Application of Solar Pasteurization for the Treatment of Harvested Rainwater

by

Brandon Reyneke

Thesis presented in fulfilment of the requirements for the degree Master of Science at Stellenbosch University

Supervisor: Prof. Wesaal Khan

Co-supervisor: Dr Sehaam Khan

Department of Microbiology

Faculty of Science

March 2017

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2017

Signature:…….. Date:……..

SUMMARY

Rainwater harvesting has been earmarked by South African governmental authorities as an intervention strategy that could alleviate the pressures on existing centralised water distribution systems, especially in rural areas and urban informal settlements, where insufficient waste removal and potable water infrastructure are available. However, numerous studies have indicated that harvested rainwater may not be safe to use for all daily water requirements, as numerous chemical and microbial contaminants may be associated with stored tank water. Rainwater treatment technologies, including solar pasteurization (SOPAS), have subsequently been investigated (**Chapter 1**).

In order to determine whether decentralised rainwater harvesting SOPAS systems may be a viable alternative in providing the inhabitants of informal settlements with a supplementary water source, two small- (Sites 1 and 2) and one large-scale (Site 3) rainwater harvesting SOPAS systems were installed in Enkanini informal settlement, Stellenbosch, South Africa (**Chapter 2**). The microbial and chemical quality of the unpasteurized and pasteurized (produced by the respective systems) rainwater was monitored using conventional water quality monitoring techniques, including the culturing of indicator organisms, screening for selected indigenous rainwater pathogens using the polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays and the monitoring of anion and cation concentrations. Additionally, the operational sustainability of the systems and water usage by the participating households were monitored. Chemical analyses indicated that all anions and cations were within the limits stipulated by various national and international drinking water quality guidelines, with the exception of zinc which contravened the respective guidelines before (mean: 3919 µg/L) and after (mean: 3964 µg/L) pasteurization at both Sites 1 and 2. In addition, the arsenic concentrations measured at Site 3 before (mean: 18.69 µg/L) and after (mean: 18.30 µg/L) pasteurization exceeded the respective drinking water guidelines. The increased zinc concentrations were attributed to the galvanised zinc roofing material installed at Sites 1 and 2, while the increased arsenic concentrations may be attributed to a roofing treatment or paint utilised to cover the catchment area at Site 3. Microbial analyses indicated that pasteurization temperatures of 53 °C (small-scale systems) and 55 °C (large-scale system) were required to reduce *Escherichia coli* and total and faecal coliforms to below the detection limit [< 1 colony forming units (CFU)/100 mL]. However, minimum pasteurization temperatures of 66 °C (small-scale systems) and 71 °C (large-scale system), were required to reduce the heterotrophic plate count (HPC) to within drinking water limits (1.0×10^4 CFU/100 mL). Of the opportunistic pathogens detected using PCR assays, *Legionella* spp. was the most prevalent pathogen detected in the small-scale systems [unpasteurized (100%) and pasteurized (91%)] and the large-scale system [unpasteurized (83%) and stored pasteurized tank water (100%)]. Quantitative PCR analysis then indicated that while the gene copies of *Legionella* spp., *Pseudomonas* spp. and *Salmonella*

spp. were reduced during SOPAS, the organisms were still detected at the highest pasteurization temperatures analysed for each site (Site 1 – 85 °C; Site 2 – 66 °C; Site 3 – 79 °C). Additionally, the application of a metabolic responsiveness adenosine triphosphate (ATP) assay (BacTiter-Glo™ Microbial Cell Viability Assay) indicated the presence of metabolically active cells in all pasteurized rainwater samples analysed. Results also indicated that the systems required limited maintenance and the small-scale systems in particular were able to provide the participating households with an alternative warm water source that could be utilised for numerous domestic purposes.

As various limitations have been associated with the use of culture-based analyses for the monitoring of water quality, the aim of **Chapter 3** was to compare molecular-based viability assays [ethidium monoazide bromide (EMA)-qPCR, propidium monoazide (PMA)-qPCR and DNase treatment in combination with qPCR] as well as the metabolic responsiveness ATP assay to culturing analysis for their ability to accurately determine cell viability in bacterial monocultures following heat treatment. Three Gram-negative (*Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp.) and two Gram-positive (*Staphylococcus* spp. and *Enterococcus* spp.) bacteria commonly associated with water sources were selected as test organisms. Of the various concentrations of EMA and PMA analysed, 6 µM EMA and 50 µM PMA were identified as the optimal dye concentrations as low log reductions were recorded (viable and heat treated samples) in comparison to the no viability treatment control. Comparison of the results obtained for all the molecular viability assays (6 µM EMA, 50 µM PMA and DNase treatment) then indicated that the 6 µM EMA concentration was comparable to both the 50 µM PMA and the DNase treatment for the analysis of most of the test organisms (viable and heat treated). In addition, the results for the culturing analysis (CFU) of the viable *S. typhimurium* as well as the viable and heat treated samples of *L. pneumophila* and *P. aeruginosa* were comparable to the gene copies detected using molecular-based viability assays. However, the CFU in the heat treated samples of *S. typhimurium* were significantly lower than the gene copies detected using DNase in combination with qPCR, with no gene copies or CFU detected in the heat treated samples of *S. aureus* and *E. faecalis*. In contrast, while the ATP assays indicated the presence of metabolically active cells in the viable and heat treated samples, the ATP assay also indicated the presence of metabolically active cells in samples that had been autoclaved (negative viability control). It was thus concluded that molecular-based assays may be used to supplement culture based analysis for the comprehensive identification of the viable microbial population in water samples (before and after treatment).

OPSOMMING

Die oes van reënwater is deur Suid-Afrikaanse regeringsowerhede as 'n ingrypingstrategie geïdentifiseer, wat die druk op die bestaande gesentraliseerde waterverspreidingstelsels kan verlig, veral vir landelike gebiede en informele nedersettings waar onvoldoende vullisverwydering en drinkwater-infrastruktuur beskikbaar is. Talle studies het egter aangedui dat ge-oeste reënwater nie veilig vir alledaagse watervereistes is nie weens talle chemiese en mikrobiese kontaminante wat met gestoorde tenkwater geassosieer word. Reënwater-behandelingstegnologieë, insluitende sonkragpasteurisasie (SOPAS), is dus ondersoek (**Hoofstuk 1**).

Om vas te stel of gedesentraliseerde reënwater-oesting SOPAS sisteme vir die inwoners van informele nedersettings ‘n aanvullende bron van water op ‘n lewensvatbare wyse kan voorsien, is twee klein- (Terrein 1 en 2) en een grootskaalse (Terrein 3) reënwater oesting SOPAS sisteme in Enkanini, Stellenbosch, Suid Afrika, geinstalleer (**Hoofstuk 2**). Die mikrobiese en chemiese kwaliteit van die gepasteuriseerde en ongepasteuriseerde (deur die onderskeie sisteme geproduseer) reënwater is met behulp van konvensionele waterkwaliteit analises gemonitor, wat die groei van indikator-organismes, die toetsing vir geselekteerde inheemse reënwaterpatogene met polimerase kettingreaksie (PKR) en kwantitatiewe PKR (kPKR) en die bepaling van anioon- en katioon konsentrasies, insluit. Daarbenewens is die operasionele volhoubaarheid van die sisteme en die waterverbruik van die betrokke huishoudings gemonitor. Chemiese analises het aangedui dat al die anioon- en katioon konsentrasies binne die limiete van die verskeie nasionale en internasionale drinkwater riglyne was, met die uitsondering van sink wat die onderskeie riglyne voor (gemiddeld: 3919 µg/L) en na (gemiddeld: 3964 µg/L) pasteurisasie by beide Terrein 1 en 2 oorskry het. Daarbenewens het die arseen konsentrasies by Terrein 3 voor (gemiddeld: 18,69 µg/L) en na (gemiddeld: 18,30 µg/L) pasteurisasie ook die onderskeie drinkwater riglyne oorskry. Die verhoogde sink konsentrasies is toegeskryf aan die gegalvaniseerde sinkplate wat as dakoppervlak by Terrein 1 en 2 gebruik is, terwyl die verhoogde arseen konsentrasies aan die verf of behandeling van die dak by Terrein 3 aangewend is, toegeskryf is. Die mikrobiese analises het aangedui dat pasteurisasie temperature van 53 °C (kleinskaalse sisteme) en 55 °C (grootskaalse sisteem) nodig is om *Escherichia coli* en totale en fekale kolivorme tot onder die opsporingslimiet [< 1 kolonie vormende eenhede (KVE)/100 mL] te verminder. Minimum pasteurisasie temperature van 66 °C (kleinskaalse sisteme) en 71 °C (grootskaalse sisteem) is egter nodig om die heterotrofiese plaattelling (HPT) tot binne die limiete van die drinkwater riglyne (1.0×10^4 KVE/100 mL), te verminder. Die PKR analises het aangetoon dat *Legionella* spp. die mees algemene patogeen in beide die kleinskaalse [ongepasteuriseerde (100%) en gepasteuriseerde (91%)] en grootskaalse sisteme [ongepasteuriseerde (83%) en gestoorde gepasteuriseerde tenkwater (100%)] was. Die kPKR analises het aangedui dat terwyl die geenkopieë van *Legionella* spp.,

Pseudomonas spp. en *Salmonella* spp. tydens SOPAS verminder is, die organismes steeds by die hoogste pasteurisasie temperatuur van elke terrein (Terrein 1 – 85 °C; Terrein 2 - 66° C; Terrein 3 - 79° C) teenwoordig was. Daarby het die metaboliese responsiwiteit adenosien trifosfaat (ATP) toets (BacTiter-Glo™ Microbial Cell Viability Assay) aangedui dat metabolies-aktiewe selle in al die gepasteuriseerde reënwatermonsters teenwoordig was. Die resultate het ook aangedui dat die sisteme minimale onderhoud nodig gehad het en dat die kleinskaalse sisteme die huishoudings met ‘n alternatiewe warm waterbron kon voorsien, wat vir verskeie huishoudelike take gebruik kon word.

