Legionella Species Persistence Mechanisms in Treated Harvested Rainwater

by

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DECLARATION

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This dissertation includes two original papers published in peer-reviewed journals and one unpublished publication. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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SUMMARY

The persistence of *Legionella* spp. at high pasteurization temperatures poses a threat to human health as a number of *Legionella* spp. are known to cause Legionnaires' disease. Research has then indicated that the primary factors that allow *Legionella* to proliferate and persist in water distribution systems are: the accessibility to nutrients in a water source, water temperature, the presence of free-living amoebae (FLA) and other aquatic bacteria. The focus of the current study was thus to investigate and functionalise selected persistence mechanisms displayed by *Legionella* spp. that aid in their survival in pasteurized and unpasteurized harvested rainwater. The overall aim of Chapter two was to isolate and identify the dominant *Legionella* spp. persisting in a domestic rainwater harvesting tank and a solar pasteurization (SOPAS) system and to identify possible FLA vectors of *Legionella* that remain viable at high pasteurization temperatures (>60°C). For this, pasteurized and unpasteurized tank water samples were screened for the dominant *Legionella* spp. using culture based techniques. In addition, as FLAs including *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* are the most frequently isolated from hot water systems, ethidium monoazide polymerase chain reaction (EMA-qPCR) was utilised for the quantification of viable *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri*. Eighty-two *Legionella* spp. were isolated from the unpasteurized tank water samples, where *L. longbeachae* (35 %) was the most frequently isolated, followed by *L. norrlandica* (27 %) and *L. rowbothamii* (4 %). This information provides pertinent knowledge on the occurrence and dominant species of *Legionella* present in the South African environment. In addition, the SOPAS system was effective in reducing the gene copies of viable *N. fowleri* (5-log) and *V. vermiformis* (3-log) to below the lower limit of detection at temperatures of 68–93°C and 74–93°C, respectively. In contrast, as gene copies of viable *Legionella* and *Acanthamoeba* were still detected after pasteurization at 68–93°C, it could be concluded that *Acanthamoeba* spp. primarily act as vectors for *Legionella* spp. in solar pasteurized rainwater.

The primary objective of Chapter three was to determine the resistance of three *Legionella* species isolated from unpasteurized rainwater [*L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.)], two *Legionella* reference strains (*L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462) and *Acanthamoeba mauritaniensis* ATCC 50676 to heat treatment (50–90°C). In addition, the resistance of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676, respectively, to heat treatment (50–90°C) was determined using EMA-qPCR. The interaction mechanisms exhibited between *Legionella* and *Acanthamoeba* during heat treatment (50–90°C) were also elucidated by monitoring the relative expression of genes associated with metabolism and virulence of

L. pneumophila ATCC 33152 (*lolA, sidF, csrA*) and *L. longbeachae* (env.) (*lolA*) in co-culture with *A. mauritaniensis* ATCC 50676, respectively. *Legionella longbeachae* (env.) and *L. pneumophila* ATCC 33152 were the most resistant to heat treatment as both organisms were still culturable (CFU/mL) following treatment at 50 and 60°C. However, the sensitivity of detection of viable cells was increased when using EMA-qPCR as all *Legionella* spp. and *A. mauritaniensis* ATCC 50676 were detected following heat treatment (50–90°C). In addition, while the heat resistance of *L. pneumophila* ATCC 33152 in co-culture with *A. mauritaniensis* ATCC 50676 improved, it is postulated that *L. longbeachae* (env.) is unable to replicate in *A. mauritaniensis* ATCC 50676 as *L. longbeachae* (env.) in co-culture was not detected following heat treatment at 80°C and 90°C. Results also showed a clear trend between genes with related function and differential expression during heat treatment (50-90°C). For example, relative to the untreated samples, the expression of *lolA* remained constant while the expression of *sidF* increased and the expression of *csrA* decreased significantly during *L. pneumophila* ATCC 33152 co-culture with *A. mauritaniensis* ATCC 50676. Results thus confirm that while heat treatment may reduce the number of viable *Legionella* spp., *L. pneumophila* is able to interact with *A. mauritaniensis* and persist during heat treatment.

The overall aim of Chapter four was to elucidate other microbial and physico-chemical characteristics that may be associated with the incidence of *Legionella* spp. and *Acanthamoeba* spp. in rainwater harvested from different roofing materials. Overall results indicated that the roofing materials did not influence the incidence of *Legionella* and *Acanthamoeba* spp. as these organisms were detected in all tank water samples collected from the Chromadek®, galvanized zinc and asbestos roofing materials. However, significant ($p < 0.05$) positive Spearman (ρ) correlations were noted between *Legionella* spp. vs. nitrites and nitrates and between *Acanthamoeba* spp. vs. barium, magnesium, sodium, silicon, arsenic and phosphate, respectively. In addition, while no significant correlations were observed between *Legionella* spp. vs. the indicator bacteria ($p > 0.05$), positive correlations were established between *Acanthamoeba* spp. vs. total coliforms and *Escherichia coli*, respectively. Results thus indicated that the incidence of *Legionella* and *Acanthamoeba* spp. in harvested rainwater may primarily be due to external pollutants such as dust and animal faecal matter present on the catchment system.

OPSOMMING

Die voortbestaan van *Legionella* spp. by hoë pasteurisasie temperature kan 'n bedreiging vir menslike gesondheid inhou deurdat 'n aantal *Legionella* spp. daarvoor bekend is om die siekte Legionnaires te veroorsaak. Navorsing dui ook aan dat die primêre faktore wat bydra tot die vermeerdering en voortbestaan van *Legionella* in waterverspreidingsisteme, die toeganklikheid tot voedingstowwe in 'n waterbron, die watertemperatuur, die teenwoordigheid van vrylewende amoeba (VLA) en die teenwoordigheid van ander akwatiese bakterieë, insluit. Die fokus van hierdie studie was dus om die voortbestaansmeganismes te ondersoek wat deur *Legionella* spp. gebruik word om in gepasteuriseerde en ongepasteuriseerde ge-oeste reënwater te oorleef.

Die oorhoofse doel van Hoofstuk twee was om die dominante *Legionella* spp. te isoleer en te identifiseer wat voorkom en oorleef in 'n huishoudelike reënwater opgaringstenk en 'n sonkrag pasteurisasie (SOPAS) sisteem. Verder was die doel ook om moontlike VLA, wat as vektore vir *Legionella* kan dien, te identifiseer en om te bepaal of hierdie vektore dan lewensvatbaar bly by hoë pasteurisasie temperature (>60°C). Hiervoor is gepasteuriseerde (45°C, 65°C, 68°C, 74°C, 84°C en 93°C) en ongepasteuriseerde tenkwatermonsters getoets vir die dominante *Legionella* spp., deur gebruik te maak van groei-gebaseerde tegnieke. Daarbenewens, aangesien VLA insluitend *Acanthamoeba* spp., *Naegleria fowleri* en *Vermamoeba* (*Hartmannella*) *vermiformis* die mees algemene amoeba spesies is wat uit watermonsters en warmwatersisteme geïsoleer word, is ethidium monoasied kwantitatiewe polimerase kettingreaksie (EMA-kPKR) aangewend om die lewensvatbare *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* en *N. fowleri* in gepasteuriseerde (68°C, 74°C, 84°C en 93°C) en ongepasteuriseerde tenkwatermonsters, te kwantifiseer. Twee-en-tagtig *Legionella* spp. is vanuit die ongepasteuriseerde tenkwatermonsters geïsoleer, met *L. longbeachae* (35%) wat die meeste geïsoleer is, gevolg deur *L. norrlandica* (27%) en *L. rowbothamii* (4%). Verder is daar bevind dat die die SOPAS sisteem die geen kopieë van die lewensvatbare *N. fowleri* (5-log) en *V. vermiformis* (3-log) effektief verminder het tot onder die onderste grens van opsporing by pasteurisasie temperature van 68-93°C en 74-93°C, onderskeidelik. In teenstelling, is daar bevind dat daar steeds lewensvatbare *Legionella* en *Acanthamoeba* spp. teenwoordig is by pasteurisasie temperature van 68-93°C, aangesien geen kopieë steeds in hierdie monsters waargeneem is. Daar kon dus afgelei word dat *Acanthamoeba* spp. hoofsaaklik as vektore dien vir *Legionella* spp. in son-gepasteuriseerde reënwater.

Die primêre doel van Hoofstuk drie was om drie *Legionella* spp. [*L. longbeachae* (env.), *L. norrlandica* (env.) en *L. rowbothamii* (env.)] geïsoleer vanuit ongepasteuriseerde reënwater;

twee *Legionella* verwysingstamme (*L. pneumophila* ATKK 33152 en *L. longbeachae* ATKK 33462) en *Acanthamoeba mauritaniensis* ATKK 50676 se weerstand teen hittebehandeling (50-90°C), te bepaal. Daarna is die weerstand teen hittebehandeling (50-90°C) van onderskeidelik *L. pneumophila* ATKK 33152 en *L. longbeachae* (env.) in samegroeïng met *A. mauritaniensis* ATKK 50676 bepaal deur gebruik te maak van EMA-kPKR. Verder is die interaksie meganismes wat uitgevoer word tussen *Legionella* en *Acanthamoeba* tydens hittebehandeling (50-90°C) ook geondersoek deur die relatiewe uitdrukking van gene wat geassosieer word met die metabolisme en virulensie van *L. pneumophila* ATKK 33152 (*lolA*, *sidF*, *csrA*) en *L. longbeachae* (env.) (slegs *lolA*) te monitor. *Legionella longbeachae* (env.) en *L. pneumophila* ATKK 33152 het die meeste weerstand getoon teen hittebehandeling aangesien beide hierdie organismes steeds op media gegroei het (KVE/ml) by onderskeidelik 50 en 60°C. Verder is bevind dat EMA-kPKR 'n meer sensitiewe tegniek is om lewensvatbare selle op te spoor, omdat alle *Legionella* spp. en *A. mauritaniensis* ATKK 50676 steeds in die monsters opgespoor kon word nadat hittebehandeling (50-90°C) toegepas is. Daarbenewens, terwyl die hitte weerstandigheid van *L. pneumophila* ATKK 33152 in samegroeïng met *A. mauritaniensis* ATKK 50676 verbeter het, word daar gepostuleer dat *L. longbeachae* (env.) nie in staat is om te vermeerder binne in *A. mauritaniensis* ATKK 50676 nie, aangesien *L. longbeachae* (env.) in samegroeïng met *A. mauritaniensis* ATKK 50676 nie met die EMA-kPKR toets opgespoor kon word na hittebehandeling by 80°C en 90°C nie. Verder het die resultate getoon dat daar 'n defnitiewe tendens tussen gene met verwante funksie en differensiële uitdrukking tydens hittebehandeling (50-90°C) is. Byvoorbeeld, relatief tot die onbehandelde (ongepasteuriseerde) watermonsters, het die uitdrukking van *lolA* konstant gebly, terwyl die uitdrukking van *sidF* toegeneem het en die uitdrukking van *csrA* beduidend afgeneem het tydens die samegroeïng van *L. pneumophila* ATKK 33152 met *A. mauritaniensis* ATKK 50676. Resultate bevestig dus dat, terwyl hittebehandeling die aantal lewensvatbare *Legionella* spp. kan verminder, *L. pneumophila* en *A. mauritaniensis* op mekaar inwerk. en kan *L. pneumophila* dus hittebehandeling oorleef.

Die oorhoofse doel van Hoofstuk vier was om vas te stel of ander mikrobiese, fisiese of chemiese eienskappe verband hou met die teenwoordigheid van *Legionella* spp. en *Acanthamoeba* spp. in reënwater wat opgevang is vanaf dakke wat uit verskillende materiale gemaak is. Daar is bevind dat die materiaal waarvan die dakke gemaak is nie die voorkoms van *Legionella* en *Acanthamoeba* spp in die watermonsters beïnvloed nie. Dit was duidelik omdat hierdie organismes in al die tenkwatermonsters teenwoordig was ongeag die materiaal (Chromadek®, galvaniseerde sink en asbestos) waarvan die dak wat gebruik is om the reënwater te oes, gemaak is. Daar is egter beduidende (p <0.05) positiewe Spearman (ρ) korrelasies opgemerk tussen *Legionella* spp., nitriete en nitrate, en tussen *Acanthamoeba* spp.

en barium, magnesium, natrium, silikon, arseen en fosfaat. Daarbenewens, terwyl geen beduidende korrelasies waargeneem is tussen *Legionella* spp. en indikator bakterieë (p> 0.05) nie, is positiewe korrelasies tussen *Acanthamoeba* spp. en totale kolivorme en *Escherichia coli* onderskeidelik waargeneem. Resultate dui dus aan dat die teenwoordigheid van *Legionella* en *Acanthamoeba* spp. in ge-oeste reënwater hoofsaaklik toegeskryf kan word aan eksterne besoedelingstowwe soos stofdeeltjies en diere fekale materiaal wat op die opvanggebied mag voorkom.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACES	N-(2-acetamido)-2-aminothane-sulfonic acid
ADWG	Australian drinking water guideline
PAS	Page amoeba saline
MPN	most probable number
PYG	peptone-yeast extract glucose
MOI	multiplicity of infection
ANK	ankyrin
C_q	quantitation cycle
r^2	correlation coefficient
AK	*Acanthamoeba* keratitis
ANOVA	analyses of variance
ARB	amoebae-resisting bacteria
ATCC	American type culture collection
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BSA	bovine serum albumin
BYCE	buffered charcoal yeast extract
CAF	Central Analytical Facility
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
CNS	central nervous system
CsrA	carbon storage regulator a
CV	coefficient of variation
CYE	charcoal-yeast extract
Da	Dalton
DAG	diacylgylcerol
Dot/Icm	defective in organelle trafficking/intracellular multiplication
DRWH	domestic rainwater harvesting
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EDCs	endocrine disrupting compounds
ELDSNet	European Legionnaires' Disease Surveillance Network
EMA	ethidium monoazide
EPF	end-point fluorescence
ER	endoplasmic reticulum
EU	European Union
F-G	Feeley Gorman
FLA	free-living amoebae
GAE	granulomatous amoebic encephalitis
GDF	guanosine nucleotide dissociation inhibitor -displacement factor
GDI	guanosine nucleotide dissociation inhibitor
gDNA	genomic DNA
GEF	guanine nucleotide exchange factor
GHI	global horizontal irradiance

GPS	global positioning system
GTPase	guanosine triphosphatase
GVBC	glycine, vancomycin, polymyxin B SO_4, cycloheximide
HPC	heterotrophic plate count
ICP	internal positive control
ICP-AES	inductively coupled plasma atomic emission spectrometry
IF	initiation factor
IM	inner membrane
kPa	kilopascal
LAMP-1	lysosome associated membrane protein-1
LCV	*Legionella* containing vacuole
LLOD	lower limit of detection
LSD	least significant difference
Lsp	*Legionella* secretion pathway
Lss	type I secretion system
MDG	Millennium Development Goals
mRNA	messenger RNA
N/A	not applicable
NNA	non-nutrient agar
NA	nutrient agar
NCBI	National Centre for Biotechnology Information
ND	not determined
NHLS	National Health Laboratory Service
NHMRC	National Health and Medical Research Council
NRMMC	Natural Resource Management Ministerial Council
OM	outer membrane
ORFs	open reading frames
PAIs	pathogenicity island loci
PAM	primary amoeboic meningoencephalitis
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
Pel	prenylated effectors of *Legionella*
PET	polyethylene-terephthalate
PEX	cross-linked polyethylene
PIs	phosphoinositides
ppGpp	guanosine tetraphosphate
qPCR	quantitative polymerase chain reaction
R2A	Reasoner´s 2A agar
SABS	South African Bureau of Standards
SANS	South African National Standards
SD	standard deviation
SNARE	soluble N-ethylaleimide-sensitive factor attachment protein receptor
SODIS	solar disinfection

SOPAS	solar pasteurization
sRNAs	small RNAs
T2SS	type II secretion system
Tat	twin – arginine
UAT	urinary antigen testing
UK	United Kingdom
UN DESA	United Nations, Department of Economic and Social Affairs, Population Division
USA	United States of America
USEPA	U.S. Environmental Protection Agency
UTR	untranslated region
UV	ultraviolet
VBNC	viable but non-culturable
WHO	World Health Organisation
WRC	Water Research Commission

Chapter 1:
Literature Review

(UK spelling is employed)

1.1. Introduction

The domestic rainwater harvesting (DRWH) process refers to the capture of rainwater from diverse catchment areas (such as a rooftop) and the storage of this water source in tanks. Stored harvested rainwater is globally accepted by a number of governmental administrations as an alternative water resource that can be utilised to aid in tackling the challenges associated with increasing water demand and climate change (Uba & Aghogho, 2000; Despins et al. 2009; Lee et al. 2010; 2012; Australian Government, 2011; Rowe, 2011). As a result of water scarcity and water quality constraints in South Africa, rainwater harvesting has been earmarked as a key, alternative strategy to supply water for irrigation, domestic and potable purposes to households and informal settlements [Department of Water Affairs (DWA), 2013]. However, rainwater may become contaminated during the harvesting process by various microbial and chemical pollutants and the quality of this water is regularly non-compliant with recommended drinking water standards (Gwenzi et al. 2015). For this reason, it is advisable that harvested rainwater is utilised for non-potable purposes such as cooking, cleaning and other domestic activities (Gwenzi et al. 2015). In order to reduce the level of contamination in harvested rainwater sources, cost-effective treatment methods, capable of treating adequate quantities of harvested rainwater, are required.

Many different methods are available to reduce or remove pollutants from harvested rainwater. These include chlorination (Sazakli et al. 2007), slow sand filtration (Dobrowsky et al. 2015a), nanofiltration (Kilduff et al. 2004; Dobrowsky et al. 2015a), solar disinfection (SODIS) and solar pasteurization (SOPAS) (Safapour & Metcalf, 1999; McGuigan et al. 2012; Dobrowsky et al. 2015b). In a previous study conducted by Dobrowsky et al. (2015b), the efficiency of a closed coupled SOPAS system was assessed with regard to improving the microbial quality of harvested rainwater. This system relied on direct heat and a thermo-siphoning effect to treat harvested rainwater directly from a DRWH tank. Results obtained from the pilot scale study indicated that rainwater samples pasteurized at 72°C and above (78–81°C and 90–91°C) were suitable for potable purposes, as the general indicator analysis showed that total coliforms, *Escherichia coli* (*E. coli*) and the heterotrophic plate count (HPC) were reduced to below the accepted detection limits. However, in the same study the screening of genomic DNA (gDNA), extracted from pasteurized rainwater samples, by the polymerase chain reaction (PCR) (utilising genus specific primers), indicated the presence of various bacterial opportunistic pathogens. For example, PCR analyses indicated that *Yersinia* spp. were detected in rainwater samples pasteurized at 78°C, while *Legionella* spp. and *Pseudomonas* spp. persisted at temperatures above 91°C. However, the PCR assays could have merely confirmed the presence of naked DNA rather than entire viable bacterial cells at high pasteurization temperatures. Thus, further studies were required in order to determine viability of the bacterial

pathogens in the solar pasteurized rainwater. Research conducted by Reyneke et al. (2016) then confirmed the viability of *Legionella* spp. in rainwater samples pasteurized at a temperature range of between 71.5°C and 95°C. In the latter study, quantitative PCR (qPCR) was used to determine the copy numbers of gDNA in samples pre-treated with the DNA-binding dye, ethidium monoazide (EMA). These findings are in agreement with a study conducted by Storey et al. (2004) who noted that conventional hyper-disinfection (treatment at 80°C and 100 mg/L chlorine) was insufficient for the long-term control of Acanthamoebae-bound *Legionella* in water supply systems.

Legionella are ubiquitous inhabitants of fresh water and soil and as natural water sources with low temperatures inhibit the proliferation of this microorganism, *Legionella* spp. from the natural environment are rarely associated with *Legionella*-induced disease (Szewzyk et al. 2000). The earliest reports of *L. pneumophila* infections were recorded in 1976 when an outbreak of severe lung inflammation (pneumonia) occurred among 200 residents in a hotel in Philadelphia (Fraser et al. 1977). The causative agent was discovered only several months after the outbreak as the bacterium could not be cultured on standard growth media. *Legionella* spp. are known to cause two types of disease, namely pneumonia (Legionnaires' disease) and a milder influenza-like illness (Pontiac fever) (Fraser et al. 1977; Glick et al. 1978). Both types of disease occur when aerosols contaminated with *Legionella* cells are inhaled. It is notable that the occurrence of infection after ingesting water contaminated with *Legionella* spp. is rare (Szewzyk et al. 2000). *Legionella pneumophila* serotypes 1, 4 and 6 cause 85% of the infections reported (Gruas et al. 2013). However, 17 other species have also been associated with disease. These include *L. longbeachae*, *L. micdadei*, *L. anisa* and *L. bozemanii* and infection with these species usually occurs in immunocompromised patients (Gruas et al. 2013). Pathogenic *Legionella* spp. have further been isolated from a number of man-made warm water systems including cooling towers, hot tubs, showerheads and spas (Fields, 1996; Atlas, 1999; Fields et al. 2002; Miquel et al. 2003)

The principal factors that enhance the proliferation of *Legionella* in water distribution systems and allow them to survive in adverse conditions are; availability of nutrients (metals including iron, zinc, manganese and organic material) in the water source (Cianciotto, 2007); water temperature - *Legionella* spp. require temperatures above 20°C to multiply and can remain viable at >80°C (Farhat et al. 2012; Schwake et al. 2015); presence of eukaryotic host organisms, including genera from the free-living amoebae (FLA) which act as hosts for the intracellular replication of *Legionella* in the environment (Donlan et al. 2005); and other aquatic bacteria, where *Legionella* are able to attach to biofilms that provide nutrients and protection from adverse environmental conditions, including water disinfection (Kim et al. 2002). Studies conducted by Murga et al. (2001), Kuiper et al. (2004) and Declerck et al. (2007) investigated

the microbial communities in tap water systems and concluded that biofilms allow for the persistence of *L. pneumophila,* while amoebae are required for the intracellular growth and proliferation of this microorganism.

Protists, particularly the protozoa, have been described by Bichai et al. (2008; pg 510) as "the Trojan Horse of microorganisms" as they can ingest pathogenic bacteria as a food source. As previously noted, the ability of *L. pneumophila* to persist and proliferate depends predominantly on the capacity of this bacterium to survive and replicate within protozoa (Barker & Brown, 1994). Examples of these protozoa include genera within the FLA such as *Acanthamoeba* spp., [1]*Vermamoeba* (*Hartmannella*) *vermiformis, Vahlkampfia* spp. and *Naegleria* spp. Other hosts of *Legionella* include human alveolar macrophages and protozoan ciliates such as the *Tetrahymena pyriformis* (Barker et al. 1992; Fields, 1993; Newsome et al. 1985; Rowbotham, 1986; Wadowsky et al. 1991). The hosts provide nutrients including amino acids for the proliferation of *Legionella* spp. and a protective environment when *Legionella* spp. are enclosed in resistant cysts formed by amoebae (Thomas et al. 2006). Storey et al. (2004) showed that *Acanthamoeba* cysts (containing *L. pneumophila* and *L. erythra*) remained viable after being treated at temperatures ranging from 40°C to 80°C. Brüggemann et al. (2006) explained that once *L. pneumophila* has gained entry into protozoan hosts such as *Acanthamoeba castellanii*, *Hartmannella* spp. and *Naegleria* spp., and into human alveolar macrophages, the microorganism survives by manipulating the host cell functions particularly the host phagocytic mechanisms. This is brought about by reprogramming the endosomal-lysosomal degradation pathway of the host cell (Brüggemann et al. 2006).

Studies performed by Burstein et al. (2016) and Hempstead and Isberg (2015) confirmed that extensive research is still required for the elucidation of the virulence genes encoded by *Legionella* spp., the host evasion mechanisms, the origin and progression of *Legionella* outbreaks and the isolation and identification of *Legionella* spp. in water supplies. The focus of the current study was thus to investigate and functionalise selected persistence mechanisms displayed by *Legionella* spp. that aid in their survival in pasteurized and unpasteurized harvested rainwater. During the current study, *Legionella* spp. capable of surviving and persisting in pasteurized and unpasteurized harvested rainwater samples were isolated. They were subsequently identified by using standard culture-based methods and conventional PCR. The viability of *Legionella* spp. and FLA including *Acanthamoeba* spp., *V. vermiformis* and

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[1] It should be noted that *Hartmannella vermiformis* was renamed *Vermamoeba vermiformis* as the species displayed significant differentiation from all other *Hartmannella* species (Smirnov et al. 2011). However, as most studies published prior to 2011 refer to *Vermamoeba vermiformis* as *Hartmannella vermiformis*, when citing previous studies, this review will refer to the genus as *Hartmannella* spp. and where applicable refer to the species as *V. vermiformis*.

Naegleria fowleri at the various pasteurization temperatures tested was also determined using EMA-qPCR. To investigate the resistance of five *Legionella* strains and *Acanthamoeba mauritaniensis* ATCC 50676 to heat treatment (50–90°C), the culturability and viability of the organisms were monitored. In addition, to examine whether *Legionella* spp. remained capable of colonising *A. mauritaniensis* ATCC 50676 after the application of high pasteurization temperatures (50–90°C), total RNA was extracted from *Legionella* and *Acanthamoeba* co-cultures. The expression of three *Legionella* genes was monitored and quantified using relative qPCR. One of the genes viz. *lolA* is involved in metabolism, and the remaining genes selected was the virulence gene *sidF,* which encodes an effector protein of the defective in organelle trafficking/intracellular multiplication (Dot/Icm) secretion system, while the *csrA* gene encodes a regulator responsible for the switch between the replicative and transmissive forms of *Legionella* spp. Finally, to determine whether microbial and physico-chemical characteristics of harvested rainwater influence the incidence of *Legionella* and *Acanthamoeba* spp., fluctuations of microbial indicator analysis and cation and anion concentrations in rainwater harvested from different roofing materials were monitored and then correlated with numbers of *Legionella* spp. and *Acanthamoeba* spp. (quantified using the qPCR) present in tank water samples.

1.2. Drinking water prospects

Water is arguably the most important resource as it is essential for many facets of life including human health, food availability, hydro-energy and the economy. It is therefore not surprising that a poor water supply and a lack of sanitation services negatively influence the environmental, economic and social sustainability of a country and its people (Mara, 2003; Moore et al. 2003; Montgomery & Elimelech, 2007; Johnson et al. 2008). The Millennium Development Goals (MDG) were established in the year 2000, and were aimed at addressing the world's concerns regarding the improvement of gender equality, health, education and the alleviation of poverty. One of the aims of the MDG was to halve the proportion of people without access to potable water and safe sanitation by 2015 (United Nations General Assembly, 2000).

The international MDG target for safe drinking water was achieved five years ahead of schedule and to date, 147 countries have met the drinking water target, 95 countries have achieved the sanitation target and 77 countries have complied with both targets. While numerous MDG regions including Eastern Asia, Latin America and the Caribbean, South-Eastern Asia, Southern Asia and Western Asia have halved the proportion of the respective populations without access to improved drinking water, Sub-Saharan Africa failed to meet the MDG target [United Nations, Department of Economic and Social Affairs, Population Division (UN DESA)., 2015; United Nations, 2015]. However, by 2015 the population of Sub-Saharan Africa with

access to an improved drinking water source increased to 68%. It is estimated worldwide, that 663 million people still do not have access to an improved drinking water source and 16% and 4% of the population living in rural communities and urban areas respectively, do not have access to improved drinking water sources (UN DESA, 2015; United Nations, 2015).

Water sources predominantly utilised for drinking purposes include public standpipes, dams, lakes, rivers and boreholes (Nieuwoudt & Mathews, 2005). Waterborne diseases are commonly caused by contamination of these water sources by, for example, human and industrial activities and bird and animal faecal matter. Through these activies, many pathogenic bacteria, viruses and parasites [World Health Organisation (WHO), 2013] as well as algal blooms, detergents, fertilisers, pesticides, chemicals, heavy metals, endocrine disrupting compounds (EDCs), pharmaceuticals, personal care products, surfactants and various industrial additives (Ritter et al. 2002; Fawell & Nieuwenhuijsen, 2003; Rodriguez-Mozaz et al. 2004; Falconer & Humpage, 2005) pollute the water sources. Moreover, with the world's population growing annually by an estimated 1.18% (expected to increase to 8.5 billion by 2030), water sources are becoming increasingly contaminated due to an escalation of anthropogenic activities (UN DESA, 2015). All of these added pressures including the pollution of water sources, the growth of the human population and climate change, have forced global authorities to consider alternative water sources such as harvested rainwater, to meet increasing water demands (Ahmed et al. 2011a).

1.3. A brief introduction into domestic rainwater harvesting

Domestic rainwater harvesting is a procedure whereby rainwater is collected from rooftops, courtyards or treatment systems and is then stored in harvesting tanks (Mwenge Kahinda et al. 2008). This age-old technology has gained increased attention during recent years as rainwater harvesting offers a cost-effective, decentralised water collection system. Countries which include the United Kingdom (UK), Spain, Australia, the United States of America (USA), Germany, Japan, Nigeria and South Africa, have investigated the use of rainwater harvesting as an alternative means of providing water for domestic, commercial and industrial purposes (Uba & Aghogho, 2000; Despins et al. 2009; Lee et al. 2010; Australian Government, 2011; Rowe, 2011; Morales-Pinzón et al. 2012; Fernandes et al. 2015). Various countries including China, are further considering rainwater harvesting as a means of providing drinking water to densely populated urban cities such as Hong Kong (An et al. 2015). In addition, favourable government policies and the availability of funding have directly stimulated the implementation of harvested rainwater systems in several countries which include Australia, the United Kingdom (UK), South Africa, the USA, Germany, Switzerland, Belgium, Denmark and Japan

(Tomaz, 2003; Aladenola & Adeboye, 2010; Way et al. 2010; Fernandes et al. 2015; Mahmoud & Tang, 2015).

In Southern Africa, decentralised water collection points together with adequate water supply infrastructures are in increasing demand. This is because South Africa has both large rural communities that are widely dispersed, as well as peri-urban informal communities, which are continuously expanding due to urbanisation. The water sector in South Africa is regulated by the Department of Water Affairs (DWA), which is governed by two Acts. These are the National Water Act (1998) and the Water Services Act (1997), which together with national strategic objectives direct effective water use and management (DWA, 2013). Rainwater harvesting projects have considerable potential to alleviate the effects of climate change and the pressures of an increasing population in South Africa (Mwenge Kahinda et al. 2007). In addition, harvesting rainwater provides an alternative water supply during periods of mandatory water restrictions. The technology also ensures a source of water at or near the point of consumption (Sazakli et al. 2007). To date, in South Africa rainwater harvesting tanks have been installed in nine provinces. These are Limpopo (5186 tanks), Mpumalanga (2592 tanks), Gauteng (1925 tanks), North West (3087 tanks), Northern Cape (123 tanks), Free State (524 tanks), KwaZulu-Natal (9238 tanks), Western Cape (1529 tanks) and Eastern Cape (45542 tanks) (Malema et al. 2016). However, information available on the quality of harvested rainwater in sub-Saharan Africa and more specifically in South Africa is limited (Gwenzi et al. 2015).

As rainwater harvesting involves the catchment of rainwater from rooftops and other catchment areas into domestic rainwater tanks, various chemical and microbial contaminants enter the tank. Many studies have indicated that untreated harvested rainwater is often not safe to drink. These studies have detected numerous contaminants including pathogens such as enteropathogenic *E. coli* and *Cryptosporidium* spp. as well as toxic metal cations and anions in stored rainwater (Uba & Aghogho, 2000; Simmons et al. 2001; Zhu et al. 2004; Sazakli et al. 2007; Ahmed et al. 2011a; Dobrowsky et al. 2014c). Studies have also indicated that the risk of illness to consumers is greater when exposed to microbial pathogens rather than chemical pollutants in harvested rainwater, as the latter impurities in this water source have rarely been associated with the incidence of disease (Spinks et al. 2006; Ahmed et al. 2008; Lee et al. 2010; Ahmed et al. 2011a).

1.3.1. Contaminants of harvested rainwater

Although there are several advantages associated with the utilisation of harvested rainwater, it is not widely used for potable purposes. This is primarily due to a lack of information regarding the potential risks associated with chemical and microbiological pollutants, the absence of mandatory guidelines for potable or non-potable uses of this water source and the potential

public health risk associated with consuming untreated harvested rainwater contaminated with microbial pathogens (Ahmed et al. 2011a). Rainwater can be contaminated with chemical pollutants from a variety of sources. These include: leaching of metals from the roofing materials used for the catchment system, atmospheric deposition originating from traffic exhaust fumes, industrial aerosols (Lee et al. 2012) and dust from industrial areas that may contain high levels of metals including cadmium, lead, zinc, copper and aluminium (Duruibe et al. 2007; Gwenzi et al. 2015).

Research results are often conflicting regarding the chemical quality of harvested rainwater. However, extensive research has indicated that aluminium (Chang et al. 2004), manganese (Chang et al. 2004), copper (Simmons et al. 2001; Chang et al. 2004), lead (Simmons et al. 2001; Chang et al. 2004; Peters et al. 2008) and zinc (Simmons et al. 2001; Chang et al. 2004) can be present in harvested rainwater at concentrations in excess of the respective drinking water guidelines. High concentrations of these chemicals in drinking water are undesirable. For example, the presence of lead has serious health implications as it is a potent and persistent neurotoxicant. The effects of lead poisoning range from death to impaired cognitive and behavioural development that can have long-term detrimental consequences in children (Lidsky & Schneider, 2003). Studies in Australia reported lead concentrations in harvested rainwater in excess of the Australian Drinking Water Guideline (ADWG) [National Health and Medical Research Council (NHMRC) & Natural Resource Management Ministerial Council (NRMMC), 2004] value of 10 µg/L (Simmons et al. 2001; Chapman et al. 2006; 2008; Morrow et al. 2007; Huston et al. 2009; Rodrigo et al. 2009). As noted by the Australian Health Council (EnHealth Council, 2004), the increased lead concentrations were attributable principally to the roof materials and uncoated lead flashing used for the roofing process. Thus, the construction materials used influence the chemical quality of harvested rainwater. In contrast, a study conducted by Dobrowsky et al. (2014a) on the content of anions and metal cations in rainwater samples collected from 29 houses located in Kleinmond, South Africa, where the rooftops were constructed from double roman standard plus tiles, determined that the concentrations of both anions and cations in the rainwater complied with the Department of Water Affairs and Forestry (DWAF, 1996), South African National Standards (SANS) 214 [South African Bureau of Standards (SABS, 2005)], World Health Organisation (WHO, 2011) and ADWG (NHMRC & NRMMC, 2011) drinking water guidelines. Thus the components of these tiles do not appear to adversely affect the chemical quality of the harvested rainwater.

The Australian Health Council (EnHealth Council, 2004) then published guidelines for the utilisation of rainwater tanks. These state that roofing materials such as cement or terracotta tiles, Colorbond®, galvanised iron, Zincalume®, asbestos/fibro cement, polycarbonate or fibreglass sheeting and slate, are suitable materials for the construction of the catchment area

of a rainwater harvesting system. These guidelines were based on research, which indicated that these roofing materials may not influence the chemical quality of roof harvested rainwater (EnHealth Council, 2004; Chang et al. 2004; Mendez et al. 2010; Meera & Ahammed, 2011).

During the rainwater harvesting process, microbial contaminants that originate from dust and faecal matter (from birds, insects, rodents and other small animals) present on the rooftops are often washed into the rainwater harvesting tanks (Li et al. 2010; Ahmed & Toze, 2015). Vectors such as mosquitoes and flies that gain access directly to the storage tanks, can also carry pathogenic microorganisms (Mwenge Kahinda et al. 2007). It is thus common practice to use drinking water guidelines to monitor the microbial quality of the water in order to determine whether a water source is of a potable standard. For most guidelines, indicator bacteria which include *E. coli* or thermotolerant coliforms, faecal coliforms and enterococci, are enumerated [ADWG and DWAF guideline zero colony forming units (CFU)/100 mL]. The presence of these indicators suggests faecal pollution of the water (DWAF, 1996; NHMRC & NRMMC, 2011). In addition, the recommended guideline for total coliforms is <10 CFU/100 mL in 95% of samples collected (WHO, 2004) and ≤5 CFU/100 mL (DWAF, 1996). Total coliforms indicate the general hygienic quality of the water and the presence of biofilms in a water source (DWAF, 1996; De Kwaadsteniet et al. 2013).

Most studies reporting on the quality of harvested rainwater utilise faecal indicator bacteria to assess the microbiological quality of the water (**Figure 1.1**) [(adapted from De Kwaadsteniet et al. (2013)]. Dillaha and Zolan (1985) reported that 68% of harvested rainwater samples analysed in Micronesia (country composed of four island states) contained faecal coliforms (**Figure 1.1**). However, the authors suggested that the harvested rainwater could be utilised for drinking, although the numbers of faecal coliforms were high and not within the respective guidelines. In contrast, numerous studies have also reported that harvested rainwater is not suitable for potable purposes. For example, Spinks et al. (2006) sampled 49 rainwater tanks and reported that 33% of the harvested samples tested positive for *E. coli* and 73% were positive for enterococci, exceeding the ADWG of zero CFU/100 mL (**Figure 1.1**). They concluded that the rainwater sources sampled were thus not suitable for drinking. In harvested rainwater samples collected in South East Queensland, Australia, Ahmed et al. (2010) indicated that *E. coli* numbers ranged from 4 to 800 CFU/mL and enterococci ranged from 5 to 200 CFU/mL. *Escherichia coli* and enterococci were detected in 63% and 78% of the rainwater samples analysed, respectively (**Figure 1.1**). As *E. coli* was not detected in a number of samples, the authors suggested that harvested rainwater should be screened for a range of relevant faecal indicators, to obtain more accurate results regarding faecal contamination.
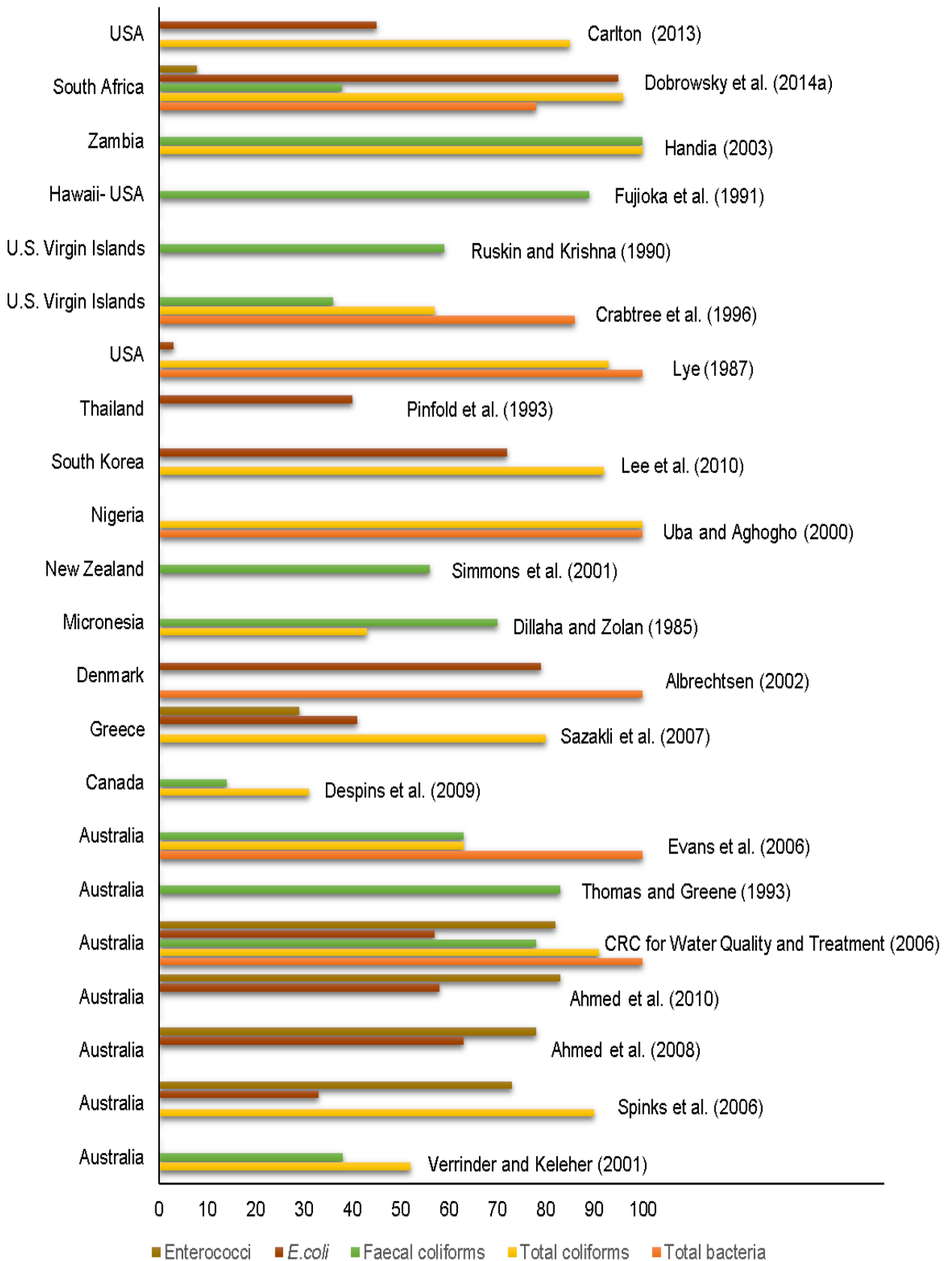
Figure 1.1. Percentage of samples positive for faecal indicators (adapted from De Kwaadsteniet et al. 2013).

Other studies have investigated the presence of numerous indicator bacteria including heterotrophic microorganisms, total coliforms, faecal coliforms, *E. coli* and enterococci and indicated that the quality of harvested rainwater does not meet drinking water guidelines (**Figure 1.1**) (Handia et al. 2003; Sazakli et al. 2007; Ahmed et al. 2008; 2011a; Dobrowsky et al. 2014b). Ahmed et al. (2011b) showed that a poor correlation existed between indicator bacteria and pathogenic bacteria in harvested rainwater samples. Subsequently, further studies have reported microbial contamination of harvested rainwater, where potential pathogenic bacteria including *Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Legionella* spp., *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. have been detected in harvested samples (Uba & Aghogho, 2000; Simmons et al. 2001; Albrechtsen, 2002; Ahmed et al. 2008; 2011a; Dobrowsky et al. 2014b). However, to date, the presence of pathogenic protozoan species in collected rainwater has not been extensively investigated. Despite a well-established zoonotic link, studies have generally focused on the presence of only two pathogenic protozoan species namely *Cryptosporidium* spp. (Crabtree et al. 1996; Simmons et al. 2001; Abo-Shehada et al. 2004; Dobrowsky et al. 2014b) and *Giardia* spp. (Crabtree et al. 1996; Ahmed et al. 2008; 2011a; Dobrowsky et al. 2014b).

Microorganisms, other organic substances and heavy metals are some of the major pollutants found in the atmosphere that affect the quality of harvested rainwater. The design of a rainwater harvesting system should thus minimise the entry of contaminants into the harvesting tank during the collection process. For example, selecting an appropriate roofing material such as galvanised zinc for the catchment area could reduce the amount of chemical contaminants. Furthermore, the use of a closed tank creates a dark environment thereby inhibiting the proliferation of algae in the system (Gould, 1999; Zhu et al. 2004).

Lee et al. (2012) noted that the installation of first flush diverters improved the physical, chemical and microbiological quality of the rainwater harvested from a galvanised steel rooftop. However, studies have indicated that even though system design and management, including periodical cleaning of the rainwater harvesting system and the installation of first flush diverters can decrease contamination, untreated harvested rainwater may still not comply with acceptable drinking water standards (Mwenge Kahinda et al. 2007; Dobrowsky et al. 2014a; 2014b; 2015a; 2015b).

1.3.2. Water treatment systems for harvested rainwater

As indicated by Burch and Thomas (1998), when selecting a suitable water treatment system, the influence of various technical and social variables must be considered. Technical variables include whether or not microbial contamination of the harvested rainwater includes viruses, fungi, bacteria and protozoa or a combination of all four, as this will affect the choice of

treatment system required to eliminate or reduce the level of contamination. In addition, the turbidity of the harvested rainwater will influence the efficiency of, for example, a SODIS system. Social factors, including proximity of the available treated water source to the household and whether the treated water is to be used to supply an entire community or a single family, will affect the volume of water produced by the treatment system. The availability of electricity will influence the choice of treatment system; a passive system will be implemented in areas without electricity, or where residents have to pay for electricity. Importantly, an awareness of disease and a familiarity with the faecal-oral cycle by community members will aid in their motivation to invest in a water treatment system. Furthermore, individuals in economically disadvantaged groups require water disinfection treatment systems that are sustainable and do not require regular expensive maintenance. It is because of these numerous and important variables that it becomes essential for water treatment systems to be evaluated and monitored to verify their efficiency and sustainability before implementation at a full-scale level (Burch & Thomas, 1998).

Currently, several cost-effective treatment methods are used for the removal or reduction of contamination in harvested rainwater. These include disinfection, where chlorination is the most common practice implemented to improve the microbiological quality of harvested rainwater (Sazakli et al. 2007). Generally, the dose of chlorine is at a level of 0.4–0.5 mg/L free chlorine and the chemical is applied after harvested rainwater has been removed from the tank (Helmreich & Horn, 2009). However, disadvantages include the presence of undesirable by-products such as carcinogenic substances (trihalomethanes) that are produced when the chlorine is exposed to organic matter. In addition, certain parasitic species (including *Acanthamoeba* spp.) have exhibited resistance to low levels of chlorine (Ellis, 1991; Li et al. 2010).

Filtration systems are also cost effective treatment methods and can be divided into two passive systems, namely slow sand filtration and nanofiltration and can be used to treat rainwater. Slow sand filtration relies on the formation of a schmutzdecke or biofilm layer that serves as a biological filter as the rainwater flows at a slow rate through the sand (for absorption, due to electrical forces) and the biofilm (Schulz & Okun, 1983; Dobrowsky et al. 2015a). However, this system relies on the 'ripening' of the biofilm for efficiency, prior to which bacteria are not removed effectively (Mwabi et al. 2011). In contrast, nanofiltration relies on Donnan exclusion and sieving separation that filter molecules with a molecular mass of between 300 and 1000 Da at pressures as low as 350 to 1000 kPa (Eriksson, 1988; Kilduff et al. 2004). One of the disadvantages of this treatment is decreased performance caused by fouling of the membrane (Cornelissen et al. 2008; Dobrowsky et al. 2015a).

Lastly, heat treatments including SOPAS, rely on the thermal inactivation of pathogens. As solar systems depend on the energy of the sun, no electricity is required and the systems do not need regular expensive maintenance (Dobrowsky et al. 2015b). Previous studies conducted by Dobrowsky et al. (2014b; 2014c; 2015a; 2015b) detected viruses, bacteria, protozoa or a combination of all three in harvested rainwater. As solar-based disinfection and pasteurization systems are effective in reducing these microbial contaminants in various water sources including harvested rainwater (McGuigan et al. 2012; De Kwaadsteniet et al. 2013; Dobrowsky et al. 2015b) this review will focus and elaborate on the use of SOPAS and SODIS systems.

1.3.3.1 Solar pasteurization (SOPAS) and solar disinfection (SODIS): treatment of harvested rainwater

Untreated harvested rainwater is used for numerous purposes including livestock watering, laundry, bathing, toilet flushing, and many other domestic activities (Mwenge Kahinda et al. 2007; Lynch & Dietsch, 2010; Ward et al. 2012). However, harvested rainwater may contain numerous potentially pathogenic bacteria and prior treatment of the harvested rainwater is thus essential if the water is to be used for potable purposes (Dobrowsky et al. 2014b; 2015a; 2015b). The inactivation of many protozoa (Moriarty et al. 2005; Cervero-Aragó et al. 2014), bacteria (Sherwani et al. 2013) and viruses (Strazynski et al. 2002) by boiling the contaminated water for several minutes has proven to be effective in eradicating these organisms. However, for rural communities, firewood, a preferred fuel source for boiling water, may be expensive and promoting the use of biomass for boiling can have a serious negative impact on the environment (Islam & Johnston, 2006). Utilising the natural free energy of the sun permits pasteurization systems to reduce the level of bacterial numbers in water without direct boiling (Dobrowsky et al. 2015b).

