considered to be a ubiquitous microorganism that is found in a wide variety of environments. [129] Furthermore, the PCR primers utilised in this study were designed for the amplification of the entire *Acanthamoeba* genus and therefore did not limit this study to the detection of a single species. [125] *N. fowleri* was not detected in any of the samples; however, this was most likely due to the nature of the samples that were tested. *N. fowleri* is a moderate thermophile that multiplies best at temperatures >30 °C. It is suggested that soil may be the preferred environment for *N. fowleri;* however, it is rarely isolated from such environments. [130, 131] Typically, *N. fowleri* is isolated from warm freshwater environments such as lakes and thermal springs, which may explain why it has been recognised as a host for *L. pneumophila*. [112, 113, 132]

It is not possible to compare our findings with those of other studies, as no other studies have been published that have investigated the presence of *L. longbeachae* and amoebal species on tree bark. However, similar studies that have investigated this relationship in alternative environments have shown that *L. longbeachae* is frequently detected in samples where *Acanthamoeba* and *Naegleria* species are also present. [69, 102] This suggests that whilst pine tree bark has been identified as a reservoir for *L. longbeachae*; it may not be a natural reservoir for *Acanthamoeba* species or *N. fowleri.* It is plausible that the interaction between *L. longbeachae* and their amoebal hosts could be occurring within a distinct environment, such as during the manufacturing process of commercial potting mix. The high heat and accumulation of moisture during this process likely provides an environment in which amoeba can freely graze upon the bacteria that has been introduced into the product. Therefore, it would be important to screen a wider variety of samples where *L. longbeachae* is present in order to completely understand the nature of this relationship.

This study was constrained by the fact that the majority of the PCR positive samples were outside of the linear range (Ct >37). As we could only extrapolate beyond this point, this made it difficult to quantify our samples. However, as a consistent threshold value was used for all assays, we can be confident that these values represent true positives and are not a result of background fluorescence. These weak positives may have been a result of low organism levels within the sample or could be a reflection of inadequate sample processing prior to amplification. Poor amplification is a complication that is regularly encountered in environmental research where PCR is used as a primary method of detection. [133] Organic compounds, mineral particles, and heavy metals can have significant inhibitory effects on the enzymatic activity of DNA polymerases and can also interfere with primer binding. [133, 134] The samples used in this study were diluted in 50 mL UltraPure water (refer to section 2.4) to homogenize microorganisms and reduce the effects of PCR inhibitors. As inhibition controls

were included in all assays, we could be confident that inhibitory substances were not interfering with our observations. However, by diluting our samples in such a large volume, the concentration of amoebal cells would have also been significantly reduced. Furthermore, DNA was only extracted from a 200 µL aliquot of this sample, and only 5 µL of the extracted DNA was added to each PCR reaction mixture. Therefore, due to these dilution factors, the results of each PCR assay were representative of <1% of the total number of organisms in each 50 mL sample. Assuming that the amoeba were evenly distributed throughout each sample, obtaining a positive PCR result would require a minimum concentration of 1 cell/µL in the original suspension (50 mL). Thus, samples with low organism loads would be subject to significant sampling error which would impact the sensitivity of the PCR and therefore, it is likely that the true number of positive trees was underestimated. Several methods for improving DNA recovery from complex samples have been discussed in the literature. [135, 136] In this instance, purification of the target DNA using immunomagnetic separation or a similar technique before extraction would have limited the effects of sampling error, reduced random sample variation, and resulted in a more reliable estimate of the number of organisms present in our samples.

It has also been suggested that the effectiveness of DNA isolation from complex samples is highly dependent on the procedure that is used. [137, 138] The DNA used in this study was extracted using the GenElute™ Bacterial Genomic DNA Kit, which is designed for the isolation of already purified bacterial DNA (Sigma Aldrich, MO, USA). This kit may have been less effective in extracting the eukaryotic DNA from our samples, and investigations into more appropriate DNA extraction kits that can simultaneously isolate high quality eukaryotic and prokaryotic DNA from complex samples are warranted.