Verskeie beperkings word geassosieër met die gebruik van groei-gebaseerde analises om waterkwaliteit te monitor. Die doel van **Hoofstuk 3** was dus om molekulêr-gebaseerde lewensvatbaarheidstoetse [ethidium monoasied bromied (EMA)-kPKR, propidium monoasied (PMA)-kPKR en DNase-behandeling in kombinasie met kPKR] en die metaboliese responsiwiteit ATP toets met groei-gebaseerde analises, te vergelyk in terme van hul vermoë om sel lewensvatbaarheid in bakteriële monokulture na hitte-behandeling te bepaal. Drie Gram-negatiewe (*Legionella* spp., *Pseudomonas* spp. en *Salmonella* spp.) en twee Gram-positiewe (*Staphylococcus* spp. en *Enterococcus* spp.) bakterieë, wat algemeen met waterbronne geassosieer word, is as toets organismes gekies. Verskeie EMA en PMA konsentrasies is getoets met 6 µM EMA en 50 µM PMA wat as die optimale konsentrasies geïdentifiseer is op grond van die lae log-vermindering wat opgemerk is (lewensvatbare en hitte-behandelde monsters) in vergelyking met die nie-lewensvatbare kontrole. Vergelyking van die resultate wat vir al die molekulêre lewensvatbaarheidstoetse (6 µM EMA, 50 µM PMA en DNase behandeling) verkry is, het aangedui dat 6 µM EMA met beide die 50 µM PMA en die DNase behandeling vir meeste van die toets organismes (lewensvatbaar en hitte behandeld) vergelykbaar was. Daarbenewens was die groei-gebaseerde analise (KVE) van *S. typhimurium* en die lewensvatbare en hitte-behandelde *L. pneumophila* en *P. aeruginosa* vergelykbaar met die geenkopieë wat met die molekulêre lewensvatbaarheidstoetse verkry is. Die KVE in die hitte-behandelde *S. typhimurium* monsters was egter beduidend laer as die geenkopieë wat met die DNase in kombinasie met kPKR analise verkry is, terwyl daar nie geenkopieë of KVE in die hitte-behandelde *S. aureus* of *E. faecalis* monsters verkry kon word nie. In teenstelling, alhoewel die ATP toets aangedui het dat metabolies-aktiewe selle in die lewensvatbare en hitte behandelde monster teenwoordig was, het die toets ook aangedui dat daar metabolies-aktiewe selle in die ge-outoklaveerde monsters was (die negatiewe lewensvatbare kontrole). Dus kan molekulêr-gebaseerde toetse gebruik word om groei-gebaseerde toetse vir die omvattende identifikasie van die lewensvatbare mikrobiese populasie in water monsters (voor en na behandeling) aan te vul.

ACKNOWLEDGEMENTS

Dr Wesaal Khan – who has been my mentor and supervisor throughout my studies. I cannot thank you enough for the amazing opportunities you have provided me with over the years and for all your help towards achieving my goals. I really appreciate all the time and effort you put into my project. Jammer oor al die grys hare wat ek soms veroorsaak het. Without your guidance and support, my studies and project would not have been a success.

Dr Sehaam Khan – for her co-supervison, guidance and knowledge throughout the project and always saying things are going to be okay when things weren't going according to plan.

Khan Lab (Penny Dobrowsky, Thando Ndlovu, Andre Strauss, Tanya Clements, Michael Tobin) – to my Khan Academy Family, I would like to say thank you for all your help in the lab, sampling in Enkanini, advise, friendship and always being interested in the project.

The Water Research Commission for funding the project.

The National Research Foundation for financial assistance throughout my postgraduate studies. *"The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF."*

Mr. Jacques de Villiers for technical assistance with the installation of the solar pasteurization systems.

Mr. Berry Wessels for assistance with the installation of the rainwater harvesting systems and his knowledge about all things Enkanini-related.

Mr. Yondela Tyawa for his on-site assistance in Enkanini during the project.

Participating households of Enkanini for their willingness to participate in the project, all the friendly "hallo's" during sampling and enthusiasm throughout the project.

The South African Weather Services for providing the total rainfall data for the months of September 2015 to October 2016.

Louw/Africander Labs (Biochemistry Department) for the use of the Veritas Luminometer.

Stellenbosch University/Department of Microbiology

My family and friends – Thank you for all your love and support.

My parents, Piet and Veronica Reyneke – Baie dankie vir al die opofferings wat Pappa en Mamma gemaak het sodat ek die geleenthede kon hê wat julle nie gehad het nie. Ek waardeer alles wat julle vir my doen en beteken!

Monique Waso – Baie dankie vir al jou liefde, ondersteuning en 01:00 oggend-koffie aandra terwyl ek opgeskryf het. Ek sou dit nie sonder jou kon doen nie. Baie lief vir jou!

TABLE OF CONTENTS

DECLARATION ..... **ii**

SUMMARY ..... **iii**

OPSOMMING ..... **v**

LIST OF ABBREVIATIONS AND ACRONYMS ..... **x**

Chapter 1: **1**

Literature Review **1**

1.1 Introduction 2

1.2 Domestic rainwater harvesting 4

1.3 Quality of harvested rainwater 8

1.4 Rainwater treatment systems 16

1.5 Viability detection systems 23

1.6 Description of the study site 29

1.7 Project aims 31

1.8 References 34

Chapter 2: **55**

Operational sustainability and efficacy of small- and large-scale solar pasteurization treatment systems installed in Enkanini informal settlement (Stellenbosch) for the treatment of rainwater **55**

2.1 Introduction 58

2.2 Materials and methods 61

2.3 Results 74

2.4 Discussion 103

2.5 Conclusion 114

2.6 References 115

Chapter 3: **128**

Molecular-based viability assays versus metabolic responsiveness assays for the accurate determination of microbial cell viability **128**

3.1 Introduction 131

3.2 Materials and methods 135

3.3 Results 142

3.4 Discussion 155

3.5 Conclusions 163

3.6 References 164

Chapter 4: 172

General Conclusions and Recommendations 172

4.1 References 183

Appendix A: 189

Construction and installation of the domestic rainwater harvesting solar pasteurization treatment systems in Enkanini informal settlement (Stellenbosch) and water usage by the participating households 189

1. Site selection and description 190

2. Design and construction of the rainwater harvesting treatment systems 191

3. Monitoring the rainwater and municipal water usage by the households utilising the solar pasteurization treatment systems 197

4. References 205

Appendix B: 206

Conventional PCR Results 206

LIST OF ABBREVIATIONS AND ACRONYMS

ADWG	Australian Drinking Water Guidelines
ARISA	Automated rRNA Intergenic Spacer Analysis
ATP	Adenosine Triphosphate
BDL	Below Detection Limit
BLAST	Basic Local Alignment Search Tool
CAF	Central Analytical Facility
CDC	Centres for Disease Control and Prevention
CFU	Colony Forming Units
CSIR	Council for Scientific and Industrial Research
DNA	Deoxyribonucleic Acids
DRWH	Domestic Rainwater Harvesting
DST	Department of Science and Technology
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EDTA	ethylenediaminetetraacetic acid
EMA	Ethidium Monoazide bromide
FAO	Food and Agricultural Organization
FIB	Faecal Indicator Bacteria
GDRC	Global Development Research Centre
HNA	High Nucleic Acid
HPC	Heterotrophic Plate Count
LLOD	Lower Limit of Detection
MDG	Millennium Development Goals
mRNA	Messenger Ribonucleic Acids
NCBI	National Centre for Biotechnology Information
NHMRC	National Health and Medical Research Council
NRMMC	Natural Resource Management Ministerial Council
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PMA	Propidium Monoazide
PVA	Poly (Vinyl Alcohol)
qPCR	Quantitative or Real-Time Polymerase Chain Reaction
r^2	Correlation Coefficient
R2A	Reasoner's 2 Agar
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RWH	Rainwater Harvesting
SABS	South African Bureau of Standards
SANS	South African National Standards
SDG	Sustainable Development Goals
SODIS	Solar Disinfection
SOPAS	Solar Pasteurization
TBE	Tris Borate Ethylene-diaminetetraacetic Acid
UK	United Kingdom
UN	United Nations
UNICEF	United Nations International Children's Emergency Fund
USA	United States of America
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
VBNC	Viable But Non-Culturable
WHO	World Health Organization
WRC	Water Research Commission

Chapter 1: Literature Review

(UK spelling is employed)

1.1 Introduction

While the target of the global Millennium Development Goals (MDG), to halve the proportion of the population without sustainable access to safe drinking water was met by 2015, it was reported that 663 million people worldwide (approximately 9% of the global population) still did not have access to a safe water source and that 2.4 billion people lacked access to improved sanitation services (United Nations MDG Report, 2015). Motivated by the success of the MDG, world leaders then gathered on 25 September 2015 at the United Nations to adopt the 2030 Agenda for Sustainable Development. The Agenda is comprised of 17 Sustainable Development Goals (SDG) including; ensuring universal access to safe and affordable drinking water by 2030. In order to achieve the latter, various alternate water sources such as on-site greywater and treated wastewater re-use, rainwater and desalinated water have been investigated as potential sources of supply. As the harvesting of rainwater has been utilised for centuries throughout the world, this method of water collection has been earmarked by many international countries as a cost-effective water source, which could provide clean and potable water directly to the consumer, thereby alleviating pressures on existing water supplies (Li et al. 2010; Mwenge Kahinda et al. 2010).