According to Nieuwoudt and Matthews (2005), the idea of heating water to below boiling has gained much attention and for this reason, the design and application of heat-based disinfection systems is fairly advanced. Solar disinfection relies on a combination of heating and exposing water to ultraviolet (UV) radiation, whereas SOPAS relies on the thermal effect at a temperature of at least 70°C without direct radiation of the rainwater (Sommer et al. 1997). While conventional SODIS refers to exposing a transparent container [usually polyethylene-terephthalate (PET) or glass] filled with contaminated water to direct sunlight, SODIS batch systems are comprised of a transparent container that is placed in a solar collector lined with an absorptive material (McGuigan et al. 2012). In contrast, the SOPAS systems rely on a thermo-siphoning effect where water circulates through the system due to a range of temperatures that causes variable water densities. As a result, heated water will rise and cooled water will descend in the system. As SOPAS systems rely on the thermo-siphoning effect to circulate

water, they are considered to be passive and do not require an electric pump (and electricity) for circulating water.

Many developed thermal collectors rely on solar energy to pasteurize water at high temperatures (> 90°C). There are generally two types: those that operate as batch systems and those that act as continuous flow-through systems (da Silva et al. 2016). For example, in a batch collector, Safapour and Metcalf (1999) indicated that pasteurization was more effective when dark containers (acting as a SOPAS system) were placed in reflectors when compared with transparent containers (acting as a SODIS system) as temperatures of approximately 70°C were attained for a duration of 30 minutes. McGuigan et al. (2006) indicated that *C. parvum* oocysts and *G. muris* cysts were non-infective towards CD-1 suckling mice after respective SODIS treatments of ≥10 and 4 hours in a batch system. In a recent study, Amsberry et al. (2015) demonstrated that in a continuous flow system consisting of cross-linked polyethylene (PEX) tubing coiled and mounted on a steel absorber plate, 55 L/day of treated water was produced on a clear day and the system reached temperatures of up to 74°C. Lastly, a closed-coupled system was utilised to pasteurize large volumes of rainwater and produced an average quantity of 13.6 kg/h, 12.0 kg/h, 9.90 kg/h, 8.94 kg/h and 7.38 kg/h at temperatures of 55–57°C, 64–66°C, 72–74°C, 78–81°C, 90–91°C, respectively (Dobrowsky et al. 2015b).

Multiple research studies have then routinely used various indicator bacteria to assess the efficiency of different treatment systems. For example, the presence of coliform bacteria after treatment implies that the treatment used was either ineffective (McFeters et al. 1997) or that there was an intrusion of contaminated water into the potable water supply after heating (Clark et al. 1996). The presence of indicator bacteria in heated water could also suggest that coliform bacteria were able to revive and regrow after treatment (LeChevallier et al. 1996). *Escherichia coli* is screened for as an indicator of faecal pollution originating from warm-blooded animals (DWAF, 1996; Dobrowsky et al. 2014a). Many regulatory health agencies suggest using HPC bacteria as indicators of possible health risks associated with the consumption of a contaminated water source (DWAF, 1996). The heterotrophic bacteria, including *Aeromonas*, *Klebsiella* and *Pseudomonas*, classified as opportunistic pathogens, can be enumerated using HPC methods. Using a SOPAS based system, Dobrowsky et al. (2015b) monitored the quality of rainwater before and after heat treatment at various temperature ranges. Results from the study indicated that rainwater samples pasteurized at 72°C and above (78–81°C and 90–91°C) were suitable for potable purposes, as the numbers of total coliforms, *E. coli* and HPC were reduced below the detection limit. It has however previously been documented that bacteria such as *Aeromonas* spp., *Klebsiella* spp., *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Shigella* spp. amongst others, are able to enter a viable but non-culturable state and may thus not be detected by using standard culturing

methods (Oliver, 2010). For this reason, further analyses were performed on pasteurized and unpasteurized rainwater samples during the Dobrowsky et al. (2015b) study. Although *Aeromonas* spp., *Klebsiella* spp. and *Shigella* spp. were undetected at temperatures in excess of 65°C, the PCR analyses indicated that *Yersinia* spp. persisted at 78°C. Furthermore, *Legionella* spp. and *Pseudomonas* spp. persisted at even higher temperatures viz. 90 to 91°C. Of particular concern was the presence of *Legionella* spp. at high temperatures as *Legionella* spp. have previously been identified in harvested rainwater samples [Albrechtsen, 2002; Cooperative Research Centre (CRC) for Water Quality and Treatment, 2006; Ahmed et al. 2008; 2014]. Reyneke et al. (2016) also showed that *Legionella* spp. remained viable at pasteurization temperatures of ± 90°C.

1.4. *Legionella* spp.

Legionella are Gram-negative, motile, rod-shaped, facultative intracellular bacteria belonging to the γ-proteobacterial lineage (Chien et al. 2004). There are currently more than 60 species of *Legionella*, with 70 distinctive serogroups (Benson & Fields, 1998; Lo Presti et al. 1999; 2001; Adeleke et al. 2001; Allombert et al. 2013; Gomez-Valero et al. 2014; Benitez & Winchell, 2016). Information regarding the presence of *Legionella* spp. in South African water habitats is limited and since 1997, the focus has been to standardise culture methods for the isolation of *Legionella* in South Africa. Bartie et al. (2003) emphasised that because there are no standardised identification methods, "there is uncertainty about the true prevalence and most common species of *Legionella* present in the South African environment" (Bartie et al. 2003; pg 1362).

To date, the majority of the genomes of *Legionella* spp. sequenced and comprehensively analysed are from *L. pneumophila* (36 genomes) and *L. longbeachae* strains (two genomes) (Cazalet et al. 2010; Kozak et al. 2010). The sequencing and analyses of genomes of other *Legionella* spp. rarely associated with human disease, include *L. oakridgensis* (Brzuszkiewicz et al. 2013) and Gomez-Valero et al. (2014) sequenced and analysed the genomes of *L. micdadei*, *L. hackeliae* and *L. fallonii*. The genomes of *L. pneumophila* that have been sequenced and assembled include *L. pneumophila* str. 2300/99 Alcoy (Genbank: CP001828) which occurs in serogroup 1. This latter strain is endemic to Spanish areas (D'Auria et al. 2010). Also included in this serogroup are the genomes of *L. pneumophila* str. Corby (Glöckner et al. 2008), *L. pneumophila* str. Paris and *L. pneumophila* str. Lens (Cazalet et al. 2004). *Legionella pneumophila* str. Lens was responsible for a major outbreak in France (Cazalet et al. 2004), while *L. pneumophila* subsp. *pneumophila* str. Philadelphia 1 was isolated during the initial outbreak of Legionellosis in Philadelphia, USA (Chien et al. 2004). *Legionella pneumophila* subsp. *pneumophila* str. LPE509 was isolated from a hospital water

system in Shanghai, China (Ma et al. 2013), and *L. pneumophila* subsp. *pneumophila* str. 570-CO-H (ATCC 43290) of serogroup 12 was a clinical isolate from the Colorado Department of Health, Denver, CO (Amaro et al. 2012). *Legionella pneumophila* isolated in France include *L. pneumophila* str. Lorraine identified in 2004 from a patient (Ginevra et al. 2008; Gomez-Valero et al. 2011) and *L. pneumophila* str. HL 0604 1035, frequently isolated from hospital water systems (Gomez-Valero et al. 2011). Recently, Mercante et al. (2016a) performed whole-genome sequencing, complete assembly and comparative analysis of *L. pneumophila* strains C1 to C11 and *L. pneumophila* strains E1 to E11, which where isolated during the 1976 Philadelphia Legionnaires' disease outbreak. Kozak-Muiznieks et al. (2016) also reported the complete genome sequences of three *L. pneumophila* subsp. *pascullei* strains (including both serogroup 1 and 5 strains) that were isolated from a health care facility in Pittsburgh, Pennsylvania, USA in 1982 and 2012. Mercante et al. (2016b) described the complete genome sequences of *L. pneumophila* serogroup 1 strains OLDA (from a sporadic Legionnaires' disease case; in 1977) and Pontiac (from an outbreak at a Michigan health department in 1968).

The sequencing and annotation of the genomes of the *L. longbeachae* clinical isolate from Oregon, isolate D-4968 (Kozak et al. 2010) and *L. longbeachae* strain NSW150 serogroup 1 (Cazalet et al. 2010) revealed that the genes encoding structural components of type II, type IV Lvh and type IV Dot/Icm secretion systems are conserved amongst species. The sequencing of the genomes also showed that *Legionella* spp. have undergone horizontal gene transfer and harbour a variety of eukaryotic-like proteins, likely to be involved in inhibiting host phagocytic functions. Moreover, the *Legionella* spp. commonly associated with human disease including *L. pneumophila* and *L. longbeachae,* have sets of genes that increase the capacity of the microbes to subvert host functions, establish a protective niche for intracellular replication and enhance their advanced ability to acquire iron and resist oxidative damage. These properties thus aid in the successful infection of mammalian and *Acanthamoeba* cells (Gomez-Valero et al. 2014).

1.4.1. *Legionella* associated with disease

Legionella spp. can cause an acute form of pneumonia as part of a multisystem disease known as Legionnaires' disease (also Legionellosis or Legion Fever) which can be fatal if not treated (Fraser et al. 1977; Newton et al. 2010). The organism can also cause a milder form of pulmonary infection known as Pontiac fever, which is a flu-like illness (Glick et al. 1978). The vulnerability of individuals to Legionnaires' disease is associated with smoking, chronic cardiovascular or respiratory disease, diabetes, alcohol misuse, cancer (especially profound monocytopenia) and immunosuppression (Plouffe & Baird, 1981; Rosmini et al. 1984; Marston et al. 1994; den Boer et al. 2008; Phin et al. 2014). *Legionella dumoffii, L. anisa*, *L. wadsworthii*

and *L. feelei* are occasionally associated with human disease (Yu et al. 2002). However approximately 90% of the clinical cases of Legionellosis reported worldwide are attributable to *L. pneumophila*. Other strains most commonly associated with disease include *L. micdadei*, *L. bozemanii* and *L. longbeachae* (Yu et al. 2002; Mercante & Winchell, 2015). *Legionella longbeachae* is associated with 2–7% of clinical cases reported worldwide. The exceptions are Australia and New Zealand where *L. longbeachae* is the causative agent of 30% of Legionnaires' disease (Yu et al. 2002). Currently, *L. pneumophila* spp. are classified serologically into 15 serogroups, of which the *L. pneumophila* serogroup 1 is responsible for most of the reported cases (84%) of disease (Marston et al. 1994; Fields et al. 2002; Yu et al. 2002). As *Legionella* spp. are intracellular pathogens, antimicrobial therapy is effective against Legionnaires' disease due to the relatively high intracellular penetration of certain antibiotics (Horwitz, 1993; Edelstein & Cianciotto, 2010). Antibiotics including most macrolides (such as Azithromycin), tetracyclines, ketolides and quinolones (including Levofloxacin) are effective. However, the β-lactams and aminoglycosides are ineffective in treating *Legionella* infections (Dunbar et al. 2004; Edelstein & Cianciotto, 2010; Garau et al. 2010; Bruin et al. 2012).

Symptoms of Legionnaires' disease include fever, non-productive coughing, headache, myalgia, rigors, dyspnoea, diarrhoea and delirium (Tsai et al. 1979). The sequencing and comparison of *Legionella* genomes has revealed that as *Legionella* spp. are capable of intracellular growth in host cells, it is likely that the majority of *Legionella* spp. could cause human disease when favourable conditions arise (Fields, 1996; Burstein et al. 2016). To date, the transmission of Legionnaires' disease from person-to-person has not been reported (Newton et al. 2010; Hilbi et al. 2011). However, a recent study reported the first case of a probable human-to-human transmission of *L. pneumophila* associated with a large outbreak of Legionnaires' disease in Portugal in 2015 (Correia et al. 2016).

There are approximately 24 species of *Legionella* associated with human illness, and infection generally occurs through the inhalation or aspiration of contaminated water aerosols produced by air conditioning systems, cooling towers, showers or other technical devices (Fields et al. 2002; Phin et al. 2014). However, it has been hypothesised that *L. longbeachae* has a route of transmission different from that of other pathogenic *Legionella* spp. To date, the mode of transmission has not been completely elucidated, although exposure to compost/soil or gardening activities is regarded as a risk factor for Legionnaires' disease caused by *L. longbeachae* (Amodeo et al. 2010; Lindsay et al. 2012). In addition, infection may occur in individuals living several kilometres away from the source of *Legionella* contamination, as *Legionella*-contaminated aerosols can spread over wide distances (Nguyen et al. 2006). Research has also indicated that specific strains have been associated with specific geographical areas; for example, *L. pneumophila* (serogroup 1) was responsible for clinical

cases in Europe and the USA, whereas *L. longbeachae* (serogroup 1) was linked to cases reported in Australia and New Zealand (Newton et al. 2010).

Legionnaires' disease is a notifiable disease in various countries. These include the USA, Canada, New Zealand, Australia, Japan, Singapore and Europe (since 1995) [Ng et al. 2009; Centers for Disease Control and Prevention (CDC), 2011; Lam et al. 2011; Graham et al. 2012; Milton et al. 2012; Ozeki et al. 2012; European Centre for Disease Prevention and Control, 2013]. In contrast, due to a lack of common definitions, diagnostics and surveillance systems, data from other parts of the world are rarely reported (Phin et al. 2014). In the United States it has been reported that 18 000 cases of Legionellosis occur each year and that 70–92% of the cases are caused by *L. pneumophila* serogroup 1 (Mercante & Winchell, 2015). On the 14th of August 2015, the Centres for Disease Control and Prevention published two reports regarding the outbreak of disease in the USA recorded during 2011 to 2012. These illnesses were associated with environmental water and drinking water sources. Of the 18 outbreaks of disease associated with environmental or undetermined water sources reported from 11 different states (including Illinois and Ohio), 15 outbreaks were due to Legionellosis (254 cases of illness) with 10 fatalities recorded (Beer et al. 2015a). A further 32 outbreaks of disease reported from 14 states (including New York and Florida) were associated with drinking water, where *Legionella* spp. were responsible for 21 of the outbreaks and 112 cases of illness (Beer et al. 2015b).

The surveillance of Legionnaires' disease in Europe is the function of the European Legionnaires' Disease Surveillance Network (ELDSNet), where all European Union (EU) States, as well as Iceland and Norway, contribute. In 2009 and 2010, totals of 5551 and 6305 cases of Legionnaires' disease were reported, respectively, by 29 European countries (Beauté et al. 2013). *Legionella pneumophila* was commonly associated with the more severe cases of this atypical pneumonia (Reingold et al. 1984; Yu et al. 2002). However, there are instances where Legionnaires' disease remained undiagnosed due to a lack of clinical awareness.

In South Africa, *Legionella* falls within the Occupational Health and Safety Act: Regulation for Biological Agents, number 85:1993 and in an attempt to reduce exposure to *Legionella*, two new national standards were published. These are the South African National Standards (SANS) 893-1, Legionnaires' disease Part 1: Risk Management and SANS 893-2, Legionnaires' disease Part 2: The control of *Legionella* in water systems. The SANS 893-1 provides guidelines for the risk management of *Legionella* bacteria, while the SANS 893-2 focuses on the design and management requirements for hot and cold water systems that could transmit *Legionella* bacteria through water droplets. The SANS 893-2 focuses predominantly on cooling towers and evaporative condensers (SABS, 2013).

1.4.2. Reports of Legionnaires' disease in South Africa

In 1980, the first reports of Legionnaires' disease in South Africa (Johannesburg) were recorded, where two cases (a 52-year-old, diabetic male and a 48-year-old female who smoked) were confirmed by using the immunofluorescent antibody test. However, no possible sources of infection were identified (Kaplan et al. 1980). In Port Elizabeth, eight sporadic cases of Legionnaires' disease in individuals whose ages ranged from 21 – 51 years have been reported (Randall et al. 1980). Although the sources of infection were not noted, the authors emphasised the unusual clinical aspects and the need for improved diagnostic procedures. Strebel et al. (1988) reported the first investigation of an outbreak of Legionnaires' disease in South Africa, where 12 cases of Legionnaires' disease were identified during the period 11 November 1985 to 21 February 1986 at a Johannesburg teaching hospital. *Legionella pneumophila* was the causative agent and in an attempt to identify the source of infection and mode of transmission, an epidemiological investigation was initiated. The authors suggested that the ventilators used in the medical and surgical intensive care units were a major risk factor for acquiring Legionnaires' disease. In addition, a study conducted in 1997 over a period of six months with the use of antibodies for detection, indicated that *L. pneumophila* (serogroups 1 - 4) were associated with 36% of mineworkers and 10% of factory workers (Bartie & Klugman, 1997). In 2013, a patient from Cape Town, South Africa, was diagnosed with Legionnaires' disease after urine samples subjected to the *Legionella* urinary antigen testing (UAT) performed at Groote Schuur National Health Laboratory Service (NHLS) tested positive for *Legionella* (National Institute for Communicable Diseases, 2013). During June 2012 to September 2014, sputum samples from patients with lower respiratory tract infections admitted to the Klerksdorp-Tshepong Hospital Complex, Klerksdorp (North West Province) and Edendale Hospital, Pietermaritzburg (KwaZulu-Natal Province) were analysed for *L. pneumophila* and *L. longbeachae* infections using real-time PCR. Of the 1805 patients tested, 21 (1.2%) tested positive for *Legionella* spp. infection (Wolter et al. 2016).

Reports on the incidences of disease associated with *Legionella* spp. in South Africa are however, still limited. Although doctors by law are required to notify the relevant authorities (National Department of Health) of diagnosed cases, few doctors routinely test for *Legionella* spp. Moreover, in South Africa, routine analysis for the diagnosis of patients with community- or hospital-acquired pneumonia does not include the detection of *Legionella* spp., unless a health professional submits appropriate specimens (including respiratory tract specimens) for the culturing of *Legionella*, or urine for the UAT. In addition, doctors need to stipulate that laboratories should specifically identify *Legionella* spp. It is also not always possible to distinguish clinically between Legionnaires' disease and other types of pneumonia (Edelstein, 1993) as the X-ray patterns of the various types of pneumonia may not differ, even

though Legionnaires' disease is commonly associated with alveolar infiltrates (Macfarlane et al. 1984). For this reason, doctors are inclined to prescribe antibiotics that target all species of bacteria linked to pneumonia in order to spare patients additional costs (SABS, 2013). Moreover, *Legionella* are fastidious and are not always detectable (Fields et al. 2002). The reliability of a diagnosis thus depends on a high index of suspicion and selective laboratory tests, where a conclusive diagnosis depends on culturing methods to determine the presence of *Legionella* spp. One of the many disadvantages associated with culturing the bacteria is that the method is not sufficiently sensitive and there is considerable variability among the tests performed. Furthermore, the expertise of the personnel performing the culturing methods may differ among the different laboratories and subsequently the sensitivity of the tests may vary (National Institute for Communicable Diseases, 2013).

Worldwide, the most common recommended diagnostic test is the UAT, which is cost-effective and detects the antigens of *L. pneumophila* serogroup 1 in urine. However, the disadvantage of this test is that no other *Legionella* spp. are reliably detected and it requires a four-fold rise in antibody titres in acute and convalescent sera thus decreasing sensitivity of the test. Although numerous studies have utilised PCR to detect *Legionella* spp. in environmental samples, in the clinical context, the occurrence of false-positive results and the lack of experience to perform the PCR analysis have delayed its use as a diagnostic tool for the identification of *Legionella* spp. (National Institute for Communicable Diseases, 2013).

1.4.3. Parameters influencing *Legionella* contamination in water distribution systems

Legionella spp. have been isolated from aquatic environments such as rivers, lakes and ponds and numerous other water sources including fabricated warm water systems such as cooling towers, hot tubs, showerheads and spas (Fields, 1996; Atlas, 1999; Fields et al. 2002; Miquel et al. 2003). *Legionella* spp. have also been isolated from fountains, heated birthing pools, supermarket mist machines, ice machines, air, plant material, compost and potting soil (Steele et al. 1990; Hughes & Steele, 1994; Fields & Moore, 2006; Montagna et al. 2016). In the environment, *Legionella* spp. exist as free-living bacteria, or within living protozoa or aquatic biofilms and have been detected by using culture methods (40%) and PCR assays (80%) (Fields et al. 2002). In man-made water systems, factors that favour *Legionella* growth include: water temperature, flow rate, stagnation, pipe materials, pipe corrosion, high water shear stress and flushing (Exner et al. 2005). In addition, investigations into the relationship between factors such as trace element concentrations, water hardness, heterotrophic bacteria and *Legionella* colonisation have been done (Edagawa et al. 2008; Völker et al. 2010).

To date, no studies have indicated the manner in which trace metals influence the persistence and growth of *Legionella* spp. in harvested rainwater systems. However, some studies

(**Table 1.1**) have indicated that certain metals enhance or inhibit the growth of *Legionella* spp. in plumbing systems of buildings. For example, recently Rakić and Štambuk-Giljanov, (2016) examined the technical and technological characteristics of four accommodation facilities located in Southern Croatia (the Split-Dalmatian County). The chemical parameters monitored included analysing the concentrations (mg/L) of iron, zinc, copper and manganese (**Table 1.1**). The authors concluded that copper at higher concentrations had an inhibitory effect (negative correlation) on the numbers of *Legionella* spp., while a positive correlation was established between iron (>0.120 mg/L) and zinc (>0.15 mg/L) and the presence of *Legionella* spp. (**Table 1.1**). It has also been reported that drinking water distribution systems require regular monitoring for microbial contamination and that the concentration of *Legionella* present in these systems is dependent on the metal concentrations rather than the type of water heating system being assessed. Research has thus indicated that cations including iron (>0.095–50 mg/L), zinc (>0.15–1.0 mg/L), potassium (1, 10 and 100 mg/L) and manganese (8.37 mg/L) appear to enhance the growth of *Legionella* spp. (States et al. 1985; Bargellini et al. 2011; Rakić et al. 2012; Serrano-Suárez et al. 2013; Rakić & Štambuk-Giljanović, 2016). However, copper at concentrations above 0.76 mg/L and iron in excess of 50 mg/L inhibited the growth of *Legionella* spp. (States et al. 1985; Serrano-Suárez et al. 2013; Rakić & Štambuk-Giljanović, 2016) (**Table 1.1**).

Iron is required for the growth of *Legionella* in the laboratory. Buffered Charcoal Yeast Extract (BYCE) Agar is supplemented with ferric pyrophosphate, although ferric chloride, ferric nitrate and ferrous sulphate may also be used. Iron acts as a cofactor for enzymes and is required for *Legionella* infection and replication in mammalian host cells (Cianciotto, 2007). During the stationary phase of *Legionella* growth, iron catalyses the formation of the homogentisic acid melanin, which is a brownish pigment (unpublished results). During the acquisition of iron, *L. pneumophila* are known to produce the siderophore legiobactin and there is evidence that a second siderophore may also be produced (Cianciotto, 2007). It has been established that ferrous/ferric iron transport occurring at low iron concentrations is mediated by the *feoB* gene, and the siderophore legiobactin. There is further evidence suggesting that the cytochrome c maturation locus also promotes iron assimilation and plays a role during the intracellular infection process (Viswanathan et al. 2002; Cianciotto, 2007).

Table 1.1 The effects of metals detected in water distribution systems in buildings on the growth of *Legionella* spp.

Legionella species	Environment (Country)	Geometric Mean			Reference
		Metals Enhancing Growth	**Metals not Affecting Growth**	**Toxic Metal Concentrations**	
Legionella pneumophila	Plumbing system of hospital and institutional hot-water tanks (Pittsburgh, Pennsylvania)	Fe (25–50 mg/L)	Mn (0.646 mg/L); Al (1.98 mg/L); Zn (0.924 mg/L); Cd (0.004 mg/L); Cu (0.469 mg/L); Cr (0.004 mg/L); Pb (0.047 mg/L); Ag (<0.001 mg/L); Ba (0.082 mg/L); Na (15.20 mg/L); K (2.29 mg/L); As (0.003 mg/L); Se (0.001 mg/L) Ni (0.019 mg/L); Co (0.008 mg/L)	Fe (50–75 mg/L)	States et al. (1985)
	Metal-supplemented tap water (Pittsburgh, Pennsylvania)	Fe (0.5, 1.0 mg/L); Zn (0.5, 1.0 mg/L); K (1, 10, 100 mg/L)	ND*	Fe (10, 100 mg/L); Zn (10, 100 mg/L)	
	Hotels, homes for the elderly and disabled (Dalmatian County of Croatia)	Fe (0.08 mg/L), Mn (8.37 mg/L)	Zn (0.17 mg/L); Cu (0.01 mg/L)	ND*	Rakić et al. (2012)
	Hot water recirculation systems in hotels and nursing homes	Fe (>0.095 mg/L)	Zn (0.391 mg/L)	Cu (>0.76 mg/L)	Serrano-Suárez et al. (2013)
Legionella spp.	Hotels, homes for the elderly and disabled (Dalmatian County of Croatia)	median Fe (>0.120 mg/L), Zn (>0.15 mg/L)	median Mn (0.01–0.1mg/L)	median Cu (0.010–0.020 mg/L)	Rakić and Štambuk-Giljanović, (2016)
	Public and private hospitals, 88 private homes (Modena, Italy)	Mn (>6 µg/L);	Zn (>375 µg/L); Fe (>42 µg/L)	Cu (>50 µg/L)	Bargellini et al. (2011)

*ND - Not Determined

Zinc is a trace element that is essential for proliferation in *Legionella* and other bacteria, as it is required for enzymes, other proteins and nucleic acid metabolism (Makarova et al. 2001; Hantke, 2005). Data regarding the involvement of manganese in *Legionella* virulence and growth are lacking, although manganese is essential for the growth and pathogenicity of other bacteria (Kehres & Maguire, 2003; Papp-Wallace & Maguire, 2006; Arirachakaran et al. 2007). The fact that copper at certain concentrations inhibits the growth of *Legionella* in water samples is not surprising as previous studies have indicated that copper is a well-known anti-infective element (Gordon et al. 1994) and methods which use copper-silver injection have previously been used to control *Legionella* in water samples (Stout & Yu, 2003).

Although numerous studies report on the significance of utilising the HPC technique to predict *Legionella* contamination, the relationship between heterotrophic bacteria and the colonisation of *Legionella* has only recently been investigated (Kusnetsov et al. 2003; Edagawa et al. 2008; Bargellini et al. 2011). The HPC has previously been utilised to monitor the development of biofilms in drinking and hot water distribution systems (Bagh et al. 2004; Moritz et al. 2010). It was then demonstrated that these biofilms may provide the habitat for the interaction between *Legionella* and protozoa, as *Legionella* require the presence of other heterotrophic bacteria, including *Pseudomonas* spp., to establish the biofilm before *Legionella* colonisation of a surface occurs (Borella et al. 2005; Lau & Ashbolt, 2009; Huang & Hsu, 2010).

1.5. Free-living amoebae (FLA)

The FLA are a polyphyletic group of amoebae found ubiquitously in soil and water (Rodríguez-Zaragoza, 1994). The principal genera of this group include *Acanthamoeba*, *Protacanthamoeba*, *Hartmannella*, *Naegleria* and *Vahlkampfia* (Hoffmann & Michel, 2001; Thomas et al. 2006; Valster et al. 2009; Magnet et al. 2015). During the 1900s, Vahlkampf (1905), Nägler (1908) and Hartmann (1912) identified and described the so-called limax, or slug-like amoebae. Sir Aldo Castellani (Castellani, 1930) isolated a FLA growing in a yeast culture of *Cryptococcus pararoseus* and this amoeba was initially named *Hartmannella castellanii* but was later re-classified as *Acanthamoeba castellanii* (Douglas, 1930). As FLA feed mainly by phagocytosing bacteria within biofilms, these amoebae are reported to harbour pathogenic bacteria. Among the pathogenic bacteria are the causal agents of pneumonia, including *Legionella*, *Chlamydiae* and *Mycobacterium*. Some of these bacteria merely persist and survive once inside the FLA host cell, while others such as *L. pneumophila* are able to multiply. Collectively, these bacteria comprise the amoebae-resisting bacteria (ARB) and develop transitory associations with FLA (Greub & Raoult, 2004). The ARB resistant to amoebae phagocytosis may also be resistant to macrophage phagocytosis, as the phagocytosis process in macrophages and amoebae is similar.

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Amoebae have two distinct stages of development commonly known as the trophozoite and the cyst. The latter is a dormant stage surrounded by two external layers and these layers enable amoebae to survive adverse treatments such as chlorination and temperatures between -20°C and +42°C (Greub & Raoult, 2004). Some FLA species are also pathogenic. Examples of these include *Naegleria* spp., *Acanthamoeba* spp., and *Balamuthia* and can cause diseases such as encephalitis in humans (Schuster & Visvesvara, 2004).

The ensuing review will focus on literature relevant to *Acanthamoeba* spp., *Naegleria* spp. and *Hartmannella* spp. as these FLA are most frequently isolated from the environment and act as hosts for *Legionella* spp. (Declerck et al. 2005; Visvesvara et al. 2007). In particular, as the relationship between *Acanthamoeba* spp. and *Legionella* spp. was thoroughly investigated during the course of this study, available published information regarding *Acanthamoeba* spp. is comprehensively reviewed.

1.5.1. *Acanthamoeba* spp.

Three genera occur in the family Acanthamoebidae viz. *Acanthamoeba* and *Protacanthamoeba* (Page, 1988) and more recently, *Luapeleamoeba* (Shadwick et al. 2016). There are 20 different species within the *Acanthamoeba* genus, and these include *A. castellanii, A. lugdunensis, A. rhysodes, A. divionensis, A. polyphaga, A. mauritaniensis, A. quina* and *A. triangularis*, amongst others (De Jonckheere, 1983; Khan, 2008).

The trophozoites and cysts of Acanthamoebidae are characterised by a large nucleus that encompasses a centrally located distinct nucleolus. The various divisions of the nuclei of the small amoebae have been described by Pussard (1973). They are the promitotic (found in *Naegleria* and *Tetramitus*) and mesomitotic (found in *Hartmannella*) or metamitotic (found in *Acanthamoeba*) patterns. During promitosis the nucleolus and nuclear envelope are present throughout karyokinesis whereas during mesomitotis the nuclear envelope is only present until the commencement of the anaphase. Metamitosis is similar to the process which occurs in higher animals where the nuclear envelope degrades early, prior to prophase or metaphase, and the nucleolus then disintegrates (Schuster, 1979).

During the trophozoite stage, the amoeba is motile and able to feed. However, the cyst is surrounded and protected by a double wall structure that confers both resistance and dormancy. The outer wall of the cyst, known as the ectocyst, is wrinkled and proteinaceous, while the inner wall, the endocyst, is either stellate, polygonal, round or oval in shape and contains cellulose (Page, 1967; 1988b). Based on the morphology of their cysts, generally > 18 μm in diameter, A*canthamoeba* spp. have been divided into three morphological groups (Pussard & Pons, 1977). Group I is comprised of four non-pathogenic species, wherein the

cysts are characterised by a star-like endocyst enclosed by a round ectocyst. Group II consists of ten species including *A. castellanii,* characterised by a thick or thin ectocyst that is frequently wrinkled or mamillated. The endocyst may be stellate, polygonal, triangular, round or oval and usually there is an absence of well-developed arms or rays. The *Acanthamoeba* cysts belonging to group III are characterised by a round endocyst surrounded by a thin rippled or unrippled ectocyst. A number of studies have indicated that in the cyst stage *Acanthamoeba* spp. are resistant to extreme physical and chemical conditions and various harsh treatments. These treatments include a pH of 2.0, freezing, freeze-thawing cycles, γ- (250 rads) and UV- irradiation (800 mJ/cm^2), moist heat (60°C with a contact time of 60 minutes), prolonged storage at room temperature for 24 months or 24 years at 4°C in water, and exposure to heavy metals and polychlorinated biphenyls (PCBs) (Sawyer et al. 1982; Biddick et al. 1984; Kilvington, 1989; Mazur et al. 1995; Aksozek et al. 2002).

Acanthamoeba spp. ingest bacteria and the organism has been isolated from a number of habitats. These include: soil, fresh water ponds, pools, lakes, brackish water, seawater, dust, heating and ventilation systems, air-conditioning filters and medical equipment including gastric wash tubing, dental irrigation units, used eye contact lens solutions and other paraphernalia, vegetables, cell cultures and human and animal tissues (Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004; Khan, 2006; Visvesvara et al. 2007; Caumo et al. 2009; Costa et al. 2010; Ahmed et al. 2014). Sakamoto et al. (2009) also identified *Acanthamoeba* (utilising PCR assays) in rainwater puddles found on road surfaces.

Acanthamoeba spp. have been isolated from numerous habitats in South Africa. For example, in one of the first cases reporting on the identification of limax amoebae, Lastovica (1980) isolated over 240 strains of *Naegleria*, *Acanthamoeba* and other slug-like amoebae. The study was four years in duration and samples were collected from a variety of habitats in both South Africa and South West Africa (now known as Namibia). Moreover, the author reported that seven potentially pathogenic *Naegleria fowleri* strains and one potentially pathogenic strain of *Acanthamoeba* were isolated from the Eerste River located in the Western Cape, South Africa (Lastovica, 1980). In other studies, carried out in South Africa, *A. mauritaniensis* has been isolated from; the corneas of patients with *Acanthamoeba* keratitis (East London, Eastern Cape), sewage sludge (Northern Sewage Works, Gauteng Province), contact lenses (Johannesburg, Gauteng Province) and fluid from contact lens cases (Pretoria, Gauteng Province) (Schroeder et al. 2001; Ledee et al. 2003; Niszl, 2011).

1.5.1.1. *Acanthamoeba* associated with disease

Acanthamoeba spp. were described by Khan (2009) as opportunistic, incidental or accidental protozoan pathogens known to have severe negative human health implications. These species can infect other animals including gorillas, monkeys, dogs, ovines, bovines, horses and kangaroos as well as birds, reptiles, amphibians and fish. In humans, infections caused by these protozoa include keratitis, an infection of the eye that may occur due to contact lens use or to cutaneous infections. Furthermore, a fatal brain infection or granulomatous amoebic encephalitis (GAE), an infection of the skin frequently associated with immunocompromised individuals, has been described (Khan, 2003; 2006; Qvarnstrom et al. 2013). As indicated by Kilic et al. (2004) the precise mechanisms of pathogenesis of *Acanthamoeba* infections are not understood. For this reason, despite advancements in antimicrobial chemotherapy and supportive care, *Acanthamoeba* infections have remained problematic and infections continue to increase in frequency (Khan, 2003; Marciano-Cabral & Cabral, 2003). To date, the recommended treatment for *Acanthamoeba* encephalitis is a combination of ketoconazole, fluconazole, sulfadiazine, pentamidine isethionate, rifampicin, amphotericin B. However, the mortality rate remains high at 90% (Trabelsi et al. 2012).

Both pathogenic and non-pathogenic species occur in *Acanthamoeba* and differentiating between the two is imperative for clinic diagnosis. Thus far, studies have identified 18 different genotypes, namely T1 – T18 (Reyes-Batlle et al. 2014). However, establishing the pathogenicity of each particular genotype is still under investigation (Kilic et al. 2004; Tawfeek et al. 2016). Thus far, 95% of the keratitis-causing *Acanthamoeba* isolates occur within the T4 genotype and for this reason, it is hypothesised that pathogenicity may be limited to closely related genotypes (Stothard et al. 1998; Walochnik et al. 2000; Khan et al. 2002; Kilic et al. 2004). It is well established that *Acanthamoeba* cause corneal infections in some individuals wearing contact lenses. Of interest is a study conducted by Donzis et al. (1989) which demonstrated that 75% of infected lens wearers used hydrogen peroxide to disinfect their contact lenses and that many of the microorganisms isolated from the lens fluid exhibited catalase activity.

1.5.2. *Naegleria* spp.

The protozoan genus *Naegleria* belongs to the family Vahlkampfiidae and the organism is a free-living amoeboflagellate detected in lakes, fresh water, swimming pools, aquaria, sewage, irrigation canals, ponds, hot springs, thermally polluted streams and rivers (Marciano-Cabral et al. 2003; Maclean et al. 2004; Schuster & Visvesvara, 2004; Trabelsi et al. 2012; Tung et al. 2013). The life cycle of *Naegleria* spp. occurs in three stages, namely the trophozoite (10-25 μm), the resistant cyst (8–20 μm) and a transitory pear-shaped flagellate stage

(10-16 µm). During the trophozoite stage, the amoeba is able to feed and replicate asexually and constantly changes in size and shape (Trabelsi et al. 2012). During the flagellate stage, *Naegleria* forms two flagella at the broad end of the cell when placed in a nutrient-poor environment. However, the microorganism is able to revert to the trophozoite stage when conditions are favourable (Trabelsi et al. 2012). There are approximately 30 species of *Naegleria* but only *N. fowleri* has been associated with a rare but fatal human disease, primary amoeboic meningoencephalitis (PAM), an infection of the central nervous system (CNS) (Trabelsi et al. 2012). Additionally, *N. fowleri* is thermophilic and has been shown to survive and grow at a temperature of 45°C (Trabelsi et al. 2012).

1.5.3. *Hartmannella* spp.

Hartmannella spp. occur in the family Hartmannellidae and are widespread in nature, where specifically *Hartmannella vermiformis* has been isolated from soil, freshwater, air and numerous engineered water systems (Page, 1974; Walker et al. 1986; Rohr et al. 1998). However, because of its substantial differentiation from all other *Hartmannella* spp., *Hartmannella vermiformis* is now re-classified as *Vermamoeba vermiformis* (Smirnov et al. 2011). *Hartmannella* spp. including *V. vermiformis* also have two life stages, namely the trophozoite where the cell remains actively feeding and multiplying, and the cyst, where the cells become inactive and dormant (Page, 1988). *Vermamoeba vermiformis* has been directly and indirectly associated with human disease as this organism was isolated from the cerebrospinal fluid of a patient with meningoencephalitis and bronchopneumonia (Centeno et al., 1996). It is also hypothesised that *V. vermiformis* can cause keratitis (Kennedy et al. 1995; De Jonckheere & Brown, 1998). However, the major importance to public health by this organism is its ability to act as a host for *L. pneumophila* (Schwake et al. 2015).

1.6. Methods used for the detection of *Legionella* spp. and FLAs in water samples

1.6.1. Culture- based methods

Although the isolation of *Legionella* spp. from the environment is difficult, to date the culturing of *Legionella* spp. from environmental and clinical samples remains the gold standard for detection (Phin et al. 2014). Molecular analyses using PCR and qPCR methods are however proving to be more rapid and reliable for the detection of *L. pneumophila* and non-*pneumophila Legionella* spp., including *L. longbeachae* (Phin et al. 2014; Benitez & Winchell, 2016). The isolation of *Legionella* spp. from certain environmental samples often requires pre-treatment prior to culturing the sample onto a growth medium. In the case of potable water, microbial contaminants present in a volume of 1 L are concentrated by the use of filtration or centrifugation. These procedures ensure that sufficient microbial flora are present before

culturing (Centers for Disease Control and Prevention, 2005; Fiume et al. 2005). If the water source being sampled was treated initially with chlorine, 0.5 mL of 0.1 N sodium thiosulfate per 1 L is added to the sample to neutralize residual disinfectants (Centers for Disease Control and Prevention, 2005; Serrano-Suárez et al. 2013). Samples from non-potable water sources generally require a form of heat or acid treatment to reduce the presence of non-*Legionella* organisms present in the sample. Heat treatment at 50°C for 30 minutes is generally applied as *Legionella* spp. are thermally tolerant up to 63°C (Leoni & Legnani, 2001; Whiley & Taylor, 2016). As the FLA generally harbour *Legionella* in environmental samples, this pre-heat treatment is also thought to kill the amoebae and other bacteria, thus enhancing the isolation of *Legionella* (Dennis et al. 1984; Dietersdorfer et al. 2016). In the case of acid treatment, once the sample has been concentrated, an HCl–KCl buffer (pH 2.2) is added and the sample is incubated for 15 minutes (Bopp et al. 1981; Centers for Disease Control and Prevention, 2005).

The culturing of *Legionella* spp. is generally performed by spread plating an inoculum onto buffered charcoal yeast extract (BCYEα). Buffered charcoal yeast extract (BCYEα) is comprised of *Legionella* charcoal-yeast extract (CYE) agar base supplemented with *Legionella* BCYE growth supplement *N*-(2-acetamido)-2-aminothane-sulfonic acid (ACES) buffer/potassium hydroxide: 1.0 g/L, ferric pyrophosphate (0.025 g/L), alpha-ketoglutarate (0.10 g/L) and *L*-cysteine HCL (0.04 g/L). Charcoal-yeast extract (CYE) agar was first described by Feeley et al. (1979) when Feeley Gorman (F-G) Agar (Feeley et al. 1978) was modified to include yeast extract as a replacement for acid hydrolysed casein as a source of nitrogen, carbon and vitamins. As hydrogen peroxide is toxic to *Legionella* spp., activated charcoal was later also included in the medium to decompose hydrogen peroxide and superoxide radicals, inhibit light-accelerated autooxidation of cysteine and to modify surface tension (Hoffman et al. 1983). The ACES buffer maintains the optimal pH for *Legionella* growth. Ferric pyrophosphate and *L*-cysteine are also included in the medium, as *Legionella* spp. requires iron and the amino acid for growth. In addition, antibiotics may be added to the medium to prevent the growth of non-*Legionella* microorganisms. Using this method, the suspension containing the culture is inoculated by means of the spread plate technique onto BYCE agar supplemented with a *Legionella* selective supplement [ammonia free glycine (1.5 g/L), vancomycin (0.5 mg/L), polymyxin B SO$_4$ (40 000 IU) and cycloheximide (40.0 mg/L) (GVBC)] (Centers for Disease Control and Prevention, 2005). Cultures are incubated under microaerophilic conditions in a candle jar or under a 2.5% CO$_2$ atmosphere at 35°C to further reduce the growth of non-*Legionella* organisms (Centers for Disease Control and Prevention, 2005). There are however many disadvantages associated with culture-based methods for the routine screening of *Legionella* spp. These include the need for special reagents, technical skills and the pre-treatment of the environmental sample, which is often required. Furthermore, the method is

time-consuming and to obtain results requires a minimum of 10 days' incubation (Bartie et al. 2001; Fittipaldi et al. 2012).

Free-living amoebae have particular nutritional requirements although various media may be utilised to isolate the amoebae from the environment (Niszl, 2011). Two types of cultivation methods, namely monoxenic and axenic culture are commonly used for artificial culture media. In the case of monoxenic culture of FLAs, an inoculum is generally cultured on Page amoeba saline (PAS) [NaCl (0.12 g/L), $MgSO_4.7H_2O$ (0.004 g/L), $CaCl_2.2H_2O$ (0.004 g/L), Na_2HPO_4 (0.142 g/L), KH_2PO_4 (0.136 g/L)] containing 1.5% agar, also referred to as non-nutrient agar (NNA). The NNA is seeded with heat-killed or live (depending on downstream applications) *E. coli* as a food source for the amoebae (Page, 1976; Schuster & Visvesvara, 2004). When axenic culture is the method of choice, a nutrient-enriched broth medium is used without the addition of bacteria. However, the medium may be supplemented with ampicillin (Niszl, 2011). Although culture methods are cost-effective, the use of molecular methods for the identification of *Acanthamoeba* spp*., Naegleria* spp. and *Hartmannella* spp. and others have been implemented by a number of researchers, as the use of morphological criteria for identification has proven time-consuming and laborious (Pussard & Pons, 1977; Visvesvara, 1991).

1.6.2. Molecular based methods

Multiple assays for the detection of *Legionella* spp., *Acanthamoeba* spp*., Naegleria* spp. and *Hartmannella* spp. that employ the use of different target genes and qPCR, have been published (**Table 1.2**). Conventional PCR methods allow for the detection of specific organisms within approximately seven hours by the amplification of a particular DNA sequence. With the development of qPCR, which relies on the general principle of conventional PCR, the DNA of a specific organism is amplified and quantified in real time after the completion of each cycle. Generally, there are two methods used to quantify DNA. Firstly those that incorporate fluorescent dyes that intercalate with double-stranded DNA and secondly, those that rely on DNA oligonucleotide probes that fluoresce when hybridised with complementary DNA (Fittipaldi et al. 2012).

Table 1.2. Studies that have used culturing or PCR-based methods or a combination of both for the detection of *Legionella* spp., *Acanthamoeba* spp., *Hartmannella* spp. and *Naegleria* spp. in water samples.

Organism	DNA Extraction Method	Conventional test method		Conventional PCR		Quantitative PCR			Reference
		Method	No. of positive samples	Target (Primers)	No. of positive samples	Chemistry	Target (Primers)	No. of positive samples	
Legionella spp.	UltracleanTM Soil DNA isolation kit (Mo Bio Laboratories, Belgium)	*ND	*N/A	*ND	*N/A	Taqman chemistry	23S-5S spacer region (LegF and LegR)	38/45	Declerck et al. (2007)
	MagNA Pure LC equipped with MagNA Pure LC DNA isolation kit III (Roche, Nutley, NJ, USA)	*ND	*N/A	*ND	*N/A	SYBR Green	23S-5S rRNA	77/140	Ji et al. (2014)
	Fast DNA® Kit (MP Biomedicals, Illkkrich, France)	Acid shock, culture method (GVPC)	4/70	(L858 and L448)	41/70	*ND	*N/A	*N/A	Magnet et al. (2015)
	High Pure PCR template preparation kit; Roche Diagnostics, Meylan, France	Culture method (GVPC)	105/256	16S rRNA gene (16S1-A and 16S2-A)	229/256	Dual FRET hybridization probes	16S rRNA gene (16S1-A and 16S2-A)	182/256	Joly et al. (2006)
Legionella pneumophila	UltracleanTM Soil DNA isolation kit (Mo Bio Laboratories, Belgium)	*ND	*N/A	*ND	*N/A	Taqman chemistry	*mip* gene (LPQF and LPQR)	34/45	Declerck et al. (2007)

*ND – Not Determined; *N/A – Not Applicable

Table 1.2. (Continued). Studies that have used culturing or PCR-based methods or a combination of both for the detection of *Legionella* spp., *Acanthamoeba* spp., *Hartmannella* spp. and *Naegleria* spp. in water samples.

Organism	DNA Extraction Method	Conventional test method		Conventional PCR		Quantitative PCR			Reference
		Method	No. of positive samples	Target (Primers)	No. of positive samples	Chemistry	Target (Primers)	No. of positive samples	
Legionella pneumophila	MagNA Pure LC equipped with MagNA Pure LC DNA isolation kit III (Roche, Nutley, NJ, USA)	*ND	*N/A	*Mip, rpoB, dotA,*	12/140	*ND	*N/A	*N/A	Ji et al. (2014)
	Three freeze-thaw cycles (−75°C for 10 min and 94°C for 10 min)	Immunomagnetic separation (anti-*Legionella pneumophila* polyclonal antibody)	35/60	*DotA* gene (DotA F and DotA R)	8/60	Taqman chemistry	*DotA* gene (DotA F and DotA R)	35/60	Yáñez et al. (2005)
Acanthamoeba spp.	UltracleanTM Soil DNA isolation kit (Mo Bio Laboratories, Belgium)	*ND	*N/A	JDP primers	32/45	*ND	*N/A	*N/A	Declerck et al. (2007)
	MagNA Pure LC equipped with MagNA Pure LC DNA isolation kit III (Roche, Nutley, NJ, USA)	Non- Nutrient Agar (NNA)	55 (All FLA)	*ND	*N/A	Taqman chemistry	18S rRNA	37/140	Ji et al. (2014)
	Fast DNA® Kit (MP Biomedicals, Illkkrich, France)	Non- Nutrient Agar (NNA)	85.7/70	18S rDNA (AcantF900 and AcantR1100)	61/70	*ND	*N/A	*N/A	Magnet et al. (2015)

*ND – Not Determined; *N/A – Not Applicable

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Table 1.2. (Continued). Studies that have used culturing or PCR-based methods or a combination of both for the detection of *Legionella* spp., *Acanthamoeba* spp., *Hartmannella* spp. and *Naegleria* spp. in water samples.