The relationship between *Legionella* species and their amoebal hosts has been well established in the literature through the use of co-culture models. However, the varying rates of success of

these studies suggest that this interaction is both highly dependent on strain selection and the conditions under which the co-culture is performed. Previous studies have shown that *Acanthamoeba* species have very little preference for which *Legionella* species they consume. [139] However, the ability of *Legionella* to subvert host cell signalling and initiate replication within an amoebal host maybe both species and strain-specific. [139]

This study examined the uptake of *L. longbeachae* and *L. pneumophila* by *A. polyphaga* using fluorescence microscopy. After two hours, both *L. longbeachae* and *L. pneumophila* were co-localised with *A. polyphaga*. However, as there was evidence of non-internalised bacilli in some samples, we were unable to determine whether this co-localisation was a result of internalisation or whether *L. longbeachae* was only transiently associated with the amoebal membrane. Flow cytometry is one technique that has been used to quantify internalised *Legionella* populations after co-culture with *Acanthamoeba* species. [140] For future investigations, performing these two techniques in parallel is one way that the association between *L. longbeachae* and *A. polyphaga* could be validated.

Furthermore, the incubation time allocated for this experiment was not sufficient enough for us to conclude whether *L. longbeachae* was replicating within *A. polyphaga* or being degraded following ingestion. As it cannot be assumed that all *Legionella* species are capable of replication within the same hosts, repetitive experiments using the methods developed in this study would need to be conducted over an extended period in order to characterise this relationship further.

Both *A. castellanii* and *A. polyphaga* have been used in co-culture experiments with *L. longbeachae* with varying degrees of success. [34, 102, 110, 141] However, it is important to note that all of these experiments were performed under different conditions, which could have

affected the replication potential of *L. longbeachae* within these hosts. As our results are only preliminary and the findings in the literature are contradictory, it is difficult to compare our findings with those of other studies at this stage.

The use of culture-based methods to isolate *L. longbeachae* in environmental samples has had limited success. [37-39, 41] The methods developed in this study were a result of a lengthy process in which various methodologies were reviewed and used to develop a more simplified approach. *L. longbeachae* was first isolated from potting mix in 1989 by Steele *et al*., who demonstrated that the use of both acid and heat treatments (>50 °C) were necessary for its isolation. [37-39] However, we found that a more straightforward approach, which included the dilution and centrifugation of samples prior to culture, was just as successful. This suggests that the laborious pre-treatments outlined in the literature may not be necessary to isolate *L. longbeachae*. Furthermore, these newly developed methods may represent a way in which commercial potting mixes can be easily screened for viable legionellae.

Identifying amoebal species that could serve as laboratory hosts for *L. longbeachae* was the primary focus of this research. Using the methods developed by Amaro and Shuman, we successfully isolated viable amoeba from all samples in which *L. longbeachae* had been previously isolated. [126] To the best of our knowledge, very few studies have successfully isolated amoebal species from environments other than natural water-systems. As soil is a unique and highly diverse environment we cannot compare our findings with these studies, as it is likely that these environments have very distinctive microbial compositions. Encouraging the growth and migration of amoebal species within our samples whilst restricting the spread of other microorganisms was the main limitation of this method. Several techniques were employed, however many of these were found to inhibit the growth of the amoeba just as rapidly as they did other microorganisms. Therefore, although the repetitive sub-culture method was

both a prolonged and tedious process, it appeared to be the best method for isolating viable cells. However, as sub-culturing was required before fungal contaminants had a chance to spread (~3 days), this method was highly favourable towards the most abundant species in the sample. This is potentially why all of our isolates appeared to be the same species, despite sub-cultures being taken from several distinct regions of the original cultures. However, as we were unable to identify any of our isolates in the given time frame, we are unable to confirm this based on morphology alone. Further experiments to identify these isolates using 18S-ITS2 universal primers have been planned. These primers have been effective in identifying *Naegleria* species to the genus level in several studies. However, obtaining species-specific identifications have been less successful. [142-144] Furthermore, there is limited information concerning the use of these primers in identifying non-vahlkampfiidae (*Naegleria* spp.) amoeba. Nevertheless, a successful genus-level identification of these isolates could be the key to identifying the environmental host for *L. longbeachae*.

4.2 Technical limitations

In this study, using PCR as our primary method of detection had one major disadvantage, that being the inability for us to distinguish between viable and non-viable cells. [145-147] In nature, it has been shown that DNA is relatively stable and can persist for up to several weeks following cellular degradation. [145] As PCR is a technique that relies upon DNA to identify and quantify target organisms within a sample, it therefore has the potential to lead to an inaccurate representation of the viable biomass. [145] Furthermore, in adverse conditions, amoeba have the ability to enter into a dormant state in which they become metabolically inactive. [148] As PCR is unable to provide information on the condition or state in which an organism exists, the potential for *Acanthamoeba* species to serve as hosts to *L. longbeachae* could not be assessed using this method alone. [149] However, as our sample size was not sufficient enough for us to observe whether there was a relationship between these species, these factors would not have

had a significant impact on our findings. Nevertheless, it is important to consider these limitations for future research.