Research has however indicated that numerous chemical and microbial contaminants are associated with stored tank water sources including harvested rainwater (Ahmed et al. 2008, 2011; Helmreich & Horn, 2009; Li et al. 2010). While chemical contaminants have not been directly associated with the incidence of disease (Sazakli et al. 2007; Chapman et al. 2008; Huston et al. 2012), microbial contaminants detected in harvested rainwater include traditional faecal indicators and various other bacterial and protozoan species, many of which are associated with human disease (Uba & Aghogho, 2000; Lye, 2002; Ahmed et al. 2008; 2010a; 2010b; 2011; De Kwaadsteniet et al. 2013; Dobrowsky et al. 2014). Research has also linked sporadic outbreaks of disease to the utilisation of tank water sources (Merritt et al. 1999; Simmons et al. 2008; Franklin et al. 2009).

Thus in order to ensure that harvested rainwater is safe to utilise for all daily water requirements, time- and cost-effective treatment technologies need to be implemented. Technologies that have been used for the treatment of rainwater include poly (vinyl alcohol) (PVA) nanofiber membranes, activated carbon and slow sand filtration systems, solar disinfection (SODIS), solar pasteurization (SOPAS) and chlorination (Chapman et al. 2008; McGuigan et al. 2012; De Kwaadsteniet et al. 2013; Dobrowsky et al. 2014; Abraham et al. 2015). In particular, SOPAS is considered a reliable system for the effective treatment of large volumes of water (Helmreich & Horn, 2009), where the removal of most pathogens is independent of turbidity, pH and other parameters that may influence water treatment systems (Burch & Thomas, 1998; Abraham et al. 2015; Dobrowsky et al. 2015). Furthermore, as SOPAS

is associated with the generation of high temperatures to disinfect contaminated water, the time period required to treat large volumes of water is less than that associated with other water treatment systems (Caslake et al. 2004).

In order to determine if the treated rainwater may be used for potable purposes, it is common practice to assess the quality of the water by comparing it to prescribed guidelines for drinking water which specify microbial and chemical parameters (Ahmed et al. 2011). The microbial parameters specified within various drinking water guidelines are usually limited to the presence of indicator organisms. However, research has shown that there is a poor correlation between the presence of indicator and pathogenic organisms in water (Lemarchand & Lebaron, 2003; Hörman et al. 2004; Harwood et al. 2005; Ahmed et al. 2008; Dobrowsky et al. 2014). Additionally, certain pathogenic microorganisms may be better adapted to surviving water treatment processes than are the indicator groups. Moreover, traditional culture-based methods cannot always be used to accurately monitor the presence and number of possible viable microbial contaminants, as microorganisms often occur in a viable but non-culturable (VBNC) state and thus remain undetected (Ahmed et al. 2008; Dusserre et al. 2008). Certain pathogenic microorganisms are also extremely difficult to culture from environmental samples and therefore most laboratories have resorted to using molecular based techniques to confirm the presence of the organisms [Centres for Disease Control and Prevention (CDC), 2013]. Molecular-based techniques targeting nucleic acids, such as polymerase chain reaction (PCR) assays, overcome the major drawbacks associated with using culturing techniques by detecting specific pathogenic microorganisms as well as organisms present in a VBNC state within an environmental sample (Li et al. 2015). However, merely confirming the presence of pathogenic organisms following treatment is not sufficient to accurately assess the risk associated with using treated rainwater for domestic purposes as only the viable portion of microbial contaminants poses a health risk to the consumer. A need therefore arises for viability assays that would allow for the rapid and sensitive detection of the viable portion of microbial contaminants in water sources following disinfection treatment. In order to achieve this, researchers have suggested targeting three indicators of bacterial viability, viz. metabolic activity or responsiveness, the presence of nucleic acids and membrane integrity (Keer & Birch, 2003). It is important to note that targeting these individual properties may not provide a definitive yes or no answer to viability. However, by utilising assays that target multiple properties and relating results to the water treatment method used [mode of action – ultra-violet light that damages deoxyribonucleic acid (DNA) or heat that causes cell membrane to lyse etc.], an improved understanding will be obtained for both the efficiency of the water treatment and the potential health risk associated with using the treated rainwater.

The primary aim of the current study was thus to construct and monitor small- and large-scale rainwater harvesting SOPAS treatment systems in a local informal settlement in Stellenbosch

(Western Cape). The inhabitants of the informal settlement would thus be provided with an alternative water source, other than the overburdened municipal standpipe water supplies (refer to section 1.6). To achieve this aim, the microbial and chemical quality of the rainwater before and after SOPAS treatment was monitored on-site in the local informal settlement. This was achieved by using conventional water quality monitoring techniques including the culturing of indicator organisms and screening for pathogens commonly associated with rainwater sources using conventional PCR. The pathogens most readily detected in the untreated and treated rainwater samples were then quantified using quantitative PCR (qPCR). Additionally, the BacTiter-Glo™ Microbial Cell Viability Assay was utilised to detect the presence of metabolically active cells in the untreated and treated water samples. The operational sustainability of the systems were also monitored to determine whether the systems are efficient in providing a sufficient volume of water to the users and whether the system components are durable. However, a need exists for viability assays that enable the rapid detection and quantification of viable microbial pathogens in water sources. An additional aim of the study was thus to compare the efficacy of viability assays targeting cellular integrity and the presence of nucleic acids (viability-qPCR and DNase enzyme-based assay) and the metabolic activity or responsiveness of biological contaminants (BacTiter-Glo™ Microbial Cell Viability Assay).

1.2 Domestic rainwater harvesting

1.2.1 A brief history and scope of implementation

With the demands on water supplies continually increasing, rainwater harvesting, which refers to the collection and storage of the natural resource rainwater, has been considered a cost-effective water source that is ideal for domestic water uses such as toilet flushing, car washing, laundry, watering of gardens and various applications in agriculture (Helmreich & Horn, 2009; Li et al. 2010; Ahmed et al. 2011; Mwenge Kahinda & Taigbenu, 2011). Rainwater harvesting is not a new technology and it can be traced back as far as 2000 BC when Roman cities were designed to capture rainwater for use in various domestic activities [Bruins et al. 1986; Global Development Research Centre (GDRC), 2015]. Similarly, even earlier evidence of rainwater harvesting for domestic or agricultural purposes can be found in Africa (Egypt) and Asia (Thailand and Turkey) as far back as 7000 BC (Bruins et al. 1986; GDRC, 2015). Currently, rainwater harvesting is being utilised globally as an alternative water source for both potable and non-potable purposes, with most governmental organisations recognising its potential as an additional water source. Countries that have investigated the use of domestic rainwater harvesting and utilise the technology include Australia, Bangladesh, Bermuda, Brazil, Canada, China, Denmark, Germany, India, Indonesia, Ireland, Italy, Japan, New Zealand, Philippines, Singapore, Thailand and the United States, amongst many others (Uba & Aghogho, 2000;

Evans et al. 2006; Despins et al. 2009; Li et al. 2010; Ahmed et al. 2011; Mwenge Kahinda & Taigbenu, 2011; GDRC, 2015).

Significantly contributing to the acceptance of harvested rainwater as a water source are the establishment of government subsidies and tax incentives, such as those employed in Australia, France, New Zealand and the United Kingdom (Albrechtsen, 2002; Ahmed et al. 2011). For example, the “Home Water Wise Rebate Scheme” provides subsidies for households that use rainwater for non-potable domestic purposes in Queensland, Australia and 260 000 households joined the programme during the first two years of implementation. In addition, a 50% increase in rainwater harvesting was observed in countries such as France and the United Kingdom once the respective governments had established tax incentives for the utilisation of this water source. Numerous examples exist which show the marked impact that rainwater harvesting has had on developing regions of the world. One of the most notable examples is the collaboration between a group of non-profit organisations and the Brazilian government to construct one million rainwater tanks over a five year period. It is estimated that these should supply approximately five million people with water. Due to the success of the programme, the Brazilian Rainwater Catchment Systems Association was established, as were educational programmes pertaining to rainwater harvesting (GDRC, 2015).

The progress in implementing rainwater harvesting systems in Africa has been slow. This is associated with low annual rainfall and the seasonal variability of precipitation. Furthermore, when taking into account the average household income in Africa, costs associated with constructing catchment systems are expensive (GDRC, 2015). Nevertheless a rapid expansion in the technology has been evident in recent years and rainwater harvesting has been introduced in various African countries as most governments are aware of the potential of this technology. Rainwater harvesting projects have thus been successfully used in Botswana, Kenya, Malawi, Mali, Mozambique, Namibia, Sierra Leone, South Africa, Tanzania, Togo, Uganda and Zimbabwe, amongst others (Hartung, 2006; 2007; Mwenge Kahinda et al. 2007; Sturm et al. 2009; Mwenge Kahinda & Taigbenu, 2011; Mosler et al. 2013; GDRC, 2015).