Organism	DNA Extraction Method	Conventional test method		Conventional PCR		Quantitative PCR			Reference
		Method	No. of positive samples	Target (Primers)	No. of positive samples	Chemistry	Target (Primers)	No. of positive samples	
Naegleria spp.	UltracleanTM Soil DNA isolation kit (Mo Bio Laboratories, Belgium)	*ND	*N/A	ITS primers (400–450 bp)	38/45	*ND	*N/A	*N/A	Declerck et al. (2007)
	MagNA Pure LC equipped with MagNA Pure LC DNA isolation kit III (Roche, Nutley, NJ, USA)	Non- Nutrient Agar (NNA) agar plates seeded with killed *Escherichia coli*	55 (All FLA)	*ND	*N/A	Taqman chemistry	ITS	25/140	Ji et al. (2014)
Vermamoeba vermiformis	FastDNA spin kit for soil (BIO 101)	*ND	*N/A	*ND	*N/A	SYBR green	18S rRNA gene (Hv1227F and Hv1728R)	21/28	Kuiper et al. (2006)
	MagNA Pure LC equipped with MagNA Pure LC DNA isolation kit III (Roche, Nutley, NJ, USA)	Non- Nutrient Agar (NNA) agar plates seeded with killed *Escherichia coli*	55 (All FLA)	18S rRNA	22/140	*ND	*N/A	*N/A	Ji et al. (2014)

*ND – Not Determined; *N/A – Not Applicable

An example of a fluorescent dye is SYBR Green. This is a non-specific dye which binds to double stranded DNA in a sample, including to non-specific amplification products and primer dimers. A benefit of utilising SYBR Green rather than oligonucleotide probes is that the protocols require less optimisation. In addition, a melt curve analysis of PCR products is included for SYBR green protocols and for each sample analysed, which generates a specific profile for the variation of the fluorescence rates over time as a function of temperature. Using this, the success of the PCR assay is monitored. Furthermore, SYBR Green master mixes for qPCR are cost-effective, simple to use and results are available within approximately seven hours (Fittipaldi et al. 2012; Lombard, 2016).

As the FLAs such as *Acanthamoeba* spp., *Naegleria* spp. and *Hartmannella* spp. in the environment can harbour potentially pathogenic bacteria including *Legionella*, numerous studies have employed the use of PCR and/or qPCR for the detection of these organisms (**Table 1.2**). In particular, *Acanthamoeba* has been extensively investigated over the last two decades (Khan, 2006) and many research groups have indicated that PCR methods are sensitive enough for the detection of *Acanthamoeba* in both clinical and environmental samples (Vodkin et al. 1992; Kong & Chung, 1996; Howe et al. 1997; Lehmann et al. 1998; Dyková et al. 1999; Mathers et al. 2000). With the use of PCR-based methods, 1 - 10 trophozoites of *Acanthamoeba* spp. (Schroeder et al. 2001), 5 - 75 cells/L of *H. vermiformis* (Kuiper et al. 2006) and less than 1 *Naegleria* cell are detected per total volume of sample processed (Qvarnstrom et al. 2006).

Numerous studies have compared the use of culture-based techniques and conventional PCR, with the use of qPCR for the enumeration of *Legionella* spp., *Acanthamoeba* spp., *Naegleria* spp. and *Hartmannella* spp. in water samples (**Table 1.2**). For example, Magnet et al. (2015) collected 70 water samples from three drinking water treatment plants, three wastewater treatment plants and five natural pools in Spain. *Legionella* spp. were detected in 58.6% (41/70) of the water samples using conventional PCR. In contrast only 5.7% (4/70) were detected when using agar culturing methods (**Table 1.2**). However, the sensitivity of detection of *Legionella* spp. was increased by co-culturing with *Acanthamoeba*, and *Legionella* spp. were found in 75.7% (53/70) of the water samples screened. Furthermore, Declerck et al. (2007) sampled biofilms from eight anthropogenic and 37 natural aquatic environments between June and August 2005. Real-time PCR methods were used, rather than culture-based methods. This was because in preliminary sampling sessions, when culture-based methods were compared with real-time PCR, no *Legionella* were isolated using conventional culture-based techniques (**Table 1.2**). However, the use of molecular methods resulted in the detection of both *Legionella* spp. and *L. pneumophila* in all of the anthropogenic aquatic systems. *Legionella* spp.

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were also detected in 81% of the natural floating biofilm samples, 70% of which were positive for *L. pneumophila*. Overall, *Legionella* numbers recorded were in the range of 10^1-10^2 cells/cm^2 floating biofilm. *Naegleria* spp. (ITS primers) and *Acanthamoeba* spp. (JDP primers), were also present in 50–92% and 67–72%, respectively, of the floating biofilm samples (**Table 1.2**). Ji et al. (2014) used SYBR green chemistry and real-time PCR for the quantification of *Legionella* spp. in river water, water treatment plants and hot springs (**Table 1.2**). Overall, *Legionella* spp. were detected in 55% (77/140) of the samples and *L. pneumophila* was detected at a lower rate, with an overall detection of 8.6%. Among the FLA assessed of which *Vermamoeba* spp. *Acanthamoeba* spp. and *Naegleria* spp. were included, the authors suggested that *V. vermiformis* was most likely to coexist with *Legionella* spp.

Although the use of PCR and qPCR assays may be more sensitive and reliable for the detection of these microorganisms, there are disadvantages to their use. These include the presence of PCR inhibitors in the form of humic and fulvic acids and metals in environmental samples that can cause false negatives, and the need for trained personnel to perform the assays (Yáñez et al. 2005). However, caesium chloride density centrifugation methods can be used to improve general DNA purity (Abbaszadegan et al. 1993). In addition, the availability of commercial DNA extraction kits including the QIAampR DNA Stool Mini Kit, allow for the removal of DNA-damaging substances and PCR inhibitors during the extraction of DNA from environmental samples (Johnson et al. 2005; Lombard, 2016). Some authors have also suggested the adaption of DNA extraction protocols to include a Taqman® exogenous internal positive control (ICP) and the addition of bovine serum albumin (BSA) to the PCR mixture to ensure that the amplification is not repressed by PCR inhibitors (Behets et al. 2007; Declerck et al. 2007).

1.7. Associations of *Legionella* with protozoa

Amoebae, like many protists, graze mainly on bacteria through phagocytosis and several bacterial genera are able to survive after ingestion. As indicated in **Table 1.3**, numerous studies have reported that various species of *Legionella* remain viable and colonise a variety of environmental protozoa. Hosts include amoebae such as *Acanthamoeba* (Rowbotham, 1980; 1986; Tyndall & Domingue, 1982; Fields et al. 1989; Breiman et al. 1990)*, Hartmannella* (Rowbotham, 1986; Fields et al. 1989; Breiman et al. 1990)*, Naegleria* (Rowbotham, 1980; Tyndall & Domingue, 1982; Newsome et al. 1985), *Echinamoeba* (Fields et al. 1989)*, Vahlkampfia* (Rowbotham, 1986; Breiman et al. 1990) and ciliates such as *Tetrahymena* spp. (Fields et al. 1984; Kikuhara et al. 1994).

Table 1.3. Intracellular *Legionella* pathogens occurring in free-living amoebae (FLA)

Free-Living Amoebae	*Legionella* species	Reference
Acanthamoeba castellani	*Legionella pneumophila, Legionella gormanii, Legionella micdadei, Legionella steigerwaltii, Legionella longbeachae, Legionella dumoffii, Legionella erythra*	Rowbotham (1980); Neumeister et al. (2000); Storey et al. (2004)
Acanthamoeba culbertsoni	*Legionella pneumophila, Legionella dumoffii*	Fields et al. (1989); Ohnishi et al. (2004)
Acanthamoeba hatchetti	*Legionella pneumophila*	Breiman et al. (1990)
Acanthamoeba polyphaga	*Legionella pneumophila, Legionella drancourtii, Legionella micdadei*	Rowbotham (1980, 1986); La Scola et al. (2004)
Acanthamoeba palestinensis	*Legionella pneumophila*	Rowbotham (1986)
Acanthamoeba royreba	*Legionella pneumophila*	Tyndall and Domingue (1982); Zeybek and Binay (2014)
Amoeba proteus strain x D	*Legionella jeonii*	Park et al. (2004)
Comandonia operculata	*Legionella pneumophila*	Breiman et al. (1990)
Echinamoeba exudans	*Legionella pneumophila*	Fields et al. (1989)
Filamoeba nolandi	*Legionella pneumophila*	Breiman et al. (1990)
Hartmannella spp.	*Legionella pneumophila*	Fields et al. (1989)
Hartmannella cantabrigiensis	*Legionella pneumophila*	Rowbotham (1986); Breiman et al. (1990)
Hartmannella vermiformis	*Legionella pneumophila, Legionella micdadei*	Rowbotham (1986); Fields et al. (1989); Breiman et al. (1990)
Naegleria fowleri	*Legionella pneumophila*	Newsome et al. (1985)
Naegleria gruberi	*Legionella pneumophila*	Rowbotham (1980)
Naegleria jadini	*Legionella pneumophila*	Rowbotham (1980)
Naegleria lovaniensis	*Legionella pneumophila*	Tyndall and Domingue (1982)
Paratetramitus jugosis	*Legionella pneumophila*	Breiman et al. (1990)
Vahlkampfia spp.	*Legionella pneumophila*	Breiman et al. (1990)
Vahlkampfia jugosa	*Legionella pneumophila*	Rowbotham (1986)
Vahlkampfia ustiana	*Legionella pneumophila*	Breiman et al. (1990)

The co-culturing of *L. erythra* and *L. pneumophila* with *A. castellanii* and *Acanthamoeba* IA (an environmental thermotolerant Acanthamoebae isolate) increased the resistance of the *Legionella* spp. to thermal treatment by 10- (at 40–50°C) to 100- (at 60–80°C) fold after being subjected to heat treatment (**Table 1.3**) (Storey et al. 2004). Neumeister et al. (2000) also indicated that intracellular replication of *L. gormanii, L. micdadei, L. steigerwaltii, L. longbeachae* and *L. dumoffii* within human monocytic leukaemia cells (MM6) was greatly enhanced after being co-cultured with *A. castellanii* (**Table 1.3**).

In 2014, Ji et al. demonstrated that *Vermamoeba* (*Hartmannella*) *vermiformis* probably coexists with *Legionella* spp. in surface water (river), intake areas of drinking water treatment plants and recreational hot spring complexes. In addition, *Legionella* spp. and amoebae have been detected simultaneously in rainwater samples (Lye, 1992; 2002). Moreover, *L. drancourtii* and *L. jeonii* are obligate intracellular pathogens of *A. polyphaga* and *Amoeba proteus* strain x D, respectively, and cannot be cultivated axenically in culture media (**Table 1.3**) (La Scola et al. 2004). Other potentially pathogenic bacteria ingested by *A. castellanii* include *Salmonella typhimurium, Yersinia enterocolitica, Shigella sonnei* and *E. coli* (King et al. 1988).

The discovery of a parasitic relationship between amoebae and *Legionella* spp. led to the hypothesis that the ecology and pathogenesis of *Legionella* spp. are intimately linked (Buchrieser & Hilbi, 2013). It was proposed therefore that the adaptation strategies evolved by *Legionella* to infect amoebae have enabled the bacteria to infect and replicate in mammalian cells, specifically in alveolar lung macrophages (Greub & Raoult, 2004; Molmeret et al. 2005; Brüggemann et al. 2006). *Legionella* spp. enter eukaryotic hosts by means of phagocytosis and they inhibit phagosome maturation. To achieve this, *Legionella* spp. are dependent on innate secretion systems to establish a replicative vacuole within the amoeba cells.

1.8. Secretion systems of bacteria

Secretion systems in bacteria, including Gram-positive, Gram-negative, cell wall-less bacteria and some archaea, have evolved to aid in their survival and virulence (Alvarez-Martinez & Christie, 2009). Cellular DNA, macromolecules such as monomeric proteins, multimeric toxins and DNA-protein complexes are transferred across the cell envelope from the interior of the bacterial cell to the external milieu under the control of various secretory pathways (i.e. the series of steps a cell uses to transfer effector molecules). Understanding the delivery of toxins or effector proteins produced by bacteria to the host and the mechanisms of horizontal spread of antibiotic resistance among bacteria is of clinical significance (Waksman & Orlova, 2014).

To date, secretion systems have been classified into seven major classes (**Table 1.4**) of which the type IV secretion systems are the most functionally versatile (Cornelis & Van Gijsegem, 2000; Omori & Idei, 2003; Filloux, 2004; Henderson et al. 2004; Abdallah et al. 2007). All seven classes of secretion systems transport proteins (effector molecules) from the interior of the cell across the cell envelope and hence into the external environment by means of the Sec or Twin – arginine (Tat) pathways, or a combination of both. In other instances, the mechanism of translocation is unknown (**Table 1.4**).

Table 1.4. The division of secretion systems into the seven major classes in Gram-negative and Gram-positive bacteria.

Type of secretion pathway	Signal Required	Pathway utilised for translocation	No of proteins in system	Reference
I	No signal required	Sec pathway	Two/three	Kanonenberg et al. (2013)
II	N-terminal signal peptide	twin-arginine translocation system, Sec system	multi-protein complexes	Voulhoux et al. (2001)
III	No signal required	translocation mechanism unknown	multi-protein complexes	Perrett and Zhou (2011)
IV	No signal required	translocation mechanism unknown	multi-protein complexes	Lawley et al. (2003); Ding et al. (2003)
V	N-terminal signal peptide	Sec pathway and Tat machinery	Two/three	Newman and Stathopoulos (2004)
VI	N-terminal signal peptide	Sec pathway	Two/three	Filloux et al. (2008)
VII	No signal required	translocation mechanism unknown	multi-protein complexes	Houben et al. (2014)

The type IV secretion systems play a critical role in the pathogenicity of many important pathogens, including *Agrobacterium tumefaciens*, *Helicobacter pylori* and *L. pneumophila* (Nagai & Kubori, 2011). In particular, *L. pneumophila* expresses the type I, type II and type IV secretion systems and all the genes required for the Sec pathway, with the exception of *secG,* have been identified. However, the Sec pathway of *L. pneumophila* has not yet been fully characterised (De Buck et al. 2007). A Tat pathway was discovered in *L. pneumophila* and this appears to play a role in bacterial replication in macrophages, biofilm formation and motility (De Buck et al. 2004; De Buck et al. 2005). However, the contribution of the Tat pathway to the replication of *Legionella* within the amoebae appears to be more complex, where it was determined that one *L. pneumophila* strain required the Tat pathway and another strain did not

(De Buck et al. 2005; Rossier & Cianciotto, 2005). In addition, *L. pneumophila* encodes all of the components of a type I secretion system (Lss). Although the Lss may have a role in *Legionella* biology, it does not appear to be essential for host-pathogen interactions (Jacobi & Heuner, 2003) and has thus not been extensively studied. The type II secretion system (T2SS) known as the *Legionella* secretion pathway (Lsp) occurs in all strains of *L. pneumophila* from which the genomes have been sequenced (Rossier et al. 2004). The Lsp appears to be involved in the infection of *H. vermiformis, A. castellanii* and human macrophages (Rossier et al. 2004; Rossier et al. 2009). Furthermore, the T2SS appears to be essential for *L. pneumophila* survival at low temperatures, an important consideration in environmental persistence (Söderberg et al. 2008).

Type IV secretion systems produce a variety of substrates that include single proteins, protein complexes, DNA and nucleoprotein complexes. The type IV secretion systems are further divided into three functional groups (Waksman & Orlova, 2014). The first type IV secretion system functional group (type IVA) mediates conjugation, which plays a pivotal role in bacterial genome plasticity and diversity by allowing for the transfer of DNA from one bacterial cell to another. The second group mediates the translocation of proteins; these proteins range from being small effector proteins to large protein complexes. For example, the type IV secretion system is used by various Gram-negative bacteria such as *H. pylori*, *Brucella suis* and *L. pneumophila* to inject virulence proteins into mammalian host cells (Backert & Meyer, 2006; Ninio & Roy, 2007; De Jong et al. 2008) and for this reason this secretion system is sometimes referred to as the type IVB secretion system. The third group is involved in the mediation of DNA release and uptake and this mechanism is observed typically in *H. pylori* and *Neisseria gonorrhoeae* (Lederberg & Tatum, 1953). In an ATP-dependent process, nucleoprotein and protein complexes are secreted through a specific channel that traverses the cell envelope. Additionally, the type IV secretion systems are evolutionarily related (Lessl & Lanka, 1994) as the components and the processes of these secretion systems found in numerous bacteria have many common features (Christie & Cascales, 2005).

1.8.1. The type IVB Dot/Icm secretion system

The type IV secretion systems of bacteria are related to bacterial conjugation systems. They are divided into type IVA and type IVB subgroups of which the type IVB secretion system is closely related to conjugation systems of IncI plasmids (Nagai & Kubori, 2011). To date, the type IVB secretion system has been reported to be present in two other bacterial species, namely, *Coxiella burnetii* (zoonotic pathogen) and *Rickettsiella grylli* (arthropod pathogen) both of which carry a type IVB secretion system similar to that of *Legionella* (Nagai & Kubori, 2011).

Although the stoichiometry of the various components in a native fully assembled type IV system is unknown, the type IV secretion systems are all located internally along the bacterial cell envelope and these allow for the translocation of various substrates. Based on the nomenclature used for the *Agrobacterium tumefaciens* type IV secretion system, almost all the type IV secretion systems of Gram-negative bacteria consist of a minimum of 12 proteins named VirB1 to VirB11 and VirD4. As indicated in **Figure 1.2** [adopted from Waksman & Orlova, (2014)], these proteins span the envelope to form a transport apparatus. There are multiple copies of each of these proteins and the 12 components are organised as three major subcomplexes.
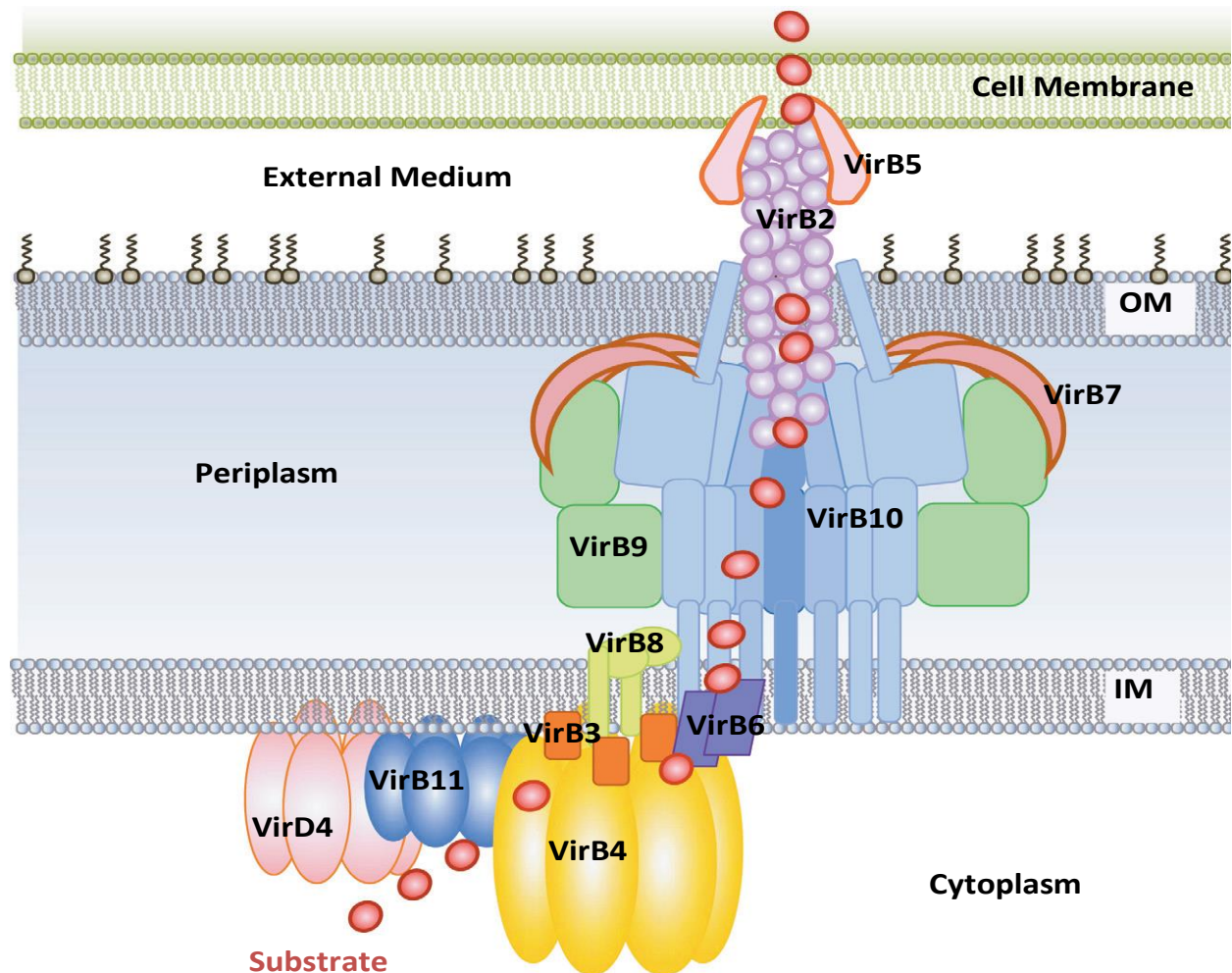
Figure 1.2. The three major subcomplexes of the transport apparatus of the type IV system of Gram-negative bacteria, related to bacterial conjugation systems [adopted from Waksman & Orlova, (2014)]. OM: Outer Membrane. IM: Inner Membrane.

One of these is a cytoplasmic-inner membrane subcomplex composed of three ATPases (VirB4, VirB11 and VirD4), the VirB3 and VirB6, and sections of the VirB8 and VirB10 proteins (**Figure 1.2**). The ATPases are responsible for powering the system assembly and translocation of the substrates (Gomis-Rüth et al. 2001; Savvides et al. 2003; Atmakuri et al.

2004; Hare et al. 2006; Walldén et al. 2012). The VirD4 homologues are termed "coupling proteins" and are crucial type IV secretion system ATPases involved in delivering the substrate to the translocation channel (Gomis-Rüth et al. 2001). A coupling protein, the DotL, is an inner membrane component of the Dot/Icm secretion system of *L. pneumophila.* This protein functions as a receptor for substrates and requires adaptor proteins for the secretion of a major class of substrates (Sutherland et al. 2012). The DotL protein is an analogue of VirD4 and TraB in *A. tumefaciens* and *E. coli*, respectively (Buscher et al. 2005).

The second major component is the large central core complex and is composed of three proteins known as VirB7, VirB9 and VirB10 (**Figure 1.2**). This complex serves as scaffolding for the remainder of the type IV secretion system components and forms a trans-membrane pore that spans the outer and inner membrane of Gram-negative bacteria. This complex therefore actively allows for the transfer of substrates across the bacterial cell envelope (Llosa et al. 2003; Christie & Cascales, 2005; Alvarez-Martinez & Christie, 2009; Jakubowski et al. 2009).

The third structural complex, formed by the VirB2 and VirB5 proteins, is an extracellular pilus that spans the outer membrane, thus permitting delivery of the substrate to the recipient cell. The overall organisation of these proteins is well conserved among the different type IV secretion systems. However, some variations in the composition have been noted (Alvarez-Martinez & Christie, 2009).

In the case of *L. pneumophila,* the establishment of a replicative niche, intracellular replication or macrophage killing, is dependent on the Dot/Icm type IVB secretion system. The genes encoding the system are divided independently into two groups: either *dot* (for defect in organelle trafficking) or *icm* (for intracellular multiplication). The Dot/Icm type IVB secretion system is encoded by 25 genes localised on two separate regions of the genome. Region I is comprised of seven genes (*icmV, W* and *X* and *dotA, B, C* and *D*).

Region II consists of 18 genes (*icmT, S, R, Q, P, O, N, M, L, K, E, G, C, D, J, B, F* and *H*) (Segal & Shuman, 1997; Andrews et al. 1998; Purcell & Shuman, 1998; Segal et al. 1998; Vogel et al. 1998; Zusman et al. 2004; Nagai & Kubori, 2011). These genes encode for;

(i)   a multiprotein apparatus for secretion where the Dot/Icm core complex has a minimum of five Dot/Icm lipoproteins - DotC, DotD, DotF, DotG and DotH - that function as major components (Vincent et al. 2006),

(ii) coupling ATPases (*IcmO/DotL*; *IcmP/DotM*; *IcmJ/DotN*) (Buscher et al. 2005) and

(iii) chaperone proteins for the recognition of substrates for the translocon (*IcmS*; *IcmW*; *LvgA*) (Bardill et al. 2005).

By expressing approximately 27 proteins, this apparatus connects the bacterial cytoplasm to the extracellular environment and in this manner an excess of 330 effector proteins of *L. pneumophila* are delivered into both protozoan and mammalian host cells (Nagai & Roy, 2003). Once these effectors have reached the cytosol of the host, the cell biology of the eukaryote is modulated to acquire nutrients, block microbial degradation, subvert host defences and allow the transmission of the pathogen to other hosts (Ensminger, 2016).

1.9. Phagocytosis by protozoa

The term "phagocytosis" was first described by the Russian biologist Ilya Ilyich Mechnikov in the 1880's, when he observed that the phagocytes of the larvae of starfish (*Hydra*) were able to attack foreign material (Metchnikoff, 1884). As defined by Brown and Neher (2012), the term phagocytosis describes a process occurring during physiological and pathological conditions where a cell recognises, engulfs and digests a target (dead or dying cells) that is ≥1 μm in size (Brown & Neher, 2012; Hochreiter-Hufford & Ravichandran, 2013). Phagocytosis is therefore a specialised form of endocytosis where phagosomes undergo sequential maturation similar to endosomes and ultimately fuse with degradative lysosomes.

During phagocytosis, most proteins involved in vesicle formation, motor recruitment and vesicle binding must be recruited from the cytosol and transported to the relevant organelles. In eukaryotic cells, donor organelles have compartments that form transport vesicles and during membrane trafficking the transport vesicles are able to move and fuse with the correct acceptor organelle. This process is mediated by a number of proteins belonging to three major categories. These include coat proteins that form transport vesicles, motor proteins that move vesicles to a particular area within the cell and protein complexes and long coiled-coil proteins involved in the tethering of the vesicles to the correct acceptor organelle (Munro, 2002).

As indicated in **Figure 1.3**, during phagocytosis biogenesis of the plasma membrane forms a phagosome that harbours an invading bacterium. The Rab families belong to the Ras superfamily of small guanosine triphosphatases (GTPases) (Brumell & Scidmore, 2007). After ingestion of the avirulent bacterium, the phagosome undergoes a series of endocytic developmental phases commencing with an early phagosome, under the control of Rab-5 (**Figure 1.3**) (Enari et al. 1998; Sakahira et al. 1998; Porter & Jänicke, 1999). This is succeeded by the formation of a late phagosome, regulated by Rab-7. The late phagosome then fuses to a lysosome wherein the microorganism is subsequently degraded (**Figure 1.3**) (Sturgill-Koszycki et al. 1996; Meresse et al. 1999; Duclos & Desjardins, 2000; Fratti et al. 2001; Mukherjee et al. 2011).

After endocytosis, phagocytosis and autophagy from the cytoplasm, lysosomes are required to degrade macromolecules originating from the extracellular space (**Figure 1.3)**. Lysosomes are acidic membrane-bound organelles rich in hydrolytic enzymes (Eskelinen et al. 2003). The lysosomal membrane glycoproteins, namely, lysosome associated membrane protein-1 (LAMP-1) and LAMP-2 are transmembrane proteins that constitute approximately 50% of the proteins of the lysosomal membrane (Hunziker et al. 1996). Their functions are largely unknown although they were considered originally to assist in protection against hydrolytic enzymes as well as the maintenance of structural integrity of the lysosomal compartment (Eskelinen, 2006). However, Eskelinen, (2006) suggested that other functions may exist, as mutant mice deficient in both LAMPs show an increase in the accumulation of autophagic vacuoles and unesterified cholesterol. However, once ingested through phagocytosis many pathogenic organisms including *L. pneumophila,* have an increased ability to cause disease by preventing endocytic maturation of the host phagosomes during the endosomal-lysosomal degradation pathway (Meresse et al. 1999; Duclos & Desjardins, 2000).
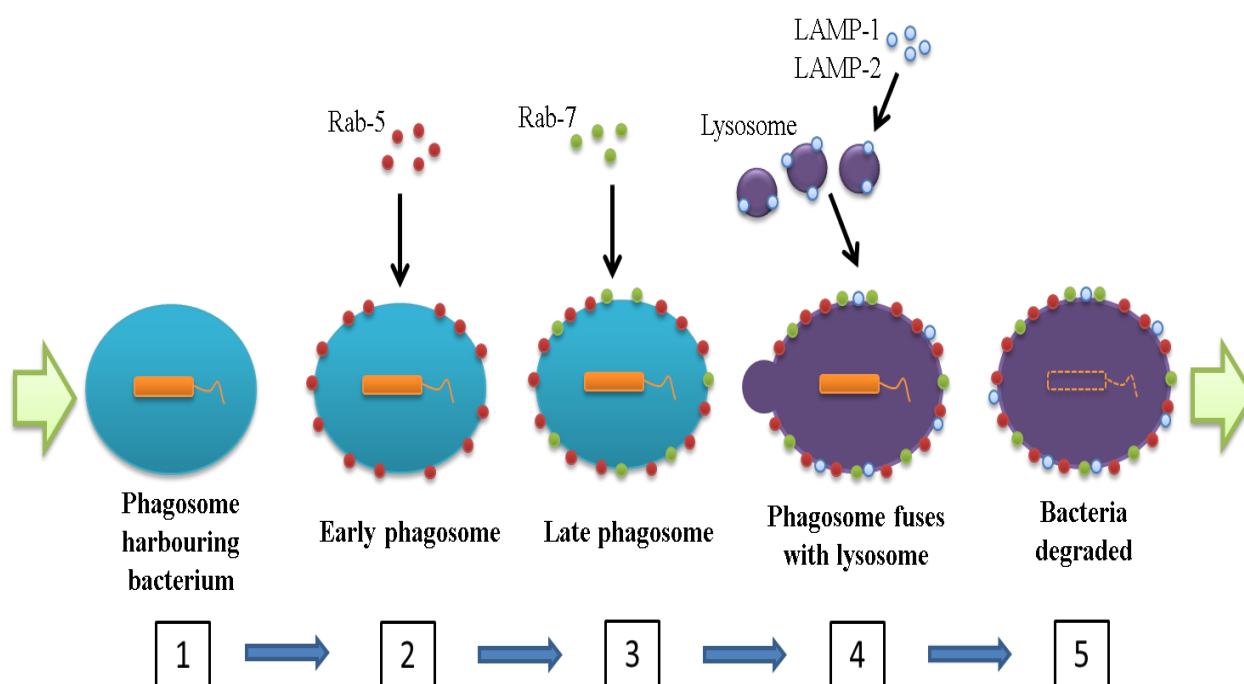
Figure 1.3. During phagocytosis early phagosome formation is controlled by the GTPase Rab-5, succeeded by Rab-7 that controls the late phagosome. The phagosome then fuses with the lysosome and the bacterium is degraded.

1.9.1. Vesicle trafficking and phagocytosis of *L. pneumophila*

Legionella pneumophila is able to control biogenesis of the phagosome and once inside the eukaryotic host, produces the *Legionella* containing vacuole (LCV) replicative niche. This is done by modulating the recruitment of Rab-5, Rab-7 and LAMP-1 to the phagosome. As a

result, the phagosome does not undergo full maturation through the default endosomal-lysosomal degradation pathway (Horwitz, 1983a; Roy et al. 1998; Wiater et al. 1998; Clemens et al. 2000a; 2000b).

During the early stages of infection (**Figure 1.4A**) and within 15 minutes of biogenesis of the plasma membrane, secretory vesicles from the endoplasmic reticulum (ER) exit sites and mitochondria are recruited to the LCV (Horwitz, 1983b) as indicated in **Figure 1.4B** and **1.4C**. During the following hours, the LCV is covered by host-cell ribosomes. This allows for modification of the phagosome membrane by the *Legionella* such that a rough ER-derived membrane forms (Horwitz, 1983b; Swanson & Isberg, 1995; Tilney et al. 2001; Roy & Tilney, 2002; Kagan & Roy, 2002).

Under these circumstances, *Legionella* spp. proliferate within the rough ER-like compartment. In this compartment, *L. pneumophila* is able to stabilise the pH of the LCV to maintain a value of approximately pH 6.1. This is in contrast to a vacuole containing *E. coli* where the pH is generally below 5 (Horwitz & Maxfield, 1984). Once the nutrients within the vacuole become limiting, the bacterium transforms from a replicative form into the transmissive phase and bacterial cell replication ceases. It is notable that after intracellular replication, *L. pneumophila* enhances the synthesis of virulence traits including flagella-induced motility and other virulence factors which aid in host cell lysis. After the lytic process, the free bacteria are capable of invading other phagocytic cells where they establish a new replicative niche (Allombert et al. 2013). Hence, *L. pneumophila* has the ability to kill and escape from the host cells, where some of these *Legionella* cells will establish a new replicative niche within a new host cell and others will continue to survive as planktonic and/or within biofilms as sessile cells until a new replicative niche within a new host cell is required (Molofsky & Swanson, 2004).

Several studies demonstrated that *Legionella* spp. have several virulence factors essential for the infection cycle. The genes coding for these factors have been characterised and reviewed (Cianciotto, 2001; Shevchuk et al. 2011; Zhan et al. 2015) and are required for the entire infection process including bacterial cell attachment to host cells, survival, intracellular replication and cell-to-cell movement. Some of the genes possibly associated with the pathogenicity of *Legionella* are encoded by distinct regions of DNA within the genome known as the pathogenicity island loci (PAIs). Moreover, the infection cycles of *Legionella* spp. within amoebae and macrophages are similar as both hosts are eukaryotic and share conserved molecular pathways.
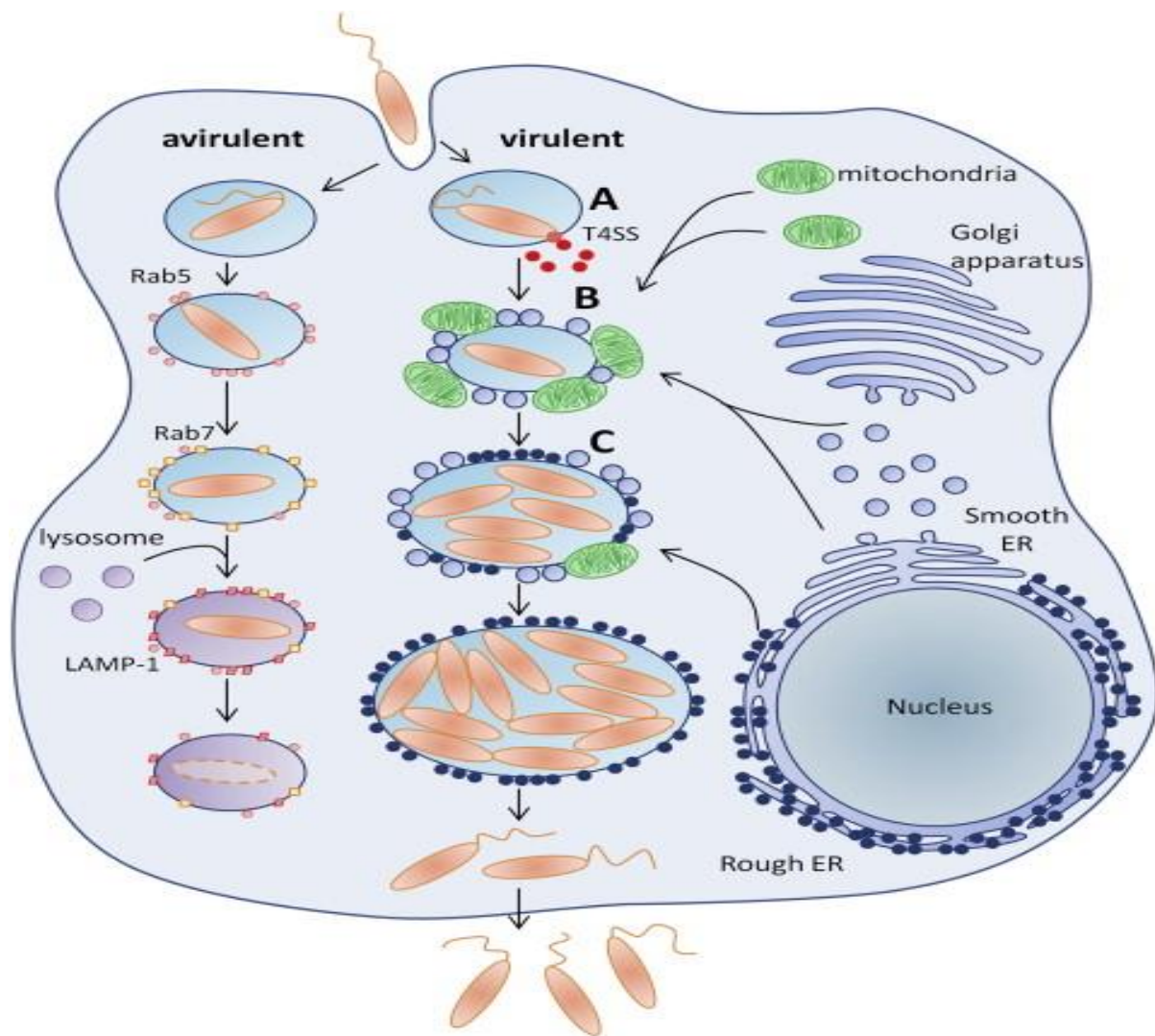
Figure 1.4. A comparison of the phagocytosis of virulent and avirulent *Legionella* strains. Virulent: A: *Legionella pneumophila* uses type IV secretion system proteins to control the development of the LCV. B: During early stages of infection, secretory vesicles of the host are recruited to the LCV. C: The LCV develops into a rough ER-derived membrane vesicle and *L. pneumophila* replicates in this protective niche [adopted from Allombert et al. (2013)].

These cellular processes are controlled by proteins and substrates of the type IV secretion system of *Legionella*. For this reason, it has been suggested that environmental amoebae act as the "trainers" for *Legionella* to replicate intracellularly in mammalian macrophages, as amoebae aid in triggering expression of the bacterial invasive phenotype (Al-Quadan et al. 2012; Escoll et al. 2013; Richards et al. 2013; Price et al. 2014).

1.10. The development of the LCV for effective *L. pneumophila* replication

Once *L. pneumophila* has gained entry into the eukaryotic host cell, its survival depends on the biogenesis of a replication-permissive vacuole, controlled by substrates transported by the

Dot/Icm type IV secretion system. This is responsible for the translocation of more than 250 effector proteins which manipulate signal transduction and vesicle trafficking. To avoid degradation by the host, *L. pneumophila* ensures that the LCV fuses with the host ER–vesicles that are hijacked either during the trafficking of the vesicles between the ER and the Golgi apparatus or during the interception of smooth ER vesicles. In this manner, the LCV avoids the phagocytic pathway by exhibiting a membrane with ER characteristics. Moreover, by utilising the membrane materials, the LCV is able to expand and allow for the intracellular proliferation of *L. pneumophila* (Allombert et al. 2013). Thus within the LCV, *L. pneumophila* depends on the effectors of the Dot/Icm type IV secretion system to:

(i)   enrich the vacuole with phosphatidylinositol 4-phosphate (PI(4)P) on the membrane,

(ii)   recruit Dot/Icm effectors to the LCV and

(iii)   recruit or activate various host proteins including GTPases, which allows for the fusion with the smooth ER vesicles (Allombert et al. 2013).

1.10.1. The enrichment of the LCV with phosphatidylinositol 4- phosphate (PI(4)P)

Cellular compartments within eukaryotic cells are distinguished by many factors, including cellular function and phosphoinositides (PIs). Plasma membranes are generally characterised by an abundance of $PI(4,5)P_2$, where early endosomes contain $PI(3,4,5)P_3$ and $PI(3,4)P_2$ and the Golgi apparatus contains PI(4)P. During phagocytosis, 4- or 5-phosphatases including SHIP-1 and Inpp4A dephosphrylate $PI(3,4,5)P_3$ and $PI(3,4)P_2$ resulting in a membrane that is rich in PI(3)P (Shin et al. 2005). Lysosomes then have a high affinity for PI(3)P (Flannagan et al. 2009). However, one of the mechanisms *L. pneumophila* has developed to ensure stability within the host is the biosynthesis of a vacuole membrane that mimics that of the Golgi compartment i.e. a membrane that contains (PI(4)P), and once the membrane is synthesised the LCV is able to fuse with ER-vesicles (**Figure 1.5**).

One of the Dot/Icm substrates, SidF encoded for by the *sidF* gene, is situated on the surface of the vacuole containing *Legionella* and together with SidF, OCRL1 (a host phosphoinositide phosphatase) binds to the membrane in association with LpnE, a further Dot/Icm substrate (**Figure 1.5A**). As SidF is a phosphatidylinositol polyphosphate 3-phosphatase, it is able to dephosphorylate $(PI(3,4,5)P_3)$ and $(PI(3,4)P_2)$ to produce $(PI(4,5)P_2)$ and (PI(4)P) (Hsu et al. 2012). Along with SidF, OCRL1 also dephosphorylates $(PI(3,4,5)P_3)$ and $(PI(3,4)P_2)$ (Weber et al. 2009). In this manner, SidF acts synergistically with OCRL1 to produce (PI(4)P) as the sole final product (**Figure 1.5A**).
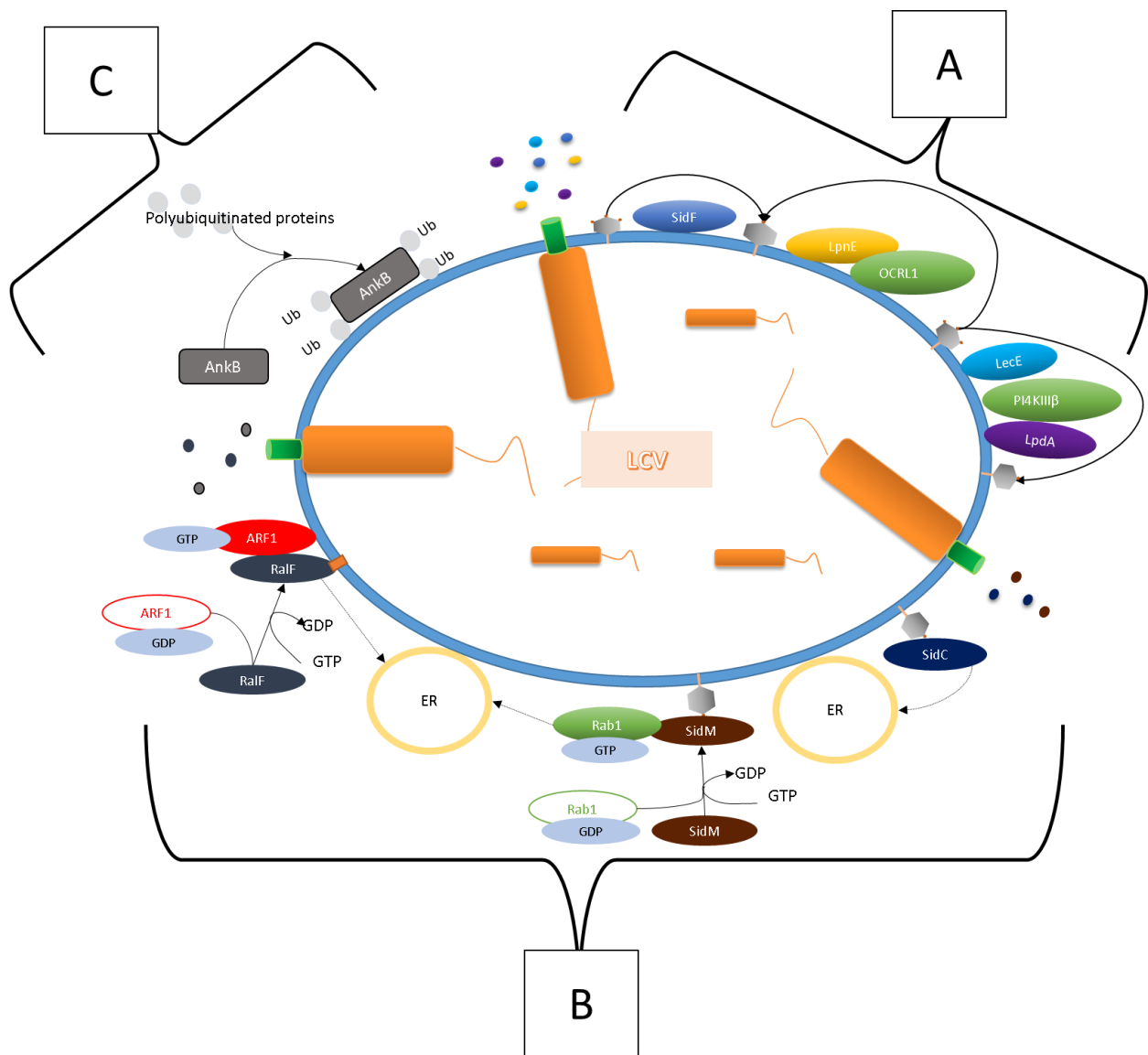
Figure 1.5. Illustration of the known functions of selected *L. pneumophila* Dot/Icm effector proteins. A: The membrane of LCV is first lined with phosphatidylinositol 4-phosphate (PI(4)P). B: Substrates are anchored to the LCV by means of the (PI(4)P). C: Free amino acids, essential as energy and carbon sources, within the proteasome are provided by polyubiquitinated proteins [adapted from Allombert et al. (2013)].

A further enzyme within the host that catalyses the production of (PI(4)P), is PI4KIIIβ (**Figure 1.5A**). Studies have indicated that inhibition of PI4KIIIβ results in a decreased accumulation of (PI(4)P) on the LCV (Brombacher et al. 2009). The recruitment of PI4KIIIβ to the LCV, or the dependence that the Dot/Icm system has on the enzyme has not been completely elucidated. As indicated in **Figure 1.5A**, it is hypothesised that LecE and LpdA may be indirectly associated with the activation of PI4KIIIβ, as these two Dot/Icm effectors manipulate host phospholipid biosynthesis. This is done by the proteins, which act synergistically to produce diacylglycerol (DAG) from phosphatidylcholine which is localised to the LCV (Viner et al. 2012; Allombert et al. 2013).

1.10.2. Recruiting the Dot/Icm substrates to the LCV

For efficient recruitment of the ER, many of the effectors of the Dot/Icm system are localised on the membrane and *L. pneumophila* is able to use the (PI(4)P) accumulated on the membrane, to anchor some of the Dot/Icm substrates to the cytoplasmic expanse of the LCV. An example of substrates anchoring to the membrane are the Dot/Icm substrate, SidC and its paralogue SdcA which attach to the LCV by means of the specific binding of the 20 kDa domain near the C-terminus to (PI(4)P). This in turn promotes ER vesicle recruitment (**Figure 1.5B**) (Ragaz et al. 2008).