The second limitation of this study related to the efficiency of the PCR. The PCR assays for both *Acanthamoeba* spp. and *N. fowleri* exhibited relatively low efficiencies (<80%). As optimisation steps were carried out, these low efficiencies were most likely a result of poor primer design that subsequently led to inefficiencies in primer binding to the target DNA. As the PCR efficiency decreases, so does the sensitivity of the assay. Due to this, it is likely that the exact number of positive samples was underestimated. However, as the aim of this study was to observe a trend rather than detect every positive, these values were considered as acceptable for this investigation. Furthermore, this study relied upon the use of pre-designed primers that had published in two other studies. [69, 125] Therefore, increasing the efficiency of these assays would have required us to design and validate new primers, which was outside of the scope of the project.

4.3 Future directions

Future work will be aimed at continuing to identify environmental hosts for *L. longbeachae* that also have the ability to serve as laboratory hosts. Identifying these species will help us gain a greater understanding of *L. longbeachae* persistence in the environment and provide a mechanism in which *L. longbeachae* can be easily recovered from complex environments. In the immediate future, co-culture experiments with *A. polyphaga* will be repeated and extended over periods of 24 and 48 hours. In doing this, the replicative potential of *L. longbeachae* within this host could be assessed using qPCR and direct plate count methods.

Furthermore, a more extensive range of environments, such as commercial potting mixes, will be screened for *Acanthamoeba* and other species of interest. As previously mentioned, both

V. vermiformis and *T. pyriformis* are potential hosts of *L. longbeachae* that warrant further investigation. Once a potential host has been identified, we will assess their ability to serve as laboratory hosts to *L. longbeachae* using the co-culture methods that have been developed in this study.

Once this relationship has been better characterised and these methods have been well established, this work may be expanded into assessing the infectivity potential of *L. longbeachae* in human macrophage cell lines. This will allow us to study the effects of amoebal co-culture on *L. longbeachae* virulence and subsequently compare these findings to *L. longbeachae* isolates that have been cultured *in vitro*. Recognising these differences is critical in understanding how *L. longbeachae* persists in the environment and the conditions under which it multiplies. In turn, this will improve our understanding of how *L. longbeachae* acquires its pathogenicity and virulence that contributes to human disease.

4.4 Concluding statement

This research project was an exploratory project that intended to identify amoebal strains that could serve as hosts to *L. longbeachae*. This project has allowed us to expand and strengthen our *Legionella* research by opening a new line of enquiry into the fundamental ecology of *L. longbeachae*. Although we were unable to fulfil the aims of this study in the given time frame, we have established the methods to investigate other potential hosts that may be unique to *L. longbeachae* and have eliminated some of the previously described hosts of *L. pneumophila*.

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Chapter 6: Appendices

6.1 Media and buffers used in this study

All products used for media preparation were obtained from Sigma Aldrich (MO, USA) unless otherwise stated.

BCYE	Aces buffer	5 g	0.97 %	(w/v)
	Agar	8.5g	1.6 %	
	Charcoal activated	1 g	0.2 %	
	Yeast extract	5 g	0.97 %	
	a-Ketoglutarate monopotassium salt	0.5 g	0.1 %	
	*L-cysteine	0.2 g	0.03 %	
	*Ferric pyrophosphate	0.125 g	0.02 %	
	ddH_2O	490 mL	96 %	(v/v)
±GVPC	Oxoid™ Legionella GVPC Selective Supplement (Thermo Fisher Scientific, MA, USA)	10 mL	1.95 %	(v/v)

Balance to pH 6.9 ± 0.2 using KOH (~8-10 pellets) and autoclave at 121 °C for 35 minutes.
***** Dissolve the L-cysteine and Ferric pyrophosphate in 5 mL ddH_2O and filter sterilize into the media after autoclaving (final volume - 500mL). Filter sterilize a 10 mL solution of GVPC into the solution if required.

Freshwater amoeba medium (ATCC 997)	Bacto™ Malt extract (Thermo Fisher Scientific, MA, USA)	0.05 g	0.01 %	(w/v)
	Yeast extract	0.05g	0.01 %	
	Agar	5 g	0.98 %	
	ddH_2O	500 mL	99 %	(v/v)

Autoclave at 121ºC for 35 minutes.