As 2.48 million South Africans (approximately 4.8% of the total population) do not have access to an adequate water supply [Department of Water Affairs (DWA), 2013], rainwater harvesting has been earmarked by the South African Government as a possible alternative and sustainable water source that would provide water directly to households and thereby help alleviate the pressures on existing water systems (DWA, 2009; 2012). In 2010 it was estimated that only 0.4% of households in the country utilised rainwater tanks. As a result, efforts to promote the use of rainwater harvesting systems in South Africa particularly in rural communities have been increased (Statistics South Africa, 2010). These efforts include the collaboration between the Department of Water Affairs (DWA) and the Department of Science

and Technology (DST) to distribute rainwater harvesting tanks throughout all nine provinces in South Africa (Mwenge Kahinda & Taigbenu, 2011; Malema et al. 2016). Other projects include collaborations between the Department of Science and Technology (DST) and the Council for Scientific and Industrial Research (CSIR) through which sustainable housing schemes that use alternative technologies such as rainwater harvesting tanks, low-energy fittings and solar hot water geysers, were established in Mdantsane (Eastern Cape) and Kleinmond (Western Cape) (De Villiers, 2011). As a result, approximately 70 000 households throughout South Africa now use rainwater as their primary water source (Malema et al. 2016). Additionally, the South African government has announced that they are in the process of compiling guidelines aimed at specifying parameters for acceptable rainwater quality [Water Research Commission (WRC) Reference Group Meeting, 2015, personal communication].

1.2.2 Rainwater harvesting principle and system components

Rainwater harvesting is a technique used throughout the world for the collection of rainwater from rooftops, land surfaces or other artificial catchments into storage tanks (Helmreich & Horn, 2009; Mwenge Kahinda & Taigbenu, 2011; Campisano & Modica, 2012). The most commonly used rainwater harvesting system design includes three basic components; the catchment area, the conveyance system and a storage tank (Gould, 1999; Sazakli et al. 2007). Factors that need to be taken into consideration when constructing domestic rainwater harvesting systems include the type of catchment area, storage tank (material), the proximity of possible sources of pollution, the location of the system and weather/climate conditions (Gould, 1999; Sazakli et al. 2007).

Catchment areas can be divided into two principal categories viz. land surface catchments (ground catchments) and rooftop catchments (**Fig. 1.1**) (Mwenge Kahinda et al. 2007). Land surface catchment systems allow for the collection of rainwater that falls to the ground and this water enters drainage systems and storage tanks. These systems have the advantage of collecting water from a large surface area. However, some of the water is lost as it is absorbed into the ground and by plant material in the area. This technique is commonly used in agriculture where drainage systems lead to underground storage tanks or man-made storage dams (Helmreich & Horn, 2009; Li et al. 2010; Mwenge Kahinda & Taigbenu, 2011). The volume of water captured using this method can be increased by reducing soil permeability and/or increasing the land slope that leads to the storage system and clearing vegetative cover.

Due to the quality of water required (low chemical and biological contamination), limited space availability and lack of infrastructure in certain urban and rural areas (informal settlements), rooftop catchment systems are frequently used (Krishna et al. 2005). As one millimetre of rainwater collected per one square metre of collection surface yields one litre of water [Food

and Agriculture Organisation (FAO), 1985], the volume and quality of the water collected from rooftop catchments will depend mainly on the surface area of the rooftop and the material utilised for its construction. The most commonly used materials include roof tiles and metal sheeting, for example galvanized zinc or steel sheets, in an urban environment and organic materials such as wood, grass, palms and mud, which are more frequently used in rural communities (Gould & Nissen-Peterson, 1999; Mwenge Kahinda & Taigbenu, 2011).

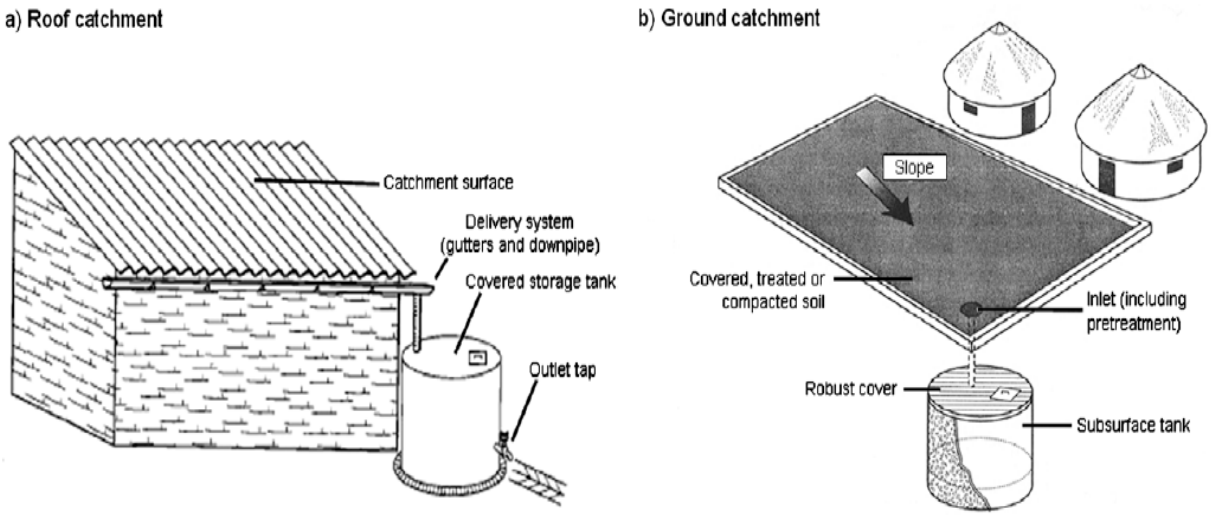

Fig. 1.1. Schematic illustration of **(a)** roof and **(b)** ground catchment systems used for rainwater harvesting (adopted from Sturm et al. 2009).

The rainwater that is collected from the rooftop catchment system is transported to a storage tank by means of a conveyance system i.e. gutters and pipes which channel the water from the roof directly into the storage tank. The conveyance system affords a convenient means of decreasing the likelihood of contaminants entering the storage tank (Martinson & Thomas, 2005). One example is the installation of a first-flush diverter. At the start of a rain event, all the possible contaminants such as debris, plant material, dust and animal faecal matter deposited on the rooftop or gutter system could be washed into the storage tank and thus contaminate the harvested rainwater (Sazakli et al. 2007; Ahmed et al. 2008). In order to prevent this from occurring, first-flush diverters that direct the initial in-flow of rain at the start of a rain event (containing most of the contaminants) away from the storage tank, can be installed (Mwenge Kahinda et al. 2007). This process can either be performed manually or an automated system can be installed. However, in a study conducted by Gikas and Tsihrintzis (2012), it was noted that while the use of a first-flush diverter improved the physico-chemical properties of the harvested rainwater it did not improve the microbial quality of this water source.

The final component in a rainwater harvesting system is the storage tank. Storage tanks collecting water from rooftop catchment systems can be located either above or below the ground (Mwenge Kahinda et al. 2007). When a tank is located below the ground, a pump-

system needs to be installed in order to extract the water from the tank (Helmreich & Horn, 2009). Limiting the possibility of contamination from animal or human sources is an important consideration when installing a storage tank in order to prevent the breeding of mosquitoes or algal growth in the system. As was noted for the catchment systems, a wide variety of materials are used for the construction of storage tanks; however, the most commonly used storage tanks are constructed from a high-grade polyethylene.

Limited maintenance is required for a rainwater harvesting system and usually includes inspecting and cleaning the system components, particularly before the start of the high rainfall season (Gould, 1999). The regular removal of organic debris (dust and plant material) and other accumulated materials from the rooftop and gutter system will decrease the possibility of contamination and it has been recommended that rainwater storage tanks are cleaned annually (Ahmed et al. 2008).

1.3 Quality of harvested rainwater

The quality of harvested rainwater depends on many factors which include the location of the collection and storage system (proximity to pollution sources) and the susceptibility of the collected water to the atmosphere (air pollution), roof cleanliness, rain intensity and the number of dry days before a rain event (Abdulla & Al-Shareef, 2009; Li et al. 2010; De Kwaadsteniet et al. 2013). As indicated in **Fig. 1.2**, rainwater may become contaminated as rain droplets travel through the air or when the rainwater comes into contact with the catchment or conveyance system or even in the storage tank.

In order to determine if harvested rainwater may be used for potable purposes, it is thus common practice to assess the quality of the water source by comparing it to recommended drinking water guidelines that specify microbial and chemical parameters (Ahmed et al. 2011). There are however conflicting conclusions regarding the quality of harvested rainwater (Mwenge Kahinda et al. 2007). Some studies have indicated that harvested rainwater satisfies required international drinking water guidelines (Handia et al. 2003; Zhu et al. 2004; Sazakli et al. 2007) whereas others have indicated that harvested rainwater does not comply with drinking water standards, due to the presence of microbial and/or chemical contaminants which could pose a serious health risk (Vasudevan & Pathak, 2000; Simmons et al. 2001; Abbott et al. 2006; Helmreich & Horn, 2009; Li et al. 2010; Dobrowsky et al. 2014). To date, chemical contaminants of harvested rainwater have not been directly associated with the incidence of disease (Sazakli et al. 2007; Chapman et al. 2008; Huston et al. 2012; Dobrowsky et al. 2014).