Based on functional similarities, small GTPases occur in five major categories viz. Ras, Rho, Rab, Ran and Arf (Wennerberg et al. 2005). Two of these GTPases, Arf1 and Rab1 are activated on the LCV. These are recruited to the LCV by the Dot/Icm effectors, RalF and SidM, respectively and are crucial for the regulation of vesicles transferring between the ER and Golgi apparatus (**Figure 1.5B**) (Nagai et al. 2002; Brombacher et al. 2009). The substrate SidM (also called DrrA) is a multidomain protein that binds to (PI(4)P) by means of a 12 kDa binding domain. The three other activity-associated domains of SidM include GEF (guanine nucleotide exchange factor), GDF [guanosine nucleotide dissociation inhibitor (GDI)-displacement factor] and AMPylation that promote ER vesicle recruitment by recruiting and aiding in the control of the Rab1 GTPase of the host cell (Murata et al. 2006; Müller et al. 2010). These structural features allow for the; interaction between SidM and Rab1 on the LCV (Machner & Isberg, 2006; Murata et al. 2006), the release of Rab1 from GDI (Ingmundson et al. 2007) and the association of GTP-coupled Rab1 with the LCV membrane (**Figure 1.5B**). The activation of Rab1 therefore is carried out by SidM. In turn, this promotes firstly, securing of ER vesicles to the plasma membrane of the LCV and secondly, the functional association between Sec22b on ER-derived vesicles with the plasma membrane Stx3 protein on the LCV, to form a functional SNARE (soluble N-ethylaleimide-sensitive factor attachment protein receptor) complex. Finally,

the activation stimulates the fusion of the membranes of the ER vesicles to the plasma membrane of the LCV (**Figure 1.5B**) (Arasaki et al. 2012).

An additional mechanism used by *L. pneumophila* to insert effectors into the LCV membrane is the expression of the Pel proteins (prenylated effectors of *Legionella*). These proteins contain CAAX (C denotes cysteine, A is an aliphatic amino acid and X is the amino acid that determines which enzyme acts on the protein) motifs, a four-amino acid sequence at the carboxyl terminus of the protein. This creates a site where a group of prenyltransferase enzymes catalyse a post-translational lipid modification or prenylation (Wright & Philips, 2006). Encoded by at least 11 genes, *L. pneumophila* employs the prenylation apparatus of the host to modify these proteins, which in turn permits either the anchoring of hydrophilic proteins to lipid membranes, or the association with other hydrophobic proteins. Thus, in this manner *L. pneumophila* localises these proteins to the LCV (**Figure 1.5C)**.

Together with the CAAX farnesylation motif, ankyrin B (AnkB) contains two ankyrin (ANK) protein-protein interaction domains and an F-box domain that are considered to act as a platform for the attachment of polyubiquitinated proteins to the LCV membrane (**Figure 1.5C**) (Price et al. 2011). The recruitment of the polyubiquitinated proteins to the LCV thus promotes the generation of free amino acids, essential as energy and carbon sources, within the proteasome, which in turn allows for the intracellular proliferation of *Legionella* spp. (Dorer et al. 2006).

Although the six Dot/Icm effectors SidM, SidD, LepB, AnkX, Lem3 and LidA are involved in the recruitment, activation and deactivation of Rab1, inactivation of the corresponding genes does not show any obvious defects in the intracellular replication of *L. pneumophila* (O'Connor et al. 2011). Therefore, the control of Rab1 activity and its involvement in LCV biogenesis may be initiated by additional mechanisms (Allombert et al. 2013). Evidence for this exists as SidJ (Liu & Luo, 2007) and LegK2 (Hervet et al. 2011), two Dot/Icm effectors, are known to be involved in ER-recruitment to the LCV and thus, the intracellular multiplication of *L. pneumophila*, although the host cell targets of these two effectors are not yet characterised.

1.11. Methods used to determine the effector proteins of the Dot/Icm secretion system

Advances in the understanding of mechanisms *L. pneumophila* uses to manipulate host cells were made by creating sophisticated experimental designs (Luo & Isberg, 2004; Campodonico et al. 2005; Shohdy et al. 2005). Previous studies implemented a number of methods to identify, characterise and understand the roles of effector proteins of the Dot/Icm system (Newton et al. 2010). The general approaches used to identify Dot/Icm substrates incorporate a systematic

approach based on β-lactamase translocation assays of all *L. pneumophila* open reading frames (ORFs) coupled to other methods. The latter include:

(i)   bioinformatics approaches which enable the prediction of eukaryotic-like proteins that have similar structural or functional features (Cazalet et al. 2004; de Felipe et al. 2005) or the presence of a C-terminal secretion signal of proteins that physically interacts with the Dot/Icm complex or chaperones (Bardill et al. 2005; Burstein et al. 2009),

(ii)   genetic assays which monitor proteins capable of disrupting cellular processes in yeast (*Saccharomyces cerevisiae*) (Campodonico et al. 2005; Shen et al. 2009; Xu et al. 2010) and

(iii)   translocation assays which monitor reporter activity (Luo & Isberg, 2004; Burstein et al. 2009).

For example, to measure the Dot/Icm-mediated protein translocation, the first method involved the immunostaining of infected cells and LCVs with antibodies specific for effector proteins (Nagai et al. 2002; Luo & Isberg, 2004). All these approaches have confirmed the presence of at least 275 Dot/Icm substrates (Zhu et al. 2011; Allombert et al. 2013). However, in spite of recent progress, a comprehensive understanding of the manner in which *Legionella* spp. impact on host cell functions at the mechanistic level is not yet elucidated (Brüggemann et al. 2006).

From the analyses of the functionality of the 330 effectors of the Dot/Icm secretion system, it is apparent that the inactivation of only one gene encoding for an effector does not always result in an intracellular growth defect (O'Connor et al. 2011). Moreover, the effectors are not necessarily expressed together and although they are involved in various host processes, the fact that there are so many substrates involved in the establishment of a replicative niche, as well as intracellular replication and killing, renders the purpose of the substrates difficult to understand (Allombert et al. 2013). One factor influencing determination of the functions of the substrates includes timeous control of the secretion of the Dot/Icm substrates. This is probably caused firstly by the trafficking of proteins to the translocon[2] by chaperone proteins, secondly by the stability of the substrate protein and thirdly, by minor translocation regulations. The secretion of the apparatus of the Dot/Icm system requires no external signal as it is

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[2] *Membrane proteins that are synthesised by ribosomes may remain either within the cell as these are soluble, or they may be translocated for transmembrane export, as these are generally hydrophobic membrane proteins. In order to prevent the aggregation of these hydrophobic membrane proteins in cellular compartments, the translocon is the principal mechanism that works in conjunction with the ribosomes to manage the methodical insertion of α-helical membrane proteins directly into the endoplasmic reticulum membrane of eukaryotes or into the plasma membrane of bacteria (White & von Heijne, 2008).*

constitutively synthesised (Gal-Mor et al. 2002) and *L. pneumophila* expresses the effectors before the bacterium comes into contact with its host cell (Charpentier et al. 2009). Therefore, the functional redundancy of the 275 substrates makes conclusive genetic studies difficult and as a result, only 15% of the Dot/Icm substrates have been functionally characterised (Allombert et al. 2013).

1.12. Factors that control the virulence of *Legionella* spp.

In order for bacterial pathogens to survive in the external environment and subsequently in a host, their metabolism, physiology and virulence factors must be responsive to changes in a given environment (Vakulskas et al. 2015). A number of genes responsible for an organism's central metabolism may be constitutively synthesised. An example of a metabolic substrate includes lipoproteins that anchor to a cell membrane by N-terminally linked fatty acids. Lipoproteins are chaperoned to the outer membrane by LolA (encoded by the *lolA* gene) (Buse et al. 2015). However, the pathogenicity of *Legionella* spp. is attributed to two categories of factors viz. regulatory factors that control the transcriptional pattern in response to a particular environment, and effector molecules which mediate interactions once *Legionella* spp. are inside the host.

The infectious lifecycle of *Legionella* occurs in two phases viz. replication and transmission. Replication occurs when there is an abundance of nutrients and transmission traits therefore are not expressed. Transmission occurs when nutrients are limiting and this induces the expression of required traits (Vakulskas et al. 2015). As the post-exponential growth phase of *Legionella* correlates markedly with virulence phenotypes, the study of the replicative or transmission phase of *Legionella* requires that the bacteria are cultured to either the exponential or stationary phase respectively (Vakulskas et al. 2015). In addition, studies have indicated that the ability of *Legionella* to infect a host is dependent on the manner in which the inoculum is prepared in the laboratory. For example, the growth of *Legionella* on BYCE agar maintains virulence of the microorganism whereas culturing on high-salt agar and limiting iron levels causes loss of virulence (Nowicki et al. 1987; Catrenich & Johnson, 1989; Yamamoto et al. 1993; James et al. 1995).

Transmission traits exhibited by cultures grown in a broth during the stationary phase include motility/flagella expression, resistance to heat and osmotic pressure, sodium sensitivity, type IV secretion and the ability to avoid phagosome-lysosome fusion (Byrne & Swanson, 1998; Alli et al. 2000; Molofsky & Swanson, 2004). This is termed the transmissive phase, as each trait is predicted to enhance the movement of *Legionella* from one cell to another (Dalebroux et al. 2009). Generally, it is hypothesised that these observations are due to nutrient depletion which

disrupts fatty acid biosynthesis. As a result, the production of guanosine tetraphosphate (ppGpp) by the bifunctional synthetase/hydrolase SpoT is stimulated (Dalebroux et al. 2009). Guanosine tetraphosphate (ppGpp) is an alarmone (intracellular molecules produced by harsh environmental factors) that acts as a general signal of bacterial starvation and stress (Srivatsan & Wang, 2008).

This increase in ppGpp levels permits the cooperation of the stationary-phase sigma factor RpoS (alternative sigma factor of RNA polymerase) and LetS/LetA to induce the expression of transmission genes in *Legionella* (Bachman & Swanson, 2001; Hammer et al. 2002; Dalebroux et al. 2009; Edwards et al. 2009). Hence, during the exponential/replicative phase, RpoS down regulates the transcription of virulence genes including *csrA*, *letE* and *flaA* and this represses bacterial motility, infectivity and cytotoxicity. The RpoS also regulates the expression of various genes including those for ankyrin (used for intracellular growth within amoebae) and LqsR-regulated genes (involved in virulence, motility and cell division) and while RpoS exerts a minor effect on the expression of the Dot/Icm genes, many of the Dot/Icm genes are dependent on RpoS for full expression (Zusman et al. 2002; Tiaden et al. 2007; Habyarimana et al. 2008; Hovel-Miner et al. 2009).

In addition, monitoring the up- or down-regulation of the *csrA* gene that encodes for carbon storage regulator A (CsrA) proteins may indicate the manner in which virulence is regulated during heat disinfection. Previous studies have indicated that CsrA proteins (the global regulators of post-transcriptional gene expression) play an important part in the regulation of virulence gene expression in many Gram-positive and Gram-negative bacteria (Mercante et al. 2006; Timmermans & Van Melderen, 2010; Vakulskas et al. 2015). Carbon storage regulator A (CsrA) proteins inhibit translation of certain genes by binding to the 5' untranslated region (UTR) and prevent ribosomal access to the Shine-Dalgarno sequence[3] of mRNAs thereby altering translation, mRNA turnover and/or transcript elongation. This mode of post-transcriptional modification is exhibited by many species of bacteria including *E. coli* (Baker et al. 2002; Dubey

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[3] *During translation initiation (mRNA to proteins) in prokaryotes, the small ribosomal subunit separates from the large ribosomal subunit as mediated by two initiation factors viz. Initiation Factor (IF)1 and IF3 (Gualerzi & Pon, 1990). The small ribosomal subunit then binds to a purine-rich region known as the Shine-Dalgarno sequence which is located on the 5' UTR approximately seven nucleotides upstream from the start codon (AUG) of the mRNA strand (De Smit & Van Duin, 1990; Hartz et al. 1991;). As the base pair sequence of Shine-Dalgarno sequence is complementary to the base pair sequence of the 16S rRNA component of the small ribosomal subunit, this alignment ensures that the start codon is in the correct position within the ribosome. Initiation Factor 2 (IF2) then transports the initiator tRNA charged with the initiator amino acid N-formyl-methionine to the mRNA strand. The large ribosomal subunit then binds to the complex whereafter all initiation factors are released and elongation commences (Gualerzi & Pon, 1990).*

et al. 2003; Bhatt et al. 2009; Pannuri et al. 2012), *Pseudomonas aeruginosa* (Brencic & Lory, 2009), *Bacillus subtilis* (Yakhnin et al. 2007), *Salmonella enterica* serovar *Typhimurium* (Jonas et al. 2010) and *L. pneumophila* (Nevo et al. 2014). In *Legionella* spp. the LetS/LetA dual-component system then activates the transcription of inhibitory small RNAs (sRNAs), a group of small regulatory non-coding RNA molecules, as the primary method of controlling CsrA activity (Romeo et al. 2013).

1.13. Project Aims

Research has indicated that harvested rainwater is often contaminated by many viral, bacterial and protozoan pathogens, and therefore treatment to disinfect the rainwater is required before its use as a primary potable resource (Ahmed et al. 2011b; 2014; Dobrowsky et al. 2014b; 2014c; 2015a; 2015b). Cost-effective treatment methods that are used for the treatment of harvested rainwater include sand- and nanofiltration, SODIS and SOPAS systems (Joyce et al. 1996; McGuigan et al. 2006; Dobrowsky et al. 2015a; 2015b). Although SOPAS systems offer the advantage of treating substantial volumes of harvested rainwater, potential pathogens including *Pseudomonas* spp. and *Legionella* spp. can survive and persist at the high pasteurization temperatures associated with the process (Dobrowsky et al. 2015b; Reyneke et al. 2016). Of concern is the fact that various *Legionella* spp. have been detected in treated and untreated harvested rainwater samples. Although *L. pneumophila* serotype 1 is the major causative agent of 90% of the incidences of Legionnaires' disease associated with water distribution systems worldwide, other species frequently associated with disease include *L. micdadei*, *L. bozemanii* and *L. longbeachae* (Yu et al. 2002; Mercante & Winchell, 2015).

Factors that allow *Legionella* spp. to proliferate in water environments include: nutrient availability (Cianciotto, 2007), water temperature - *Legionella* require temperatures above 20°C (Farhat et al. 2012; Schwake et al. 2015), the presence of eukaryotic host organisms including genera of the FLA (Donlan et al. 2005) and other aquatic bacteria (Kim et al. 2002). Numerous studies have thus indicated that amoebae are required for the growth and proliferation of *L. pneumophila* (Murga et al. 2001; Kuiper et al. 2004; Declerck et al. 2007). However, to date, no studies have identified the persistence mechanisms used by *Legionella* spp. for survival and proliferation in harvested rainwater and SOPAS systems after treatment at high temperatures.

As reported in the foregoing, *Legionella* spp. are well known parasites of FLAs such as *Acanthamoeba* spp., *Hartmannella* spp. and *Naegleria* spp. in fresh water environments and some of these microorganisms cause disease in humans and animals (Zhu et al. 2011; Dobrowsky et al. 2015a; 2015b). The sequencing of the genomes of *Legionella pneumophila* and other *Legionella* spp. has indicated the presence of many genes that encode eukaryotic-

like proteins (Cazalet et al. 2004; Burstein et al. 2016). *Legionella* makes use of cell biological features similar to the features used to infect amoebae, to infect mammalian cells. Therefore, it is hypothesised that amoebae assist in the ability of *Legionella* to colonise and infect higher organisms (Swanson & Hammer, 2000). Moreover, many of the genetic determinants which aid in *Legionella*'s ability to multiply within amoebae are also crucial for its growth in mammalian cells (Solomon et al. 2000).

The primary aim of the current study was thus to investigate and functionalise the persistence mechanisms displayed by *Legionella* spp. that enhance survival of the bacterium in pasteurized and unpasteurized harvested rainwater and was successfully achieved by completing the following objectives:

- **Chapter Two:** Molecular detection of *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* as vectors for *Legionella* spp. in untreated and solar pasteurized harvested rainwater (published in Parasites and Vectors).

The dominant *Legionella* spp. associated with harvested rainwater samples and pasteurized rainwater samples were isolated and identified. The viability of *Legionella* spp. and various FLA such as *Acanthamoeba* spp., *V. vermiformis* and *Naegleria fowleri* at the pasteurization temperatures screened was also determined using EMA-qPCR. The co-detection of *Legionella* spp. and *Acanthamoeba* spp., *V. vermiformis and N. fowleri* was determined by performing Spearman Rank correlations.

- **Chapter Three**: Heat resistance of *Legionella* species and *Acanthamoeba mauritaniensis* and altered gene expression of *L. pneumophila* and *L. longbeachae* during co-culture with *A. mauritaniensis* (submitted to Science of the Total Environment).

To demonstrate that *Legionella* spp. isolated from harvested rainwater samples were able to survive at high pasteurization temperatures, *Legionella* spp. were co-cultured with *Acanthamoeba* spp. The growth of planktonic *Legionella* and *Legionella* within the LCV of the *Acanthamoeba* following heat treatment (50–90°C) was confirmed utilising EMA-qPCR. The transcriptional responses of *Legionella* during infection were analysed by using relative qPCR, which is a reliable technique for the study of gene expression profiles. One gene associated with metabolism (*lolA*) and two virulence genes (*sidF, csrA*) from *Legionella* spp. were monitored during heat treatment. The *lolA* gene encodes for a molecular chaperone involved in recruiting lipoproteins to the outer membrane of the LCV (Section 1.12). The post-transcriptional repressor CsrA encoded by the *csrA* gene is a global regulator responsible for

the switch between the replicative and transmissive forms of *Legionella* spp. and the SidF protein (encoded by *sidF*) is a type IVB Dot/Icm secretion system effector (Section 1.10.1).

- **Chapter Four**: Microbial and physico-chemical characteristics associated with the incidence of *Legionella* spp. and *Acanthamoeba* spp. in rainwater harvested from different roofing materials (submitted to Environmental Science and Pollution Research).

A third objective was to determine whether metal concentrations and microbiological parameters of harvested rainwater affected the incidence of *Legionella* spp. and *Acanthamoeba* spp. in harvested rainwater. To do this, harvested rainwater samples were collected from catchment areas constructed from different roofing materials viz. galvanised zinc, Chromodek® and asbestos. The presence of *Legionella* spp. and *Acanthamoeba* spp. was confirmed in the harvested rainwater samples by using molecular methods, including qPCR. The data obtained from these analyses were compared statistically in order to ascertain whether the concentrations of *Legionella* and *Acanthamoeba* spp. in harvested rainwater samples were dependent on the quantities of metals and/or other biotic factors (including indicator analysis) in the system.

1.14. References

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Chapter 2:

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Molecular detection of *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* as vectors for *Legionella* spp. in untreated and solar pasteurized harvested rainwater

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Abstract

Background: *Legionella* spp. employ multiple strategies to adapt to stressful environments including the proliferation in protective biofilms and the ability to form associations with free-living amoebae (FLA). The aim of the current study was to identify *Legionella* spp., *Acanthamoeba* spp., *Vermamoeba* (*Hartmannella*) *vermiformis* and *Naegleria fowleri* that persist in a harvested rainwater and solar pasteurization treatment system.

Methods: Pasteurized (45 °C, 65 °C, 68 °C, 74 °C, 84 °C and 93 °C) and unpasteurized tank water samples were screened for *Legionella* spp. and the heterotrophic plate count was enumerated. Additionally, ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) was utilized for the quantification of viable *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri* in pasteurized (68 °C, 74 °C, 84 °C and 93 °C) and unpasteurized tank water samples, respectively.

Results: Of the 82 *Legionella* spp. isolated from unpasteurized tank water samples, *Legionella longbeachae* (35 %) was the most frequently isolated, followed by *Legionella norrlandica* (27 %) and *Legionella rowbothamii* (4 %). Additionally, a positive correlation was recorded between the heterotrophic plate count vs. the number of *Legionella* spp. detected ($\rho = 0.710$, $P = 0.048$) and the heterotrophic plate count vs. the number of *Legionella* spp. isolated ($\rho = 0.779$, $P = 0.0028$) from the tank water samples collected. Solar pasteurization was effective in reducing the gene copies of viable *N. fowleri* (5-log) and *V. vermiformis* (3-log) to below the lower limit of detection at temperatures of 68–93 °C and 74–93 °C, respectively. Conversely, while the gene copies of viable *Legionella* and *Acanthamoeba* were significantly reduced by 2-logs ($P = 0.0024$) and 1-log ($P = 0.0015$) overall, respectively, both organisms were still detected after pasteurization at 93 °C.

Conclusions: Results from this study indicate that *Acanthamoeba* spp. primarily acts as the vector and aids in the survival of *Legionella* spp. in the solar pasteurized rainwater as both organisms were detected and were viable at high temperatures (68–93 °C).

Keywords: Rainwater harvesting, solar pasteurization, *Legionella*, *Acanthamoeba*, *Vermamoeba*, *Naegleria*

2.1. Background

The demand on fresh water supplies is intensifying as a result of an increase in the world's population and urbanization, coupled with the negative effects of climate change [1-3]. Domestic rainwater harvesting systems can be utilized to augment existing surface- and groundwater supplies and in many countries it is utilized as a primary potable water source as well as for domestic and irrigation purposes. Several studies have however, highlighted that rainwater may become contaminated, especially during the harvesting process when debris, animal excreta, dust and leaves, which have accumulated on the roof catchment surface, are washed into the rainwater storage tank [4-6]. It is thus recommended that harvested rainwater is disinfected as numerous pathogens, including those that are opportunistic in nature, have previously been detected in this water source and are of a human health concern [7-12].

Dobrowsky et al. [13], indicated that a closed-coupled solar pasteurization system operating at temperatures greater than 72 °C can be utilized to treat harvested rainwater as the level of heterotrophic bacteria, *Escherichia coli* (*E. coli*) and total coliforms were reduced to below the detection limit and were within the respective drinking water guidelines [14-16]. However, PCR assays confirmed the presence of *Yersinia* spp., *Legionella* spp., and *Pseudomonas* spp., at temperatures greater than 72 °C, with *Legionella* spp. persisting at temperatures greater than 90 °C. A follow-up study performed by Reyneke et al. [17], indicated that *Legionella* spp. may be entering a viable but non-culturable (VBNC) state as intact *Legionella* cells were detected at temperatures of up to 95 °C using ethidium monoazide (EMA) quantitative PCR (EMA-qPCR).

Legionella spp. exhibit a number of mechanisms enabling them to withstand environmental stresses such as heat treatment. These include associations with at least 20 protozoan hosts including *Acanthamoeba* spp., *Naegleria* spp., *Vermamoeba* (*Hartmannella*) *vermiformis* and *Vahlkampfia* spp. and two species of ciliated protozoa, including *Tetrahymena* spp. and *Cyclidium* spp. [18]. Their association with free-living amoebae (FLA) is especially effective as the amoeba host provides nutrients including, amino acids for the proliferation of *Legionella* spp. and a protective environment when *Legionella* spp. are enclosed in the cysts of the amoeba species [19, 20].

Of the genera belonging to the FLA, *Acanthamoeba* spp., *Naegleria fowleri* and *V. vermiformis* are the most frequently isolated from water samples [21-23], including samples from hot water systems [19, 24, 25]. Moreover, *Acanthamoeba* spp. and *N. fowleri* are associated with human and animal infections, including amoebic keratitis and severe brain pathologies [26-29]. The life-cycles of these FLAs are then divided into two stages. First, in the form of a vegetative trophozoite, the organism is able to feed and replicate. Secondly, a cyst is formed under

unfavourable environmental conditions and this allows the organism to withstand nutrient starvation, heat, cold, desiccation and biocidal treatments [29-31]. Although there is limited data regarding FLA resistance to various disinfection procedures, they are a potential risk to public health not only because of the transmission of the protozoa themselves, but because they harbour a range of microbial pathogens including *Legionella* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Mycobacterium* spp., amongst other species [29, 32, 33].

Generally during the process of phagocytosis, the amoeba will engulf avirulent bacterial cells and form a phagosome. The phagosome then fuses with the lysosome, containing lysozymes, which degrade the bacterial cells [34]. Although there are differences in host-cell trafficking processes amongst *L. pneumophila*, *L. micdadei* and *L. longbeachae*, virulent *Legionella* spp. have the ability to halt the phagosome-lysosomal degradation pathway of the amoeba. This implies that the phagosome (containing the *Legionella*) does not undergo sequential maturation and therefore does not ultimately fuse with degradative lysosomes [35, 36]. The *Legionella* instead form a *Legionella* containing vacuole by recruiting secretory vesicles from the endoplasmic reticulum exit sites and mitochondria of the amoeba to the plasma membrane of the *Legionella* containing vacuole [36-38]. Owing to the proteins of the Type IVB defect in organelle trafficking/intracellular multiplication (Dot/Icm) secretion system, that aid in the establishment and the preservation of the *Legionella* containing vacuole, *Legionella* are then able to proliferate in this protective rough endoplasmic reticulum-like compartment [38, 39]. Once nutrients within the *Legionella* containing vacuole become limiting, *Legionella* will kill the amoeba and escape, where after they either establish a new replicative niche within a new host or continue to survive as planktonic cells and/or within biofilms as sessile cells [39, 40]. It has then been suggested that the growth of *Legionella* within amoeba hosts in the environment, is required to select or maintain virulent strains of *Legionella* able to cause Legionnaires' disease [18, 39].

Numerous *Legionella* spp. (e.g. *L. pneumophila* and *L. longbeachea*, amongst others) have been known to cause the acute potentially fatal form of pneumonia as part of a multisystem disease known as Legionnaires' disease (also referred to as Legionellosis or Legion Fever) [41] or a milder form of pulmonary infection known as Pontiac fever, which is a flu like illness [42]. Since the outbreak of Legionnaires' disease has previously been linked to roof-harvested rainwater systems [8, 43] and hot water distribution systems [44], the aim of the current study was to isolate and identify the primary *Legionella* spp. contaminating a harvested rainwater and a solar pasteurization (SOPAS) system (used for the treatment of roof-harvested rainwater) and to identify possible vectors including *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri*, enabling their resistance and persistence. The viability of the FLA's as well as *Legionella* spp.

at temperatures greater than 68 °C was also determined using EMA-qPCR. The enumeration of heterotrophic bacteria was included as previous studies have suggested that a correlation may exist between the heterotrophic plate count and *Legionella*, as *Legionella* multiply in biofilms as a survival strategy in the environment [45-47]. Additionally, the heterotrophic plate count was utilized to monitor the change in the number of viable heterotrophic bacteria before and after pasteurization [48]. Unpasteurized and pasteurized rainwater samples treated at temperatures of 45 °C, 65 °C, 68 °C, 74 °C, 84 °C and 93 °C, were collected for the enumeration of the heterotrophic plate count and the isolation of *Legionella* spp. However, as previous research has indicated that the indicator counts are reduced to below the detection limit at temperatures greater than 70 °C [13,17], the samples collected at temperatures of 68 °C, 74 °C, 84 °C and 93 °C only were utilized for the EMA-qPCR experiments.

2.2. Methods

2.2.1. Sample site and collection

The Apollo™ solar pasteurization system previously described by Dobrowsky et al. [13] was utilized for the treatment of harvested rainwater stored in a polyethylene rainwater harvesting (RWH) tank (2000 l). The RWH tank and pasteurization system were installed at the Welgevallen experimental farm (33°56′36.19″S, 18°52′6.08″E), Stellenbosch University, Western Cape, South Africa, during July 2013. Samples (5 l) pasteurized at 45 °C, 65 °C, 68 °C, 74 °C, 84 °C and 93 °C were collected from the Apollo™ solar pasteurization system during September and October 2015, with six corresponding unpasteurized tank water samples (5 l) collected from the connecting RWH tank. The pH and temperature of the tank water samples were recorded at the sampling site, using a using a handheld pH55 pH/temperature meter (Martini Instruments, North Carolina, USA) and an alcohol thermometer, respectively.

Ambient temperature for the Stellenbosch area during 2015 were obtained from the South African Weather Services (Pretoria, South Africa), while global horizontal irradiance (GHI; W/m^2) data were obtained from Stellenbosch Weather Services, Engineering Faculty, Stellenbosch University (http://weather.sun.ac.za/).

2.2.2. Enumeration of the heterotrophic plate count

For the enumeration of the heterotrophic plate count, a serial dilution (1:10) was prepared for each unpasteurized and pasteurized tank water sample, respectively, and 100 µl of each undiluted and diluted (10^{-1}–10^{-2}) sample was spread plated onto Reasoner´s 2A agar (R2A agar; Difco Laboratories, Detroit, Michigan, USA), with the plates incubated at 37 °C for

up to 4 days in accordance with Standard Methods 9215 C, American Public Health Association [49].

2.2.3. Isolation of *Legionella* spp. from pasteurized and unpasteurized tank water samples

Legionella spp. were recovered from the pasteurized (45 °C, 65 °C, 68 °C, 74 °C, 84 °C and 93 °C) and unpasteurized tank water samples according to the procedure outlined by the Centers for Disease Control and Prevention [50]. Briefly, 500 ml of each sample collected from the domestic rainwater harvesting tank and from the pasteurization system, respectively, was filtered through a sterile GN-6 Metricel® S-Pack Membrane Disc Filter (Pall Life Sciences, Michigan, USA) with a pore size of 0.45 µm and a diameter of 47 mm. The filtration flow rate was approximately ≥ 65 ml/min/cm^2 at 0.7 bar (70 kPa). The filters were then aseptically removed from the filtration system and were placed into sterile 50 ml centrifuge tubes containing sterile water (5 ml). If more than one filter was required, additional filters were added to the same sample tube. The centrifuge tubes were then vortexed to detach the cells from the filters. As *Legionella* detection and isolation may be hampered by the growth of non-*Legionella* background flora [51], the selective detection for the unpasteurized tank water samples was increased by pre-incubating the cell suspension at 50 °C for 30 min before cultivation [50]. Thereafter, 100 µl of the cell suspension was spread plated onto buffered charcoal yeast extract (BCYE) agar containing ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] buffer/potassium hydroxide (1.0 g/l), ferric pyrophosphate (0.025 g/l), alpha-ketoglutarate (0.10 g/l) and *L*-cysteine HCL (0.04 g/l) (Oxoid, Hampshire, England), BCYE agar supplemented with glycine, vancomycin, polymyxin B and cycloheximide (GVPC) and charcoal yeast extract (CYE) agar base without supplements (Oxoid, Hampshire, England).

All plates were then incubated at 35 °C for approximately 10 days. Colonies exhibiting a convex and round with entire morphology that appeared on the BCYE and GVBC media and not on the CYE medium were selected for further analysis. To confirm the presence of presumptive *Legionella* spp., colonies were streaked onto BCYE agar and Nutrient Agar (NA; Merck, Gauteng, South Africa) as a preliminary identification strategy. Colonies that grew only on BCYE agar and not NA were presumptively classified as *Legionella* spp. and were utilized for further analysis.

2.2.4. Total genomic DNA extractions from presumptive *Legionella* isolates

Total genomic DNA (gDNA) extractions were performed for presumptive *Legionella* spp. isolated from the tank water samples present on the BCYE and GVBC media and not on the CYE media. Before the extraction of DNA, presumptive *Legionella* colonies were inoculated into buffered yeast extract (BYE) broth supplemented with ACES buffer/potassium hydroxide

(1.0 g/l), ferric pyrophosphate (0.025 g/l), alpha-ketoglutarate (0.10 g/l) and *L*-cysteine HCL (0.04 g/l) according to Edelstein & Edelstein [52]. Presumptive *Legionella* cultures were then incubated at ± 35 °C for 4 days. Total gDNA was extracted from the cultures using the boiling method previously described by Ndlovu et al. [53].

2.2.5. Ethidium monoazide (EMA) treatment and total gDNA extractions from pasteurized and unpasteurized tank water samples

For the detection and quantification of *Legionella* spp., *Acanthamoeba* spp., *N. fowleri* and *V. vermiformis* total gDNA was extracted from pasteurized (68 °C, 74 °C, 84 °C, 93 °C) and the corresponding unpasteurized tank water samples. For this, 1 l of each sample was first subjected to flocculation as previously described by Dobrowsky et al. [54]. Briefly, 2 ml of $CaCl_2$ (1 M) and 2 ml of Na_2HPO_4 (1 M) were added to each sample before the samples were stirred (room temperature) for 5 min. The samples were then concentrated by filtration as outlined above. For the detachment of cells from the filters, the filters were transferred to 4 ml citrate buffer (0.3 M, pH 3.5) and were vortexed. The filters were subsequently removed and the 4 ml suspension was centrifuged at 16 000× *g* for 10 min. After the removal of the supernatant the pellet was re-suspended in 1 ml sterile MilliQ water before EMA treatment.

Ethidium monoazide (2.5 µg/ml) was added to the 1 ml of concentrated sample according to Delgado-Viscogliosi et al. [55] and Chang et al. [56]. The suspension was then vortexed vigorously and placed on ice for 10 min in the dark. To cross link the EMA to the naked DNA, the samples were kept horizontal on ice and were exposed to a 500 W halogen light for 15 min at a distance of 20 cm. Following centrifugation (16 000× *g*, 5 min), the supernatant was removed and the pellet was washed with 1 ml NaCl (0.85 %). The sample was centrifuged (16 000× *g*, 5 min) and the pellet was re-suspended in 700 µl lysis buffer. The extraction of total gDNA was then performed using the Soil Microbe DNA MiniPrep™ Kit (Zymo Research, Irvine, USA) according to manufacturer's instructions.

2.2.6. Conventional PCR assays for the identification of *Legionella* isolates

For the identification of the presumptive *Legionella* isolates, DNA was extracted from each isolate as outlined above. The primer set LEG 225/LEG 858 was then utilized to amplify 634 bp of the 16S rRNA sequence as previously described by Miyamoto et al. [57] (**Table 2.1**). The PCR mix consisted of 10 µl of 5× Green GoTaq® Flexi Buffer (1×; Promega, Madison, USA), 4 µl $MgCl_2$ (2.0 mM; Promega), 0.5 µl of each dNTP (0.1 mM; Thermo Fischer Scientific, Waltham, USA), 2 µl of each PCR primer (LEG 225 and LEG 858; 0.4 µM), 0.3 µl of GoTaq® Flexi DNA Polymerase (1.5U; Promega) and 2 µl of template DNA. All conventional PCR mixtures consisted of a final volume of 50 µl. The PCR cycling parameters were as follows:

initial denaturation at 95 °C (1.5 min) followed by 30 cycles of denaturation at 94 °C (10 s), annealing at 64 °C (1 min) and elongation at 74 °C (1 min). A final extension was included at 72 °C (10 min).

2.2.7. Quantification of viable *Legionella* spp., *Acanthamoeba* spp., *N. fowleri* and *V.* vermiformis in pasteurized and unpasteurized tank water samples

For the quantification of viable *Legionella* spp., *Acanthamoeba* spp., *N. fowleri* and *V. vermiformis* in pasteurized (68 °C, 74 °C, 84 °C, 93 °C) and unpasteurized tank water samples, quantitative PCR (qPCR) was performed using a LightCycler ® 96 (Roche, Gauteng, South Africa) following EMA treatment. For all qPCR assays, to a final reaction volume of 20 µl, using the FastStart Essential DNA Green Master Mix (Roche Applied Science, Mannheim, Germany), the following were added: 10 µl FastStart Essential DNA Green Master Mix (2×), 5 µl template DNA, and 0.4 µl of each primer (0.2 µM).

For the quantification of *Legionella* spp. in pasteurized and unpasteurized tank water samples, the primers and qPCR parameters according to Herpers et al. [58] were utilized (**Table 2.1**). To generate a standard curve for the quantification of *Legionella* spp., the purified conventional PCR product obtained by amplifying the 256 bp product from *L. pneumophila* ATCC 33152 was utilized.

For the quantification of *Acanthamoeba* spp. in pasteurized and unpasteurized tank water samples, the primers and qPCR parameters as previously described by Qvarnstrom et al. [59] were utilized (**Table 2.1**). To generate the standard curve for the quantification of *Acanthamoeba* spp., the 180 bp PCR product amplified from gDNA of *A. mauritaniensis* ATCC 50677 was cloned into the pGEM T-easy vector system (Promega Corp.) according to the manufacturer's instructions. Once the plasmid had been sequenced, the plasmid containing the correct insert was used to generate the standard curve.

Additionally, for the quantification of *N. fowleri* in pasteurized and unpasteurized tank water samples, the primers and qPCR parameters as outlined by Qvarnstrom et al. [59] were utilized (**Table 2.1**). To generate the standard curve for the quantification of *N. fowleri* a purified 153 bp PCR product obtained by screening a 1 l tank water sample from a domestic rainwater harvesting tank located at Stellenbosch University (GPS coordinates: 33°55'51.1"S, 18°51'56.7"E) using the NaeglF192/NaeglR344 primer set was cloned into the pGEM T-easy vector system (Promega Corp.) according to the manufacturer's instructions. Once the plasmid had been sequenced, the plasmid containing the correct insert was used to generate the standard curve for the quantification of *N. fowleri*.

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Table 2.1 Primers and amplification conditions utilized in the current study for the identification and quantification of *Legionella* spp., *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* in pasteurized and unpasteurized tank water samples.

Organism	Primer name	Primer sequence (5'-3')	Gene (Size, bp)	Amplification conditions	Reference
Legionella spp. (Identification)	LEG 225	AAGATTAGCCTGCGTCCGAT	16S rRNA (634)	95 °C (1.5 min) followed by 30 cycles of 94 °C (10 s), 64 °C (1 min) and 74 °C (1 min). Final extension: 72 °C (10 min).	Miyamoto et al. [57]
	LEG 858	GTCAACTTATCGCGTTTGCT			
Legionella spp.	Leg F	CTAATTGGCTGATTGTCTTGAC	23S–5S rRNA (259)	95 °C (1 min) followed by 45 cycles of 95 °C (15 s), 60 °C (15 s) and 72 °C (11 s)	Herpers et al. [58]
	Leg R	CAATCGGAGTTCTTCGTG			
Acanthamoeba spp.	AcantF900	CCCAGATCGTTTACCGTGAA	18S rDNA (± 180)	95 °C (1 min) followed by 45 cycles of 95 °C (15 s), 60 °C (1 min) and 72 °C (40 s)	Qvarnstrom et al. [59]
	AcantR1100	TAAATATTAATGCCCCCAACTATCC			
Naegleria fowleri	NaeglF192	GTGCTGAAACCTAGCTATTGTAACTCAGT	18S rDNA (153)	95 °C (1 min) followed by 45 cycles of 95°C (15 s), 64°C (1 min) and 72°C (1 min)	Qvarnstrom et al. [59]
	NaeglR344	CACTAGAAAAAGCAAACCTGAAAGG			
Vermamoeba (*Hartmannella*) *vermiformis*	Hv1227F	TTACGAGGTCAGGACACTGT	18S rRNA (502)	95 °C (3 min) followed by 45 cycles of 95 °C (20 s), 58 °C (30 s) and 72 °C (40 s)	Kuiper et al. [60]
	Hv1728R	GACCATCCGGAGTTCTCG			

For the quantification of *V. vermiformis* in pasteurized and unpasteurized tank water samples, the primers and qPCR parameters according to Kuiper et al. [60] were utilized (**Table 2.1**). To generate a standard curve for the quantification of *V. vermiformis*, the purified conventional PCR product (502 bp) obtained by screening a 1 l tank water sample from a domestic rainwater harvesting tank located at Stellenbosch University (GPS co-ordinates: 33°55'51.1"S, 18°51'56.7"E) using the Hv1227F/Hv1728R primer set was utilized.

The concentration of the purified PCR products (*Legionella* spp. and *V. vermiformis*) and plasmid DNA (*Acanthamoeba spp.* and *N. fowleri*) were quantified using the NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA) in triplicate at CAF. Serial 10-fold dilutions (10^9 to 10^1) of the sequenced conventional PCR products and plasmid DNA were prepared in order to generate the standard curves for each respective organism. A concentration of 1.00×10^9 gene copies/µl was prepared for the dilution with the highest copy number and a concentration of 1.00×10^1 gene copies/µl was prepared for the dilution with the lowest copy number. Standard curves generated by plotting quantitative cycle (Cq) values *vs* the log concentrations of standard DNA as previously described by Chen and Chang [61], were then used to determine the number of gene copies of each of the organisms. Melt curve analysis was included for all SYBR green real-time PCR assays in order to verify specificity of the primer set by ramping the temperature from 65 to 97 °C at a rate of 0.2 °C/s with continuous fluorescent signal acquisition at 5 readings/ °C.

2.2.8. Sequencing of PCR amplicons

The PCR amplicons of each presumptive *Legionella* isolate, the PCR products used as positive controls to generate the standard curves for each qPCR assay and representative products of each of the qPCR assays of each organism were then purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research) and were sent for sequencing at the CAF, Stellenbosch University. Chromatograms of each sequence were examined as outlined in Dobrowsky et al. [12] and all sequences were submitted as a query to BLAST for a sequence similarity search against the NCBI databases (blast.ncbi.nlm.nih.gov).

2.2.9. Inter- and intra-assay reproducibility and the lower limit of detection

To establish the inter-assay reproducibility of the qPCR assays optimized for each respective organism, the coefficient of variation (CV) was determined using the concentrations of nine dilutions (10^9 to 10^1) of conventional PCR products and plasmid DNA that were quantified in duplicate during three separate qPCR experiments. In addition, the CV for intra- assay repeatability was calculated using the concentrations of nine dilutions (10^9 to 10^1) for each

qPCR assay [62-65]. In order to eliminate any PCR inhibitors, samples resulting in end-point fluorescence (EPF) values of less than 3.15 were diluted (10×) and the qPCR experiment was repeated for these samples. The minimum number of gene copies (highest dilution) that could be measured accurately within an assay was considered the lower limit of detection for each organism [63].

2.2.10. Statistical analysis

The data obtained from the microbial and physical analysis of the tank water samples collected, were assessed using the Statistical software package, Statistica^{TM} version 13.0 (Statsoft Inc.). Before the data analysis, the Shapiro-Wilk test was used to test the normality of data sets. The gene copies of *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri* obtained for the pasteurized and unpasteurized tank water samples were assessed for nonparametric differences using the Mann-Whitney U Test. Thus, the temperature of the tank water samples, before and after pasteurization was used as a single, ordinal variable. Spearman Rank (ρ) correlation tests were performed to establish correlations between different microbiological (the heterotrophic plate count, number of *Legionella* isolates, the gene copies of *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri* obtained before and after pasteurization) and physical parameters (pH and temperature of tank water samples) as previously described by Wang et al. [66]. Significance was set at a *P*-value of ≤ 0.05 for all statistical analyses performed.

2.3. Results

2.3.1. Physical parameters of pasteurized and unpasteurized tank water samples collected during the sampling period (September-October 2015)

Pasteurized (45 °C, 65 °C, 68 °C, 74 °C, 84 °C and 93 °C) tank water samples were collected from the Apollo™ solar pasteurization system with corresponding unpasteurized tank water samples collected from the RWH tank during September and October 2015. The average daily ambient temperature ranged from 15.7 °C (September 2015) to 18.5 °C (October 2015). Additionally, as the Apollo™ solar pasteurization system relies on radiation from the sun to heat the tank water, the average total GHI was recorded at 8288.9 W/m^2 during September 2015 and 11574.6 W/m^2 during October 2015. The temperature of the water samples collected from the RWH tank ranged from the lowest temperature of 18 °C (15.09.2015) to the highest recorded temperature of 31 °C (27.10.2015). An average pH of 8.0 (range: 7.9–8.1) was recorded for unpasteurized tank water samples which then increased to pH 8.3 (range: 8.2–8.5) after pasteurization (**Table 2.2**).

2.3.2. The heterotrophic plate count and culturing of *Legionella* spp.

For all unpasteurized tank water samples ($n = 6$), the heterotrophic plate count numbers ranged from 2.7×10^5 CFU/ml to 1.5×10^6 CFU/ml and were above the Department of Water Affairs and Forestry (DWAF) [14] guideline of 100 CFU/ml (**Table 2.2**). Additionally, the heterotrophic plate count were above the DWAF [14] guidelines following pasteurization at 45 °C (1.5×10^5 CFU/ml) and 65 °C (4.7×10^2 CFU/ml), respectively (results not shown). However, after the pasteurization treatment for the temperatures ranging from 68 to 93 °C, heterotrophic plate counts were reduced to below the detection limit (< 1 CFU/ml) and were within the DWAF guidelines (**Table 2.2**).

2.3.3. Conventional PCR for the identification of *Legionella* isolates

Culture based methods were then utilized to isolate *Legionella* spp. from all pasteurized ($n = 6$) and unpasteurized ($n = 6$) tank water samples. While no *Legionella* spp. were isolated from pasteurized tank water samples (45 °C to 93 °C), *Legionella* spp. were isolated from all the unpasteurized tank water samples utilizing culturing methods (**Table 2.2**). A total of 82 *Legionella* isolates were obtained overall from all the unpasteurized samples and all the resulting DNA sequences of the *Legionella* isolates displayed similarities to sequences of *Legionella* spp. recorded on NCBI. *Legionella longbeachae* ($n = 29$; GenBank accession no: FN650140.1, JN606078.1, NR_102800.1) was the species most frequently isolated from the unpasteurized tank water samples (results not shown), followed by *Legionella norrlandica* ($n = 22$) and *Legionella rowbothamii* ($n = 3$) (**Fig. 2.1**; accession numbers included), the remaining 28 isolates were undetermined *Legionella* species (results not shown) and BLAST analysis indicated the presence of uncultured *Legionella* spp. (GenBank accession no: HQ111985.1, HQ111937.1, GU185995.1) and *Legionella* spp. (GenBank accession no: JN380993.1, JN380988.1).

Table 2.2 Microbiological parameters and physical parameters determined for pasteurized and unpasteurized harvested rainwater samples

Date	Unpasteurized and pasteurized sample temp. (°C)	pH	Heterotrophic plate count (CFU/ml)	No. of *Legionella* isolates obtained	Gene copies/ml			
					Legionella spp.	*Acanthamoeba* spp.	*Vermamoeba vermiformis*	*Naegleria fowleri*
22.10.2015	24	8.4	1.5×10^6	9	6.5×10^4	9.8×10^4	5.7×10^6	1.6×10^5
	68[a]	8.4	BDL[b]	BDL[b]	3.2×10^3	8.4×10^3	9.4×10^3	LLOD[d]
22.10.2015	25	8.1	1.0×10^6	2	4.5×10^4	8.3×10^4	3.9×10^4	6.4×10^4
	74[a]	8.5	BDL[b]	BDL[b]	9.2×10^3	5.2×10^3	LLOD[c]	LLOD[d]
19.10.2015	21	8.3	6.8×10^5	1	5.7×10^6	1.3×10^5	1.4×10^5	9.2×10^4
	84[a]	8.2	BDL[b]	BDL[b]	2.3×10^3	1.7×10^4	LLOD[c]	LLOD[d]
27.10.2015	31	8.4	2.7×10^5	3	8.2×10^6	6.5×10^4	3.2×10^5	1.0×10^6
	93[a]	8.2	BDL[b]	BDL[b]	1.1×10^3	1.4×10^4	LLOD[c]	LLOD[d]

[a] Pasteurized rainwater sample

[b] BDL, below detection limit

[c] LLOD, lower limit of detection: *Vermamoeba vermiformis* (< 5–8 gene copies/µl)

[d] LLOD, lower limit of detection: *Naegleria fowleri* (< 12–17 gene copies/µl)
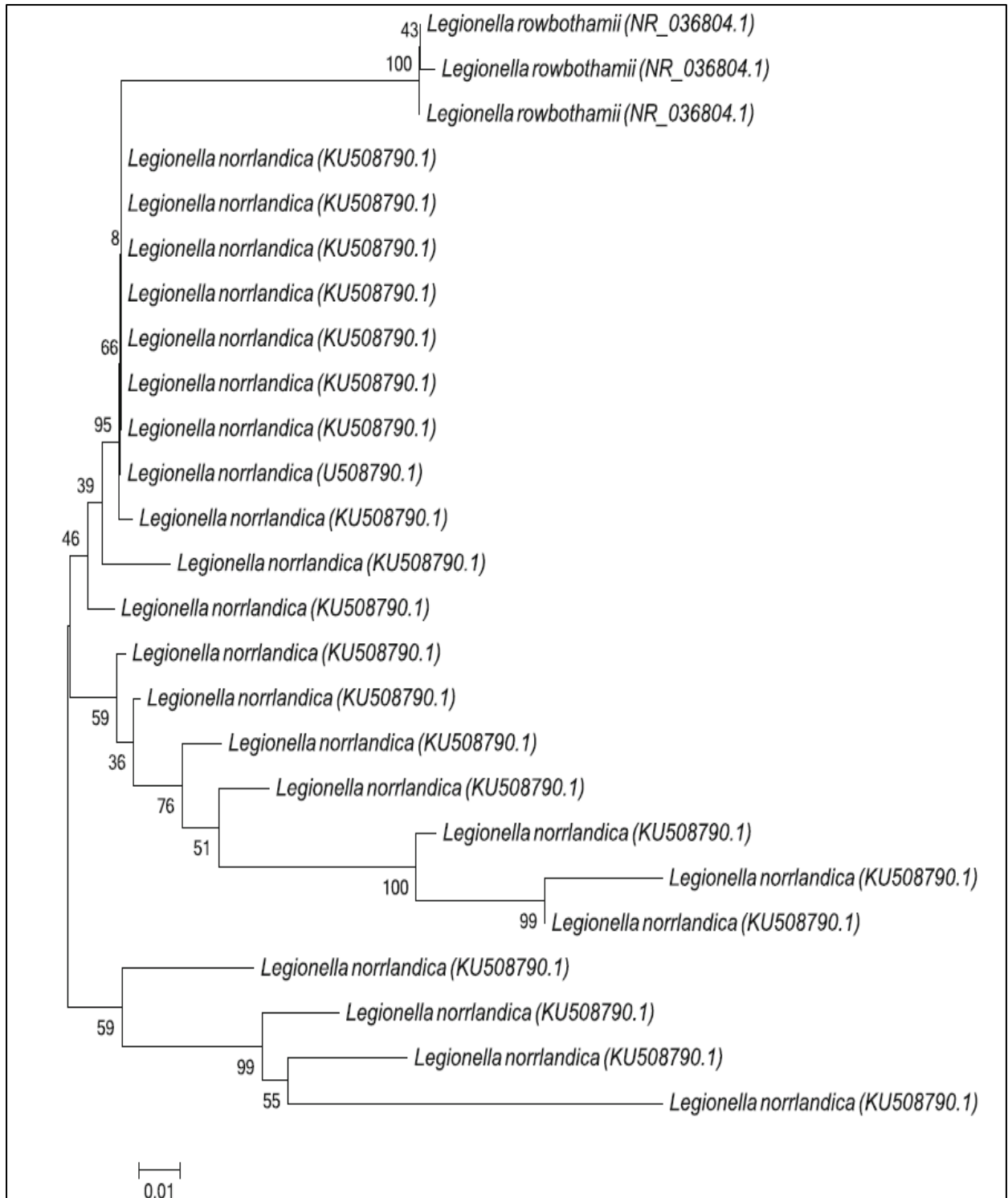
Fig. 2.1. Phylogenetic tree constructed from sequences of PCR products of *Legionella norrlandica* and *Legionella rowbothamii* isolates by means of the maximum composite likelihood function (evolutionary history) and neighbor-joining method using the program MEGA 5. The numbers found adjacent to the nodes represent the data (percentages) from 1,000 exploratory bootstrap trials. Bar: 0.002 changes per site. The *L. norrlandica* and *L. rowbothamii* isolates were obtained from unpasteurized tank water samples.