Page's saline (PAS/PBS)

Solution 1	Na_2HPO_4	0.142 g	0.02 %	(w/v)
	KH_2PO_4	0.136 g	0.02 %	
	ddH_2O	500 mL	99.9 %	(v/v)
Solution 2	$MgSO_4 \times 7H_2O$	4.0 mg	0.0007 %	(w/v)
	$CaCl_2 \times 2H_2O$	4.0 mg	0.0007 %	
	NaCl	0.120 g	0.02 %	
	ddH_2O	500 mL	99.9 %	(v/v)

Prepare solutions in separate bottles and autoclave at 121ºC for 35 minutes. Combine when cooled to room temperature.

PYG (Basal medium)	Bacto™ Proteose peptone (Thermo Fisher Scientific, MA, USA)	10 g	2.0 %	(w/v)
	Yeast extract	0.5g	0.1 %	↓
	Na Citrate x $2H_2O$	0.5 g	0.1 %	↓
	ddH_2O	450 mL	88 %	(v/v)
Stock solutions				
	0.05 M $CaCl_2$	4.0mL	0.8 %	(v/v)
	0.4 M $MgSO_4$	5.0mL	1.0 %	↓
	0.25 M Na_2HPO_4	5.0mL	1.0 %	↓
	0.25 M KH_2PO_4	5.0mL	1.0 %	↓
	*0.005 M $Fe(NH_4)_2(SO_4)_2$ x $6H_2O$	5.0mL	1.0 %	↓
	*2.0 M Glucose	25 mL	4.9 %	(v/v)

Prepare each stock solution separately and add to the basal medium to avoid precipitation and autoclave at 121ºC for 35 minutes. *Filter sterilize a 5 mL $Fe(NH_4)_2(SO_4)_2$ x $6H_2O$ solution and 2.0 M glucose solution and add to the above.

Tryptic soy broth (TSB)	Bacto™ TSB powder (Thermo Fisher Scientific, MA, USA)	12.5 g	2.5 %	(w/v)
	ddH_2O	500 mL	97.5 %	(v/v)

Autoclave at 121ºC for 35 minutes.

Non-nutritive agar	PAS solution	500 mL	98.5 %	(v/v)
	Agar	7.5 g	1.5 %	(w/v)

Autoclave at 121ºC for 35 minutes.

6.2 DNA extraction protocol

GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich, MO, USA)

Specimen Preparation

1. Preheat dry heat block to 55°C.
2. Add 20µL of Proteinase K to 200µL of pre-prepared sample
3. Vortex and pulse spin
4. Add 200µL of Lysis solution C
5. Vortex and pulse spin
6. Incubate at 55°C on dry heat block for 10 minutes
7. Pulse spin

   Samples are now ready for loading onto binding columns (Sigma kit)

Column preparation

1. Add 500µL of Column Preparation Solution to each pre-assembled column and centrifuge at 8000rpm for 1 minute. Discard flow-through liquid.
2. Number the column with the sequential sample numbers

Addition of lysate

1. Add 200µL of 100% Ethanol to the specimen lysate
2. Vortex thoroughly and pulse spin
3. Transfer to the binding column, using a transfer pipette
4. Centrifuge at 8000rpm for 1 minute
5. Discard the collection tube and place the binding column in a new tube, which has been labelled with the corresponding number
6. Add 500µL of wash solution (I) to the column
7. Centrifuge at 8000rpm for 1 minute
8. Discard the collection tube and place the binding column in a new tube, which has been labelled with the corresponding extraction number
9. Add 500µL of wash solution (II) to the column
10. Centrifuge at 13000rpm for 3 minutes The binding column must be free of ethanol before eluting the DNA. If residual ethanol is seen, spin the column for an additional minute at maximum speed
11. Discard the collection tube and place the binding column in a new tube, which has been labelled with the corresponding extraction number

Elution of DNA

1. Pipette 100µL of Elution Solution directly into the centre of the binding column
2. Incubate at room temperature for 5 minutes
3. Centrifuge at 8000rpm for 1 minute
4. Discard the binding column
5. DNA is now ready for PCR testing or storage

6.3 Inter-assay variability (PCR)

Table 6.1 Inter-assay variation of positive control DNA used in all Rt-qPCR assays

	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**CT Range**
Acanthamoeba	27.03424	27.17623	26.49364	27.4833	
	Assay 5	**Assay 6**	**Assay 7**	**Assay 8**	**= 0.98966**
	26.85545	27.33468	27.28276	27.20584	
	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**CT Range**
Naegleria	29.2482	29.55473	29.52961	29.76183	
fowleri	**Assay 5**	**Assay 6**	**Assay 7**	**Assay 8**	**= 0.51363**
	29.68554	29.43644	29.6097	29.60579	

Values were used to monitor the efficiency of each PCR.