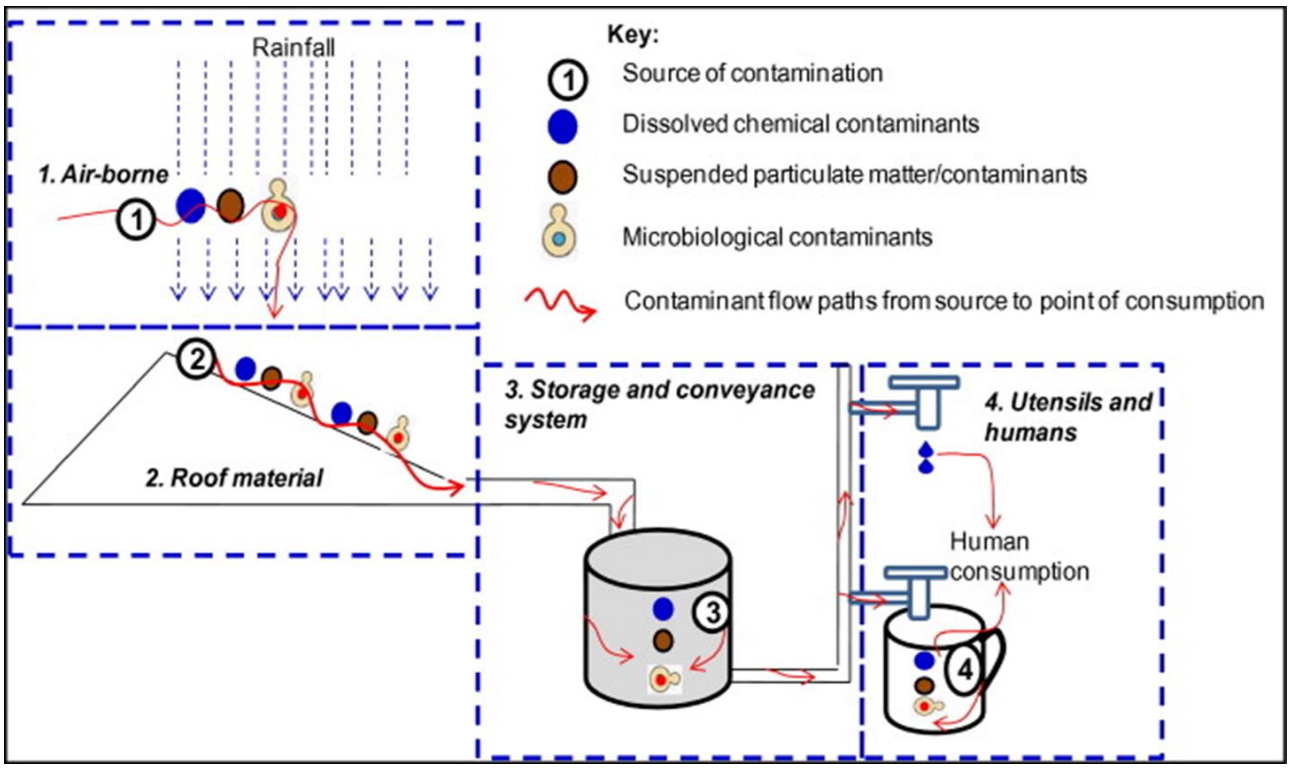

Fig. 1.2. Schematic illustration of the potential sources of microbial and chemical contamination in a typical rainwater harvesting system (adopted from Gwenzi et al. 2015).

However microbial contaminants identified include the commonly described traditional faecal indicators, various opportunistic bacterial pathogens, including *Legionella* spp. and *Pseudomonas* spp. and protozoan species such as *Cryptosporidium* and *Giardia*. Many of these contaminants are associated with human disease (Uba & Aghogho, 2000; Lye, 2002; Ahmed et al. 2008; 2010a; 2011; De Kwaadsteniet et al. 2013; Dobrowsky et al. 2014), with individuals with compromised immune systems, young children and the elderly being at the greatest risk of infection (Mwenge Kahinda et al. 2007). This is of concern as rainwater harvesting has been identified as an intervention strategy to provide individuals residing in informal settlements and rural areas with an alternative water source, where residents are at an increased risk of waterborne disease as a result of poor living conditions (Rao et al. 2010).

1.3.1 Chemical quality of harvested rainwater

Currently there are no international guidelines specifying indicators of chemical or microbial quality for harvested rainwater and those investigating the use of this water source use various national or international drinking water guidelines as reference sources [Department of Water Affairs and Forestry (DWAF, 1996); South African National Standards (SANS) 241 (South African Bureau of Standards (SABS), 2005); Australian Drinking Water Guidelines (ADWG) (NHMRC and NRMMC, 2011); World Health Organisation (WHO, 2011)]. However, due to discrepancies in available water quality guidelines, there are conflicting conclusions regarding

the chemical quality of harvested rainwater. Certain studies have indicated that various cations and anions in harvested rainwater comply with drinking water standards, while others indicated that there were elevated levels of anions and cations that exceeded the specific water guidelines (Peters et al. 2008; Morrow et al. 2010; Huston et al. 2012; Dobrowsky et al. 2014). Using the concentration of iron as an example, according to the South African Department of Water Affairs Drinking Water Guidelines, iron concentrations should not exceed 100 µg/L (DWAF, 1996) while the South African Bureau of Standards SANS 241 (SABS, 2005) recommends a concentration limit of 200 µg/L. The acceptable iron concentration increases even further with the ADWG (NHMRC and NRMMC, 2011) where the limit specified is 300 µg/L. Conversely the WHO (2011) drinking water guidelines do not stipulate any levels for iron concentrations. Factors that also contribute to the variability observed for chemical quality of water include differences in rainwater harvesting system designs (Ahmed et al. 2011), the type of roofing material used (WRC Project K5/2368//3 Report, 2016) and roof cleanliness (Chang et al. 2004; Ahmed et al. 2008). The location of the system is also important as urban, industrial or rural areas all record differing levels of atmospheric pollution (Huston et al. 2009).

Particles, microorganisms, heavy metals and various organic substances constitute some of the major pollutants found in the atmosphere that may adversely affect harvested rainwater. Hence the location of the system and catchment cleanliness both contribute markedly to the chemical quality of harvested rainwater. Typically, industrial areas have greater levels of atmospheric pollution than do rural areas. The use of fossil fuels for transportation may also contribute to atmospheric pollution in urban areas (Huston et al. 2009). While trying to establish the sources of chemical pollution in harvested rainwater Huston et al. (2012) attributed 65% of the chemical contaminants found in the rainwater to originate from system components and atmospheric pollution and the remaining 35% of these contaminants could be traced to the lead paint used on the roof catchment area. Chemical contamination of harvested rainwater may also result from natural sources. The latter include contaminated organic materials (plant debris containing insecticides/pesticides) on rooftops which are washed into the storage tank.

The rainwater harvesting system design can thus also influence the chemical quality of harvested rainwater as the material used for the construction of the system (catchment material, pipes and storage tanks) contributes to the leaching of chemicals (such as heavy metals) into the harvested rainwater (Mwenge Kahinda et al. 2007; Morrow et al. 2010; Huston et al. 2012; Dobrowsky et al. 2014). Metal roofing materials have also been shown to be major contributors to metal contamination as the acidity of rainwater (pH 5.0 – 5.6) in combination with the exposure of the roof surface to the sun, facilitate possible leaching of metals from the roofing material (Chang et al. 2004). Handia et al. (2003) observed that harvested rainwater collected from roofs constructed from galvanized zinc sheets contained higher zinc concentrations (0.14 to 3.16 mg/L) than did harvested rainwater collected from asbestos cement roofs (< 0.001 to

0.025 mg/L). It has also been suggested that the corrosion of galvanized iron sheets contributes to lead contamination of harvested rainwater (Simmons et al. 2001). It is thus hypothesised that the selection of the appropriate roofing material such as clay or concrete roof tiles rather than the use of metal-based materials would reduce chemical contamination (Handia et al. 2003).

1.3.2 Microbial quality of harvested rainwater

Possible sources of microbial contamination include faecal matter originating from various animals and birds that access the collecting surface and/or organic debris deposited on a roof. Following a rain event, faecal and organic matter are often washed into the collection tank and contaminate the harvested rainwater (Heyworth et al. 2006; Ahmed et al. 2008). The proliferation of pathogenic microorganisms in harvested rainwater may then depend on various factors such as ambient temperature and rainfall intensity (Schets et al. 2010; Kaushik et al. 2012; De Man et al. 2014). The role of bioaerosol particles in rainwater contamination has also been explored (Bauer et al. 2003; An et al. 2006; Turkum et al. 2008; Ekström et al. 2010). It has been suggested that bioaerosol particles act as cloud condensation nuclei which enable the transfer of microbial pathogens into rainwater through cloud droplets as the cloud and rain droplets traverse the atmosphere (Bauer et al. 2003; Ekström et al. 2010; Kaushik et al. 2012). This suggestion was further supported by the detection of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae*) in freshly captured rainwater (Bauer et al. 2003; Korzeniewska et al. 2008; Ekström et al. 2010; Kaushik et al. 2012). As no guidelines for rainwater quality have been formulated, studies investigating the microbial quality of harvested rainwater use drinking water guidelines as a reference (Rompré et al. 2002; Noble et al. 2003; Pitkänen et al. 2007; De Kwaadsteniet et al. 2013). These guidelines require the monitoring of multiple indicator bacteria such as enterococci, *E. coli*, faecal coliforms and total coliforms (DWAF, 1996; SABS, 2005; NHMRC and NRMMC, 2011; WHO, 2011).

1.3.2.1 Indicator bacteria

Requirements for an ideal indicator microorganism include indicating the presence of pathogens, being absent in uncontaminated water, being present in higher numbers than pathogens, having a higher survival rate than that of pathogens in water and being relatively simple to enumerate, isolate and identify (Edberg et al. 2000; Rompré et al. 2002; Noble et al. 2003; WHO, 2003). Organisms that are not necessarily pathogenic but commonly present in large numbers in the intestinal flora of warm-blooded animals were thus seen as ideal indicator candidates as their detection in water sources would indicate faecal contamination and the presence of possible pathogenic microorganisms (Rompré et al. 2002; Noble et al. 2003;

Pitkänen et al. 2007). The most commonly used indicators are *E. coli*, enterococci, faecal coliforms and total coliforms.