2.3.4. Quantification of viable *Legionella* spp. present in pasteurized and unpasteurized tank water samples

As the heterotrophic plate count was reduced to below the detection limit following pasteurization at 68 °C to 93 °C, viable *Legionella* spp. were quantified in samples pasteurized at 68 °C, 74 °C, 84 °C, 93 °C and the four corresponding unpasteurized tank water samples (**Table 2.2**). For all unpasteurized tank water samples, the concentration of viable *Legionella* ranged from 4.5×10^4 gene copies/ml (25 °C) to 8.2×10^6 gene copies/ml (31 °C) (**Table 2.2**). After pasteurization treatment, the highest concentration of *Legionella* was detected at 74 °C (9.2×10^3 gene copies/ml) which then decreased to 1.1×10^3 gene copies/ml following pasteurization at 93 °C as indicated in **Table 2.2**. The number of *Legionella* gene copies then decreased by 1-log (87.2 %) following pasteurization at 68 °C and 74 °C, respectively. In contrast, a 3-log reduction (> 99.9 %) in *Legionella* gene copies was observed following pasteurization at 84 °C, while a 4-log (> 99.9 %) reduction in *Legionella* gene copies was observed following pasteurization at 93 °C. Overall, the number of viable *Legionella* gene copies decreased significantly ($Z = 3.034$; $P = 0.0024$) by an average of 2-logs (93.6 %) following pasteurization at 68–93 °C. Representative qPCR products were sequenced and *Legionella anisa* (GenBank accession no: JN001853.1, Z24682.1) and *Legionella monrovica* (GenBank accession no: Z24729.1) were detected in representative pasteurized and unpasteurized tank water samples following BLAST analysis, respectively.

2.3.5. Quantification PCR efficiency, reproducibility and lower limit of detection

A linear range of quantification from 10^9 to 10^1 gene copies per µl of DNA extracts was observed for all standard curves produced for the quantification of *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri*, respectively. As indicated in **Table 2.3**, the qPCR assays had amplification efficiencies that ranged from 1.86 to 1.94 (*Legionella* spp.), 1.92 to 1.95 (*Acanthamoeba* spp.), 1.85 to 1.89 (*V. vermiformis)* and 1.90 to 2.04 (*N. fowleri*). The optimum amplification efficiency is measured at 2.00 and corresponds to a doubling of copy number for every PCR cycle [67]. The correlation coefficient (r^2) ranged from 0.99 to 1.00 for all qPCR assays performed for *Legionella* spp., *Acanthamoeba* spp. and *N. fowleri* and 0.98 to 1.00 for all qPCR assays performed *V. vermiformis*, respectively (**Table 2.3**). The qPCR lower limit of detection was recorded at 8–12 gene copies/µl for *Legionella* spp., 2–6 gene copies/µl for *Acanthamoeba* spp., 5–8 gene copies/µl for *V. vermiformis* and 12–17 gene copies/µl for *N. fowleri* (**Table 2.3**). The qPCR assays demonstrated good reproducibility as the mean inter-and intra- assay coefficient of variation (CV) values and standard deviations (SD) were less than 1 % and 5 % for all qPCR assays, respectively (**Table 2.3**).

Table 2.3 The lower limit of detection (LLOD), amplification efficiency, correlation coefficient (r^2), intra- and inter-assay reducibility within the range of 10^9 to 10^1 gene copies/µl of each qPCR assay

Organisms assayed	LLOD (gene copies/µl)	Amplification efficiency (%)	Correlation coefficient (r^2)	Mean ± SD of CV	
				Intra-assay	Inter-assay
Legionella spp.	8–12	1.86 (93)–1.94 (97)	0.99–1.00	0.160 ± 0.257	0.251 ± 0.220
Acanthamoeba spp.	5–11	1.92 (96)–1.95 (98)	0.99–1.00	0.142 ± 0.283	0.192 ± 0.225
Vermamoeba vermiformis	5–8	1.85 (93)–1.89 (95)	0.98–1.00	0.09 ± 0.112	0.129 ± 0.092
Naegleria fowleri	12–17	1.90 (95)–2.04 (102)	0.99–1.00	0.195 ± 0.410	0.348 ± 0.251

Abbreviations: CV, coefficient of variation; LLOD, lower limit of detection; SD, standard deviation

2.3.6. Quantification of FLAs present in pasteurized and unpasteurized tank water samples

Following EMA treatment and DNA extractions, qPCR was performed for the quantification of viable *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri* in samples pasteurized at 68 °C, 74 °C, 84 °C, 93 °C and the corresponding unpasteurized tank water samples, respectively (**Table 2.2**). As indicated in **Table 2.2**, gene copies of *Acanthamoeba* ranged from 6.5×10^4 gene copies/ml (31 °C) to 1.3×10^5 gene copies/ml (21 °C) for all unpasteurized tank water samples. After pasteurization treatment, the gene copies of *Acanthamoeba* spp. decreased and ranged from 5.2×10^3 gene copies/ml (74 °C) to 1.7×10^4 gene copies/ml (84 °C) and 1.4×10^4 gene copies/ml (93 °C) (**Table 2.2**). Overall, the number of *Acanthamoeba* gene copies decreased significantly ($Z = -3.183$; $P = 0.0015$) by 1-log (87.3 %) following pasteurization at 68–93 °C. *Acanthamoeba* genotype T4 (GenBank accession no: KT892923.1), genotype T15 (GenBank accession no: KT892848.1) and *Acanthamoeba lenticulata* (GenBank accession no: KX018047.1) were detected in representative pasteurized and unpasteurized tank water samples, respectively.

For all unpasteurized tank water samples, the gene copies of viable *V. vermiformis* ranged from 3.9×10^4 gene copies/ml (25 °C) to 5.7×10^6 gene copies/ml (24 °C) (**Table 2.2**). Following pasteurization, the gene copies of *V. vermiformis* decreased and ranged from 9.4×10^3 gene copies/mL (68 °C) to below the lower limit of detection (< 5–8 gene copies/µl) for the remainder of the pasteurized samples (74 °C to 93 °C). A 3-log reduction (99.9 %) in

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V. vermiformis gene copies was observed following pasteurization at 68 °C while a 5-log reduction (> 99.9 %) in gene copies of viable *V. vermiformis* was observed following pasteurization at 74 °C to 93 °C, respectively. Overall, the number of gene copies of *V. vermiformis* (GenBank accession no: KT185625.1) decreased significantly (Z = 3.067; *P* = 0.0021) by 5-log (> 99.9 %) following pasteurization at 68–93 °C.

For all unpasteurized tank water samples, gene copies of viable *N. fowleri* ranged from 6.4×10^4 gene copies/ml (25 °C) to 1.0×10^6 gene copies/ml (31 °C) (**Table 2.2**). Following pasteurization, the gene copies of *N. fowleri* decreased to below the lower limit of detection (< 12–17 gene copies/µl) for all tank water samples pasteurized at 68 °C, 74 °C, 84 °C and 93 °C, respectively (**Table 2.2**). A 5-log reduction (> 99.9 %) in *N. fowleri* gene copies was observed following pasteurization at 68 °C to 84 °C, respectively, while a 6-log reduction (> 99.9 %) in gene copies of *N. fowleri* was observed following pasteurization at 93 °C. Overall, the number of gene copies of viable *N. fowleri* (GenBank accession no: JQ271702.1, JQ271704.1) decreased significantly (Z = 3.308; *P* = 0.001) by 5.2-log (> 99.9 %) following pasteurization at 68–93 °C.

2.3.7. Associations of microbiological parameters and abiotic factors

As indicated in **Table 2.4**, Spearman (ρ) correlations were noted between parameters measured throughout the study. For example, positive correlations were observed between the heterotrophic plate count and the number of *Legionella* isolates obtained (ρ = 0.779, *P* = 0.0028) and the gene copies of *Legionella* spp. (ρ = 0.710, *P* = 0.048), *Acanthamoeba* spp. (ρ = 0.862, *P* = 0.006), *V. vermiformis* (ρ = 0.858, *P* = 0.006) and *N. fowleri* (ρ = 0.810, *P* = 0.015), respectively. Additionally, significant positive correlations were observed between the number of *Legionella* isolates obtained *vs* the gene copies of *Legionella* spp. (ρ = 0.812, *P* = 0.0014), *Acanthamoeba* spp. (ρ = 0.761, *P* = 0.028), *V. vermiformis* (ρ = 0.936, *P* = 0.001) and *N. fowleri* (ρ = 0.946, *P* = 0.0004), respectively. The number of *Legionella* spp. gene copies/ml were also positively correlated to the gene copies of the amoeba detected including *V. vermiformis* (ρ = 0.854, *P* = 0.001), *N. fowleri* (ρ = 0.913, *P* = 0.002) and *Acanthamoeba* spp. (ρ = 0.643, *P* = 0.085). Moderate to high correlations were then detected between the number of *Acanthamoeba* spp. gene copies *vs* the number of *V. vermiformis* (ρ = 0.756, *P* = 0.03) and *N. fowleri* gene copies (ρ = 0.845, *P* = 0.028), respectively. A high correlation was also established between the gene copies of *V. vermiformis* and of *N. fowleri* (ρ = 0.936, *P* = 0.001).

Table 2.4 Spearman rank order correlation coefficients (ρ) of parameters investigated in this study

Parameter	Temperature (°C)	pH	HPC/ml	No. of isolates (Culture)	*Legionella* spp.	*Acanthamoeba* spp.	*Vermamoeba vermiformis*
Temperature (°C)	–						
pH	0.367	–					
HPC/ ml	-0.847**	-0.246	–				
No. of isolates (Culture)	-0.885**	-0.374	0.779**	–			
Legionella spp.	-0.833**	0.306	0.710*	0.812*	–		
Acanthamoeba spp.	-0.809*	-0.356	0.862**	0.761*	0.643	–	
Vermamoeba vermiformis	-0.854**	0.176	0.858**	0.936**	0.854**	0.756*	–
Naegleria fowleri	-0.761**	0.118	0.810*	0.946**	0.913**	0.761*	0.936**

$*P < 0.05$, $**P < 0.01$

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2.4. Discussion

The heterotrophic plate count for all unpasteurized and tank water samples pasteurized at 45 °C and 65 °C were above the DWAF guideline for drinking water, with the heterotrophic plate count reduced to below the detection limit (< 1 CFU/ml) following pasteurization at 68 °C to 93 °C. The heterotrophic plate count represents only the culturable portion of the general bacterial community that are present in a water source and results indicated that pasteurization treatment at temperatures above 68 °C are effective as heterotrophic plate count counts in the treated samples corresponded to drinking water guidelines [14]. Previously, Sommer et al. [68] indicated that fecal coliforms were inactivated in river water at temperatures above 70 °C utilizing solar pasteurization. Moreover, Dobrowsky et al. [13] indicated that a closed-coupled solar pasteurization system operating at temperatures above 72 °C reduced the level of the heterotrophic plate count, *E. coli* and total coliforms to below the detection limit in harvested rainwater.

In the current study a positive correlation between the heterotrophic plate count and the number of culturable *Legionella* present and the gene copies of viable *Legionella* spp. was also established. These results are in agreement with a study conducted by Serrano-Suárez et al. [69], where *Legionella* spp. were isolated when the corresponding heterotrophic plate count concentrations were above 1×10^5 CFU/100 ml, indicating that the frequency at which *Legionella* spp. are isolated may depend on the presence of culturable heterotrophic bacteria. Additionally, positive correlations were established between the heterotrophic plate count and the number of gene copies of viable *Acanthamoeba* spp., *V. vermiformis and N. fowleri*. This is expected as these FLAs are heterotrophs and known grazers of bacteria and the heterotrophic plate count represents the general bacterial microbiota [66, 69, 70]. Furthermore, while results indicated that the pH of the unpasteurized and pasteurized tank water samples did not significantly influence the microbiological quality, an increase in temperature of the pasteurized harvested rainwater significantly reduced the heterotrophic plate count, the number of *Legionella* isolates obtained, and the gene copies of viable *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri* detected, respectively.

Legionella spp. were also isolated using culture based techniques and during the current study, the majority of the *Legionella* isolates were obtained from unpasteurized tank water samples at temperatures of 18 °C (52 *Legionella* isolates) and 19 °C (15 *Legionella* isolates), respectively. Molecular analysis of the *Legionella* isolates obtained then indicated that *L. longbeachae* was the dominant *Legionella* spp. isolated from the unpasteurized tank water samples. While *L. longbeachae* is generally isolated from soil, including potting soil [71], this microorganism has

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previously been isolated from water samples collected from hospital reticulation systems and cooling towers and is able to proliferate in *Acanthamoeba polyphaga* [72-74]. In addition, while *L. pneumophila* serotype 1 is responsible for most of the human reported infections, 17 additional species have also been associated with disease and these include *L. longbeachae, L. micdadei*, *L. anisa* and *L. bozemanii* [75]. BLAST analysis also revealed that *L. norrlandica* was of the dominant *Legionella* spp. isolated from unpasteurized tank water samples. This *Legionella* spp. harbours the majority of the *L. pneumophila* virulence factors and has only recently been described, where Rizzardi et al. [76] isolated a novel *Legionella* genus from the biopurification systems of wood processing plants. Moreover, the study group revealed that *L. norrlandica* could establish a replicative vacuole in *A. castellanii*. Three isolates were also identified as *Legionella rowbothamii.* Adeleke et al. [77] reported on the characterization of a novel *Legionella* spp., namely *L. rowbothamii*, and to date no studies have reported on the isolation of *L. rowbothamii* from environmental samples. Moreover, no studies have indicated whether *L. rowbothamii* proliferates in protozoa. As *L. rowbothamii* was isolated during the current study, future research should thus elucidate whether *L. rowbothamii* is able to colonize and proliferate in amoeba species.

Although *Legionella* spp. were isolated from the unpasteurized tank water samples, no *Legionella* spp. were detected using the culture based methods in the pasteurized tank water samples (45 °C to 93 °C). Numerous studies have indicated that temperatures below 50 °C are not sufficient to eradicate *Legionella* spp. from water distribution systems [13, 17, 46, 69] and it is therefore unexpected that *Legionella* spp. were not isolated from particularly the 45 °C pasteurized tank water sample. However, in agreement with previous studies that have focused on the thermal inactivation of *Legionella* spp., the culturability of *Legionella* from the pasteurized tank water samples may have been affected by the heat treatment and by the nutrient shock of going from a nutrient poor environment such as rainwater, onto the nutrient rich environment provided by the media, as this induces *Legionella* cells to enter a viable but non-cultivable (VBNC) state [78, 79].

EMA-qPCR assays were then performed for all unpasteurized and tank water samples pasteurized at 68 °C, 74 °C, 84 °C and 93 °C to determine whether *Legionella* and the FLA's were viable. Although viable *V. vermiformis* was detected in all the unpasteurized tank water samples, results indicated that solar pasteurization at 74–93 °C was effective in reducing the gene copies of *V. vermiformis* to below the lower limit of detection (< 5–8 gene copies/µl). Additionally, *N. fowleri* were not detected in any of the pasteurized tank water samples and results of the current study thus indicate that the thermal treatment of tank water at 68–93 °C is sufficient for the removal of *N. fowleri* as the gene copies of viable *N. fowleri* were reduced to

below the lower limit of detection (< 12–17 gene copies/µl) for all pasteurized tank water samples. Although discrepancies, such as the presence of multicellular communities, may arise when analysing environmental samples, Fouque et al. [31] indicated that *V. vermiformis* cysts were completely inactivated at 70 °C, which is in agreement with the current study, where *V. vermiformis* was more sensitive to heat treatment than *Acanthamoeba* spp. Additionally, previous studies have indicated that *Naegleria* spp. are considered more sensitive to heat treatments compared to thermotolerant *Acanthamoeba* and *V. vermiformis* [24, 80, 81]. Results obtained in the current study do however, indicate that rainwater harvesting tanks are vulnerable to *N. fowleri* and *V. vermiformis* colonization and amoeba, including *N. fowleri* and *V. vermiformis,* should be included in the surveillance of pathogens in drinking water distribution systems [82].

Viable *Legionella* spp. and *Acanthamoeba* spp. were then detected in all the pasteurized and unpasteurized tank water samples. It has been well established that Legionellae are facultative intracellular parasites of amoeba including, *Acanthamoeba* spp., *Naegleria* spp. and *V. vermiformis* [83]. Although significant positive correlations were observed between the number of *Legionella* gene copies and the gene copies of *V. vermiformis* and *N. fowleri*, it is hypothesized that *Legionella* spp. may primarily be associating with *Acanthamoeba* spp. during thermal treatment as viable *Legionella* spp. and *Acanthamoeba* spp. persisted in all pasteurized (68 °C to 93 °C) and unpasteurized tank water samples. This is not surprising, as previous studies have detected *Legionella* at high pasteurization temperatures (> 90 °C) in solar pasteurization systems using molecular based techniques, including EMA-qPCR [13, 17]. Although this has not been demonstrated for all *Legionella* spp., *L. pneumophila* has been known to survive and proliferate on the debris of dead microbial cells such as heat-killed *Pseudomonas putida*, *E. coli*, *Bacillus subtilis*, *Lactobacillus plantarum*, *A. castellanii* and *Saccharomyces boulardii* [84]. Moreover, *Acanthamoeba* spp. are able to graze on heat killed bacteria including *E. coli* and *Klebsiella* spp. [70]. In agreement with Thomas et al. [81], it is thus hypothesized that the solar pasteurization system may be indirectly providing favourable conditions for *Legionella* and *Acanthamoeba* spp. and these organisms may thus be surviving on the dissolved organic constituents, available through the decay of the microorganisms at high pasteurization temperatures. However, while the presence of dissolved organic constituents may allow for the survival of *Legionella* spp., it is hypothesized that *Acanthamoeba* cysts may be harbouring *Legionella* and allow the *Legionella* spp. to proliferate and grow in harvested rainwater and during the treatment process. This is in agreement with Storey et al. [85] who indicated that Acanthamoebae cysts remained viable after heat treatment at 80 °C for 10 min. It is further hypothesized that during the DNA extraction process the cysts may be lysed and *Legionella* are released and detected using molecular methods including EMA-qPCR.

Additionally, the qPCR assays utilized in the current study indicated high reproducibility as the mean inter-assay CV values and SD were less than 5 % and 1 %, respectively, which were comparable to CV and SD values obtained by Ahmed et al. [65]. The current study therefore highlights the value of EMA-qPCR for the detection of viable *Legionella* and their protozoan hosts as opposed to culture based techniques that may yield false negative results [82].

2.5. Conclusions

Although incidences of Legionnaires' disease are well documented for regions including Europe, the USA, New Zealand and Australia, limited information is available on the environmental distribution of *Legionella* spp. as well as incidences of Legionnaires' disease in developing countries such as South Africa [83, 86-88]. The surveillance of *Legionella* in water distribution systems is thus vital as *Legionella* have been described as "new or emerging pathogens in drinking water" [89]. The current study demonstrated that culture-based methods for the detection of *Legionella* are less sensitive and with the use of EMA-qPCR, viable *Legionella* spp.*, Acanthamoeba* spp.*, V. vermiformis* and *N. fowleri* were detected in untreated tank water samples, while viable *Legionella* spp. (93 °C)*, Acanthamoeba* spp. (93 °C) and *V. vermiformis* (68 °C) were detected after pasteurization.

Additionally, insight into the presence and persistence of *Legionella* spp., and amoeba including *Acanthamoeba*, *V. vermiformis* and *N. fowleri* in a representative rainwater harvesting tank and a solar pasteurization treatment system was provided. The occurrence of these pathogens in harvested rainwater is of particular concern as they are frequently detected in water distribution systems and residential plumbing [45]. For example, *N. fowleri* is the causative agent of the disease, primary amoebic meningoencephalitis (PAM) and although PAM infections are rare, the mortality rate is extremely high [90].

The presence of viable *Legionella* spp. and *Acanthamoeba* spp. highlights the need for further investigation as solar pasteurization may be insufficient for the long-term control of pathogenic *Acanthamoeba* and Acanthamoebae-bound Legionellae in harvested rainwater.

Abbreviations

ACES: *N*-(2-acetamido)-2-aminoethanesulfonic acid; BCYE: buffered charcoal yeast extract; BLAST: basic local alignment search tool; BYE: buffered yeast extract; CAF: central analytical facility; CV: coefficient of variation; CYE: charcoal yeast extract; Dot/Icm: defect in organelle trafficking/intracellular multiplication; DWAF: Department of Water Affairs and Forestry; EMA: ethidium monoazide; EMA-qPCR: Ethidium monoazide quantitative polymerase chain reaction; EPF: end-point fluorescence; FLA: free-living amoebae; gDNA: genomic DNA; GHI: global

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horizontal irradiance; GVPC: glycine, vancomycin, polymyxin B and cycloheximide; NA: nutrient agar; NCBI: national centre for biotechnology information; qPCR: quantitative PCR; R2A: Reasoner´s 2A agar; RWH: rainwater harvesting; SD: standard deviation; VBNC: viable but non-culturable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions:

WK, SK and PD conceived and designed the experiments. PD performed the experiments and analyzed the data. WK and TE acquired funding for the study. WK and SK contributed

reagents/materials/analysis tools. WK and PD wrote the paper. All authors edited the drafts of the manuscript and approved the final version of the manuscript.

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Chapter 3:

(Chapter 3 is compiled in the format of the Science of the Total Environment journal and US spelling is employed)

Heat resistance of *Legionella* species and *Acanthamoeba mauritaniensis* and altered gene expression of *L. pneumophila* and *L. longbeachae* during co-culture with *A. mauritaniensis*

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Abstract

The resistance of *Legionella pneumophila* ATCC 33152, *L. longbeachae* ATCC 33462, three strains isolated from rainwater (*L. longbeachae*, *L. norrlandica* and *L. rowbothamii*) and *Acanthamoeba mauritaniensis* ATCC 50676 to heat treatment (50–90 °C) was determined using culturability and viability (EMA-qPCR) assays. The viability and expression of metabolic (*lolA*) and virulence genes (*sidF, csrA*) of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676 during heat treatment (50–90 °C) was also monitored. While the culturability and viability of the *Legionella* species was significantly ($p < 0.05$) reduced following heat treatment (60–90 °C), *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 were culturable after treatment at 50–60 °C. Dormant cysts of *A. mauritaniensis* ATCC 50676 were observed in samples treated at 60 to 90 °C, while metabolically active trophozoites were detected in samples treated at 50 °C. Relative qPCR confirmed a significant increase in the expression of *sidF* in *L. pneumophila* ATCC 33152 during co-culture with *A. mauritaniensis* ATCC 50676 ($p = 0.0006$), while the expression of *csrA* decreased. Results thus confirm that while heat treatment may reduce the number of viable *Legionella* spp., *L. pneumophila* is able to interact with *A. mauritaniensis* and persist during heat treatment.

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3.1. Introduction

In developing and arid countries, where water scarcity is a major problem, the global practice of domestic rainwater harvesting (DRWH) has become a popular alternative to provide households with a decentralized potable and non-potable water source. Domestic rainwater harvesting has thus successfully been employed worldwide in many countries including Australia (Ahmed et al., 2011), Jordan (Rabi and Abo-Shehada, 1995), Bermuda Islands (Lévesque et al., 2008) and Greece (Sazakli et al., 2007). In South Africa, DRWH tanks have also been implemented by the Department of Water Affairs (DWA) in all nine provinces as an alternative water supply and for food production (Malema et al., 2016). However, as microbial pathogens, such as virulent pathogenic *Escherichia coli* strains, *Legionella* spp., *Salmonella* spp., and adenovirus, have previously been detected in DRWH tanks, health risks are associated with the consumption of this water source and treatment is required before rainwater is utilized as a potable water resource (Spinks et al., 2006, Sazakli et al., 2007, Ahmed et al., 2012; Dobrowsky et al., 2014a; 2014b; 2015).

Dobrowsky et al., (2015) utilized a closed-coupled solar pasteurization (SOPAS) system to treat harvested rainwater. Results indicated that the level of microbial indicator bacteria heterotrophic bacteria, *E. coli* and total coliforms were reduced to below the detection limit at temperatures greater than 72 °C. A follow-up study, aimed at identifying *Legionella* spp. and possible vectors, including *Acanthamoeba* spp., *Vermamoeba vermiformis* and *Naegleria fowleri,* in the SOPAS system, was then conducted (Dobrowsky et al., 2016). Results indicated that, while high pasteurization temperatures were effective in reducing viable *N. fowleri* (5-log; gene copies/mL) and *V. vermiformis* (3-log; gene copies/mL) to below the lower limit of detection at temperatures of 68–93 °C and 74–93 °C, respectively, gene copies of viable *Legionella* and *Acanthamoeba* spp. were detected after pasteurization at 93 °C. Moreover, Reyneke et al. (2016) indicated that *Legionella* spp. may be entering a viable but non-culturable state during pasteurization, as viable *Legionella* spp. were still detected at 95 °C. This is a matter of concern as *L. pneumophila* serotype 1 is the major causative agent of Legionnaires' disease associated with water distribution systems worldwide. Moreover, other *Legionella* spp. commonly associated with disease include *L. micdadei*, *L. bozemanii*, and *L. longbeachae* (Yu et al., 2002; Zhu et al., 2011; Mercante and Winchell, 2015). While *L. pneumophila* (gene copies/mL) has been detected in harvested rainwater (Hamilton et al., 2016), in the study conducted by Dobrowsky et al. (2016) *L. longbeachae* (29/82) was the species most frequently isolated from unpasteurized harvested rainwater, followed by *L. norrlandica* (22/82) and *L. rowbothamii* (3/82).

Acanthamoeba spp. including *A. castellani*, *A. royreba* and *A. mauritaniensis* are also the etiological agents of sub-acute to chronic granulomatous amoebal encephalitis (Scheid, 2015). *Acanthamoeba* have at least two developmental stages, namely, a metabolically active trophozoite and a dormant cyst form (Rivière et al., 2006). When harsh environmental conditions arise, it is well documented that *Acanthamoeba* cysts have a thick cellulose wall and are able to protect various microorganisms from certain disinfection procedures, including heat treatment (Cordingley et al., 1996; Aksozek et al., 2002; Greub and Raoult, 2004).

Among the factors that allow *Legionella* spp. to withstand environmental stresses such as heat treatment, genera of the free living Amoebae, including *Acanthamoeba* spp. (Donlan et al., 2005) have been shown to enhance the intracellular growth and proliferation of *Legionella* spp. (Murga et al., 2001; Kuiper et al., 2004; Declerck et al., 2007). Studies have demonstrated that once ingested by amoeba, *Legionella* spp. form a specialized, endoplasmic reticulum (ER)-derived, replicative vacuole known as a *Legionella* containing vacuole within the amoeba (Rowbotham, 1980; Isberg et al., 2009). *Legionella* spp. then rely on their Dot/Icm (defective organelle trafficking/intracellular multiplication) type IV secretion system to translocate different effector proteins into host cells, some of which anchor to the *Legionella* containing vacuole by binding to phosphoinositide (PI) lipids (Hilbi et al., 2011; Haneburger and Hilbi, 2013). The effector proteins of *Legionella* spp. manipulate the host cell functions including the protozoan's phagocytic mechanisms and in this manner *Legionella* spp. alter the innate endosomal-lysosomal degradation pathway (Brüggemann et al, 2006) and avoid degradation by the amoeba (Clemens et al., 2000a; 2000b; Brüggemann et al., 2006).

The multi-component Dot/Icm secretion and virulence system of *L. pneumophila,* used to avoid the phagocytic pathway of amoeba, has been shown to be regulated at the gene expression level by the LetAS-RsmYZ-CsrA regulatory cascade and the sigma factor RpoS (Zusman et al., 2007; Rasis and Segal, 2009; Dong and Schellhorn, 2010). One of the components of the regulatory cascade, designated CsrA (carbon storage regulator A) is encoded by the *csrA* gene and is a global regulator responsible for the switch between the replicative and transmissive forms of *Legionella* spp. (reviewed in Romeo et al., 2013). In contrast, metabolically active genes are not regulated by external stimuli including LolA, encoded by *lolA*, which is a periplasmic chaperone that forms a complex with lipoproteins during their release from the inner membrane to the outer membrane in an ATP-dependent manner (Matsuyama et al., 1995; Yakushi et al., 1998). Literature pertaining to the Dot/Icm type IV secretion system of *L. pneumophila* is extensive, with more than 300 Dot/Icm substrates identified (Hubber and Roy, 2010; Zhu et al., 2011; Lifshitz et al., 2013) and the mechanisms of 15% of these substrates have been characterized (Allombert et al., 2013; Dolinsky et al., 2014). For example,

effector proteins, SidF (encoded by *sidF*) and SdhA are involved in inhibiting host cell death (Laguna et al., 2006; Banga et al., 2007) while RalF, LidA, DrrA/SidM and SidJ manipulate host cell vesicular trafficking (Nagai et al., 2002; Machner and Isberg, 2006; Murata et al., 2006; Liu and Luo, 2007). In contrast, while information regarding the formation of *Legionella* containing vacuoles by *L. longbeachae* is limited, studies have indicated that the process appears to differ between the two *Legionella* spp. (Asare and Abu Kwaik, 2007). Although none of the effector proteins have been mechanistically characterized to date, *L. longbeachae* is predicted to produce more than 110 Dot/Icm substrates (Cazalet et al., 2010, Lifshitz et al., 2013). Very few of these effectors are conserved between *L. pneumophila* and *L. longbeachae,* and research has indicated that more than 66 effectors are unique to *L. pneumophila* (Dolinsky et al., 2014). In addition, *L. longbeachae* harbors 50 novel Dot/Icm substrates that have been identified (Dolinsky et al., 2014).

Detailed understanding of how *Legionella* spp. survive heat treatment and manipulate host cell functions on the mechanistic level is still largely unknown. The aim of the current study was thus to determine the culturability and viability of two *Legionella* reference strains (*L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462), three *Legionella* environmental strains [*L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.)] isolated from harvested rainwater and *Acanthamoeba mauritaniensis* ATCC 50676 following heat treatment (50–90 °C). The viability of *L. pneumophila* ATCC 33152, *L. longbeachae* ATCC 33462, *L. longbeachae* (env.), *L. norrlandica* (env.), *L. rowbothamii* (env.) and *A. mauritaniensis* ATCC 50676, before and after heat treatment (50-90 °C), was determined using ethidium monoazide (EMA) quantitative polymerase chain reaction (EMA-qPCR). In addition, as *Acanthamoeba* and *Legionella* spp. have previously been detected at high SOPAS temperatures (Dobrowsky et al., 2016), *L. pneumophila* ATCC 33152 and *L. longbeachae* [environmental strain (env.) isolated from harvested rainwater] were co-cultured with *A. mauritaniensis* ATCC 50676 and were subsequently heat treated. The transcriptional responses of genes associated with metabolism and virulence of *L. pneumophila* ATCC 33152 (*lolA*, *sidF*, *csrA*) and *L. longbeachae* (env.) (*lolA*) during infection and heat treatment (50–90 °C) were determined by performing relative qPCR on cDNA transcribed from isolated RNA. The *lolA* gene was selected for the current study as an indicator of metabolically active cells as the *lolA* gene encodes for a molecular chaperone involved in recruiting lipoproteins to the outer membrane of the *Legionella* containing vacuole (Tanaka et al., 2001). The *csrA* gene encodes CsrA, a global regulator responsible for the switch between the replicative and transmissive forms of *L. pneumophila* (Romeo et al., 2013). The expression of *crsA* was not evaluated for *L. longbeachae* (env.) as the ability of *L. longbeachae* to replicate intracellularly is independent of the exponential and stationary bacterial growth phase (Cazalet

et al., 2010). The SidF protein (encoded by *sidF*) is a type IVB Dot/Icm secretion system effector (Hsu et al., 2012). During absolute quantification the exact copy concentration of the target gene was calculated by relating the C_q value to a generated standard curve. Relative quantification determined the amount of a target gene in a sample relative to a calibrator sample (a constant ratio of both target and reference genes) (Yu et al., 2005).

3.2. Materials and Methods

3.2.1. Enumeration of *Legionella* spp. before and after heat treatment

Legionella pneumophila ATCC 33152 and *L. longbeachae* ATCC 33462 were obtained from Microbiologics® (St. Cloud, Minnesota, USA). Additionally, three environmental *Legionella* spp., previously isolated from a rainwater harvesting tank installed at Welgevallen experimental farm (33°56′36.19″S, 18°52′6.08″E), Stellenbosch University, Western Cape, South Africa, were utilized in the current study (Dobrowsky et al., 2016). These environmental strains were identified using sequencing analysis as *L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.) (Dobrowsky et al., 2016). All *Legionella* isolates were cultured on buffered charcoal yeast extract (BCYE) [CYE agar base supplemented with ACES buffer/potassium hydroxide (1.0 g/L), ferric pyrophosphate (0.025 g/L), alpha-ketoglutarate (0.10 g/L) and *L*-cysteine HCL (0.04 g/L)] (Oxoid, Hampshire, England). Cultures were incubated at 35 °C for 72 hours.

To obtain liquid cultures for subsequent heat treatment assays, single colonies of the respective *Legionella* strains were inoculated from the BCYE plates into 50 mL Lennox Broth [tryptone (10 g/L); yeast extract (5 g/L); NaCl (5 g/L)] supplemented with ACES buffer/potassium hydroxide (1.0 g/L), ferric pyrophosphate (0.025 g/L), alpha-ketoglutarate (0.10 g/L) and *L*-cysteine HCL (0.04 g/L) (Oxoid, Hampshire, England) according to Delgado-Viscogliosi et al. (2009). Cultures were grown at 37 °C on an orbital shaker for 5 days. Bacterial cultures were grown to an OD_{660} of 0.2 to 0.4, determined by measuring the optical density at 660 nm (OD_{660}) with a T60 UV Visible Spectrophotometer (PG Instruments Limited, Beijing China) according to Aurass et al. (2009). Six milliliter aliquots of the *Legionella* spp. cultures where then pipetted into test tubes which were subsequently heat treated for 30 min at each temperature (50 °C, 60 °C, 70 °C, 80 °C and 90 °C) in a recirculating water bath (FMH instrument, supplied by Labotec, Johannesburg, South Africa). Untreated bacterial cultures (6 mL) were included in all assays as positive controls, while untreated bacterial cultures (6 mL) that were autoclaved at 121 °C for 20 min were included as negative controls in all assays.

Following heat treatment, to determine the culturability of *L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462 and the three environmental spp. including *L. longbeachae*,

L. norrlandica and *L. rowbothamii*, 1 mL of each treated and autoclaved cell culture was centrifuged (16 000 × g for 10 min) (Boulanger and Edelstein, 1995). The pellet was resuspended in 100 µL cell culture and spread plated onto BCYE agar. This was performed in duplicate. For all untreated (positive control) *Legionella* cultures, a serial dilution (10^{-3}–10^{-5}) was prepared, after which 100 µL was spread plated onto BCYE agar in duplicate. All plates were then incubated at 35 °C for approximately 10 days.

3.2.2. Enumeration of *Acanthamoeba mauritaniensis* ATCC 50676 before and after heat treatment

Acanthamoeba cultures were obtained from the University of the Witwatersrand, Johannesburg. The isolates (*Acanthamoeba* cysts) were preserved on sections of non-nutrient agar (NNA) and stored in parafilm sealed bottles. The isolates had previously been isolated by Niszl (2011) and included *Acanthamoeba mauritaniensis* ATCC 50676 that was isolated from the eyes of patients with Acanthamoeba Keratitis (AK). These isolates had all previously been identified as *Acanthamoeba mauritaniensis* (www.atcc/org) and a multi-locus sequencing strategy was utilized by Schroeder et al. (2001) to determine the genotypes of each isolate.

For the revival and culturing of *A. mauritaniensis* ATCC 50676, Erlenmeyer flasks containing 250 mL Page Amoeba Saline (PAS; Page, 1976) and heat killed *E. coli* ATCC 417561 [cultured in Luria Bertani (LB; Merck, Damstadt, Germany) broth for 12 hours and heat treated for 30 minutes at 80 °C] were inoculated with a segment (approximately 0.5 x 0.5 cm) of the NNA containing the *Acanthamoeba* cysts. The cultures were then incubated at 30 °C without shaking for three weeks. Page Amoeba Saline [NaCl (0.12 g/L), MgSO$_4$.7H$_2$O (0.004 g/L), CaCl$_2$.2H$_2$O (0.004 g/L), Na$_2$HPO$_4$ (0.142 g/L), KH$_2$PO$_4$ (0.136 g/L)] contains minimal nutrients and thus inhibits the growth of undesirable contaminating organisms (Khan et al., 2002).

Acanthamoeba mauritaniensis ATCC 50676 (cultured in PAS) was then inoculated into Erlenmeyer flasks containing 70 mL of peptone-yeast extract glucose (PYG) [NaCl (1 g/L), K$_2$HPO$_4$ (1 g/L), MgSO$_4$ (1 g/L), CaCl$_2$ (1 g/L), glucose (10 g/L), proteose-peptone (7.5 g/L), yeast extract (2.5 g/L)] supplemented with 100 µg ampicillin/mL and 100 µg streptomycin sulfate/mL. All flasks were incubated at 30 °C without shaking for five days (Gao et al., 1997; Buerano et al., 2014). As previously indicated by La Scola et al. (2001), the doubling time of this organism under these conditions is approximately 24 hours. After 5 days, and once a concentration of 1 × 10^6 amoeba cells was obtained (quantified using a haemocytometer), 9 mL aliquots of the *A. mauritaniensis* ATCC 50676 cultures were pipetted into test tubes which were subsequently heat treated for 30 min at each temperature (50 °C, 60 °C, 70 °C, 80 °C and 90 °C) in a recirculating water bath (FMH instrument, supplied by Labotec, Johannesburg,

South Africa). Untreated *A. mauritaniensis* ATCC 50676 cultures (9 mL) were included in all assays as positive controls. In addition, untreated cultures (9 mL) of *A. mauritaniensis* were autoclaved at 121 °C for 20 min and were included as negative controls. All heat treatment assays were performed in triplicate.

For the enumeration of *A. mauritaniensis* ATCC 50676 before and after heat treatment, 1 mL, 100 μL and 10 μL of each sample including the untreated (positive control), heat treated and autoclaved (negative control) sample, were inoculated onto NNA plates seeded with heat-killed *E. coli* in quintuplicate. All plates were then incubated at 30 °C for approximately 10 days and the most probable number (MPN) method was utilized to quantify *A. mauritaniensis* ATCC 50676 according to Beattie et al. (2003). All plates were examined using a light microscope (Nikon Eclipse E200, Nikon, Japan) and a plate showing *A. mauritaniensis* trophozoites and cysts was scored as 1 while no growth was scored as 0.

3.2.3. Co-cultures of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) with *A. mauritaniensis* ATCC 50676

Acanthamoeba mauritaniensis ATCC 50676 was inoculated into Erlenmeyer flasks containing 80 mL PYG media (with no antibiotics). The flasks were incubated for five days in the dark at 30 °C with no shaking. *Legionella pneumophila* ATCC 33152 and *L. longbeachae* (env.) were inoculated into 10 mL Lennox broth and incubated at 35 °C for 72 hours. The *A. mauritaniensis* ATCC 50676 cultures (1 × 10^5 amoeba cells) were then inoculated with 10 mL *L. pneumophila* ATCC 33152 (3 × 10^5 CFU/mL) and 10 mL *L. longbeachae* (env.) (5 × 10^5 CFU/mL) respectively, to achieve a multiplicity of infection (MOI) of 30 (*L. pneumophila*) and 50 [*L. longbeachae* (env.)], respectively (García et al., 2007). For infection to occur, all samples were incubated at 30 °C for 24 hours (Dupuy et al., 2011). Thirteen milliliter aliquots of the co-cultures where then pipetted into test tubes which were subsequently heat treated for 30 min at each temperature (50 °C, 60 °C, 70 °C, 80 °C and 90 °C) in a recirculating water bath (FMH instrument, supplied by Labotec, Johannesburg, South Africa). As the co-cultures contained both *Legionella* that had infected the trophozoites, as well as free *Legionella*, 50 μg/mL gentamicin (Sigma–Aldrich Inc., USA) was added to the cultures which were then incubated for 1 h at 37 °C to kill extracellular bacteria (García et al., 2007). The antibiotic was then removed by washing three times by centrifugation (500 × g, 15 min) with phosphate buffer (50 mM, pH 8) after which the pellet was resuspended in phosphate buffer (50 mM, pH 8).

3.2.4. Ethidium monoazide (EMA) treatment and total DNA extractions from *Legionella* spp. and *A. mauritaniensis* ATCC 50676 cultures

Total DNA was isolated from the positive control, negative control and heat treated (50–90 °C) cultures of *L. pneumophila* ATCC 33152, *L. longbeachae* ATCC 33462, *L. longbeachae* (env.), *L. norrlandica* (env.), *L. rowbothamii* (env.) and *A. mauritaniensis* ATCC 50676. In addition, total DNA was isolated from *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676 before and after each heat treatment (50–90 °C). For this, 2 mL of each culture was centrifuged (16 000 × g for 10 min) after which the pelleted sample was resuspended in 1 mL sterile MilliQ water before EMA (2.5 µg/mL; Biotium, Inc., California) was added to the 1 mL of concentrated sample according to Delgado-Viscogliosi et al. (2009) and Chang et al. (2013).

The EMA was then cross linked to the naked DNA as outlined in Delgado-Viscogliosi et al. (2009) and Dobrowsky et al. (2016). The extraction of total gDNA was subsequently performed using the Soil Microbe DNA MiniPrep™ Kit (Zymo Research, Irvine, USA) according to manufacturer's instructions.

3.2.5. Total RNA extraction

Ribonucleic acid was extracted from 2 mL of untreated and heat treated (50–90 °C) samples of *L. pneumophila* ATCC 33152, *L. longbeachae* (env.) and co-cultures containing *A. mauritaniensis* ATCC 50676 with *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.), respectively, using TRI reagent (Sigma–Aldrich Inc., USA) according to the manufacturer's instructions. Ribonucleic acid concentration and purity were determined spectrophotometrically at 260 and 280 nm (Packer and Sen, 2002; Tay et al., 2010). Subsequently 2 µg of total RNA was treated with DNAse (Fermentas, Thermo Scientific Inc., USA) and transcribed into cDNA using the Imprompt II reverse transcriptase (Promega, Madison, USA) kit and oligo dT primer as described by the manufacturer.

A no template control and an additional no reverse transcriptase control were included to confirm complete removal of contaminating genomic DNA from each sample. This control contained all the Imprompt II reverse transcriptase (Promega, Madison, USA) reagents, with the exception of the reverse transcriptase enzyme (Leal et al., 2015). Two microliters of each no reverse transcriptase control was added to the respective quantitative PCR (qPCR) assay as described in section 2.6 and no signal was generated after amplification for these control samples.

3.2.6. Absolute and relative qPCR assays

All absolute and relative qPCR assays were performed on a LightCycler® 96 (Roche Applied Science, Mannheim, Germany). To a final reaction volume of 20 µL, the following were added: 10 µL FastStart Essential DNA Green Master Mix (2X; Roche Applied Science, Mannheim, Germany), 5 µL template DNA [gDNA for absolute qPCR and cDNA (5 ng/µL) for relative qPCR], and 0.4 µL of each primer (10 µM), as previously described by Dobrowsky et al. (2016). All primer sequences used in this study are listed in **Table 3.1**.

For the absolute quantification of *Legionella* spp. before and after heat treatment, the primers Leg F and Leg R were utilized to amplify a 259 bp product of the 23S–5S rRNA gene according to Herpers et al. (2003). The amplification conditions for the quantification of *Legionella* spp. were as follows: 95 °C (1 min) followed by 45 cycles of 95 °C (15 s), 60 °C (15 s) and 72 °C (11 s). The standard curve for the quantification of *Legionella* spp., was generated as outlined by Dobrowsky et al. (2016).

For the absolute quantification of *A. mauritaniensis* ATCC 50676 in untreated and heat treated samples, the primers AcantF900 and AcantR1100 were utilized to amplify a 180 bp product of the 18S rRNA gene as previously described by Qvarnstrom et al. (2006). The amplification conditions for the quantification of *A. mauritaniensis* ATCC 50676 were as follows: 95 °C (1 min) followed by 45 cycles of 95 °C (15 s), 60 °C (1 min) and 72 °C (40 s). The standard curve for the quantification of *A. mauritaniensis* ATCC 50676 was generated as outlined by Dobrowsky et al. (2016).

For the relative quantification of the *csrA, lolA* and *sidF of L. pneumophila* (*csrA, lolA, sidF*) and *L. longbeachae* (env.) (*lolA*) before and after heat treatment, the primers described in **Table 3.1** were utilized. The primers lolAFL and lolARL (**Table 3.1**) were designed using DNAman™ (version 4.1.2.1; Lynnon Biosoft, CA, USA) software to amplify a 213 bp region of the *lolA* gene of *L. longbeachae*. The amplification conditions for relative qPCR assays using the csrAF/csrAR, lolAF/lolAR, lolAFL/lolARL, sidF/sidFR and rpsLF/rpsLR primer sets were as follows: 95 °C (10 min) followed by 40 cycles of 95 °C (15 s) at, 64 °C (30 s) and 72 °C (30 s).

The *rpsL* gene was utilized as a reference gene as it is a single-copy housekeeping gene (Schroeder et al., 2010; Buse et al., 2015). Moreover, the specificity and detection limits of this assay have been described previously (Fettes et al., 2001; Lu et al., 2013; Buse et al., 2015). The quantitation cycle (C_q) values of the reference gene, *rpsL*, were utilized to normalize the calculated C_q values of the target genes amplified from the corresponding samples (ΔC_q), and the fold change ($2^{-\Delta\Delta C_q}$) compared to the control were calculated (Livak and Schmittgen, 2001).

Table 3.1. Primers and qPCR cycling parameters utilized in the current study

Organism (qPCR Assay)	Primer name	Primer sequence (5'- 3')	Target Gene	References
Legionella spp. (Absolute quantification)	Leg F	CTAATTGGCTGATTGTCTTGAC	23S–5S rRNA	Herpers et al. (2003)
	Leg R	CAATCGGAGTTCTTCGTG		
Acanthamoeba mauritaniensis ATCC 50676 (Absolute quantification)	AcantF900	CCCAGATCGTTTACCGTGAA	16S rRNA	Qvarnstrom et al. (2006)
	AcantR1100	TAAATATTAATGCCCCCAACTATCC		
L. pneumophila (Relative quantification)	csrAF	TTGATTTTGACTCGGCGTATAG	*csrA*	Fettes et al. (2001)
	csrAR	GATTCTTTTTCTTGTTGTATGCGTA		
	lolAF	GCCGAAGTACTGCAGAGTAAA	*lolA*	Buse et al. (2015)
	lolAR	AATGCCATGGAACCTGAAGA		
	sidFF	ATTGTTCGCGAGGGTATGAAAGCG	*sidF*	Faucher et al. (2011)
	sidFR	TCTTTCCAAGACAGACTCTCGCGT		
L. longbeachae (env.) (Relative quantification)	lolAFL	CAGTAGTCACTTGCCCTAATTG	*lolA*	This Study
	lolARL	GTTTAGGAGGTACTGCTGC		
Legionella spp. (Relative quantification)	rpsLF	GAAAGCCTCGTGTGGACGTA	*rpsL*	Lu et al. (2013)
	rpsLR	CAACCTTACGCATAGCTGAGTTA		

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Melt curve analysis was included for all SYBR green real-time PCR assays in order to verify specificity of the primer set by ramping the temperature from 65 to 97 °C at a rate of 0.2 °C/s with continuous fluorescent signal acquisition at 5 readings/ °C (Dobrowsky et al., 2016). Samples expressing end-point fluorescence (EPF) values of less than 3.15 were diluted (10X) and the qPCR experiment was repeated for these samples. The minimum number of gene copies (highest dilution) that could be measured accurately within an assay was considered the Lower Limit of Detection (LLOD) for each organism (Bustin et al., 2009).

3.2.7. Heat treatment efficiency

The heat treatment efficiency was calculated by comparing data (CFU/mL, gene copies/mL, MPN) obtained from the samples before heat treatment to the samples after heat treatment. The log reduction was calculated using equation 1 according to Brözel and Cloete (1991).

Equation 1:

Log reduction = (Log$_{10}$ bacterial count $_{before\ pasteurization}$ – Log $_{10}$ bacterial count $_{after\ pasteurization}$)

3.2.8. Statistical analysis

The gene copies of *Legionella* spp., and *Acanthamoeba* spp. obtained for the heat treated and untreated samples (positive controls) were assessed for nonparametric differences using the Mann-Whitney U Test. While the statistical significance of the relative qPCR data set was determined using two-way analysis of variance (ANOVA) with Bonferroni test correction for multiple comparisons. Significance was set at a *p* value of ≤ 0.05 for all statistical analyses performed.

3.3. Results

3.3.1. Culturability of *Legionella* spp. and *A. mauritaniensis* ATCC 50676 before and after heat treatment

The culturability (CFU/mL) of three environmental *Legionella* spp., previously identified as *L. norrlandica* (env.)*, L. rowbothamii* (env.) and *L. longbeachae* (env.), and the reference strains *L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462 (obtained from Microbiologics®) was analyzed before and after heat treatment (**Fig. 3.1**). In addition, the number of cysts and trophozoites of *A. mauritaniensis* ATCC 50676 was determined before and after heat treatment using the MPN method (**Appendix A, Figure 1**). Untreated cultures of each of the five *Legionella* spp. and *A. mauritaniensis* ATCC 50676 were included in all assays as positive controls while autoclaved cultures were included as negative controls for all

experiments. All the *Legionella* spp. and *A. mauritaniensis* ATCC 50676 were reduced to below the detection limit (*Legionella* spp.: <1 CFU/mL; *A. mauritaniensis* ATCC 50676; <1 MPN/mL) in the autoclaved samples.

As indicated in **Fig. 3.1**, the mean CFU/mL for *L. norrlandica* (env.) decreased significantly ($p = 0.0171$) from 2.3×10^5 to 1.7×10^3 CFU/mL (2.1-log) following heat treatment at 50 °C. Similarly, the mean CFU/mL of *Legionella rowbothamii* (env.) decreased significantly ($p = 0.0001$) from 2.2×10^3 to 3.5×10^1 CFU/mL (1.8-log). In contrast, while still significant ($p = 0.0202$), the mean CFU/mL for *L. longbeachae* ATCC 33462 only decreased from 4.0×10^5 to 6.9×10^4 CFU/mL (0.8-log) after heat treatment at 50 °C. However, after heat treatment at 60–90 °C, the mean CFU/mL of *L. longbeachae* ATCC 33462 (5.6-log), *L. norrlandica* (env.) (5.4-log) and *L. rowbothamii* (env.) (3.3-log) was reduced to below the detection limit (<1 CFU/mL).

Legionella longbeachae (env.) and *L. pneumophila* ATCC 33152 were the most resistant to heat treatment as both organisms were still culturable following treatment at 50 and 60 °C (**Fig. 3.1**). The mean CFU/mL of *L. longbeachae* (env.) decreased significantly from 2.7×10^5 to 2.4×10^4 CFU/mL (1.1-log) at 50 °C ($p = 0.0068$), and then decreased further to 2.4×10^1 CFU/mL (4.1-log) at 60 °C ($p = 0.0091$). The mean CFU/mL of *L. pneumophila* ATCC 33152 decreased from 8.7×10^5 to 8.6×10^4 CFU/mL (1-log) at 50 °C ($p = 0.0254$), and then to 2.0×10^2 CFU/mL (3.7-log) at 60 °C ($p = 0.0085$). After heat treatment at 70–90 °C, the mean CFU/mL of *L. longbeachae* (env.) (5.4-log) and *L. pneumophila* ATCC 33152 (5.9-log) were reduced to below the detection limit (<1 CFU/mL).

The MPN method was used to enumerate the number of *A. mauritaniensis* ATCC 50676 trophozoites and cysts before and after heat treatment (**Appendix A, Figure 1**). Results indicated that *A. mauritaniensis* ATCC 50676 was not significantly ($p > 0.05$) reduced after heat treatment at 50 °C as >1.8×10^4 MPN/mL was recorded before and after heat treatment at 50 °C. However, following treatment at 60 °C to 90 °C the number of *A. mauritaniensis* ATCC 50676 decreased significantly from >1.8×10^4 to 1.2×10^3 MPN/mL (1.2-log) at 60 °C ($p = 0.0003$), to 2.8×10^2 MPN/mL (1.8-log) at 70 °C ($p = 0.000013$), to 1.1×10^2 (2.2-log) at 80 °C ($p = 0.000003$) and to 2.7×10^1 (2.8-log) at 90 °C ($p = 0.0000001$). It should be noted that while trophozoites of *A. mauritaniensis* ATCC 50676 were observed for the untreated and treated samples at 50 °C, only cysts were observed for samples treated at 60 to 90 °C.

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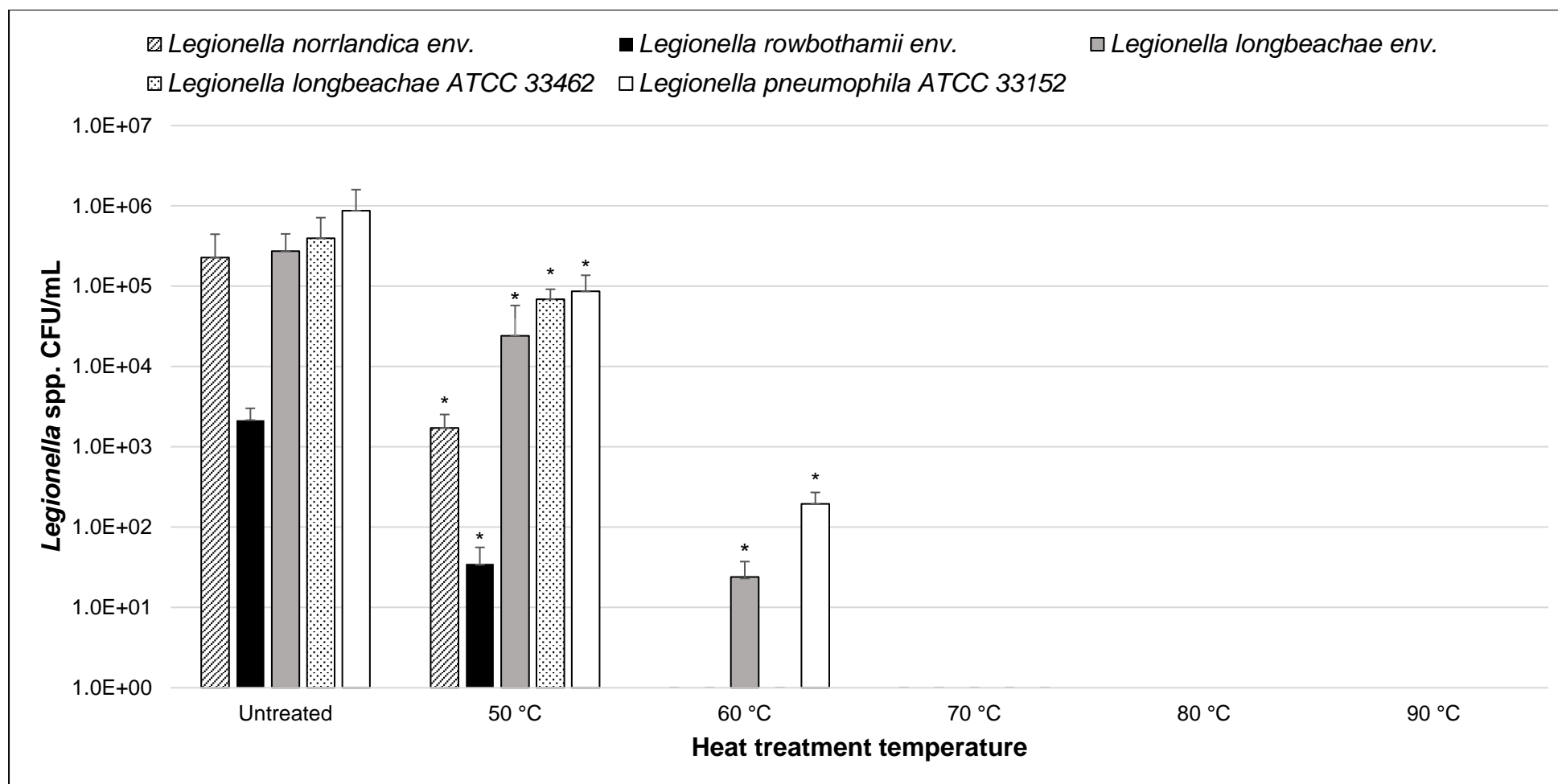
Fig. 3.1. Enumeration of *L. norrlandica* (env.)*, L. rowbothamii* (env.)*, L. longbeachae* (env.), *L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462 before (untreated positive control) and after heat treatment (50–90°C). The data are expressed as the mean of total CFU/mL and error bars indicate standard deviation (n = 3). * denotes $p < 0.05$.

3.3.2. Absolute quantification of all viable *Legionella* spp., *A. mauritaniensis* ATCC 50676 and *L. pneumophila* ATCC 33152 and *L. longbeachae* (environmental strain) during co-culture with *A. mauritaniensis* ATCC 50676

Gene copies of viable *L. norrlandica* (env.), *L. rowbothamii* (env.), *L. longbeachae* (env.), *L. pneumophila* ATCC 33152, *L. longbeachae* ATCC 33462 and *A. mauritaniensis* ATCC 50676 were quantified before and after heat treatment (50 °C, 60 °C, 70 °C, 80 °C and 90 °C) using EMA-qPCR. In addition, as *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) were detected following heat treatment at 60 °C using culture based assays, *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) were co-cultured with *A. mauritaniensis* ATCC 50676 and subsequently heat treated. The gene copies of viable *L. pneumophila* ATCC 33152, *L. longbeachae* (env.) and *A. mauritaniensis* ATCC 50676 in co-culture before and after heat treatment (50–90 °C) were then also quantified using EMA-qPCR.

A linear range of quantification from 10^9 to 10^0 gene copies per µL of DNA extracts was observed for the standard curves. For the quantification of *Legionella* spp., the qPCR assays had amplification efficiencies that ranged from 1.94 (94%) to 1.98 (98%) with errors of 0.29 and 0.32, respectively (**Appendix A, Figure 2A and 2B**). For the quantification of *A. mauritaniensis* ATCC 50676, the qPCR assays had amplification efficiencies that ranged from 1.99 (99%) to 2.02 (102%) with errors of 0.41 and 0.31, respectively (**Appendix A, Figure 3A and 3B**). For all absolute qPCR assays the correlation coefficient (r^2) was measured at 1.00. The qPCR LLOD was recorded at 6.27 to 8.93 gene copies/µL for *Legionella* spp. and 1.02 to 1.30 gene copies/µL for *Acanthamoeba* spp. It should also be noted that all *Legionella* spp. (<6.27 to 8.93 gene copies/µL) and *A. mauritaniensis* ATCC 50676 (<1.02 to 1.30 gene copies/µL) were reduced to below the LLOD for the autoclaved negative control samples.

Before heat treatment the mean gene copies/mL of *L. norrlandica* (env.) were recorded at 3.26×10^7 gene copies/mL (**Appendix A, Figure 4A**). Following heat treatment at 50 °C (1.6×10^7 gene copies/mL), 60 °C (6.8×10^6 gene copies/mL) and 70 °C (4.4×10^6 gene copies/mL) the mean number of gene copies/mL of *L. norrlandica* (env.) did not decrease significantly ($p > 0.05$), with a 0.3-log ($p = 0.936$), 0.7-log ($p = 0.379$) and 0.9-log ($p = 0.129$) reduction in gene copies/mL observed, respectively. A significant reduction in gene copies/mL of 1.49-log ($p = 0.005$) and 1.50-log ($p = 0.005$) was then obtained following heat treatment of *L. norrlandica* (env.) at 80 °C (1.05×10^6 gene copies/mL) and 90 °C (1.03×10^6 gene copies/mL), respectively.

The mean gene copies/mL of *L. rowbothamii* (env.) were recorded at 7.6×10^8 gene copies/mL before heat treatment (**Appendix A, Figure 4B**). Following heat treatment at 50 °C

$(3.0 \times 10^8$ gene copies/mL) and 60 °C $(1.7 \times 10^8$ gene copies/mL) the mean number of gene copies/mL of *L. rowbothamii* (env.) did not decrease significantly ($p > 0.05$), with a 0.4-log ($p = 0.230$) and 0.7-log ($p = 0.129$) reduction in gene copies/mL observed, respectively. A significant reduction in gene copies/mL of 1.2-log ($p = 0.005$), 1.4-log ($p = 0.005$) and 2.2-log ($p = 0.005$) was then obtained following heat treatment of *L. rowbothamii* (env.) at 70 °C $(4.5 \times 10^7$ gene copies/mL), 80 °C $(3.2 \times 10^7$ gene copies/mL) and 90 °C $(3.3 \times 10^6$ gene copies/mL), respectively.

Before heat treatment the mean gene copies/mL of *L. longbeachae* (env.) were recorded at 5.2×10^7 gene copies/mL (**Appendix A, Figure 4C**). Following heat treatment at 50 °C $(3.1 \times 10^7$ gene copies/mL), 60 °C $(1.1 \times 10^7$ gene copies/mL) and 70 °C $(5.1 \times 10^6$ gene copies/mL) the mean number of gene copies/mL of *L. longbeachae* (env.) did not decrease significantly ($p > 0.05$), however a 0.2-log ($p = 0.667$), 0.7-log ($p = 0.575$) and 1.0-log ($p = 0.374$) reduction in gene copies/mL was observed, respectively. A significant reduction in gene copies/mL of 1.7-log ($p = 0.031$) and 2.6-log ($p = 0.005$) was obtained following heat treatment of *L. longbeachae* (env.) at 80 °C $(1.2 \times 10^6$ gene copies/mL) and 90 °C $(1.4 \times 10^5$ gene copies/mL), respectively.

The mean gene copies/mL of *L. longbeachae* ATCC 33462 were recorded at 3.2×10^8 gene copies/mL before heat treatment (**Appendix A, Figure 5A**). Following heat treatment at 50 °C $(8.1 \times 10^7$ gene copies/mL), 60 °C $(4.3 \times 10^7$ gene copies/mL) and 70 °C $(2.8 \times 10^7$ gene copies/mL) the mean number of gene copies/mL of *L. longbeachae* ATCC 33462 did not decrease significantly ($p > 0.05$), with a 0.6-log ($p = 0.129$), 0.9-log ($p = 0.118$) and 1.1-log ($p = 0.066$) reduction in gene copies/mL observed, respectively. A significant reduction of 2.1-log ($p = 0.005$) and 3.4-log ($p = 0.005$) in gene copies/mL was then obtained following heat treatment of *L. longbeachae* ATCC 33462 at 80 °C $(2.5 \times 10^6$ gene copies/mL) and 90 °C $(1.2 \times 10^5$ gene copies/mL), respectively.

Before the heat treatment of *L. pneumophila* ATCC 33152 the mean gene copies/mL were recorded at 7.3×10^9 gene copies/mL (**Appendix A, Figure 5B**). Following heat treatment, a significant reduction of 0.9-log ($p = 0.008$), 1.0-log ($p = 0.005$), 1.3-log ($p = 0.005$), 1.7-log ($p = 0.005$), 3.6-log ($p = 0.005$) was obtained for *L. pneumophila* ATCC 33152 at 50 °C $(9.3 \times 10^8$ gene copies/mL), 60 °C $(7.2 \times 10^8$ gene copies/mL), 70 °C $(3.3 \times 10^8$ gene copies/mL), 80 °C $(1.4 \times 10^8$ gene copies/mL) and 90 °C $(2.0 \times 10^6$ gene copies/mL), respectively.

Legionella longbeachae (env.) was then co-cultured with *A. mauritaniensis* ATCC 50676, where after the co-culture was subjected to heat treatments for 30 min at temperatures including,

50 °C, 60 °C, 70 °C, 80 °C and 90 °C (**Appendix A, Figure 6A**). Before heat treatment of co-cultured *L. longbeachae* (env.) the mean gene copies/mL were recorded at 2.1×10^5 gene copies/mL. Following heat treatment at 50 °C (1.1×10^5 gene copies/mL) the mean number of gene copies/mL of co-cultured *L. longbeachae* (env.) did not decrease significantly ($p < 0.05$) and a 0.3-log ($p = 0.1282$) reduction in gene copies/mL was observed. However, a significant reduction of 1.0-log ($p = 0.0121$) and 1.1-log ($p = 0.005$) was obtained following heat treatment of co-cultured *L. longbeachae* (env.) at 60 °C (2.1×10^4 gene copies/mL) and 70 °C (1.9×10^4 gene copies/mL), respectively. Following heat treatment of co-cultured *L. longbeachae* (env.) at 80 °C and 90 °C the mean gene copies/mL were reduced to below the LLOD (< 6.27 to 8.93 gene copies/µL).

Legionella pneumophila ATCC 33152 was also co-cultured with *A. mauritaniensis* ATCC 50676, where after the co-culture was subjected to heat treatments at 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 30 min, respectively (**Appendix A, Figure 6B**). Before the heat treatment of co-cultured *L. pneumophila* ATCC 33152 the mean gene copies/mL were recorded at 1.7×10^6 gene copies/mL. Following heat treatment at 50 °C (1.3×10^6 gene copies/mL), 60 °C (1.2×10^6 gene copies/mL) and 70 °C (7.5×10^5 gene copies/mL) the mean number of gene copies/mL of *L. pneumophila* ATCC 33152 did not decrease significantly ($p > 0.05$), however a 0.1-log ($p = 0.8105$), 0.2-log ($p = 0.5755$) and 0.4-log ($p = 0.2678$) reduction in gene copies/mL was observed, respectively. Conversely, a significant reduction of 0.8-log ($p = 0.0308$) and 1.7-log ($p = 0.005$) was obtained following heat treatment of *L. pneumophila* ATCC 33152 at 80 °C (2.6×10^5 gene copies/mL) and 90 °C (3.3×10^4 gene copies/mL), respectively.

The gene copies/mL of *A. mauritaniensis* ATCC 50676 was also determined before and after heat treatment (50–90 °C) using EMA-qPCR. In addition, the gene copies/mL of *A. mauritaniensis* ATCC 50676 in co-culture with *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.), respectively, were also determined before and after heat treatment (**Fig. 3.2**).

Before the heat treatment of *A. mauritaniensis* ATCC 50676 the mean gene copies/mL were recorded at 3.0×10^7 gene copies/mL (**Fig. 3.2**). Following heat treatment at 50 °C (2.3×10^7 gene copies/mL) and 60 °C (1.8×10^7 gene copies/mL) the mean number of gene copies/mL of *A. mauritaniensis* ATCC 50676 did not decrease significantly ($p > 0.05$), however a 0.1-log ($p = 0.2301$) and 0.2-log ($p = 0.1285$) reduction in gene copies/mL was observed, respectively. A significant reduction in gene copies/mL of 0.7-log ($p = 0.0307$), 1.1-log ($p = 0.005$) and 1.2-log ($p = 0.005$) was then obtained following heat treatment of *A. mauritaniensis* ATCC 50676 at 70 °C (6.0×10^6 gene copies/mL), 80 °C (2.5×10^6 gene copies/mL) and 90 °C (1.7×10^6 gene copies/mL), respectively.
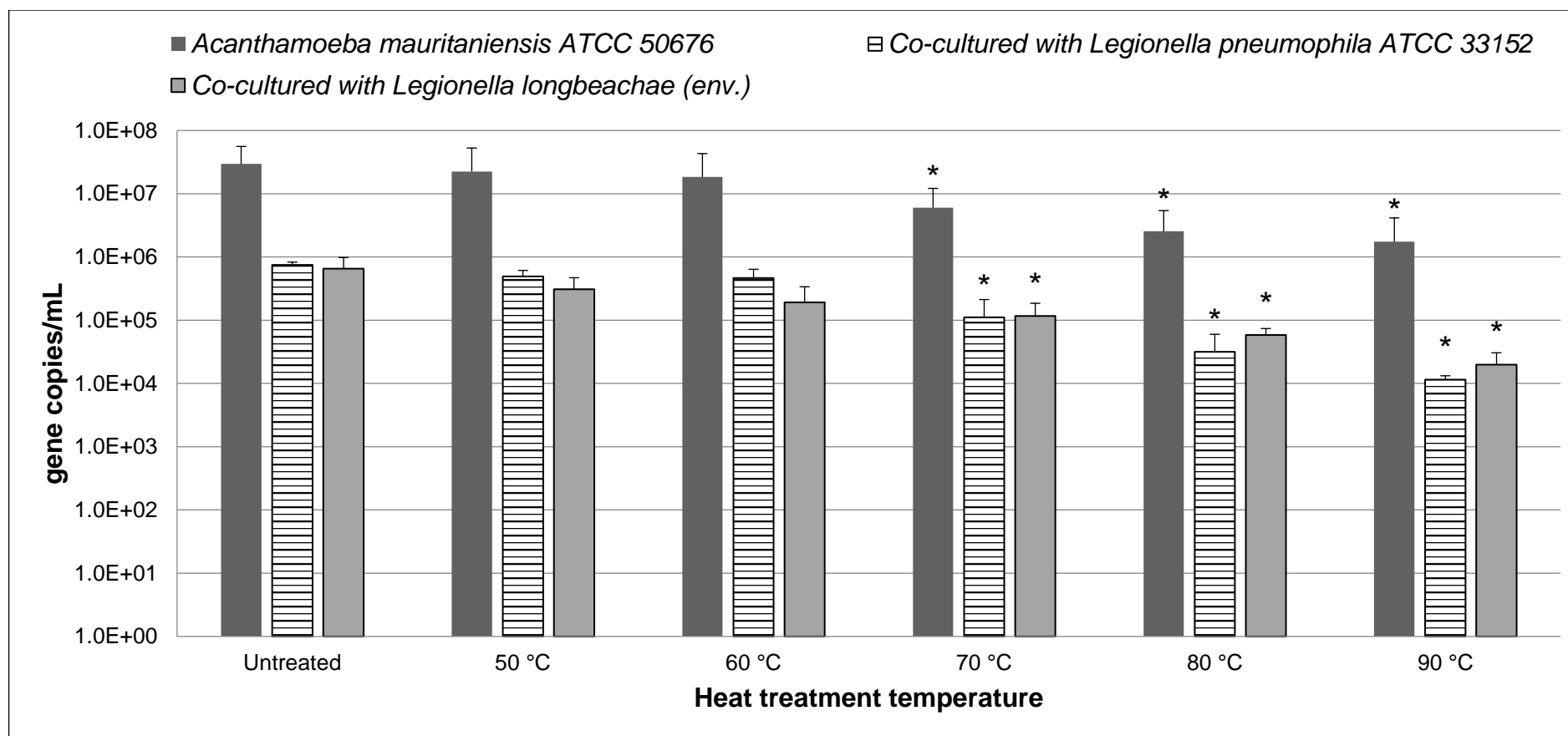
Fig. 3.2. Mean gene copies/mL of *A. mauritaniensis* ATCC 50676 before (n = 3) and after (n = 2) being co-cultured with *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.), respectively, and subsequent heat treatment. The data are expressed as the mean gene copies/mL and error bars indicate standard deviation. * denotes $p < 0.05$.

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Before the heat treatment of *A. mauritaniensis* ATCC 50676 in co-culture with *L. longbeachae* (env.) the mean gene copies/mL were recorded at 6.6×10^5 gene copies/mL (**Fig. 3.2**). Following heat treatment at 50 °C (3.1×10^5 gene copies/mL) and 60 °C (1.9×10^5 gene copies/mL) the mean number of gene copies/mL of *A. mauritaniensis* ATCC 50676 in co-culture with *L. longbeachae* (env.) did not decrease significantly ($p > 0.05$), however a 0.3-log ($p = 0.240$) and 0.5-log ($p = 0.103$) reduction in gene copies/mL was observed, respectively. A significant reduction in gene copies/mL of 0.7-log ($p = 0.018$), 1.0-log ($p = 0.0106$) and 1.5-log ($p = 0.0029$) was then obtained following heat treatment of *A. mauritaniensis* ATCC 50676 in co-culture with *L. longbeachae* (env.) at 70 °C (1.2×10^5 gene copies/mL), 80 °C (5.8×10^4 gene copies/mL) and 90 °C (2.0×10^4 gene copies/mL), respectively.

Before the heat treatment of *A. mauritaniensis* ATCC 50676 in co-culture with *L. pneumophila* ATCC 33152 the mean gene copies/mL were recorded at 7.5×10^5 gene copies/mL (**Fig. 3.2**). Following heat treatment at 50 °C (4.9×10^5 gene copies/mL) and 60 °C (4.6×10^5 gene copies/mL) the mean number of gene copies/mL of *A. mauritaniensis* ATCC 50676 in co-culture with *L. pneumophila* ATCC 33152 did not decrease significantly ($p > 0.05$), however a 0.18-log ($p = 0.072$) and 0.21-log ($p = 0.06$) reduction in gene copies/mL was observed, respectively. A significant reduction of 0.8-log ($p = 0.002$), 1.4-log ($p = 0.0005$) and 1.8-log ($p = 0.0004$) in gene copies/mL was then obtained following heat treatment of *A. mauritaniensis* ATCC 50676 in co-culture with *L. pneumophila* ATCC 33152 at 70 °C (1.1×10^5 gene copies/mL), 80 °C (3.2×10^4 gene copies/mL) and 90 °C (1.2×10^4 gene copies/mL), respectively.

3.3.3. Change in expression of virulence- and metabolism-associated genes following heat treatment and the co-culturing of *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 with *A. mauritaniensis* ATCC 50676

Culture based assays indicated that *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 were the most resistant to heat treatment as both organisms were still culturable following treatment at 50 and 60 °C. Ribonucleic acid was thus extracted from untreated and heat treated samples of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.). In addition, RNA was extracted from *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676 before and after heat treatment. The change in expression of genes including *csrA, lolA*, and *sidF* of *L. pneumophila* ATCC 33152 and *lolA* of *L. longbeachae* (env.) were then determined using relative qPCR. The fold change in gene expression was normalized to the reference gene (*rpsL*) and standardized relative to the untreated control of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.), respectively.

Following the heat treatment of *L. pneumophila* ATCC 33152 the mean fold change in gene expression decreased for all the genes analyzed (**Appendix A, Figure 7**). Overall, the expression of *sidF* decreased by a mean of 0.9-fold following heat treatment at 50-90 °C, where the decrease in gene expression ranged from 0.7-fold at 70 °C to a 1.4-fold decrease at 60 °C. Moreover, the expression of *lolA* decreased by a mean of 0.3-fold following heat treatment at 60–90 °C, where the decrease in gene expression ranged from 0.2-fold at 70 °C and 90 °C, respectively, to a 0.4-fold decrease at 80 °C. Similar results were obtained for the expression of *csrA*, where the expression of *csrA* decreased by a mean of 0.3-fold following heat treatment, and the decrease in gene expression ranged from 0.1-fold at 50 °C to a 0.4-fold decrease at 60 °C.

Before the heat treatment of *L. pneumophila* ATCC 33152 co-cultured with *A. mauritaniensis* ATCC 50676, the mean fold change in gene expression of *sidF* increased by 7.4-fold and decreased by 0.3-fold for *lolA* and 0.2-fold for *csrA*, respectively (**Fig. 3.3**). Following the heat treatment (50–90 °C) of *L. pneumophila* ATCC 33152 co-cultured with *A. mauritaniensis* ATCC 50676 the mean fold change in gene expression of *sidF* increased by a mean of 9.3-fold, where the increase in gene expression ranged from 4-fold at 70 °C to a 16-fold increase at 60 °C. However, the fold change in expression of *lolA* decreased by a mean of 0.5-fold following heat treatment at 50–90 °C, where the decrease in gene expression ranged from 0.3-fold at 80 °C to a 0.8-fold decrease at 70 °C. Similarly, the mean fold change in expression of *csrA* decreased by 2.8-fold following heat treatment at 50–90 °C, and the decrease in gene expression ranged from 1.5-fold at 60 °C to a 4.6-fold decrease at 80 °C.

Following the heat treatment of *L. longbeachae* (env.) the mean fold change in gene expression of *lolA* was analyzed, respectively (**Appendix A, Figure 8**). Results indicated that the expression of *lolA* increased by 08-fold at 50 °C, 0.9-fold at 60 °C and 0.5-fold at 70 °C. Following heat treatment at 80 °C and 90 °C, *lolA* was not detected. In addition, *lolA* was not detected before and after the heat treatment of *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676.
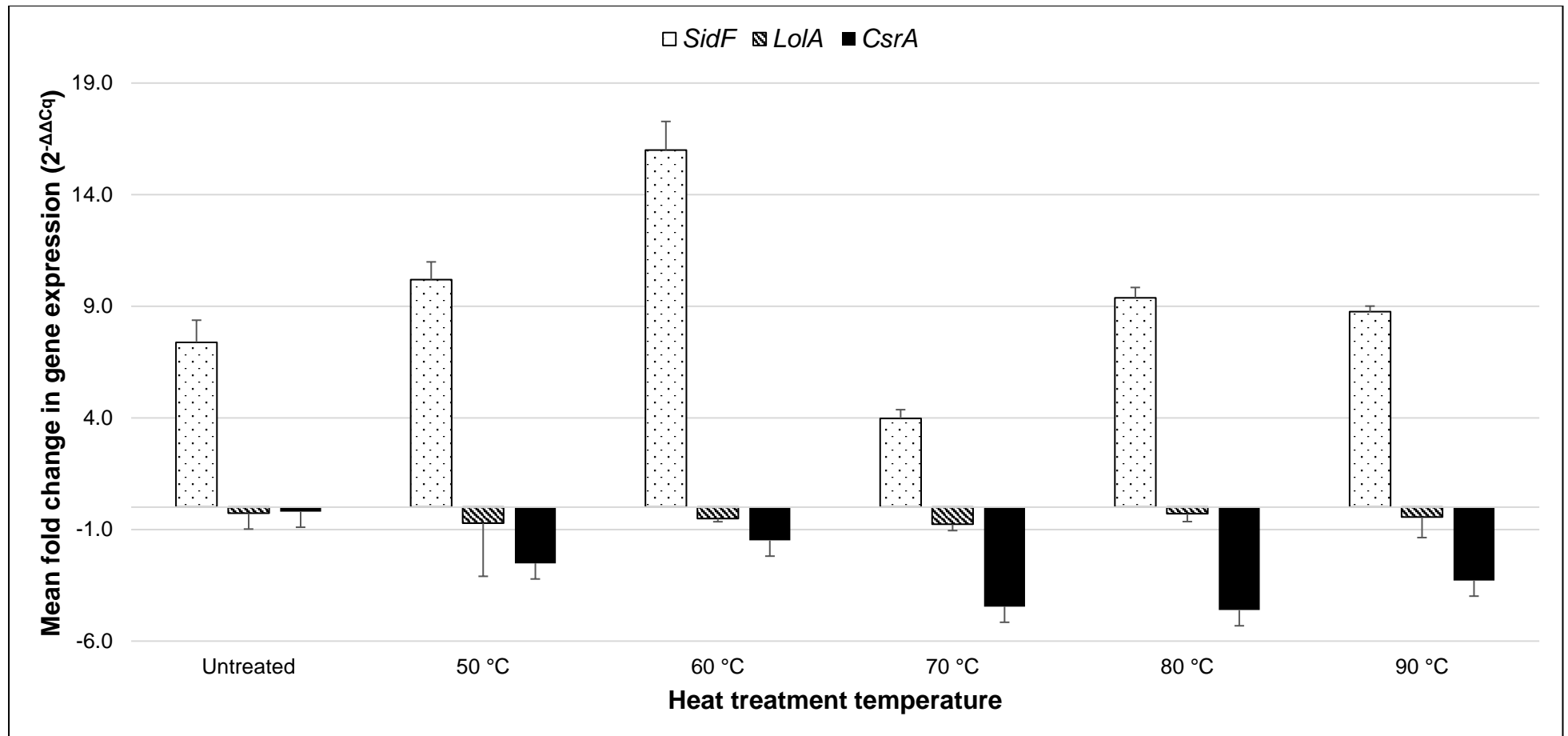
Fig. 3.3. Fold changes in gene expression before and after heat treatment of *L. pneumophila* ATCC 33152 in co-culture with *A. mauritaniensis* ATCC 50676. The data are standardized relative to the untreated positive control of *L. pneumophila* ATCC 33152 and are expressed as the mean fold change in gene expression ($2^{-\Delta\Delta Cq}$). The decrease in gene expression ($2^{-\Delta\Delta Cq} > 1$) was calculated by $-1/2^{-\Delta\Delta Cq}$.

3.4. Discussion

To determine the resistance of two *Legionella* reference strains (*L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462), three environmental strains [*L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.)] and *A. mauritaniensis* ATCC 50676 to heat treatment (50–90 °C), the culturability of the organisms was assessed. Overall results obtained for the culture based assays indicated that *L. norrlandica* (env.) was the most sensitive to heat treatment as a significant (p < 0.05) log reduction in CFU/mL was obtained following heat treatment at 50–90 °C. In contrast, while *L. longbeachae* ATCC 33462, *L. norrlandica* (env.) and *L. rowbothamii* (env.) were not detected following heat treatment at 60–90 °C, *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 were the most resistant to heat treatment as both organisms were still culturable following treatment at 50 and 60 °C. However, *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 were not detected following heat treatment at 70–90 °C. *Legionella rowbothamii* (env.) also exhibited poor growth on BCYE agar compared to the remaining *Legionella* spp. This was not surprising as of the 82 *Legionella* spp. previously isolated from unpasteurized tank water samples by Dobrowsky et al. (2016), *L. rowbothamii* (4 %) exhibited the lowest frequency of detection. Moreover, for the isolation of culturable *Legionella* spp., environmental samples often require a pre-treatment where a water sample is heated at 50 °C for 30 min to reduce the presence of non-*Legionella* organisms present (Leoni and Legnani, 2001; Whiley and Taylor, 2016). However, results of the current study indicated that heat treatment at 50–90 °C for 30 min, significantly ($p = 0.05$) reduced the culturability of all the *Legionella* spp. analyzed indicating that treating environmental samples at 50 °C for 30 min might significantly decrease the incidence of *Legionella* spp. detected in the environment.

Acanthamoeba spp. are known to function as environmental reservoirs for many *Legionella* spp. thus, characterizing their behaviour and determining their viability before and after heat treatment will aid in understanding their potential to harbour and protect *Legionella* in the environment. Results of the culture based assays then confirmed that *A. mauritaniensis* ATCC 50676 was able to withstand heat treatment and may harbor and protect *Legionella* spp. as live metabolically active trophozoites of *A. mauritaniensis* ATCC 50676 were observed following heat treatment and 50 °C, while dormant cysts were sporadically detected at 60–90 °C.

The viability of *L. norrlandica* (env.), *L. rowbothamii* (env.) *L. longbeachae* (env.), *L. pneumophila* ATCC 33152, *L. longbeachae* ATCC 33462, and *A. mauritaniensis* ATCC 50676 before (positive controls) and after heat treatment was also determined using EMA-qPCR. No significant ($p > 0.05$) difference in log reduction (gene

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copies/mL) were observed for *L. norrlandica* (env.), *L. rowbothamii* (env.) *L. longbeachae* (env.), *L. pneumophila* ATCC 33152, *L. longbeachae* ATCC 33462 treated at 50–80 °C. However, at 90 °C, *L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462 exhibited a significant (p < 0.05) log reduction of 3.6- and 3.4-log, respectively, while the lowest log reduction was observed for *L. norrlandica* (env.) (1.5-log). In addition, the gene copies/mL of *A. mauritaniensis* ATCC 50676 were detected at all the treatment temperatures (50–90 °C). The results of the EMA-qPCR analysis thus indicate that while culture based methods still represent the gold standard, many *Legionella* spp. may not be detected if culture based methods are solely used to screen water samples for potential pathogenic *Legionella* spp. (Phin et al., 2014).

While the gene copies/mL of *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676 were significantly reduced following heat treatment at 60 °C (1.0-log; *p* = 0.0120) and 70 °C (1.1-log; *p* = 0.005), the gene copies of viable *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676 were below the LLOD following heat treatment at 80 °C and 90 °C. It is thus postulated that *L. longbeachae* (env.) is unable to replicate in *A. mauritaniensis* ATCC 50676. Other studies have also indicated that while *L. longbeachae* showed moderate growth in *Tetrahymena pyriformis,* it is unable to replicate in *A. castillanii* and *V. vermiformis* (Wadowsky et al., 1991; Steele and McLennan, 1996; Neumeister et al., 1997).

In contrast the co-culturing of *L. pneumophila* ATCC 33152 with *A. mauritaniensis* ATCC 50676 improved the heat resistance of viable *L. pneumophila* ATCC 33152 as the log reduction in gene copies/mL at 90 °C, reduced by 1.7-log. Moreover, the gene copies/mL of viable *L. pneumophila* ATCC 33152 were detected at all heat treatment temperatures (50–90 °C). It has been well established that *L. pneumophila* is able to infect and replicate within amoeba, including *A. castillanii* (Rowbotham, 1980; Tyndall and Domingue, 1982; Anand et al., 1983; Holden et al., 1984) and based on the results obtained in the current study, it is thus hypothesized that *L. pneumophila* ATCC 33152 is also able to replicate in *A. mauritaniensis* ATCC 50676.

The relative gene expression of genes associated with metabolism and virulence of *L. pneumophila* ATCC 33152 (*lolA*, *sidF*, *csrA*) and *L. longbeachae* (env.) (*lolA*) during co-culturing with *A. mauritaniensis* ATCC 50676 and heat treatment (50–90 °C) were determined by performing relative qPCR. The fold change in gene expression was normalized to the reference gene (*rpsL*) and standardized relative to the untreated positive control of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.), respectively. Following the heat treatment of *L. pneumophila* ATCC 33152, the expression of the virulence gene *csrA* did not

change significantly (0.3-fold decrease). In addition, no significant ($p > 0.05$) change in the expression of *csrA* (0.2-fold decrease) was observed after the co-culturing of *L. pneumophila* ATCC 33152 with the amoeba (untreated sample). This is in agreement with research conducted by Buse et al. (2015) where it was indicated that a mean fold change in gene expression of 1 or -1 is negligible and indicates no change between the *rpsL* reference gene and the target gene. However, following the heat treatment (50–90 °C) of *L. pneumophila* ATCC 33152 in co-culture, the expression of *csrA* decreased significantly by 3.3-fold ($p = 0.001$). As studies have shown that the sigma factor RpoS (alternative sigma factor of RNA polymerase) down regulates the transcription of virulence genes including *csrA* during the exponential phase (Bachman and Swanson, 2001; 2004; Dong and Schellhorn, 2010), it is hypothesized that *L. pneumophila* ATCC 33152 remains in the exponential phase and is able to replicate in *A. mauritaniensis* ATCC 50676 during heat treatment (50–90 °C). It should be noted that the expression of *csrA* for *L. longbeachae* (env.) was not evaluated in the current study, as the ability of *L. longbeachae* to replicate intracellularly is independent of the expression of *csrA* during the exponential growth phase (Cazalet et al., 2010).

Following the heat treatment of *L. pneumophila* ATCC 33152, while a 1.4-fold decrease in *sidF* expression was observed at 60 °C, the expression of *sidF* did not change significantly ($p > 0.05$) for the remainder of the treatment temperatures (50 °C, 70–90 °C) (0.8-fold decrease). However, a significant ($p = 0.0006$) increase in the expression of *sidF* (7.4-fold increase) was observed after the co-culturing of *L. pneumophila* ATCC 33152 with *A. mauritaniensis* ATCC 50676 (untreated sample). Moreover, following the heat treatment of *L. pneumophila* ATCC 33152 in co-culture, the expression of *sidF* increased significantly by 9.7-fold. The results of the current study are in agreement with Baker et al. (2002) and Wang et al. (2005) who showed that CsrA, acts as translational repressor by binding to mRNAs of virulence genes including *sidF* that contain CsrA binding sites. In addition, the increased expression of *sidF* is expected in the amoeba as the virulence factor, SidF, is involved in the inhibition of host cell death and exhibits phosphoinositide phosphatase activity which aids in the anchoring of PI(4)P-binding effectors to the *Legionella* containing vacuole (Laguna et al., 2006; Banga et al., 2007). Moreover, Cirillo et al. (1994) also demonstrated that the growth of *L. pneumophila* in co-culture with *A. castellanii* enhances the virulence gene expression of *L. pneumophila* and the ability to infect epithelial cells *in vitro*. It should be noted that in the current study the expression of *sidF* in *L. longbeachae* (env.) was not evaluated as Cazalet et al. (2010) showed that *L. longbeachae* NSW150 did not encode the *sidF* gene.

The expression of *lolA* did not change significantly (0.3-fold decrease) following heat treatment of *L. pneumophila* ATCC 33152 ($p > 0.05$). Moreover, no significant change in the expression of

lolA (0.3-fold decrease) was observed after the co-culturing of *L. pneumophila* ATCC 33152 with *A. mauritaniensis* ATCC 50676 (untreated sample) and heat treatment (0.5-fold decrease) (*p* = 0.057). In addition, following the heat treatment of *L. longbeachae* (env.) the expression of *lolA* did not change significantly (0.8-fold increase). However, following heat treatment at 80 °C and 90 °C, the C_q signal of *lolA* was not detected. It should be noted that the *rpsL* reference gene of *L. longbeachae* (env.) was expressed during all treatment temperatures (50–90 °C). In addition, *lolA* was not detected before and after the heat treatment of *L. longbeachae* (env.) in co-culture with *A. mauritaniensis* ATCC 50676. However, the *rpsL* reference gene was detected before and after heat treatment (50–70 °C). It is thus hypothesized that although *A. mauritaniensis* ATCC 50676 may graze on *L. longbeachae* (env.), *L. longbeachae* (env.) did not exhibit active replication inside the amoeba. In addition, as the expression of *lolA* was affected at 80 °C and 90 °C, the expression of other genes associated with metabolism, such as *ccmF* involved in cytochrome c biogenesis in *L. longbeachae*, should be investigated to increase the sensitivity of the relative qPCR assay (San Francisco and Kranz, 2014).

3.5. Conclusions

The current study examined the resistance of *Legionella* reference and environmental strains and *A. mauritaniensis* ATCC 50676 to heat treatment. In addition, as *Acanthamoeba* and *Legionella* spp. have previously been detected in unpasteurized and pasteurized harvested rainwater, these two species were co-cultured and their viability was monitored after heat treatment. The expression of metabolic and virulence genes of *L. pneumophila* ATCC 33152 (*lolA, csrA, sidF*) and *L. longbeachae* (env.) (*lolA*) in co-culture with *A. mauritaniensis* ATCC 50676 during heat treatment (50–90 °C) were also examined. Results indicated that EMA-qPCR should be used in combination with culture based analysis for the routine monitoring of water supply and treatment systems as *Legionella* that were viable but non-culturable (VBNC) (due to heat treatment), internalized within amoebae and that grew poorly on conventional solid media, were detected and quantified using EMA-qPCR. The current study thus confirms that EMA-qPCR is a specific and sensitive technique that can be used to improve the sensitivity of *Legionella* and *Acanthamoeba* detection. Moreover, *L. pneumophila* ATCC 33152 was able to replicate within *A. mauritaniensis* ATCC 50676 as indicated by the down regulation of *csrA* and the increase in expression of *sidF*. As *Legionella* species differ in their ability to traffic effector proteins to the *Legionella* containing vacuole, *L. longbeachae* and *L. pneumophila* display different virulence mechanisms (Asare and Abu Kwaik, 2007). Future research should thus incorporate the relative expression of genes that encode for effector proteins that have been shown to be conserved in *L. pneumophila* and *L. longbeachae* such as SidJ, RalF, VipA, VipF, SidC, YlfA and LepB

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(Cazalet et al., 2010), which contribute to trafficking or recruitment and retention of vesicles to the *Legionella* containing vacuole. Furthermore, future studies should investigate the use of cost effective heat and pressure systems to disinfect harvested rainwater, as all *Legionella* spp. and *A. mauritaniensis* ATCC 50676 were reduced to below the detection limit and were below the LLOD in samples that were autoclaved.

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Authors' contributions:

WK and PD conceived and designed the experiments. PD performed the experiments and analyzed the data. WK acquired funding for the study. WK and SK contributed reagents/materials/analysis tools. WK and PD wrote the paper. WK, SK and PD edited the drafts of the manuscript and approved the final version of the manuscript.