Faecal coliforms are Gram-negative, rod-shaped non-spore forming bacteria belonging to the family Enterobacteriaceae, while enterococci are Gram-positive, spherical non-spore forming bacteria belonging to the family Enterococcaceae (WHO, 2003). Both faecal coliforms and enterococci serve as a direct indication of faecal contamination as these microorganisms originate from faecal sources. Enterococci include a large number of species, of which *Enterococcus faecalis* and *Enterococcus faecium* are predominant (Edberg et al. 2000). Enterococci are found in the colon of mammals at concentrations of 10^6 – 10^7 CFU/g stool sample analysed and are known to survive for longer periods of time in aquatic environments than other indicator organisms (Edberg et al. 2000).

Total coliforms serve as a general indication of water quality and water disinfection effectiveness. They include a heterogeneous group of bacteria belonging to the genera *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Serratia* and *Rahnella*, amongst others (DWAF, 1996). *Escherichia coli*, is the most common coliform among the intestinal flora of warm-blooded animals and occurs at a concentration of 10^9 CFU/g stool analysed (Rompré et al. 2002). The bacterium seldom increases in number in the environment (Edberg et al. 2000). It is therefore considered the principal indicator of faecal contamination and should be absent (0 CFU/100 mL) in drinking water (Environment Agency, 2002; Rompré et al. 2002; WHO, 2003).

The term heterotrophic plate count (HPC) refers to a variety of culture-based tests that are used to recover a wide range of microorganisms from water (WHO, 2003). The test itself does not specify the identity of microorganisms detected but it is used as an indication of water quality as it yields an estimation of the number of potential pathogenic and non-pathogenic culturable organisms present in a water source (WHO, 2003). Heterotrophic plate count analysis is recommended by numerous drinking water guidelines and water regulator authorities as an indicator of the effectiveness of a water disinfection treatment (DWAF, 1996; Ashbolt et al. 2001; WHO, 2003; 2011). This is done by comparing HPC values before and after treatment (WHO, 2003). Another common use of HPC includes monitoring for microbial re-growth in water systems following disinfection treatment (WHO, 2003).

1.3.2.2 Microbial pathogens associated with rainwater

Many studies have conducted analyses on the microbiological quality of harvested rainwater by detecting and enumerating the indicator organisms present (Evans et al. 2006; Sazakli et al. 2007; Ahmed et al. 2008; 2010b). However, while the use of indicator bacteria has become routine there is currently no single standard for bacterial indicators with regard to which species

are suitable to use. Furthermore agreed acceptable levels in various water sources remain contentious (Noble et al. 2003). In addition, numerous studies have indicated that there is a poor correlation between the presence of faecal indicators and potential pathogenic bacteria as the presence or absence of faecal indicators does not definitively indicate whether pathogens such as *Legionella* spp. (Ahmed et al. 2008; Dobrowsky et al. 2014), *Salmonella* spp. (Lemarchand & Lebaron, 2003; Ahmed et al. 2008), *Campylobacter* spp. (Hörman et al. 2004; Ahmed et al. 2008), *Cryptosporidium* spp. and *Giardia* spp. (Hörman et al. 2004; Harwood et al. 2005; Ahmed et al. 2008) and enteric viruses (Pina et al. 1998; Griffin et al. 1999; Lombard et al. 2013) are present in the rainwater samples. Moreover, numerous studies have reported waterborne disease outbreaks of microbial origin after the consumption of water which was within statutory coliform specifications (Payment et al. 1991; MacKenzie et al. 1994; Moore et al. 1994; Gofti et al. 1999; Rompré et al. 2002). Other studies have shown that certain opportunistic pathogens including *Klebsiella* spp., *Legionella* spp., *Pseudomonas* spp. and *Yersinia* spp. are resistant to water treatment technologies (Dobrowsky et al. 2014; 2015; Reyneke et al. 2016; Strauss et al. 2016).

1.3.2.2.1 *Legionella* spp.

Legionella are Gram-negative, catalase-positive motile rods with polar or lateral flagella (Benson & Fields, 1998). These bacteria utilise amino acids as carbon sources and are found in freshwater environments worldwide (Murga et al. 2001; Fields et al. 2002). The *Legionella* genus is comprised of 54 species with 70 distinct serogroups of which 39 serogroups have been associated with human disease (Stout et al. 2003). Fields (1996) suggested that most *Legionella* spp. are likely to cause human disease under appropriate conditions as it was proposed that all *Legionella* spp. are capable of intracellular growth. These organisms are thus considered pathogens as they are able to multiply within mammalian cells and cause a respiratory disease known as legionellosis in humans (Fields et al. 2002). Legionellosis may result in Legionnaires' disease, a severe multisystem disease involving pneumonia (Fraser et al. 1977) or Pontiac fever, a flu-like illness (Glick et al. 1978). Various risk factors for Legionnaires' disease include increasing age, smoking, chronic lung disease, lung cancer and diabetes (Marston et al. 1994). In the South African context, the lack of awareness and statistics pertaining to legionellosis is an important issue when attempting to quantify the risk of this disease (Milne, 2007). The estimated 6.1 million HIV-infected individuals living in South Africa, many of whom also suffer from tuberculosis, have an increased susceptibility to respiratory diseases, which makes the research into preventing the outbreak of legionellosis even more important (UNAIDS, 2013).

Legionella spp. are known to survive for long periods of time under low-nutrient conditions (Dusserre et al. 2008) including in man-made warm water environments such as cooling towers,

hot tubs, showerheads and spas (Delgado-Viscogliosi et al. 2009). Most cases of legionellosis have originated from *Legionella* contamination of man-made warm water systems (Fields et al. 2002). The ability of *Legionella* to survive at increased temperatures (50 – 65 °C) has been attributed to: firstly, the presence of heat shock proteins (Fields et al. 2002) rendering the genus more thermostable than most bacteria found in water environments (Allegra et al. 2011); secondly, the ability of *Legionella* to form associations and proliferate within biofilms (Murga et al. 2001); and thirdly, their ability to live as intracellular parasites within protozoa (Fields et al. 2002). In studies conducted by Allegra et al. (2008; 2011) it was established that ten of sixteen *Legionella* strains remained viable after heat treatment at 70 °C for 30 minutes. In addition, several studies utilising the PCR technique have identified *Legionella* spp. in harvested rainwater (Wilson et al. 2003; Abbott et al. 2006; Dusserre et al. 2008; Sakamoto et al. 2009; Ahmed et al. 2008; 2010a; Dobrowsky et al. 2014; Reyneke et al. 2016). *Legionella* spp. have also been detected in harvested rainwater in various countries. These include Australia, Denmark, Netherlands, New Zealand, Spain and the U.S Virgin Islands (Schlech et al. 1985; Simmons et al. 2001; Albrechtsen, 2002; Fields et al. 2002). Outbreaks of legionellosis due to the utilisation of harvested rainwater have been reported in all of the above-mentioned countries. In New Zealand it was reported that end-users were exposed to this pathogen by means of contaminated bathroom showers which were connected to rainwater tanks (Simmons et al. 2001). In addition, *Legionella* is frequently isolated from potting soil and is the principal cause of legionellosis in Australia amongst gardeners who use harvested rainwater for irrigation purposes (Fields et al. 2002).

1.3.2.2.2 *Klebsiella* spp.

Klebsiella are Gram-negative, catalase-positive, non-motile rods, surrounded by a capsule. These bacteria are members of the Enterobacteriaceae family and are thermotolerant coliforms. They occur ubiquitously in nature (soil, plants and water), the gastrointestinal tract of animals (Cabral, 2010) and freshly captured rainwater (Kaushik et al. 2012). *Klebsiella* spp. have been isolated from animal and human faecal matter and some species, including *K. pneumoniae* and *Klebsiella oxytoca*, are considered opportunistic pathogens, which can cause pneumonia if the bacterium enters a host through the respiratory tract. They can also cause an infection in the human bloodstream should they come into contact with an open wound (Cabral, 2010). However, a comprehensive understanding of the infection mechanisms remains unclear, although several virulence factors associated with pathogenicity have been identified in *K. pneumoniae* (Bojer et al. 2010). Immunocompromised individuals present the greatest risk of infection especially as *Klebsiella* spp. have developed antimicrobial resistance most notably against carbapenems (Cabral, 2010) and mortality rates of 20 – 70% have been reported (Bojer et al. 2010).

In a study conducted by Dobrowsky et al. (2015), *Klebsiella* spp. were detected (conventional PCR) in pasteurized rainwater samples treated at 74 °C. The PCR studies conducted by Bojer et al. (2010) and Jørgensen et al. (2016) further reported on *K. pneumoniae* isolates that demonstrated increased heat resistance. Bojer et al. (2010) showed that the isolated *K. pneumoniae* remained culturable after a heat treatment regime carried out at 60 °C. Heat resistance was attributed to the *clpK* genetic marker which has been shown to correlate positively with thermotolerant phenotypes observed among clinical *Klebsiella* isolates. Jørgensen et al. (2016) suggested that the genetic marker facilitated the survival of *Klebsiella* isolates in biofilms undergoing heat treatment which in turn contributed to the spread of an outbreak in Norway.

1.3.2.2.3 *Pseudomonas* spp.