3.6. References

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Chapter 4:

Chapter 4 has been accepted by Water, Air & Soil Pollution

(Chapter 4 is thus compiled in the format of the Water, Air & Soil Pollution journal and US spelling is employed)

Microbial and physico-chemical characteristics associated with the incidence of *Legionella* spp. and *Acanthamoeba* spp. in rainwater harvested from different roofing materials

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Abstract

The incidence of *Legionella* and *Acanthamoeba* spp. was correlated to microbial indicator analysis and physico-chemical characteristics of rainwater harvested from catchment areas constructed from galvanized zinc, Chromadek® and asbestos, respectively. Quantitative PCR (qPCR) analysis indicated that no significant difference ($p > 0.05$) in copy numbers of *Legionella* spp. and *Acanthamoeba* spp. were recorded in tank water samples collected from the respective roofing materials. However, significant positive Spearman (ρ) correlations were recorded between the occurrence of *Legionella* spp. gene copies vs. nitrites and nitrates ($p = 0.05$) in all tank water samples. Significant positive correlations were also established between *Acanthamoeba* spp. vs. barium ($p = 0.03$), magnesium ($p = 0.02$), sodium ($p = 0.02$), silicon ($p = 0.05$), arsenic ($p = 0.03$) and phosphate ($p = 0.01$), respectively. Additionally, while no significant correlations were observed between *Legionella* spp. vs. the indicator bacteria ($p > 0.05$), positive correlations were observed between *Acanthamoeba* spp. vs. total coliforms ($p = 0.01$) and *Acanthamoeba* spp. vs. *Escherichia coli* ($p = 0.02$), respectively. Results obtained in the current study thus indicate that the incidence of *Acanthamoeba* and *Legionella* spp. in harvested rainwater was not influenced by the roofing material utilized. Moreover, it is essential that the microbial quality of rainwater be assessed before this water source is implemented for potable and domestic uses as untreated harvested rainwater may lead to legionellosis and amoebae infections.

Key words: Harvested rainwater; roofing catchments; indicator analysis, cations, anions, *Legionella* spp.; *Acanthamoeba* spp.

4.1. Introduction

Domestic rainwater harvesting (DRWH) refers to the capture and storage of rainwater in tanks (Mwenge Kahinda et al. 2007). Many international governmental authorities consider stored harvested rainwater, a viable cost-effective alternative water source that can be utilized to aid in tackling the challenges associated with increasing water demand and climate change (An et al. 2015; Gwenzi et al. 2015). In South Africa, rainwater harvesting has been earmarked as supplementary water source, especially in rural areas. However, studies have indicated that the microbial and chemical quality of rainwater is substandard and should be assessed before rainwater harvesting can be implemented on the national and international level (De Kwaadsteniet et al. 2013).

The chemical quality of stored roof-harvested rainwater is influenced by metals leaching from the roofing materials utilized for the construction of the catchment areas, atmospheric deposition and the composition of the storage tanks, amongst other factors (De Kwaadsteniet et al. 2013; Gwenzi et al. 2015). The microbial quality of harvested rainwater is mainly dependent on environmental conditions that include, local climate, the amount of atmospheric pollution and rooftop cleanliness (leaves, dust, dead animals and insects and fecal matter from rodents and birds that may accumulate on the rooftop) (Lee et al. 2010; 2012; Gikas and Tsihrintzis 2012). Microbial opportunistic pathogens including *Legionella* spp., *Mycobacterium* avium complex (non-tuberculosis mycobacteria), *Pseudomonas aeruginosa, Acanthamoeba* spp. and *Naegleria fowleri* are currently the waterborne pathogens of highest concern in countries such as the United States of America and have been detected in harvested rainwater (Albrechtsen 2002; National Research Council 2006; Centers for Disease Control 2011; Bartrand et al. 2014; Dobrowsky et al. 2014a; 2016; Hamilton et al. 2016). Moreover, harvested rainwater contaminated with *L. pneumophila*, has previously been linked to outbreaks of Legionnaires' disease (Schlech et al. 1985; Simmons et al. 2008). The presence of *Acanthamoeba* spp. in harvested rainwater is of a human health concern as *Acanthamoeba* have been associated with human and animal infections, including amoebic keratitis and fatal granulomatous amoebic encephalitis (Qvarnstrom et al. 2013). In addition, *Acanthamoeba* spp. are able to enhance the growth and virulence of pathogens including *Legionella* spp. (Declerck et al. 2005; Visvesvara et al. 2007; Coulon et al. 2010).

In the environment, the proliferation of *Legionella* spp. depends on a number of factors which aids in its survival. For example, eukaryotic host organisms including *Acanthamoeba* spp. are essential for the replication of *Legionella* spp. in the environment (Donlan et al. 2005; Brüggemann et al. 2006). In addition, the risk of Legionellae colonization in plumbing systems has been positively correlated with increased manganese (8.37 mg/L), zinc (>0.15 – 1.0 mg/L),

potassium (1, 10 and 100 mg/L) and iron (>0.095 – 50 mg/L) concentrations and negatively correlated with temperature >55 °C (Farhat et al. 2012; Schwake et al. 2015) and increased copper (>0.76 mg/L) concentrations (Borella et al. 2005; Lau and Ashbolt 2009; Huang and Hsu 2010; Serrano-Suárez et al. 2013; Rakić and Štambuk-Giljanović 2016). Furthermore, *Legionella* spp. can associate with biofilm communities that essentially provide them with nutrients and protection from adverse environmental conditions, including water disinfection strategies (Kim et al. 2002; Borella et al. 2005). It has also been established that these biofilms may provide an environment for the interaction between *Legionella* and protozoa, including *Acanthamoeba* spp. (Borella et al. 2005; Lau and Ashbolt 2009; Huang and Hsu 2010).

Acanthamoeba spp. survives harsh environments by forming metabolically inactive cysts which are resistant to fluctuations in temperature and pH levels (Hsu 2016). Moreover, while seasonal distribution may be related to the occurrence of *Acanthamoeba* spp. (Kao et al. 2013), the correlation between *Acanthamoeba* spp. and environmental factors has not been well established. However, the presence of *Acanthamoeba* spp. in water distribution systems have been positively correlated with the levels of the heterotrophic bacteria ($p = 0.039$) and total coliforms ($p = 0.037$) (Kao et al. 2012; Ji et al. 2014).

Characterizing the niche that supports *Legionella* and *Acanthamoeba* colonization could assist in the implementation of effective control measures to reduce the risk associated with the incidence of *Legionella* and *Acanthamoeba* spp. in harvested rainwater. The objective of the present study was thus to determine which physico-chemical parameters and microbial indicator analysis may affect the persistence and survival of *Legionella* and *Acanthamoeba* spp. in harvested rainwater. As rainwater is harvested from various catchment materials, three different rainwater harvesting systems constructed from galvanized zinc, Chromadek® and asbestos were utilized. Physico-chemical parameters including cations, anions, pH and water temperature, were measured in the tank water samples collected from the various catchment systems. In addition, as the microbial diversity in a water source can influence the probability of *Legionella* and *Acanthamoeba* spp. detection and proliferation (Thomas 2012; Ji et al. 2014), the enumeration of indicator bacteria included total coliforms, *Escherichia coli* (*E. coli*) and heterotrophic bacteria. *Legionella* are also able to replicate within *Acanthamoeba* spp. and for this reason *Legionella* and *Acanthamoeba* spp. were quantified using quantitative real-time PCR (qPCR). All data obtained from this study was analyzed using Spearman correlations, analyses of variance (ANOVA) and the post hoc Fisher's Least Significant Difference (LSD) tests.

4.2. Materials and methods

4.2.1. Catchment areas of rainwater harvesting systems constructed from galvanized zinc, Chromadek® and asbestos roofing materials

Two pilot scale catchment systems (**Fig. 4.1A** and **Fig. 4.1B**) were established in July 2014 at Welgevallen Experimental farm, Stellenbosch University, Stellenbosch, South Africa (GPS co-ordinates: 33°56'36.19"S, 18°52'6.08"E). The catchment areas of roofing systems A and B (**Fig. 4.1A** and **Fig. 4.1B**) were constructed from corrugated galvanized zinc sheeting and Chromadek®, respectively. These roofing materials were selected for the current study as they are durable and less expensive. Furthermore, in South Africa these roofing materials are utilized to construct the catchment areas of many established buildings as well as low-cost housing. The corrugated galvanized zinc and Chromadek® sheets were 0.5 mm and 0.8 mm thick, respectively. The catchment area of each system was measured at 4 m^2 and the stands were approximately 2.5 m in height. The catchment areas were also sloped at a 20° angle to allow the rain to flow into the gutter systems that were mounted along each system. Each system included a domestic rainwater tank (1000 L) connected to the catchment area via the gutters.
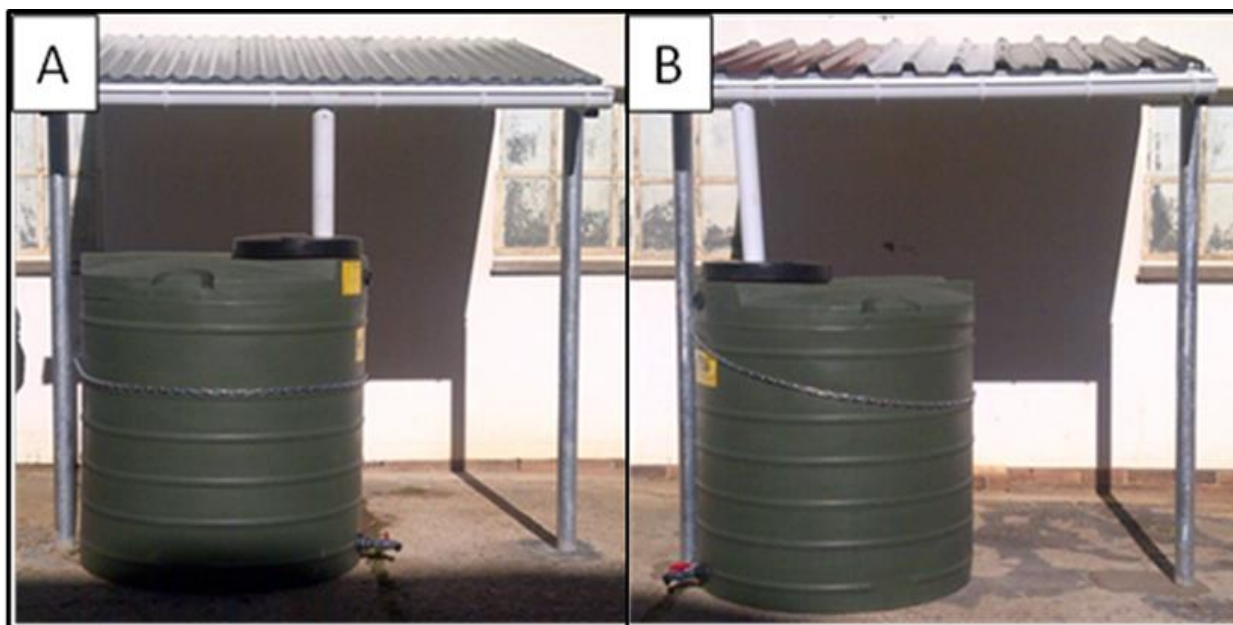
Fig. 4.1. Two rainwater harvesting systems, including catchment areas, were installed at Welgevallen Experimental Farm, Stellenbosch (GPS co-ordinates: 33°56'36.19"S, 18°52'6.08"E). A: Roofing system A, constructed from corrugated galvanised zinc sheeting. B: Roofing system B, constructed from Chromadek®. Tank water samples were also collected from the 2000 L rainwater tank connected to the asbestos roof (roofing system C; image not shown).

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An established/used roofing material of a pre-existing structure on the Welgevallen Experimental farm was also utilized in the current study (image not included). The shed is constructed from asbestos roofing and was utilized as the third catchment area (roofing system C). Asbestos has been widely used in industry as it is one of the most durable minerals and exhibits high resistance towards water, acids and other aggressive chemicals (Dyczek 2006). The shed (roof area: 267.08 m^2) was reported to have a roofing material that was approximately 30 years old (personal communication). A rainwater harvesting tank (2000 L; tank C) was installed at the asbestos roof building approximately a year before the study commenced.

4.2.2. Tank water collection

For microbial and chemical analysis, tank water samples were collected in sterile Schott bottles (1 L) from the rainwater harvesting tanks (A, B and C) connected to the respective roofing systems A, B (**Fig. 4.1**) and C (image not included) during seven sampling sessions (August 2014 to December 2014). The pH and temperature of the tank water samples were measured at the sampling locations using a hand held pH55 pH/temperature meter (Martini Instruments, North Carolina, USA).

Rainfall data was obtained from Stellenbosch Weather Services, Faculty of Engineering (http://weather.sun.ac.za/) and the daily ambient temperature ranges were obtained from the South African Weather Services (Pretoria, South Africa). During the high rainfall period (August 2014), tank water samples were collected two to four days after a rainfall event, and during the low rainfall period (September 2014 to December 2014), tank water samples were collected every two weeks.

4.2.3. Chemical analysis

Chemical analysis was performed for tank water samples collected from the roofing systems A, B and C, from sampling one to seven. The concentrations of 25 metal cations including aluminum (Al), vanadium (V), chromium (Cr), iron (Fe), amongst others, were determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) according to Saleh et al. (2000) at the Central Analytical Facility (CAF), Stellenbosch University.

Anion concentrations in tank water samples collected from roofing systems A, B, and C were determined by the PathCare Reference Laboratory (PathCare Park, Cape Town, South Africa). All anions including, chloride, fluoride, nitrates and nitrites and phosphate were measured utilizing a Thermo Scientific Gallery™ Automated Photometric Analyzer.

4.2.4. Recovery of indicator organisms from tank water samples collected from different roofing materials

For each of the tank water samples collected from tank A (galvanized zinc, n = 7), tank B (Chromadek®, n = 7) and tank C (asbestos roofing, n = 7), a serial dilution (1:10) was prepared and by use of the spread plate method, 100 µL of an undiluted sample and each dilution (10^{-1}-10^{-2}) was cultured, in triplicate, to enumerate heterotrophic bacteria, as outlined in Dobrowsky et al. (2014b). Total coliforms and *E. coli* were enumerated simultaneously as outlined by Dobrowsky et al. (2015) and the U.S. Environmental Protection Agency (USEPA 2009).

4.2.5. Extraction of total DNA from tank water samples

For total DNA extractions, each of the tank water samples (900 mL) were filtered as outlined in Dobrowsky et al. (2014a). Genomic DNA (gDNA) was then extracted according to the manufacturer's instructions using the ZR™ Soil Microbe DNA Miniprep kit (Zymo Research, Irvine, USA). Total gDNA was visualized on a 0.8% agarose gel stained with ethidium bromide (0.5 µg/mL) and electrophoresis was conducted according to Sambrook et al. (1989).

4.2.6. Quantitative real – time polymerase chain reaction (qPCR)

Quantitative real-time PCR was performed on a LightCycler ® 96 (Roche Applied Science, Mannheim, Germany) using FastStart Essential DNA Green Master Mix (Roche Applied Science, Mannheim, Germany). To a final reaction volume of 20 µl, the following was added: 10 µL FastStart Essential DNA Green Master Mix (2X), 5 µL template DNA, and 0.4 µL of each primer (10 µM), as previously described by Herpers et al. (2003).

For the quantification of *Legionella* spp. in harvested rainwater, the primers Leg F and Leg R were utilized to amplify a 259 bp product of the 23S – 5S rRNA gene according to Herpers et al. (2003) as indicated in **Table 4.1**. The standard curves for the qPCR assays were produced by amplifying the 23S – 5S rRNA gene of *Legionella pneumophila* ATCC 33152, using primers Leg F and Leg R and subsequently purifying the PCR product using the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, USA) (Dobrowsky et al. 2016).

For the quantification of *Acanthamoeba* spp. in harvested rainwater, the primers AcantF900 and AcantR1100 were utilized to amplify a 180 bp product of the 18S rRNA gene according to Qvarnstrom et al. (2006) as indicated in **Table 4.1**. To generate the standard curve for the quantification of *Acanthamoeba* spp., the 180 bp PCR product amplified from gDNA of *Acanthamoeba mauritaniensis* ATCC 50677 was cloned into the pGEM T-easy vector system

(Promega Corp., USA) according to the manufacturer's instructions. Once the plasmid had been sequenced, the plasmid containing the correct insert was used to generate the standard curve (Dobrowsky et al. 2016).

Table 4.1. Primers and qPCR cycling parameters for the quantification of *Legionella* spp. and *Acanthamoeba* spp.

Organism	Primer name	Primer sequence (5'-3')	PCR Cycling Parameters	Gene (Size bp)	References
Legionella spp.	Leg F	CTAATTGGCTGAT TGTCTTGAC	95 °C (1 min) followed by 45 cycles of 95 °C (15 s), 60 °C (15 s), and 72 °C (11 s)	23S – 5S rRNA (259)	Herpers et al. (2003)
	Leg R	CAATCGGAGTTC TTCGTG			
Acanthamoeba spp.	AcantF900	CCCAGATCGTTT ACCGTGAA	95 °C (1 min) followed by 45 cycles of 95 °C (15 s), 60 °C (1 min) and 72 °C (40 s)	16S rRNA (180)	Qvarnstrom et al. (2006)
	AcantR1100	TAAATATTAATGC CCCCAACTATCC			

Melt curve analysis was included for SYBR green real-time PCR assays in order to verify specificity of the primer set by ramping the temperature from 65 to 97 °C at a rate of 0.2 °C/s with continuous fluorescent signal acquisition at 5 readings/°C. In order to eliminate any PCR inhibitors, samples resulting in end-point fluorescence (EPF) values of less than 3.15 were diluted (10X) and the qPCR experiment was repeated for these samples.

The PCR products (*Legionella* qPCR assays) and plasmid DNA (*Acanthamoeba* qPCR assays) were quantified using the NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA) in triplicate. Serial 10-fold dilutions (10^8 to 10^0) of the respective gene copies were prepared in order to generate the standard curve. A concentration of 1.00×10^8 gene copies/µL was utilized for the dilution with the highest copy number and a concentration of 1.00×10^0 gene copies/µL was utilized for the dilution with the lowest copy number [lower limit of detection (LLOD)]. Products of representatives of the samples were purified and concentrated using the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, USA) as per manufacturer's instructions and sent to the Central Analytical Facility (CAF) at Stellenbosch University for sequencing. Chromatograms of each sequence were then examined as outlined in Dobrowsky et al. (2014a).

4.2.7. Statistical analysis

The data obtained from the microbial and chemical analysis of the collected tank water samples was assessed using the Statistical software package, Statistica™ version 12.0 (Statsoft Inc.). To establish whether the catchment roofing material would influence the incidence of *Legionella* and *Acanthamoeba* spp. in harvested rainwater, the microbial and chemical analysis of the tank water samples collected were compared using Spearman (ρ) correlations, ANOVA and post hoc Fisher's LSD tests (when ANOVA reached significance) to calculate the smallest significant differences between two means. The LSD test refers to a two-step testing technique for pairwise comparisons of several treatment groups (Meier 2006). In all hypothesis tests, a significance level of 5% was used as a standard.

4.3. Results

4.3.1. Physico-chemical parameters

During the high rainfall period (August 2014), tank water samples were collected two to four days after a rainfall event and during the low rainfall period (September 2014 to December 2014) tank water samples were collected every two weeks. The total monthly rainfall (mm) recorded during the sampling period for August (146 mm), September (44.2 mm), October (14.3 mm), November (43 mm) and December (7.2 mm) decreased as the ambient temperature increased from an average of 13.9 °C (August 2014) to 20.9 °C (December 2014) (ρ = -0.90; p = 0.037).

The pH and temperature of the rainwater collected from tanks A (galvanized zinc), B (Chromadek®) and C (asbestos), were monitored during the seven sampling sessions. Overall results indicated that the highest mean pH of 8.0 was recorded for tank A (galvanized zinc roofing). While, a mean pH of 7.8 and 7.6 was recorded for tanks B (Chromadek® roofing) and C (asbestos), respectively. For all tank water samples collected from the rainwater tanks A, B and C, a mean tank water temperature of 22.4 °C, 22.3 °C and 21.7 °C was recorded, respectively. No significant difference (p > 0.05) was noted between the temperature of the water samples collected from the respective tanks. However, while no significant difference was recorded between the pH of the tank water samples collected from tank B compared to tank A (p = 0.24) and C (p = 0.39), respectively, a significant difference was observed for the pH of the tank water collected from tank C compared to tank A (p = 0.045).

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4.3.2. Cation and anion analysis of tank water samples

The concentrations of cations and anions were analyzed in the tank water harvested from the various roofing catchment materials (**Table 4.2**). Anion concentrations did not differ significantly ($p > 0.05$) between tank water samples. However, a significant difference ($p < 0.05$) in certain cation concentrations was recorded between the tank water samples collected from tank A (galvanized zinc), B (Chromadek®) and C (asbestos), respectively. For example, the concentration of total zinc differed significantly between the tank water samples collected from tank A (812.2 µg/L), in comparison to tanks B (62.29 µg/L; $p = 0.000037$) and C (42.56 µg/L; $p = 0.000028$), respectively (**Table 4.2**). In addition, the concentration of total chromium differed significantly between the tank water samples collected from tank B (1.269 µg/L), in comparison to tanks A (0.524 µg/L; $p = 0.006$) and C (0.333 µg/L; $p = 0.001$), respectively (**Table 4.2**).

4.3.3. Indicator bacteria detected in tank water samples

Total coliforms, *E. coli* and heterotrophic bacteria were enumerated in tank water samples harvested from the different catchment systems (**Fig. 4.2**). Throughout the sampling period the total coliform counts ranged from the lowest count of 3.5×10^2 CFU/100 mL to the highest count of 1.9×10^4 CFU/100 mL for tank A, 1.7×10^2 to 1.2×10^4 CFU/100 mL for tank B and 3.0×10^2 to 3.3×10^4 CFU/100 mL for tank C (**Fig. 4.2A**). *Escherichia coli* numbers then ranged from the lowest count of 2 CFU/100 mL to the highest count of 8.8×10^1 CFU/100 mL for tank A, 1 to 1.7×10^2 CFU/100 mL for tank B and 1 to 2.0×10^2 CFU/100 mL for tank C (**Fig. 4.2B**). In addition, the heterotrophic plate count ranged from the lowest count of 9.6×10^4 CFU/100 mL to the highest count of 5.0×10^5 CFU/100 mL for tank A, 5.1×10^4 to 6.6×10^5 CFU/100 mL for tank B and 3.0×10^4 to 1.7×10^6 CFU/100 mL for tank C (**Fig. 4.2C**). No significant difference ($p > 0.05$) was observed between the mean total coliforms, *E. coli*, and heterotrophic bacterial counts obtained in all tank water samples collected from galvanized zinc, Chromadek® and asbestos roofing, respectively, throughout the sampling period (**Fig. 4.2**).

Table 4.2. The mean concentrations of all cations and anions measured for tank water samples collected from galvanized zinc, Chromadek® and asbestos catchment areas.

Cations	Mean (range)		
	Tank A (Galvanized Zinc)	Tank B (Chromadek®)	Tank C (asbestos)
Boron as B (mg/L)	0.024 (0 ± 0.05)	0.024 (0 ± 0.05)	0.183 (0 ± 1.15)
Barium as Ba (mg/L)*	*0.005 (0 ± 0.01)*	*0.005 (0 ± 0.01)*	*0.063 (0 ± 0.12)*
Calcium as Ca (ug/L)*	*1891 (1070 ± 4590)*	*2500 (2030 ± 2960)*	*6545 (4370 ± 10470)*
Potassium as K (ug/L)	370 (220 ± 520)	442.9 (0 ± 750)	2807 (0 ± 16890)
Magnesium as Mg (ug/L)	527.1 (360 ± 600)	605.7 (400 ± 720)	631.4 (250 ± 1020)
Sodium as Na (ug/L)*	*3297 (2630 ± 3870)*	*3594 (2830 ± 4300)*	*4391 (2610 ± 6540)*
Silicon as Si (ug/L)	100.0 (60 ± 140)	118.6 (50 ± 190)	2393 (520 ± 11400)
Aluminum as Al (µg/L)	13.56 (3 ± 32.29)	13.55 (2.94 ± 25.09)	42.47 (1.31 ± 220.6)
Titanium as Ti (µg/L)	0.321 (0.06 ± 0.7)	0.35 (0.1 ± 0.62)	0.916 (0 ± 3.68)
Vanadium as V (µg/L)	0.267 (0.13 ± 0.36)	0.23 (0.11 ± 0.33)	0.289 (0.05 ± 1.08)
Chromium as Cr (µg/L)*	*0.524 (0.3 ± 0.98)*	*1.269 (0.7 ± 2.34)*	*0.333 (0.09 ± 0.78)*
Manganese as Mn (µg/L)*	*4.707 (1.24 ± 12.91)*	*3.054 (0.31 ± 7.74)*	*12.35 (2.47 ± 20.67)*
Iron as Fe (µg/L)*	*5.187 (0.66 ± 12.44)*	*6.547 (2.13 ± 10.11)*	*281 (25.04 ± 1234.5)*
Cobalt as Co (µg/L)	0.119 (0.04 ± 0.4)	0.094 (0.01 ± 0.4)	0.206 (0.08 ± 0.4)
Nickel as Ni (µg/L)*	*0.321 (0.05 ± 1.47)*	*0.29 (0.05 ± 1.03)*	*19.32 (0.44 ± 65.53)*
Copper as Cu (µg/L)	1.869 (0.61 ± 6.77)	1.883 (1 ± 6.14)	132.0 (1 ± 622.7)
Zinc as Zn (µg/L)*	*812.2 (533.9 ± 969.3)*	*62.29 (39.27 ± 71.81)*	*42.56 (27.15 ± 83.06)*
Arsenic as As (µg/L)*	*0.329 (0.1 ± 0.71)*	*0.484 (0.15 ± 1.21)*	*0.904 (0.32 ± 2.09)*
Selenium as Se (µg/L)	0.53 (0 ± 1.53)	0.486 (0 ± 1.53)	0.559 (0.2 ± 1.53)
Molybdenum as Mo (µg/L)	0.421 (0.01 ± 2.14)	0.221 (0.006 ± 0.73)	0.167 (0.04 ± 0.54)
Cadmium as Cd (µg/L)	0.040 (0.01 ± 0.12)	0.046 (0.01 ± 0.16)	0.038 (0 ± 0.1)
Lead as Pb (µg/L)*	*0.241 (0.08 ± 0.46)*	*0.349 (0.05 ± 0.66)*	*4.724 (0.66 ± 12.42)*
Anions			
Phosphate as PO_4 (mg/L)	0.137 (0.02 ± 0.4)	0.156 (0.02 ± 0.41)	0.153 (0.02 ± 0.43)
Chloride as Cl^- (mg/L)	11 (3 ± 22)	13.43 (4 ± 31)	11.29 (3 ± 22)
Nitrate + Nitrite as $NO_3 + NO_2$ (mg/L)	0.91 (0.2 ± 1.7)	0.951 (0.2 ± 1.8)	0.417 (0.06 ± 1.2)
Sulfate as SO_4 (mg/L)	1.571 (0 ± 5)	1.429 (0 ± 6)	0.857 (0 ± 2)
Fluoride as F (mg/L)	0.1 (0 ± 0.2)	0.086 (0 ± 0.1)	0.1 (0 ± 0.2)

* The cations and anions indicated in italics differed significantly ($p < 0.05$) between tank water samples collected from the respective roofing materials.
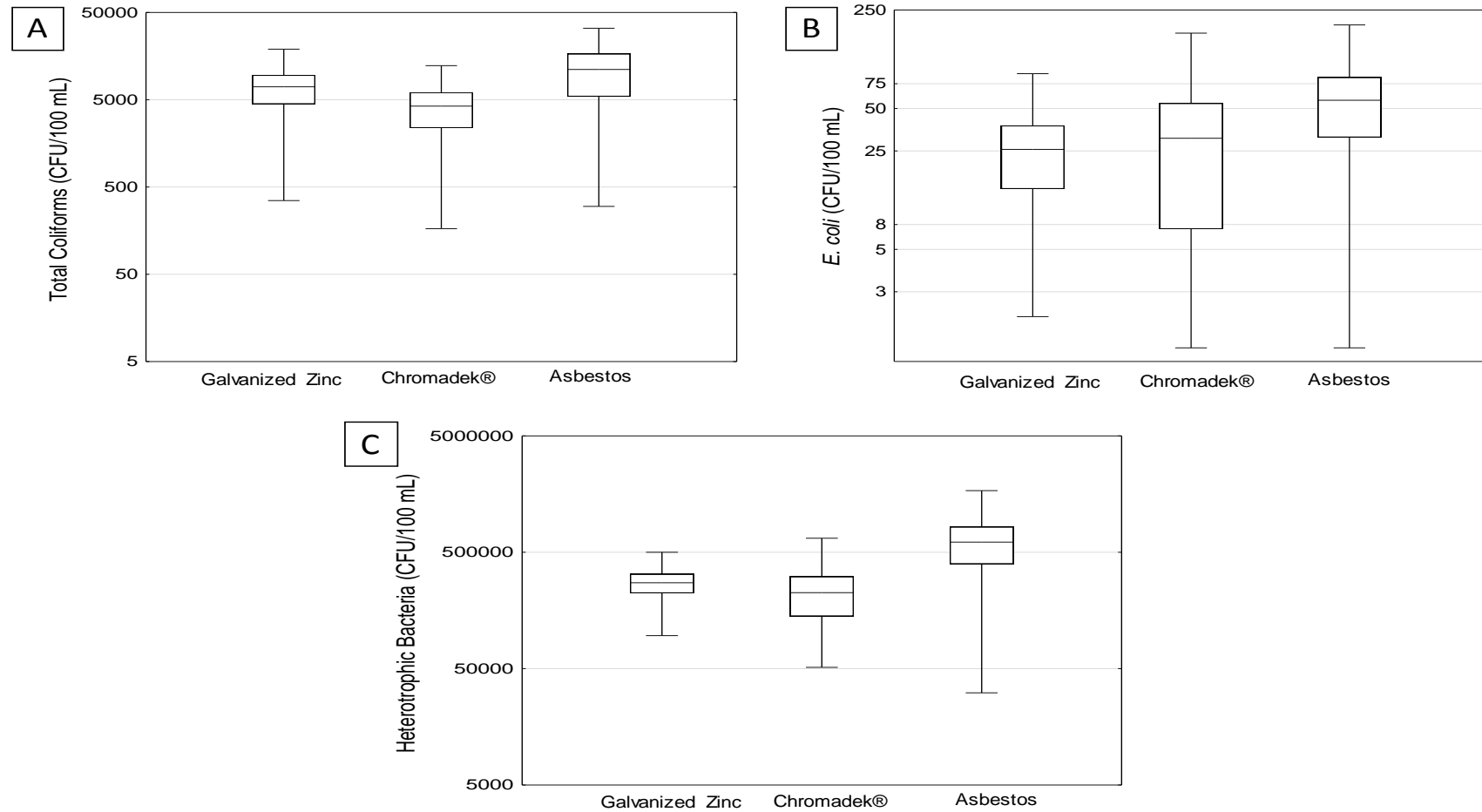
Fig. 4.2. Indicator bacteria enumerated for tank water samples collected from tank A (galvanized zinc), tank B (Chromadek®) and tank C (asbestos). A: Total coliforms. B: *Escherichia coli*. C: Heterotrophic bacteria. Box = mean ± standard error, middle line = mean, whiskers = minimum and maximum values.

4.3.4. Quantification of *Legionella* spp. and *Acanthamoeba* spp. present in tank water samples

The presence of *Legionella* spp. and *Acanthamoeba* spp. was verified in all tank water samples using qPCR assays (**Fig. 4.3**). A linear range of quantification from 10^8 to 10^0 gene copies per µL of DNA extracts was observed for the standard curves. For the quantification of *Legionella* spp., the qPCR assays had amplification efficiencies that ranged from 1.86 to 1.93 with errors of 0.37 and 0.60, respectively. For all absolute qPCR assays the correlation coefficient (r^2) was measured at 1.00. For the quantification of *Acanthamoeba* spp., the qPCR assay had an amplification efficiency of 1.95 with an error of 0.70. The qPCR LLOD was recorded at 11.43 gene copies/µL for *Legionella* spp. and 1.89 gene copies/µL for *Acanthamoeba* spp.

Overall results indicated that the highest mean concentration for *Legionella* spp. was detected in tank C (asbestos roofing), with a mean of 1.74×10^5 gene copies/mL obtained (**Fig. 4.3A**). For tank water samples collected from tank C, the gene copies of *Legionella* ranged from 1.06×10^3 gene copies/mL during sampling seven to 7.28×10^5 gene copies/mL during sampling two. A mean of 1.61×10^5 gene copies/mL was then recorded for tank B (Chromadek® roofing), with the concentration of *Legionella* spp. ranging from 6.22×10^3 gene copies/mL during sampling one to 5.03×10^5 gene copies/mL during sampling six. The lowest mean concentration of *Legionella* spp. (1.59×10^5 gene copies/mL) was recorded for tank A (galvanized zinc), with a range of 8.01×10^3 gene copies/mL (sampling four) to 7.37×10^5 gene copies/mL (sampling five) recorded.

Additionally, the presence of *Acanthamoeba* spp. was verified in all tank water samples using qPCR assays (**Fig. 4.3B**). Overall results indicated that the highest mean concentration of *Acanthamoeba* spp. were detected in tank C (asbestos roofing), with a mean of 1.27×10^6 gene copies/mL obtained. For tank water samples collected from tank C, the numbers of *Acanthamoeba* spp. ranged from 1.22×10^4 gene copies/mL during sampling one to 8.08×10^6 gene copies/mL during sampling four. Moreover, a mean of 1.39×10^5 gene copies/mL was recorded for tank A (galvanized zinc), with the concentration of *Acanthamoeba* spp. ranging from 1.33×10^4 gene copies/mL during sampling four to 4.22×10^5 gene copies/mL during sampling five. The lowest mean concentration of *Acanthamoeba* spp. (3.52×10^4 gene copies/mL) was recorded for tank B (Chromadek®), with a range of 3.87×10^3 gene copies/mL (sampling three) to 9.28×10^4 gene copies/mL (sampling seven) recorded.
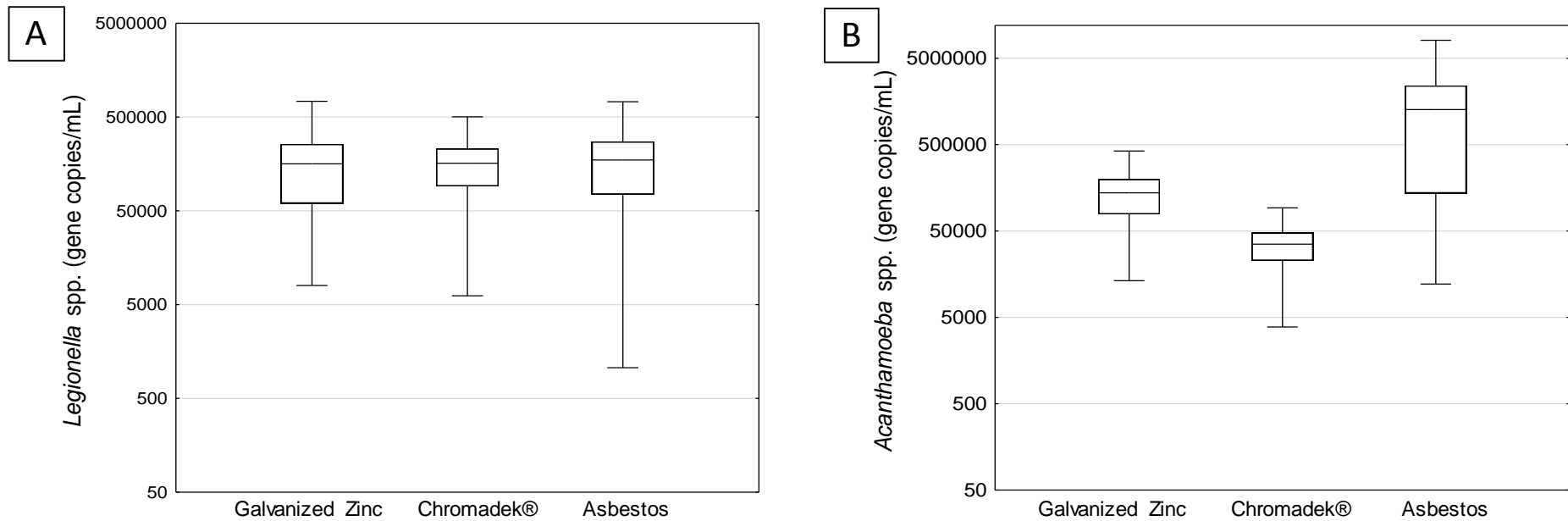
Fig.4.3. Quantification of *Legionella* spp. in tank water samples collected from tank A (galvanized zinc), tank B (Chromadek®) and tank C (asbestos) using qPCR. B: Quantification of *Acanthamoeba* spp. in tank water samples collected from tank A (galvanized zinc), tank B (Chromadek®) and tank C (asbestos) using qPCR. Box = mean ± standard error, middle line = mean, whiskers = minimum and maximum values.

Although, the highest mean concentrations of *Legionella* spp. (gene copies/mL) and *Acanthamoeba* spp. (gene copies/mL) were detected in tank C (asbestos roofing), overall no significant difference (p > 0.05) was observed in the average *Legionella* spp. and *Acanthamoeba* spp. gene copies/mL obtained for tank water samples collected from galvanized zinc, Chromadek® and asbestos roofing, respectively.

4.3.5. Correlations between *Legionella*, *Acanthamoeba* spp. and parameters detected in harvested rainwater from the galvanized zinc, Chromadek® and asbestos catchment areas

No significant Spearman (ρ) correlations ($p > 0.05$) were noted between the recorded parameters including seasonal fluctuations in rainfall and ambient temperature vs. the occurrence of *Legionella* spp., with the exception of nitrites and nitrates ($\rho = 0.44$, $p = 0.05$). In addition, while parameters including the monthly rainfall and ambient temperature did not correlate significantly ($p > 0.05$) with the occurrence of *Acanthamoeba* spp., significant correlations ($p < 0.05$) were, however, established between *Acanthamoeba* spp. vs. six chemical parameters, namely barium ($\rho = 0.47$, $p = 0.03$), magnesium ($\rho = 0.52$, $p = 0.02$), sodium ($\rho = 0.52$, $p = 0.02$), silicon ($\rho = 0.44$, $p = 0.05$), arsenic ($\rho = 0.47$, $p = 0.03$) and phosphate ($\rho = 0.55$, $p = 0.01$). Additionally, positive correlations were observed between *Acanthamoeba* spp. vs. total coliforms ($\rho = 0.56$, $p = 0.01$) and *Acanthamoeba* spp. vs. *E. coli* ($\rho = 0.52$, $p = 0.02$), respectively. Furthermore, although a positive correlation ($\rho = 0.19$) was observed for *Legionella* spp. vs. *Acanthamoeba* spp., this correlation was not significant ($p = 0.41$).

4.4. Discussion

The aim of the current study was to determine whether the incidence of *Legionella* and *Acanthamoeba* spp. could be correlated to the physico-chemical and microbial parameters associated with rainwater harvested from asbestos, galvanized zinc and Chromadek® roofing catchments. Although it has previously been hypothesized that the incidence of *Acanthamoeba* spp. in environmental water sources may be dependent on seasonal distribution (Kao et al. 2013), results of the current study indicated that the occurrence of *Legionella* spp. and *Acanthamoeba* spp. in harvested rainwater did not correlate significantly with seasonal fluctuations in monthly rainfall and ambient temperature ($p > 0.05$). Moreover, the pH and temperature did not influence the incidence of *Legionella* and *Acanthamoeba* spp. detection in harvested rainwater, as no significant correlations ($p > 0.05$) were observed for pH and temperature vs. the gene copies/mL of *Legionella* or *Acanthamoeba* spp., respectively.

Results of the qPCR analysis indicated that the highest mean concentrations of *Legionella* spp. (gene copies/mL) and *Acanthamoeba* spp. (gene copies/mL) were detected in tank C (asbestos roofing). However, no significant difference ($p > 0.05$) was observed in the mean *Legionella* spp. and *Acanthamoeba* spp. gene copies/mL recorded in tank water harvested from the respective roofing materials. In addition, while positive correlations were noted between nitrites and nitrates vs. the gene copies/mL of *Legionella* spp., no significant difference ($p > 0.05$) was observed for the nitrite and nitrate concentrations recorded in tank water samples collected from the various roofing materials. Nitrates and nitrates are jointly included in the quality analyses of water due to the inter-conversion of one form to the other in the environment (Iscen et al. 2008). High nitrate and nitrite levels are associated with plant and animal debris and fecal contamination of a water source (Ntengwe 2006; DWAF 1996; NHMRC and NRMMC 2011). As previously noted, manure and the decay of plant and animal matter are among the most significant sources of nitrate and nitrite in water (NHMRC and NRMMC 2011) and although *Legionella* spp. are unable to reduce nitrates to nitrites (Orrison et al. 1981; Benson et al. 1996), the correlation noted between nitrate and nitrite concentrations and *Legionella* in harvested rainwater may indirectly indicate that the water source is rich in nitrogen from amino acids (originating from manure and decaying plants and animals). This is essential as *Legionella* requires amino acids such as L-cysteine, for carbon, nitrogen and energy sources (Declerck 2010). However, the correlation between nitrates and nitrites vs. the concentration of amino acids in an environmental water source requires further investigation. It should also be noted that while no studies have investigated the correlation between nitrates and nitrates vs. *Legionella* spp. in a water source, Nogueira et al. (2016) indicated that biological treatment systems rich in organic nitrogen (protein) may be a possible source of *Legionella* infection as a positive correlation was noted between the occurrence of *Legionella* and organic nitrogen.

Additionally, positive correlations were established between *Acanthamoeba* spp. gene copies/mL vs. barium, magnesium, sodium, silicon, arsenic and phosphate concentrations, respectively. Dust, dead or decaying plants and animals and fecal matter may be the source of contamination of these cations and anions in the harvested rainwater as barium, magnesium, sodium, silicon, arsenic and phosphate have all been detected in various materials and are readily taken up by living organisms (Chien 1977; Kojola et al. 1978; Aikawa 1980; Nordstrom 2002; Jugdaohsingh 2007; NHMRC and NRMMC 2011). It should however be noted that the concentrations of barium ($p = 0.001$), sodium ($p = 0.049$) and arsenic ($p = 0.045$) differed significantly between tank water samples collected from the respective roofing materials and were detected at significantly higher concentrations in tank water samples collected from the asbestos roofing material. However, while the highest mean gene copies/mL of *Legionella* or *Acanthamoeba* spp. were recorded in the tank water harvested from the asbestos roofing, no

significant difference ($p > 0.05$) in copy numbers of *Legionella* spp. and *Acanthamoeba* spp. were recorded in tank water samples collected from the different roofing materials. Limited literature describing the influence of barium, magnesium, sodium, silicon, arsenic and phosphate on the metabolic processes of *Acanthamoeba* spp. is also available. However, barium may be involved in the formation of *Acanthamoeba* membranes as barium is used as a mediator that binds to the active sites of phosphoinositide-specific phospholipases during the complexing of phospholipids (Essen et al. 1997). Magnesium, which typically occurs as Mg^{2+} is an essential element in biological systems as over 300 enzymes require magnesium ions for catalytic actions, including enzymes that utilize or synthesize ATP (Wilcox 1996; Cowan 2002). Moreover, amoeba requires sufficient concentrations of hydrogen, sodium, potassium, magnesium and calcium in order to regulate their cytoplasmic and mitochondrial pHs. The sodium in the cytoplasm is generally kept constant at 0.1 to 0.3 mM and the amoeba maintains osmotic homeostasis by using the contractile vacuole subsystem to excrete sodium (Martin et al. 1987; Chauhan and Varma 2009). In addition, silicon is an important element in biological processes and when oxidized, silicon forms silica. Microbial eukaryotes use molecular mechanisms for silica uptake as silica is utilized for the development of external and internal structures (Lahr et al. 2015). Although the nutritional importance of arsenic has not been clearly defined, research has shown that arsenic has an important role in the conversion of methionine to polyamines and all parasitic protozoa contain polyamines, which may be utilized during polyamine-induced cytokinesis (Gawlitta et al. 1981; Uthus 1992; Müller et al. 2001). Phosphate also plays a vital role in all living organisms, for example, for the formation of phosphoanhydride bonds in ATP and ADP, which contain high amounts of energy, and in structural forms such as DNA and RNA (Bieleski and Ferguson 1983).

The LSD tests then indicated no significant difference ($p > 0.05$) between indicator bacteria counts (total coliforms, *E. coli* and heterotrophic bacteria) detected in tank water samples collected from the respective roofing materials. In addition, *Legionella* gene copies/mL did not correlate significantly ($p > 0.05$) with any indicator analysis including the heterotrophic plate count. Contradictory results have however been reported in studies investigating the co-colonization of *Legionella* and heterotrophic bacteria in water sources. For example, Dobrowsky et al. (2016) indicated that a positive correlation existed between the heterotrophic bacteria and *Legionella* gene copies/mL ($\rho = 0.710$, $p = 0.048$) in harvested rainwater treated using solar pasteurization. Other studies have utilized the heterotrophic plate count as a representative of biofilms present and have also indicated that *Legionella* require the development of biofilms in drinking and hot water distribution systems before colonization can occur (Bagh et al. 2004; Moritz et al. 2010). In contrast and in agreement with the current results obtained, Duda et al. (2015) noted that no correlation existed between the heterotrophic plate count vs. *Legionella* or

ATP vs. *Legionella* in non-potable water collected from cooling towers. During the current study, significant correlations ($p < 0.05$) were however, observed between *Acanthamoeba* spp. vs. total coliforms and *Acanthamoeba* spp. vs. *E. coli*. Moreover, Huang et al. (2011) showed that total coliform concentrations correlated with the occurrence of *Acanthamoeba* spp. in spring water. It is thus hypothesized that total coliforms and *E. coli* in the harvested rainwater may originate from fecal matter and other contaminants such as dust and debris and contribute to the natural microbial populations in the rainwater harvesting tanks.

The persistence of *Legionella* spp. in rainwater harvesting systems may thus be attributed to the presence of *Acanthamoeba* spp. as numerous studies have indicated that *Legionella* spp. contain a dual host system which allows for their intracellular proliferation in protozoa (Newsome et al. 1985; Rowbotham 1986; Wadowsky et al. 1991; Barker et al. 1992). This phenomenon was corroborated in the current study where *Legionella* and *Acanthamoeba* were detected in all the tank water samples collected from the respective roofing materials. Moreover, Dobrowsky et al. (2016) showed that while solar pasteurization was effective in reducing the gene copies/mL of viable *Naegleria fowleri* and *Vermamoeba vermiformis* to below the lower limit of detection at temperatures of 68–93 °C and 74–93 °C, respectively, the gene copies of viable *Legionella* and *Acanthamoeba* spp. were still detected after pasteurization at 93 °C. Although a positive correlation was established between the gene copies of *Legionella* vs. *Acanthamoeba* spp., this correlation was not significant ($p > 0.05$), indicating that the number of *Legionella* harbored by the *Acanthamoeba* spp. in harvested rainwater requires further investigation. In the environment *Legionella* spp. may occur in many phases including within hosts such as *Acanthamoeba*, as planktonic cells and/or within biofilms as sessile cells (Molofsky and Swanson 2004). Moreover, *Legionella* and *Acanthamoeba* spp. were detected regardless of the concentration of indicator bacteria or cations and anions. Further studies will have to be conducted in order to investigate and functionalize the persistence mechanisms displayed by *Legionella* spp. in roof harvested rainwater as *Legionella* spp. are known to survive in the environment by manipulating host cell functions including the hosts' phagocytic mechanisms by reprogramming the endosomal-lysosomal degradation pathway during phagocytosis by *Acanthamoeba* spp. (Brüggemann et al. 2006).

4.5. Conclusions

Quantification of *Legionella* spp. and *Acanthamoeba* spp. in the current study indicated that the roofing material utilized for the construction of the catchment areas did not significantly ($p > 0.05$) influence the concentration of *Legionella* and *Acanthamoeba* spp. detected in harvested rainwater. In addition, as no significant difference ($p > 0.05$) in indicator bacteria numbers and *Legionella* and *Acanthamoeba* spp. copy numbers were observed for all tank

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water samples collected from the respective roofing materials, it is hypothesized that external pollutants such as dust, debris and fecal matter serve as the primary reservoirs of these microbial pathogens. Moreover, individuals that utilize harvested rainwater may be at risk of *Legionella* and *Acanthamoeba* infection as these opportunistic pathogens were detected at high concentrations in all the tank water samples collected. Obtaining quantitative information on opportunistic pathogens present in harvested rainwater, may thus aid governmental organizations, responsible for the implementation of rainwater harvesting systems, in employing strategies to protect public health (Ahmed et al. 2014).