Pseudomonas spp. are Gram-negative, catalase-positive, motile rods with polar flagella, which are able to utilise a broad spectrum of nutrients. These bacteria are found ubiquitously in nature (both soil and water). The *Pseudomonas* genus is comprised of 202 species, including the opportunistic human pathogen *Pseudomonas aeruginosa* and the plant pathogen *Pseudomonas syringae* (Özen & Ussery, 2012). *Pseudomonas aeruginosa* has been shown to cause pneumonia, keratitis, burn wound infections, gastrointestinal infections and urinary tract infections (Coutinho et al. 2008; Silby et al. 2011). Immunocompromised individuals such as those infected with HIV and tuberculosis and cystic fibrosis patients are at greatest risk of infection.

The opportunistic pathogens *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* have previously been isolated from humans and have been detected in rainwater (including freshly captured rainwater), heat-exchangers, water-systems and air-conditioners (Uba & Aghogho, 2000; Albrechtsen, 2002; Kaushik et al. 2012). Both *Pseudomonas* spp. contain various resistance factors and are able to form biofilms which assist in protection when the bacterium is exposed to stressful conditions such as antibiotic and disinfection treatments (Hauser & Ozer, 2011).

1.3.2.2.4 *Yersinia* spp.

Yersinia spp. are Gram-negative, catalase-positive, facultative anaerobic, non-motile rods. The *Yersinia* genus occurs within the Enterobacteriaceae family and consists of 11 species, of which *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are human pathogens (Perry & Fetherston, 1997). Most notably, *Yersinia pestis* causes bubonic plague, while *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* may both cause yersiniosis, symptoms of which include fever, abdominal pain and diarrhoea.

Yersinia spp. have been shown to persist in the environment as *Yersinia pseudotuberculosis* is able to survive for long periods of time in soil and water, while it has also been suggested that *Yesinia pestis* may survive outside flea or animal vectors as an intracellular parasite of protozoa. The presence of *Yersinia* in biofilms appears to enhance viability (Eisen & Gage, 2008). In a study using conventional PCR, conducted by Dobrowsky et al. (2015), *Yersinia* spp. were detected in pasteurized rainwater samples after heat treatment conducted at 81 °C. Langeland (1983) then reported on the isolation of *Yersinia* spp. (including *Yersinia enterocolitica*) in 54% of the drinking water samples tested, while Cheyne et al. (2010) detected *Yersinia enterocolitica* in 38% of surface water samples tested.

1.4 Rainwater treatment systems

Research has shown that depending on the chemical and microbial quality of the harvested rainwater, utilising this water source for potable purposes may only be achievable if pre-treatment systems are employed (Helmreich & Horn, 2009; Li et al. 2010; Dobrowsky et al. 2014). Numerous United Nations Children's Emergency Fund (UNICEF) and WHO reports have indicated that millions of children die each year in developing countries as a result of waterborne diseases (UNICEF & WHO, 2009). Therefore, if rainwater harvesting is to be successfully used in developing countries where access to safe water is lacking, it is essential that treatment systems effective for the removal of pathogenic organisms are implemented (Burch & Thomas, 1998). However, numerous factors have been identified that may influence the effectiveness of a particular water treatment system in both the ability to efficiently treat the water source and in providing sufficient volumes of treated water (**Table 1.1**).

Table 1.1: Factors that may influence the effectiveness and efficiency of water treatment systems (Burch & Thomas, 1998; Mwabi et al. 2011; McGuigan et al. 2012; De Kwaadsteniet et al. 2013).

- Water source utilised (rainwater will have a lower overall turbidity and microbial load when compared with river water, which will be more difficult to treat).
- Costs and materials associated with the treatment system (must be cost-effective and constructed from readily available materials if the system is to be implemented in developing countries).
- Ease of use (compliance will be negatively impacted if the protocol is complicated).
- System maintenance (treatment should not require consumables that are difficult or too expensive to obtain).
- Treatment time (if prolonged treatment time is required, the treatment system might not be able to meet the water demands of the consumer).

By taking these factors into consideration (**Table 1.1**), various treatment systems have been proposed to treat rainwater at the household level. These systems include; poly (vinyl alcohol) (PVA) nanofibre membrane technology (Dobrowsky et al. 2014), activated carbon filtration (Areerachakul et al. 2009; Dobrowsky et al. 2014), slow-sand filtration (Fewster et al. 2004; Liu et al. 2005; Peters-Varbanets et al. 2009), chlorination (Sobsey, 1989; Gordon et al. 1995), SODIS (Sommer et al. 1997; Lonnen et al. 2005; Martin-Dominguez et al. 2005; McGuigan et al. 2012; Strauss et al. 2016) and SOPAS (Burch and Thomas, 1998; Spinks et al. 2003, 2006; Despins et al. 2009; Dobrowsky et al. 2015; Reyneke et al. 2016).

Two primary factors that exert marked influences on the effectiveness of a rainwater treatment system are the types of microorganisms present and the turbidity of the water source. It is well documented that certain organisms undergo physiological and morphological changes (formation of survival structures) under unfavourable conditions which enable them to persist through water treatment strategies (Jones, 1997; Stortz & Zheng, 2000). This includes the Gram-positive endospore formers, protozoan species which form cysts and parasitic worms that lay eggs. These survival structures are highly resistant to commonly used chemical treatments as well as to heat and ultraviolet-radiation (UV) (Jones, 1997; Stortz & Zheng, 2000). Certain organisms also possess the necessary genes and enzymes to survive unfavourable conditions by initiating an appropriate stress response. These include the heat shock response, activation of the SOS-regulon and initiation of photoreactivation (repair of DNA following UV damage) (Jones, 1997). In addition, water turbidity which is defined as the measure of the concentration of suspended particles in water, can affect treatment in at least three ways. These include shielding microorganisms from UV-radiation, by reacting with chemical disinfectants such as chlorine and by clogging filtration systems (Servais et al. 1994; Wegelin et al. 1994; Burch & Thomas, 1998; McGuigan et al. 2012).

1.4.1 Chlorination (chemical disinfection)

Chlorination was first used as a water treatment method during the 1890s, when sanitation engineers started using chlorine, as it was considered effective, inexpensive and a simple way to treat contaminated water (Edberg et al. 2000). Over the years it has become the most common form of disinfection and is used in numerous countries (De Kwaadsteniet et al. 2013). Chlorine and chlorine-based compounds efficiently destroy microorganisms during water treatment processes and also prevent microbial re-growth after the treatment. Chlorine exposure effectively destroys the bacterial cell wall by altering its biochemical and physical properties thereby terminating certain essential cellular functions (Venkobachar et al. 1977). It has been proposed that the disruption of the cell wall by the binding of chlorine to target sites on the cell surface releases vital cellular constituents from the cell. The compound also terminates

membrane-associated functions and metabolism within the cell, leading to cell death (Venkobachar et al. 1977).

Chlorination has been used to treat harvested rainwater at a recommended dose of 0.4 - 0.5 mg/L for at least 15 min (De Kwaadsteniet et al. 2013). However, some disadvantages of utilising chlorination include: the need to continuously add chlorine to the water being treated in order for it to have a beneficial effect; determining the chlorine concentration required for effective treatment of water, as this is dependent on the concentration and types of microorganisms present; and finally, high levels of organic matter in the water may also affect chlorination efficiency (Feachem et al. 1983; Servais et al. 1994). Gordon et al. (1995) then recommended treating rainwater with chlorine after removing the water from the rainwater harvesting tank to prevent the chlorine from reacting with organic material that had settled at the bottom of the tank. These reactions must be avoided as they cause the formation of hazardous by-products including, chlorite, persulfate and perchlorate, amongst others, which are detrimental to human health (Huang et al. 2016).

1.4.2 Filtration systems

Various types of filtration systems exist. These include slow-sand filtration, activated carbon filtration and nanofiltration (De Kwaadsteniet et al. 2013). The effectiveness of these filtration systems relies on their ability to remove microorganisms from water on the basis of their size (viruses 20 – 80 nm; bacteria 0.5 – 2 µm; protozoa 4 – 20 µm). Furthermore, the increased surface area of the filters permits the formation of biofilms that act as biological filters by removing contaminating microorganisms. A major advantage of using filtration is that this method can remove both microbial and chemical contaminants from water sources. However, there are disadvantages. These include prolonged treatment time and the volume of water that can be effectively treated is small. In addition, in some systems such as the slow-sand filtration system, components may need to be replaced. The entire system becomes ineffective during this period, particularly as the biofilm/biological filter needs to re-form. However, depending on the type of filtration method used, the system may be constructed cost-effectively, by employing low cost filter materials and using gravity to move the water through the system instead of a pressure pump (De Kwaadsteniet et al. 2013).

1.4.3 Solar disinfection (SODIS)

Solar disinfection (SODIS) is based on the synergistic effects of light (UV) and heat which inactivate microbial contaminants (McGuigan et al. 2012). The process is performed by filling glass or plastic bottles with the water to be treated and exposing these containers to direct sunlight for 6 – 48 hours. The exposure time will depend on the intensity of sunlight and sensitivity of the contaminating pathogens to UV radiation and heat. Ultraviolet-radiation

inactivates the microorganisms by damaging both the cell membrane and the DNA as a result of the formation of reactive oxygen species (ROS). Simultaneous heating of the water by the ultraviolet-radiation further contributes to the disinfection process. The process of SODIS is considered an inexpensive and simple easy-to-use system which is utilised daily by approximately five million people in over 50 countries in Africa, Asia and South America (McGuigan et al. 2012).