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Authors' contributions:

WK, SK and PD conceived and designed the experiments. PD performed the experiments and analyzed the data. WK and TE acquired funding for the study. WK and SK contributed reagents/materials/analysis tools. WK and PD wrote the paper. All authors edited the drafts of the manuscript and approved the final version of the manuscript.

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Chapter 5:

General Conclusions and Recommendations

(UK spelling is employed)

General Conclusions and Recommendations

Domestic rainwater harvesting (DRWH) has been approved by many international governmental authorities as a feasible alternative water source, to be utilised in confronting the challenges associated with the escalating water demand and climate change (An et al. 2015). As South Africa harbours large rural and peri-urban informal communities that are widely distributed and continuously expanding, decentralised water collection points along with adequate water supply infrastructures are in increasing demand. In alleviating the effects of climate change and the pressures of an increasing population on water supplies (Mwenge Kahinda et al. 2007), DRWH tanks have been installed in all nine provinces of South Africa (Malema et al. 2016). However, rainwater is contaminated during the harvesting process by various microbial and chemical pollutants (Gwenzi et al. 2015). For this reason, it is advised that harvested rainwater be used for non-potable purposes, as the quality thereof is largely not within drinking water standards (Gwenzi et al. 2015). Studies conducted by our research team then indicated that solar pasteurization (SOPAS) is an effective treatment method to reduce the level of indicator bacteria (total coliforms, *Escherichia coli* and heterotrophic bacteria) and disinfect up to 120 L of harvested rainwater at 72°C. However, the presence of viable *Legionella* spp. in rainwater pasteurized at > 91°C was confirmed using ethidium monoazide quantitative polymerase chain reaction analysis (EMA-qPCR) (Dobrowsky et al. 2016; Reyneke et al. 2016). The persistence of *Legionella* spp. at such high pasteurization temperatures poses a threat to human health as a number of *Legionella* spp., including *L. pneumophila* and *L. longbeachae* are known to cause two types of disease, namely pneumonia (Legionnaires' disease) and a milder influenza like disease, Pontiac fever (Fraser et al. 1977; Glick et al. 1978). While the probability of *Legionella* spp. infection after the ingestion of contaminated water is unlikely, disease occurs through the inhalation of aerosols containing *Legionella* cells (Szewzyk et al. 2000). It is then not surprising that *Legionella* have been isolated from a number of man-made warm water systems including cooling towers, hot tubs, showerheads and spas (Fields, 1996; Atlas, 1999; Fields et al. 2002; Miquel et al. 2003).

The persistence of *Legionella* spp. under adverse conditions is determined by a number of factors that include: the availability of nutrients in a water source (Cianciotto, 2007); the water temperature (Farhat et al. 2012; Schwake et al. 2015); and while biofilms allow for the persistence of *L. pneumophila,* free-living amoebae (FLA) are required for the intracellular growth and proliferation of *Legionella* spp. in the environment (Murga et al. 2001; Kuiper et al. 2004; Declerck et al. 2007). Protozoa have been aptly described as the "Trojan Horse of microorganisms" as pathogenic bacteria including *Legionella* spp., are able to survive once ingested by the protozoa. Specifically, genera of the FLA including, *Acanthamoeba* spp.,

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Hartmannella spp. and *Naegleria* spp. provide nutrients such as amino acids and a protective environment when *Legionella* spp. are enclosed in the cysts of amoebae (Barker et al. 1992; Newsome et al. 1985; Rowbotham, 1986; Wadowsky et al. 1991; Thomas et al. 2006). The focus of the current study was thus to investigate and functionalise selected persistence mechanisms displayed by *Legionella* spp. that aid in their survival in pasteurized and unpasteurized harvested rainwater.

The aim of Chapter two (published in Parasites and Vectors) was to isolate and identify the dominant *Legionella* spp. persisting in a DRWH tank and a SOPAS system and to identify possible FLA vectors of *Legionella* that remain viable at high pasteurization temperatures. For this, pasteurized (45°C, 65°C, 68°C, 74°C, 84°C and 93°C) and unpasteurized tank water samples were screened for the dominant *Legionella* spp. using culture based techniques. The heterotrophic plate count (HPC) was enumerated as the HPC was previously shown to contribute to the occurrence, proliferation and persistence of *Legionella* spp. (Bagh et al. 2004; Moritz et al. 2010). In addition, as FLAs including *Acanthamoeba* spp., *N. fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* are the most frequently isolated from water samples and hot water systems (Rohr et al. 1998; Thomas et al. 2006; Buse et al. 2013), EMA-qPCR was utilised for the quantification of viable *Legionella* spp., *Acanthamoeba* spp., *V. vermiformis* and *N. fowleri* in pasteurized (68°C, 74°C, 84°C and 93°C) and unpasteurized tank water samples. Of the 82 *Legionella* spp. identified in the unpasteurized tank water samples, *L. longbeachae* (35%) was the most frequently isolated, followed by *L. norrlandica* (27%) and *L. rowbothamii* (4%). Additionally, positive correlations were recorded between the HPC vs the gene copies/mL of *Legionella* spp. detected in the unpasteurized and pasteurized tank water samples and HPC vs the number of *Legionella* spp. isolated from the unpasteurized tank water samples. Solar pasteurization was effective in reducing the gene copies of viable *N. fowleri* (5-log) and *V. vermiformis* (3-log) to below the lower limit of detection (LLOD) at temperatures of 68 - 93°C and 74 - 93°C, respectively. Conversely, while the gene copies of viable *Legionella* and *Acanthamoeba* were significantly reduced by 2-logs ($p = 0.0024$) and 1-log ($p = 0.0015$) overall, respectively, both organisms were still detected after pasteurization at 93°C.

The presence and persistence of *Legionella* spp., and FLA including *Acanthamoeba*, *V. vermiformis* and *N. fowleri* in a representative rainwater harvesting tank and a SOPAS treatment system was thus confirmed. The occurrence of these organisms in pasteurized harvested rainwater is of a serious human health concern as the opportunistic pathogen, *N. fowleri* is the causative agent of the disease, primary amoebic meningoencephalitis (PAM), and although PAM infections are rare, the mortality rate is rapid and extremely high (Marciano-Cabral & Carbral, 2003). Granulomatous amoebic encephalitis (GAE), a brain infection caused

by *Acanthamoeba* is almost consistently a fatal infection in immune-compromised hosts (Khan, 2003; 2006; Qvarnstrom et al. 2013). *Vermamoeba vermiformis* has also been associated with human disease including keratitis and has been isolated from the cerebrospinal fluid of a patient with meningoencephalitis and bronchopneumonia (Kennedy et al. 1995; Centeno et al. 1996; De Jonckheere & Brown, 1998).

In developed countries, Legionellae are one of the most significant water-based bacterial pathogens (Kirschner, 2016), however, owing to the cultivation-based standard techniques that are currently employed, limited information is available on the environmental distribution of *Legionella* spp. as well as incidences of Legionnaires' disease in South Africa. In the current study various *Legionella* spp. such as *L. longbeachae*, *L. norrlandica* and *L. rowbothamii* were isolated and identified in rainwater tanks, which contributes relevant knowledge on the incidence and prevailing species of *Legionella* present in the South African environment. *Legionella norrlandica* has only recently been described, where Rizzardi et al. (2015) showed that this *Legionella* spp. harbours the majority of the *L. pneumophila* virulence factors and is capable of establishing a replicative vacuole in *A. castellanii*. In addition, since Adeleke et al. (2001) reported on the characterisation of *L. rowbothamii*, no studies have reported on the isolation of *L. rowbothamii* from environmental samples. Moreover, no studies have indicated whether *L. rowbothamii* proliferates in protozoa. Future research should thus elucidate whether *L. rowbothamii* is able to colonise and proliferate in amoeba species. Moreover, routine screening of DRWH systems for pathogenic microorganisms should include the detection of *Legionella* spp., including *L. longbeachae*, *L. norrlandica* and *L. rowbothamii* and FLA including *Acanthamoeba*, *V. vermiformis* and *N. fowleri*. Additionally, the presence of viable *Legionella* spp. and *Acanthamoeba* spp. highlights the need for further investigation as solar pasteurization may be insufficient for the long-term control of pathogenic *Acanthamoeba* and Acanthamoebae-bound Legionellae in harvested rainwater. Future studies should therefore also focus on interpreting the number of gene copies when determining the occurrence, infectivity and exposure as significant risk criteria for quantitative microbial risk assessment models for *Legionella* spp. and FLAs, which currently rely on culture-based measurements.

The main objective of Chapter three (submitted to Science of the Total Environment) was to determine the resistance of three *Legionella* spp. isolated from unpasteurized rainwater [*L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.)], two *Legionella* reference strains (*L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462) and *Acanthamoeba mauritaniensis* ATCC 50676 to heat treatment (50–90°C). The resistance of each organism was determined by measuring culturability and viability (using EMA-qPCR). In addition, the resistance of *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) in co-culture

with *A. mauritaniensis* ATCC 50676, respectively, to heat treatment (50–90°C) was determined using EMA-qPCR. The relative expression of genes associated with metabolism and virulence of *L. pneumophila* ATCC 33152 (*lolA, sidF, csrA*) and *L. longbeachae* (env.) (*lolA* only) in co-culture with *A. mauritaniensis* ATCC 50676, respectively was then monitored during heat treatment (50–90°C) using relative qPCR. Results indicated that while the culturability of the *Legionella* species reduced significantly ($p > 0.05$) following heat treatment (60–90°C), *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 were the most resistant to heat treatment as both organisms were still culturable (CFU/mL) following treatment at 50 and 60°C. In contrast, while dormant cysts of *A. mauritaniensis* ATCC 50676 were observed in samples treated at 60 to 90°C, metabolically active trophozoites were detected in the samples treated at 50°C. In addition, results obtained using EMA-qPCR analysis indicated that at 90°C, *L. pneumophila* ATCC 33152 (3.6-log) and *L. longbeachae* ATCC 33462 (3.4-log) exhibited a significant log reduction, while the lowest log reduction was observed for *L. norrlandica* (env.) (1.5-log) ($p > 0.05$). Relative qPCR confirmed that the expression of *lolA* remained constant for *L. pneumophila* ATCC 33152, while the expression of *sidF* increased significantly during co-culture with *A. mauritaniensis* ATCC 50676 ($p < 0.05$), and the expression of *csrA* decreased following co-culturing and heat treatment (50–90°C). In addition, while the expression of *lolA* was observed for *L. longbeachae* (env.) after heat treatment at 50–70°C, the C_q signal of *lolA* expression was not detected at 80–90°C nor for *L. longbeachae* (env.) in co-culture.

Results of the current study thus indicated that EMA-qPCR proved to be a specific and sensitive technique that could be utilised for the routine monitoring, detection and quantification of *Legionella* spp. present in a viable but non-culturable (VBNC) state, internalised within *A. mauritaniensis* ATCC 50676 and that grow poorly on conventional solid media. Relative qPCR targeting the mRNA of heat treated *Legionella* in co-culture with *A. mauritaniensis* ATCC 50676 also proved to be an effective method that could be used to assess both viability and potential virulence of *Legionella* spp. in environmental water samples. Bacterial cells rely on functional transcriptional machinery and intact corresponding DNA segments to transcribe DNA to mRNA (Kirschner, 2016). A positive mRNA signal, measured with relative qPCR, means that the cell has just produced this mRNA, as mRNA is degraded within seconds to hours (Deutscher, 2006). The combination of absolute qPCR and relative qPCR used in the current study thus successfully determined the number of *Legionella* cells that are living, able to replicate and that are potentially infectious to humans. However, in order to measure the degree at which *Legionella* are able to cause infection after heat treatment, future studies should focus on the resuscitation of *Legionella* in amoebae and then determine whether they are able to infect macrophage-like or alveolar epithelial cells (Epalle et al. 2015).

Acanthamoeba spp. were also detected following pasteurization at 93°C (Chapters two and three) and are known to serve as environmental reservoirs for many pathogens detected in harvested rainwater. Characterising the behaviour of *Acanthamoeba* during heat treatment will thus aid in understanding their potential role in the amplification of pathogens such as *Legionella* spp. in SOPAS systems. In addition, the current study indicated that amoeba co-culture increased the virulence of *L. pneumophila,* as *L. pneumophila* ATCC 33152 was able to replicate within *A. mauritaniensis* ATCC 50676, which is evident from the decrease in expression of *csrA* and the increase in expression of *sidF.* However, for *L. longbeachae* strains, this phenomenon requires further investigation as *Legionella* spp. differ in their ability to traffic effector proteins to the *Legionella* containing vacuole and display different virulence mechanisms (Asare & Abu Kwaik, 2007). The relative expression of genes that encode for other effector proteins that are conserved in *L. pneumophila* and *L. longbeachae* should thus be incorporated into future research (Cazalet et al. 2010).

While heat treatment significantly reduces the number of viable *Legionella* spp., future studies should investigate a combination of treatment systems to improve the antimicrobial effect. For example, the use of SOPAS together with oxidizing agents, SOPAS preceding UV irradiation by solar disinfection (SODIS) and the use of SOPAS in combination with pressure systems for the treatment of harvested rainwater should be considered. In addition, future studies should investigate the use of viruses to control or eradicate *Legionella* and *Acanthamoeba* spp. as a biological control method (Wang et al. 2013). The use of phage particles and individual phage proteins have been investigated in human and veterinary medicine for the treatment of infectious diseases and have also been utilised in areas of food safety, wastewater treatment and agriculture (Hudson et al. 2005; Withey et al. 2005; Clark & March, 2006; Petty et al. 2006). Moreover, Curtin and Donlan (2006) and Sutherland et al. (2004) indicated that phages may be suitable for controlling or preventing *Staphylococcus epidermidis* biofilms in the environment and on medical devices. Lammertyn et al. (2008) demonstrated the existence of bacteriophages capable of infecting members of the Legionellaceae including *L. pneumophila*. Additionally, Thomas et al. (2011) isolated a large virus Lausannevirus that was able to infect *Acanthamoeba* spp., but not other amoebae or mammalian cells. Therefore, introducing host specific bacteriophages and viruses into water and rainwater distribution systems may be a strategy to lyse and eradicate *Legionella* and *Acanthamoeba* spp.

As indicated, in the environment, *Legionella* exist as free-living bacteria, within living protozoa or within aquatic biofilms. In addition, characteristics such as trace element concentrations, water hardness and heterotrophic bacteria may influence the persistence and growth of *Legionella* spp. in water distribution systems (Edagawa et al. 2008; Völker et al. 2010). The

overall aim of Chapter four (submitted to Environmental Science and Pollution Research) was to elucidate other microbial and physico-chemical characteristics that are associated with the incidence of *Legionella* spp. and *Acanthamoeba* spp. in rainwater harvested from different roofing materials. Chromadek®, galvanized zinc and asbestos were utilised to construct the catchment areas of the DRWH systems as these roofing materials are utilised for many established buildings as well as low-cost housing in South Africa. Overall results indicated that the roofing materials did not influence the incidence of *Legionella* and *Acanthamoeba* spp. as these organisms were detected (using quantitative PCR (qPCR) analysis) in all tank water samples collected from the Chromadek®, galvanized zinc and asbestos roofing materials. However, significant (p < 0.05) positive Spearman (ρ) correlations were recorded between *Legionella* spp. vs. nitrites and nitrates and between *Acanthamoeba* spp. vs. barium, magnesium, sodium, silicon, arsenic and phosphate, respectively. In addition, while no significant correlations were observed between *Legionella* spp. vs. the indicator bacteria (p > 0.05), positive correlations were established between *Acanthamoeba* spp. vs. total coliforms and *E. coli*, respectively. Results thus indicated that external pollutants such as dust, debris and faecal matter serve as the primary reservoirs of *Legionella* and *Acanthamoeba* spp. in harvested rainwater. In addition, the presence of these pollutants may explain the frequency of isolation of *L. longbeachae* [frequently isolated from soil (Cramp et al. 2010; Currie et al. 2014)], *L. norrlandica* [previously isolated from wood processing plants (Rizzardi et al. 2015)] and *L. rowbothamii* [previously isolated from water samples including potable well water (Adeleke et al. 2001)] from unpasteurized rainwater samples (Chapter two).

Results obtained in the current study thus indicate that harvested rainwater may be a reservoir for *Legionella* and *Acanthamoeba* infections as these organisms were detected at high concentrations in all the tank water samples collected. Future studies should thus also focus on *Acanthamoeba* spp., *N. fowleri* and *V. vermiformis* as vectors for *Mycobacterium* spp. and *Pseudomonas aeruginosa,* as these microbial opportunistic pathogens together with *Legionella* spp., are currently the waterborne pathogens of highest concern in countries such as the United States of America and have been detected in harvested rainwater (Albrechtsen, 2002; National Research Council, 2006; Centers for Disease Control, 2011; Bartrand et al. 2014; Dobrowsky et al. 2014; 2016; Hamilton et al. 2016).

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APPENDIX A
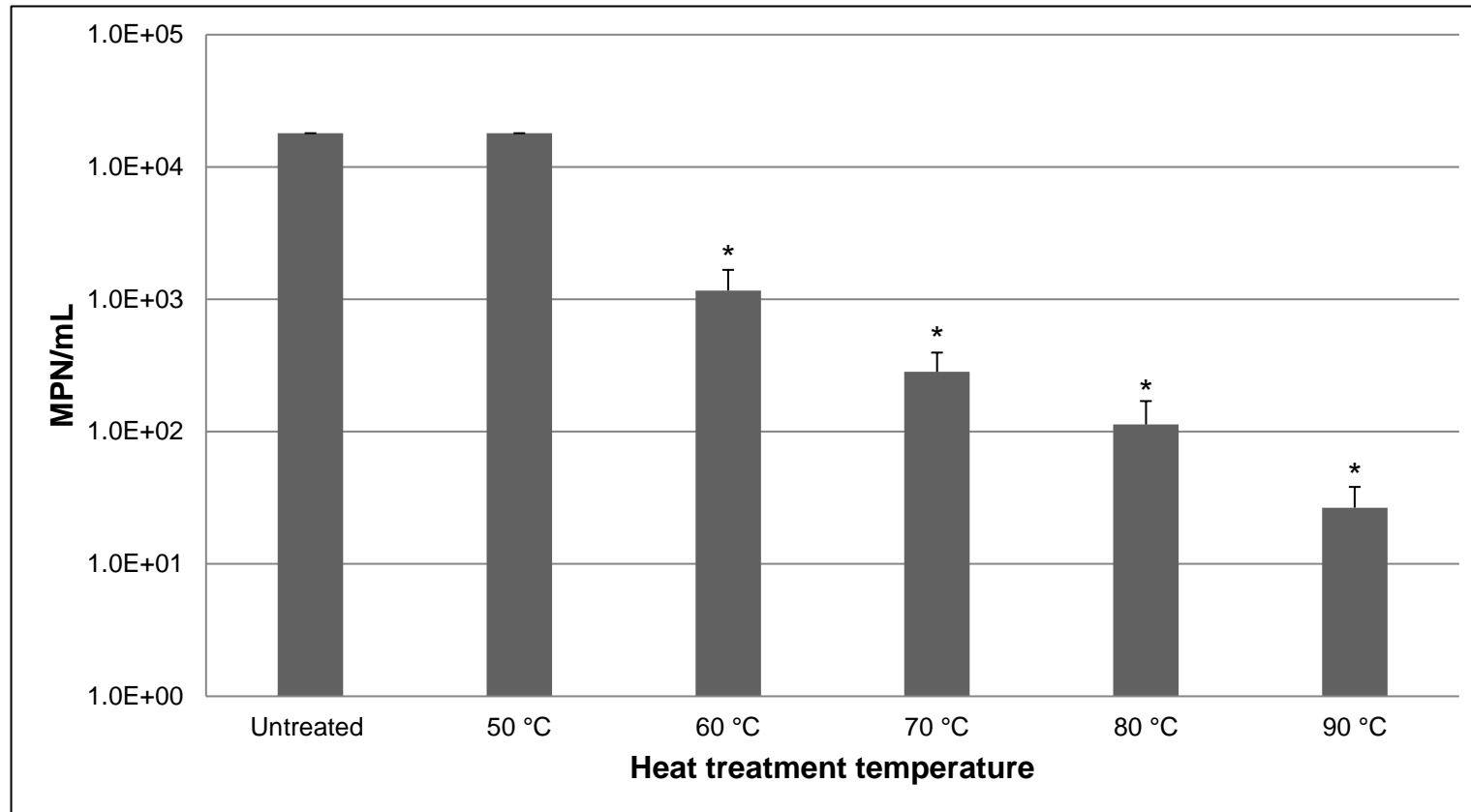
Figure 1. Most probable number (MPN) method used to enumerate trophozoites and cysts of *A. mauritaniensis* ATCC 50676 before (untreated positive control) and after heat treatment. The data are expressed as the mean of total cell numbers and error bars indicate standard deviation (n = 3). * denotes $p < 0.05$.
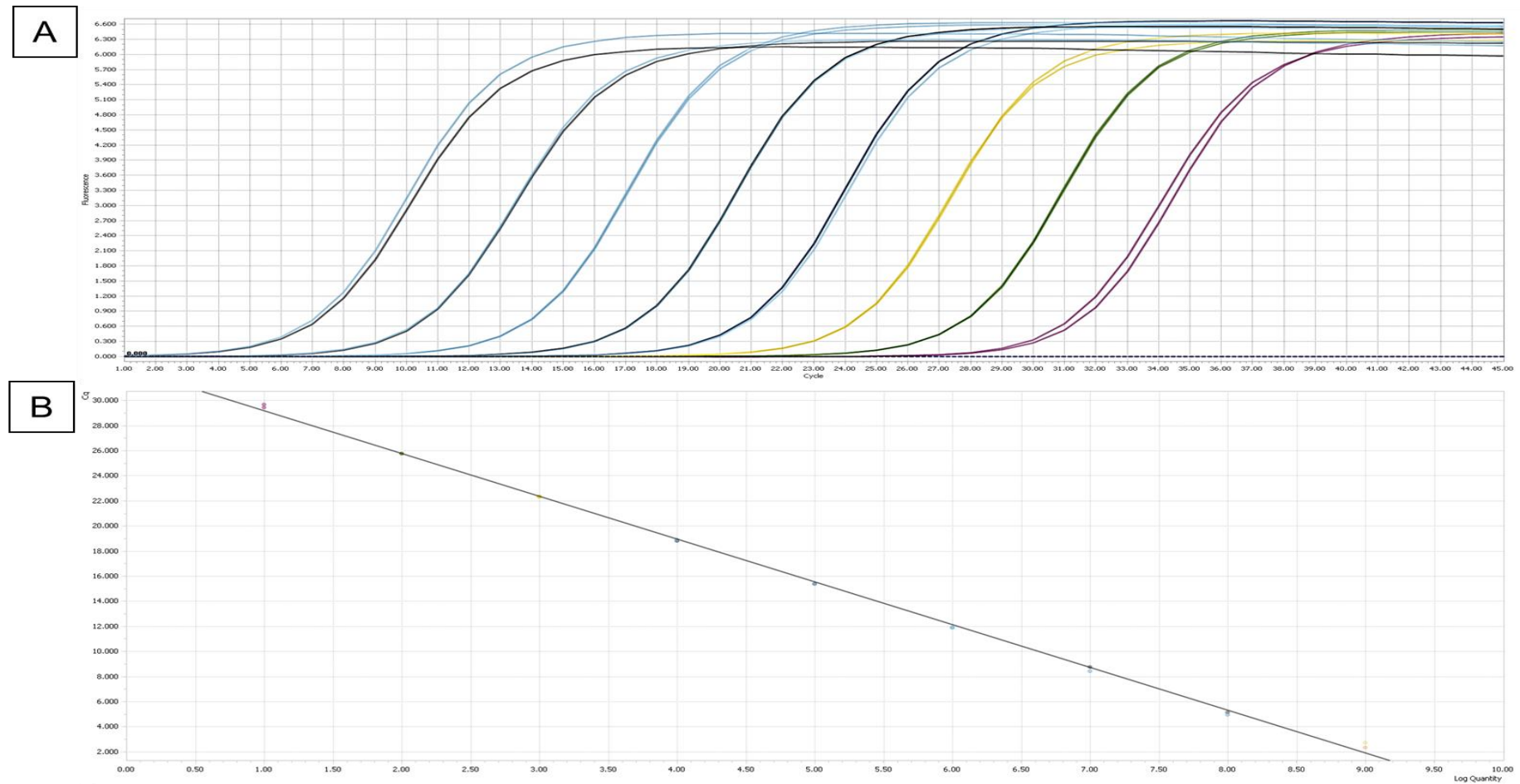
Figure 2. Ethidium monoazide (EMA) qPCR was utilized to quantify the gene copies/mL of viable *Legionella* spp. including *L. norrlandica* (env.)*, L. rowbothamii* (env.) and *L. longbeachae* (env.), and the reference strains *L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462. A.) Amplification curve and B.) standard curve generated by amplifying the 259 bp product of the 23S – 5S rRNA gene utilized to quantify *Legionella* spp.

202
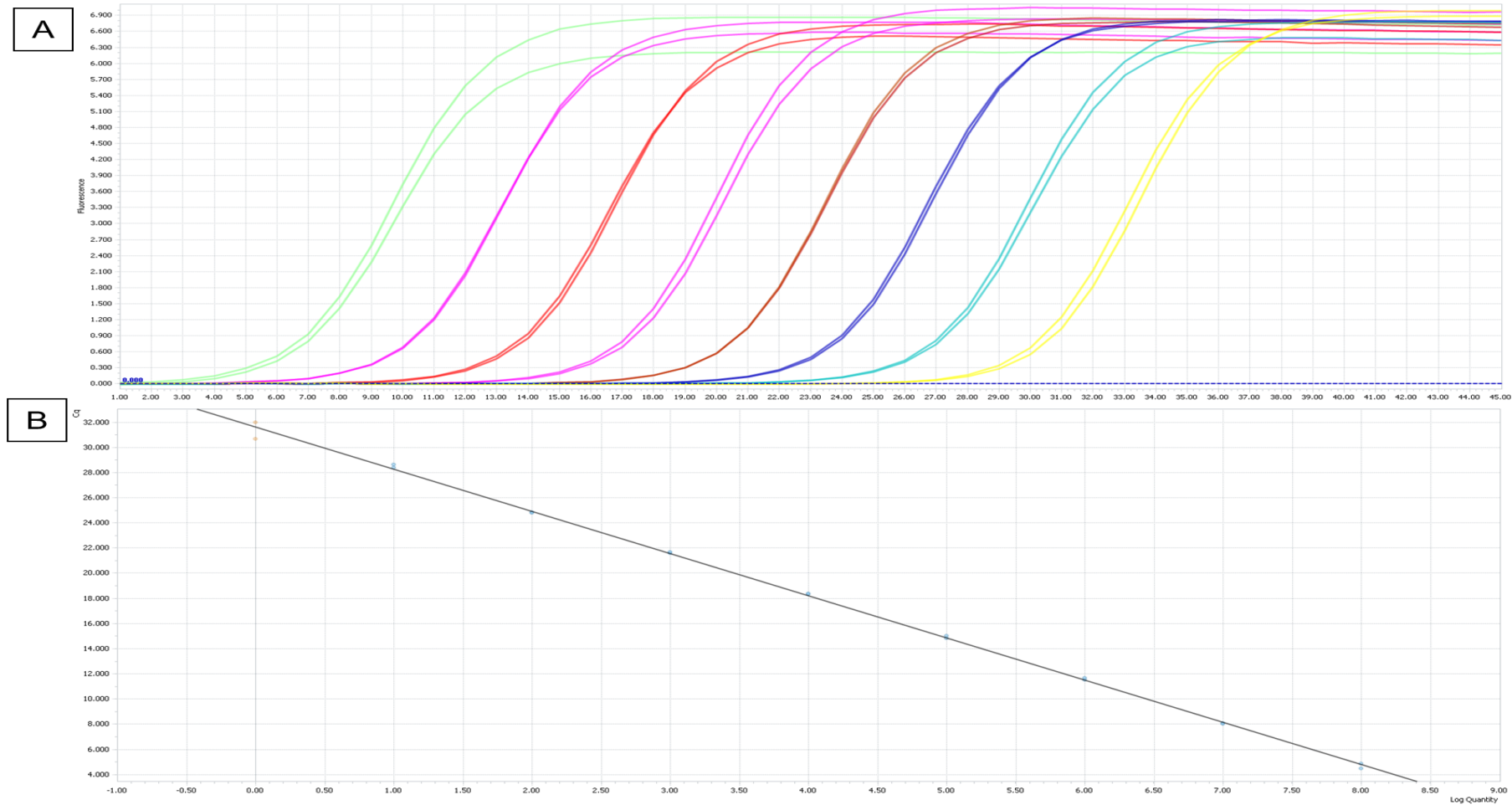
Figure 3. Ethidium monoazide (EMA) qPCR was utilized to quantify the gene copies/mL of viable *A. mauritaniensis* ATCC 50676. A.) A.) Amplification curve and B.) standard curve generate by amplifying the 180 bp product of the 18S rRNA gene utilized to quantify *A. mauritaniensis* ATCC 50676.
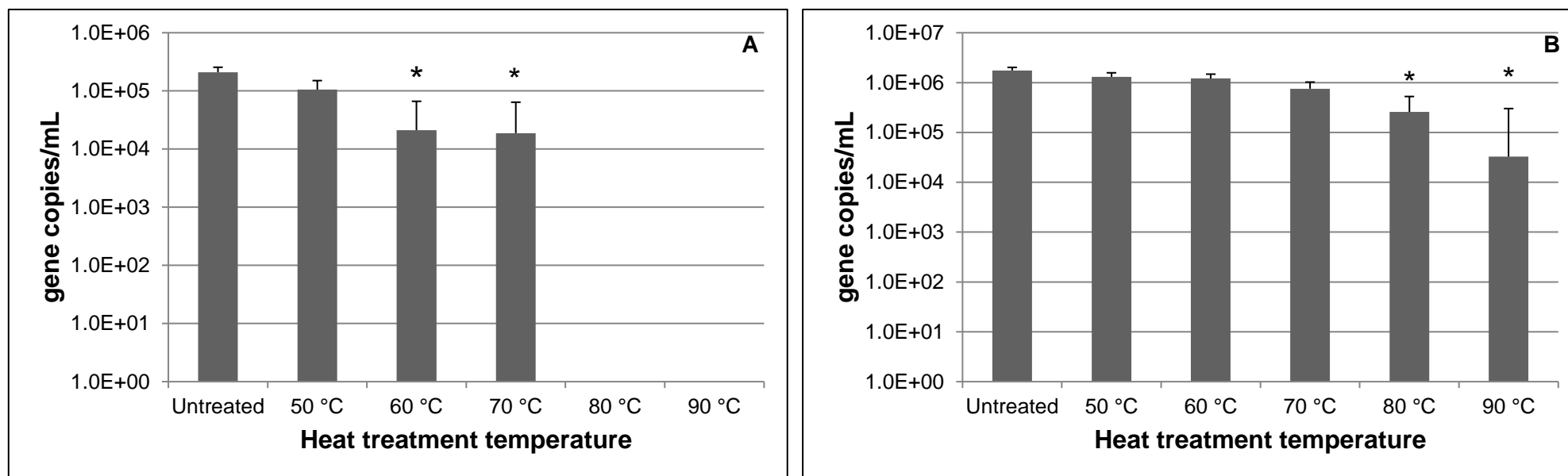
203
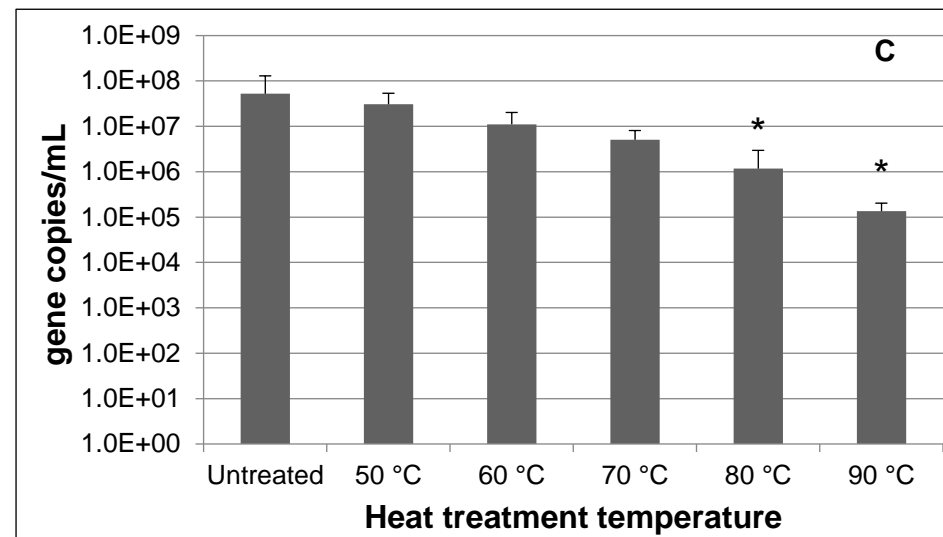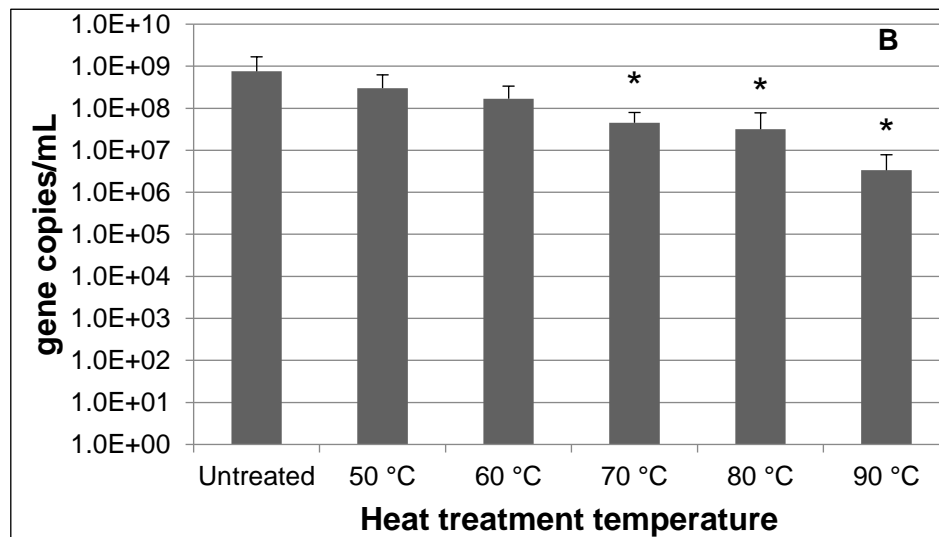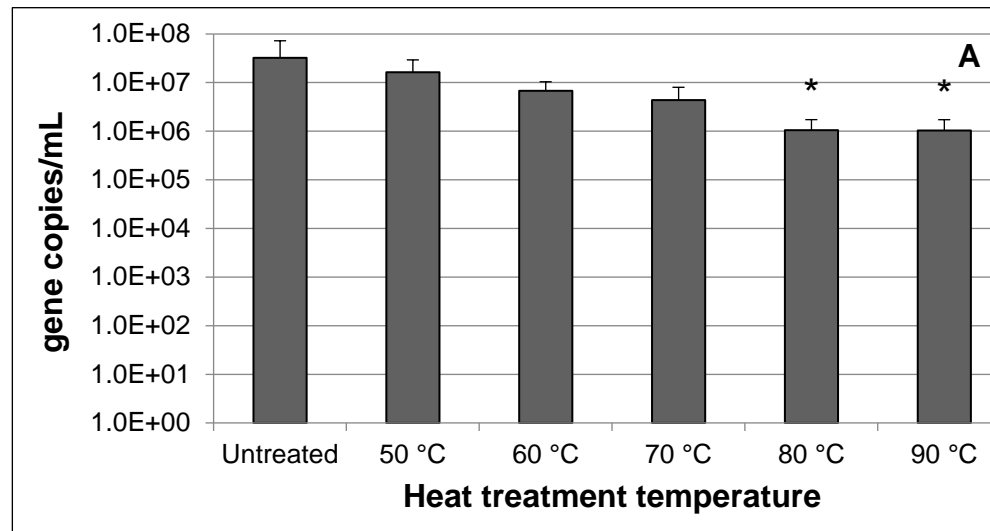
Figure 4. Mean gene copies/mL of A.) *L. norrlandica* (env.), B.) *L. rowbothamii* (env.) and C.) *L. longbeachae* (env.) quantified before (untreated positive control) and after heat treatment. The data are expressed as the mean gene copies/mL and error bars indicate standard deviation (n = 3). * denotes $p < 0.05$.
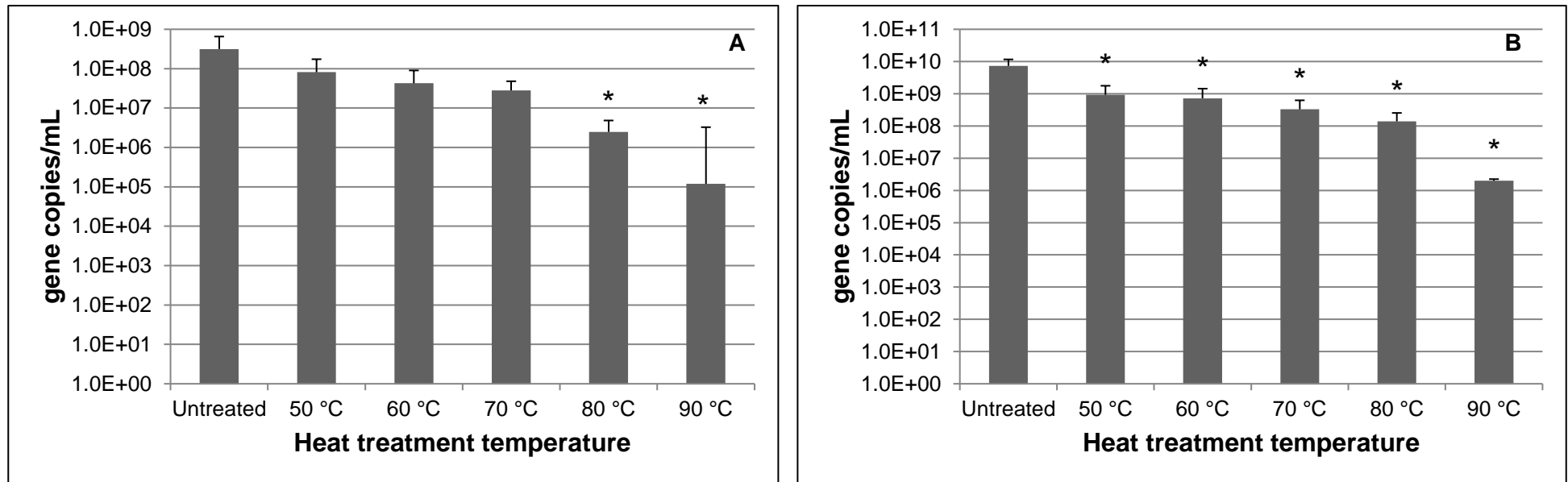
Figure 5. Mean gene copies/mL of A.) *L. longbeachae* ATCC 33462 and B.) *L. pneumophila* ATCC 33152 quantified before (untreated positive control) and after heat treatment. The data are expressed as the mean gene copies/mL and error bars indicate standard deviation (n = 3). * denotes $p < 0.05$.

Figure 6. Mean gene copies/mL of A.) *L. longbeachae* (env.) co-cultured with *A. mauritaniensis* ATCC 50676 and B.) *L. pneumophila* ATCC 33152 co-cultured with *A. mauritaniensis* ATCC 50676 quantified before (untreated positive control) and after heat treatment. The data are expressed as the mean gene copies/mL and error bars indicate standard deviation (n = 2). * denotes $p < 0.05$.
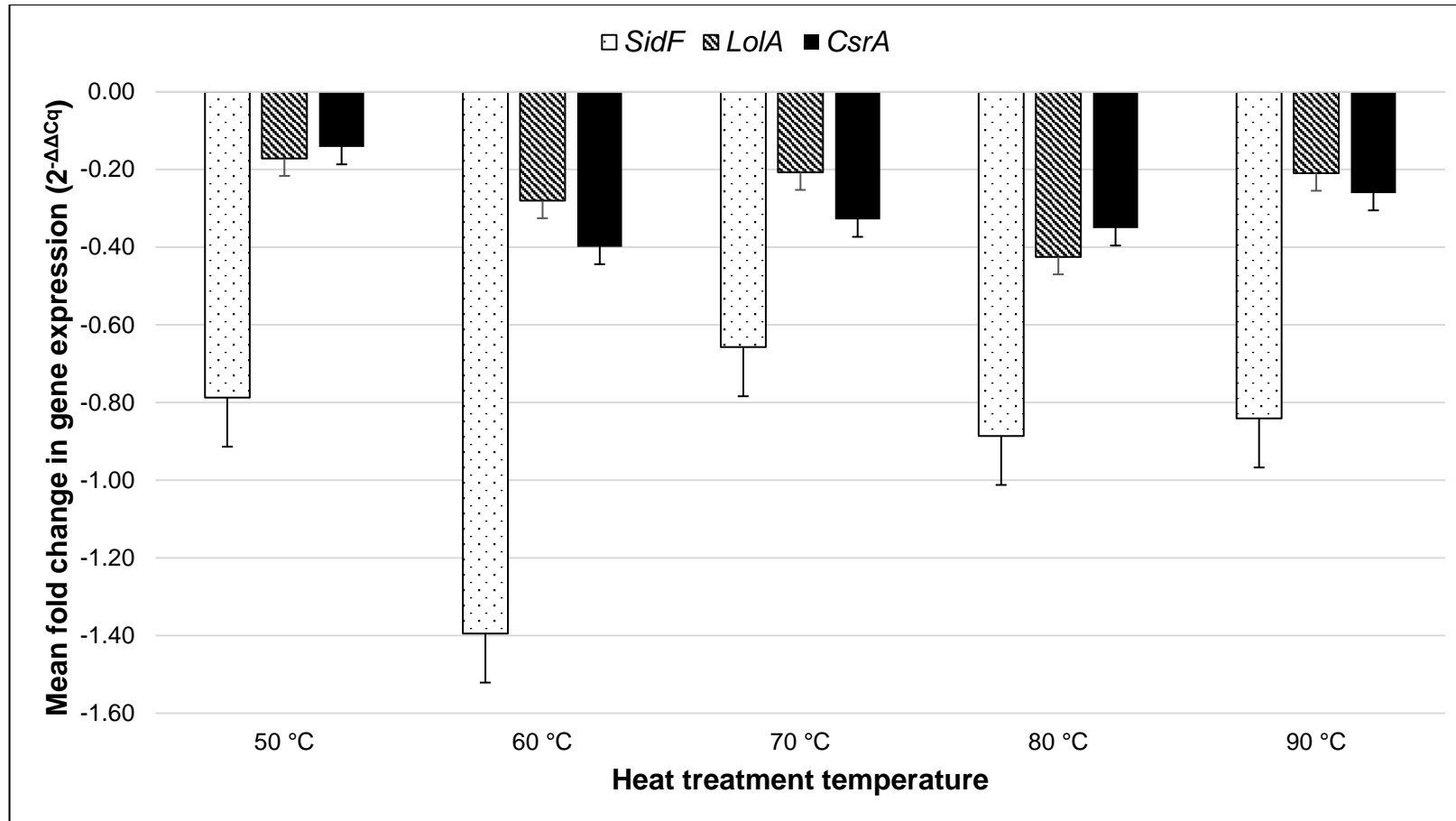
Figure 7. Mean fold change in gene expression (*SidF, LolA, CsrA*) after heat treatment of *L. pneumophila* ATCC 33152. The data are standardized relative to the untreated positive control of *L. pneumophila* ATCC 33152 and are expressed as the mean fold change in gene expression ($2^{-\Delta\Delta Cq}$). The decrease in gene expression ($2^{-\Delta\Delta Cq} > 1$) was calculated by $-1/2^{-\Delta\Delta Cq}$.
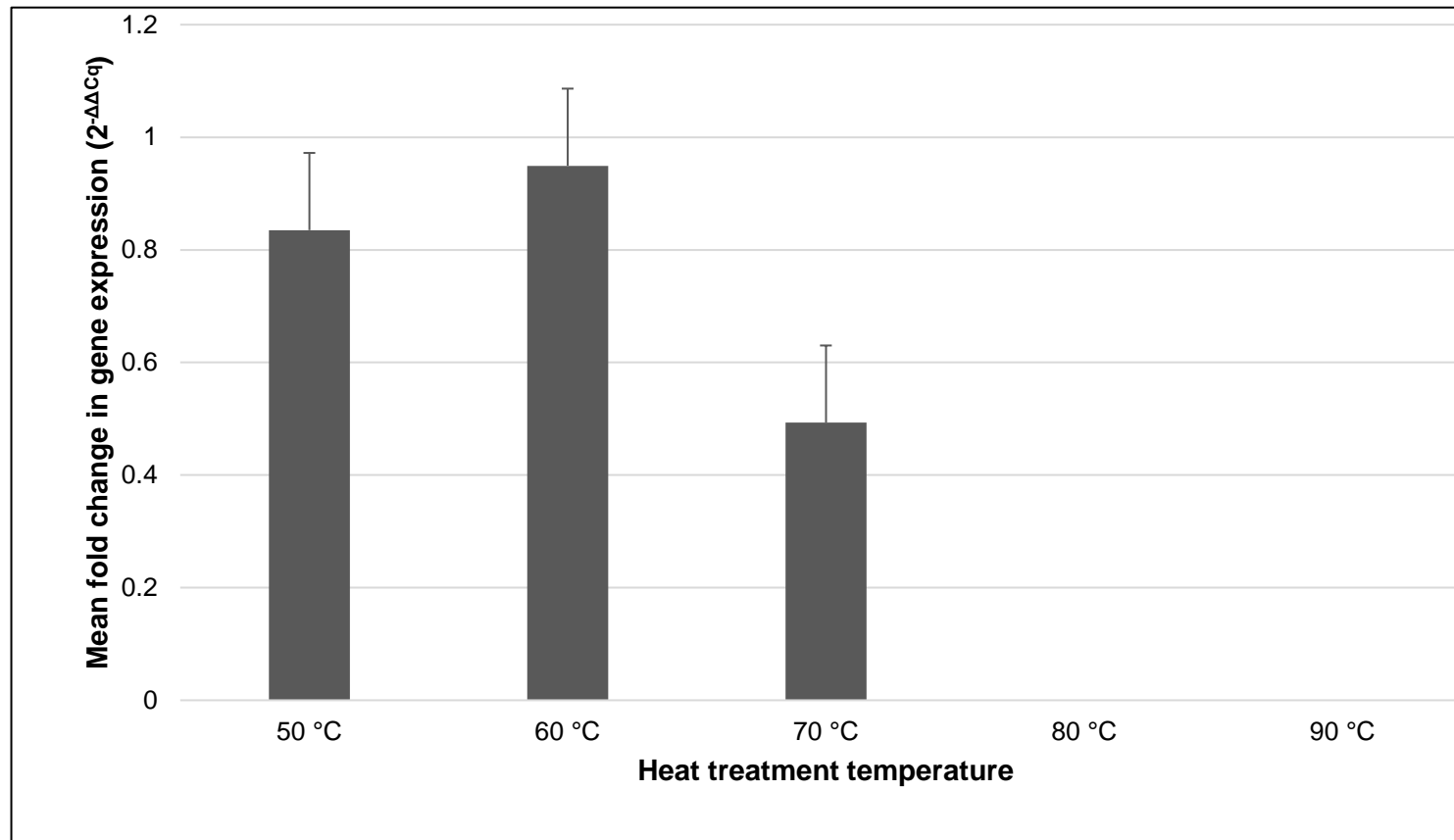
Figure 8. Fold changes in gene expression of *lolA* after heat treatment of *L. longbeachae* (env.). The data are standardized relative to the untreated positive control of *L. longbeachae* (env.) and are expressed as the mean fold change in gene expression ($2^{-\Delta\Delta Cq}$). The decrease in gene expression ($2^{-\Delta\Delta Cq} > 1$) was calculated by $-1/2^{-\Delta\Delta Cq}$.