Solar disinfection has been utilised successfully to reduce bacterial, fungal, protozoan and viral contaminants in harvested rainwater (McGuigan et al. 2012). Various waterborne pathogenic bacteria have also been reported to be susceptible to SODIS following a six-hour treatment (Wegelin et al, 1994; Dejung et al. 2007). However faecal coliforms display slower inactivation rates and thus require longer exposure times (Sommer et al. 1997; Sinton et al. 2002). In a study conducted by Boyle et al. (2008), *Bacillus subtilis* endospores could be reduced by 96.3% following a cumulative 16-hour exposure. It has also been recommended that water subjected to SODIS be used within 24 hours to avoid post-treatment re-growth (McGuigan et al. 2012).

Despite the benefits of employing SODIS to treat water, some disadvantages have been noted. For example, poor weather conditions and an increase in the turbidity of water may decrease the effectiveness of SODIS (Helmreich & Horn, 2009; Li et al. 2010; McGuigan et al. 2012). In addition, although research has demonstrated that chemicals do not leach from the plastic bottles during this treatment, the system does not have the ability to improve the chemical quality of the water. Furthermore, research has indicated that certain microorganisms and endospores are able to survive SODIS treatment and are only inhibited when the temperature is significantly increased (McGuigan et al. 2012).

In order to overcome these limitations certain enhancement technologies have been implemented to increase the treatment effectiveness of the system. These improvements include thermal enhancement, heterogeneous photocatalysis, chemical additives, flow reactors and solar mirrors. The use of thermal enhancement, flow reactors and solar mirrors all lead to an increase in both temperature and ultraviolet-radiation (Kehoe et al. 2001; Saitoh & El-Ghetany, 2002; Martin-Dominguez et al. 2005). This is achieved by using black paint (on the underside of the SODIS reactor) and solar reflectors to increase heat absorption. These technologies also aim to increase the possibility of obtaining an uninterrupted ultraviolet–radiation dose that is essential for complete inactivation of the microbial contaminants (Vidal & Diaz, 2000; Dunlop et al. 2002; Fernández-Ibánez et al. 2009). Heterogeneous photocatalysis also increases the production of ROS by using a semi-conductor such as titanium dioxide (TiO_2). This compound absorbs radiation energy and promotes ROS formation after several electron transfer reactions (Ibánez et al. 2003; Seven et al. 2004; Paspaltsis et al. 2006). The addition of chemical additives such as citrus-based compounds to the water has also led to an

increase in microbial inactivation (Fisher et al. 2012; Harding & Schwab, 2012). Research conducted by Fisher et al. (2012) showed that the use of sodium percarbonate in combination with copper or citric acid and ascorbate accelerated the inactivation of coliphages, *E. coli* and *Enterococcus* spp. In addition, by using enhancement technologies such as reflectors, Amin and Han (2009) showed that a SODIS system effectively disinfected rainwater under moderate weather conditions. The study monitored levels of *E. coli*, total and faecal coliforms and HPC.

1.4.4 Solar pasteurization (SOPAS)

Pasteurization by boiling has long been recognised as a safe method for treating contaminated water (Burch & Thomas, 1998). However, pasteurization has many disadvantages. Boiling the water may be time-consuming and is not always financially sustainable for many households (Islam & Johnston, 2006). Boiling water by utilising firewood may also be detrimental to the environment (deforestation and air pollution) (Islam & Johnston, 2006). Developing countries such as South Africa thus require a practical and inexpensive rainwater treatment protocol (Helmreich & Horn, 2009). Solar radiation offers an abundance of renewable energy that can be utilised to treat harvested rainwater in the form of SOPAS. Many countries that are in need of a clean water supply and where rainwater harvesting could be implemented, have abundant sunshine, as most of these countries are located within 20° of the equator where increased solar radiation intensity is observed (Ciochetti & Metcalf, 1984). Thus at these locations the effectiveness and efficiency of SOPAS is improved (Nieuwoudt & Mathews, 2005). South Africa is a country with an extremely high potential for solar power generation as a result of the high solar irradiation that is observed for the entire country throughout the year (**Fig. 1.3A**). An average direct normal solar irradiance of 7.0 kWh/m^2/d has been reported (Fluri, 2009). These aforementioned facts coupled with an average annual rainfall of 464 mm in South Africa (**Fig. 1.3B**), makes rainwater harvesting SOPAS systems a viable solution for the provision of an alternative water source.

Unlike SODIS, where temperatures may be insufficient to inactivate microorganisms, the use of SOPAS creates temperatures that are high enough to successfully treat contaminated water. The time required to treat the water is also reduced (Caslake et al. 2004). Furthermore, SOPAS has been considered a reliable and inexpensive treatment system (Helmreich & Horn, 2009), which is more effective for treating greater volumes of rainwater as the removal of all major pathogens is independent of turbidity, pH and additional parameters that may influence other proposed treatment systems (Burch & Thomas, 1998).

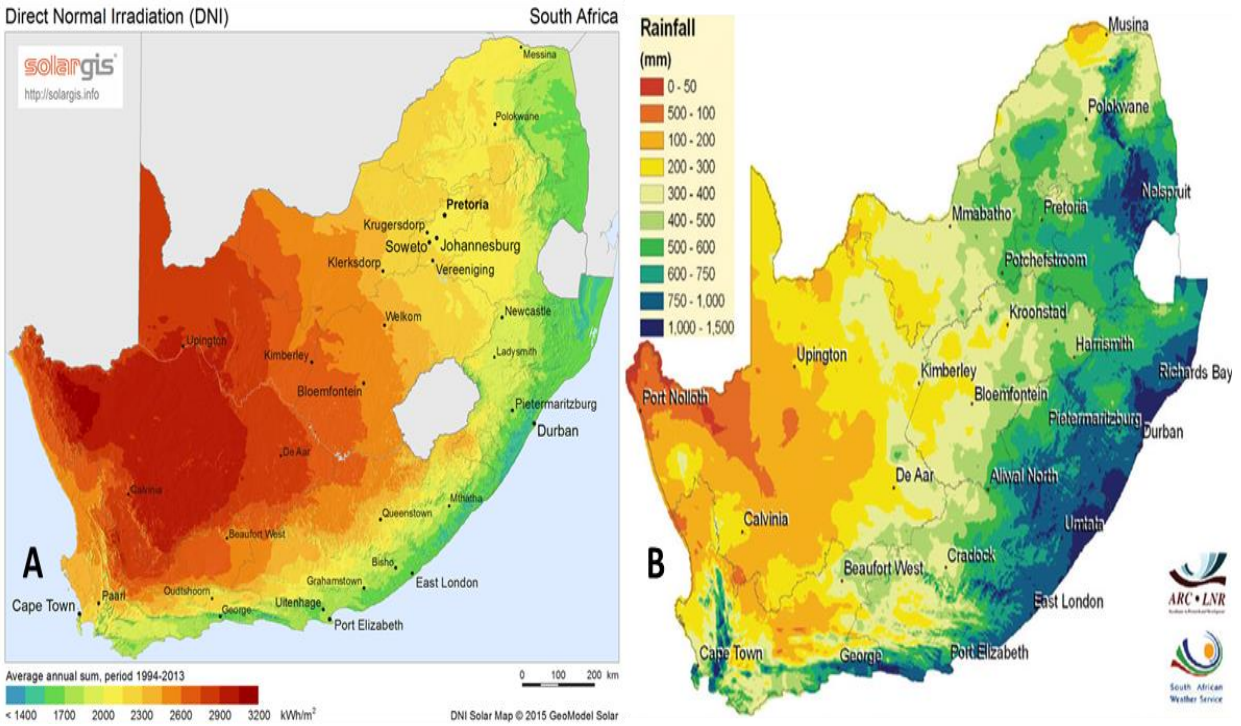

Fig. 1.3. (A) Average annual direct normal solar irradiation (DNI) in South Africa (adopted from SolarGIS © GeoModel Solar, 2016). **(B)** Average annual rainfall in South Africa (adopted from Malherbe & Olivier, 2012).

Various types of SOPAS systems are available. The most commonly used systems consist of a storage tank and a solar collector component that can heat the water either directly or indirectly (Nieuwoudt & Mathews, 2005). When installing the systems, the solar collectors must be placed in a north-facing orientation uninhibited by shade. By making use of the thermosiphoning effect, water can circulate in the system in a passive manner and this negates the need for a water pump which would increase costs associated with this technology. System storage tanks may be constructed either from metals or polymers and both materials have advantages and disadvantages. By using a polymeric storage tank, costs may be reduced and this material also provides increased resistance to corrosion and freezing. The polymeric material however has a lower temperature tolerance when compared with metal based storage tanks. In addition, although the polymeric storage tank provides increased resistance to corrosion, it may deteriorate over time as result of exposure to ultraviolet radiation. Conversely, metal storage tanks deteriorate as a result of corrosion and could therefore adversely influence the chemical quality of the treated water (Dobrowsky et al. 2015).

Fig. 1.4 illustrates the two types of SOPAS systems that are most commonly used; **(A)** direct SOPAS system and **(B)** indirect SOPAS system. The direct system uses evacuated tubes that absorb solar irradiation to heat the water. As the water temperature increases the density decreases and thus water rises to the top of the evacuated tubes and into the main storage

tank. Cooler water from the storage tank then moves into the evacuated tubes and is heated. In the indirect system, flat bed solar panels absorb solar irradiation and transfer the heat to a secondary liquid (water mixed with glycol). The secondary liquid then flows from the panels to the outer compartment of the storage tank. The heated secondary liquid in the outer compartment transfers the heat to the inner compartment containing the water (Nieuwoudt & Mathews, 2005).

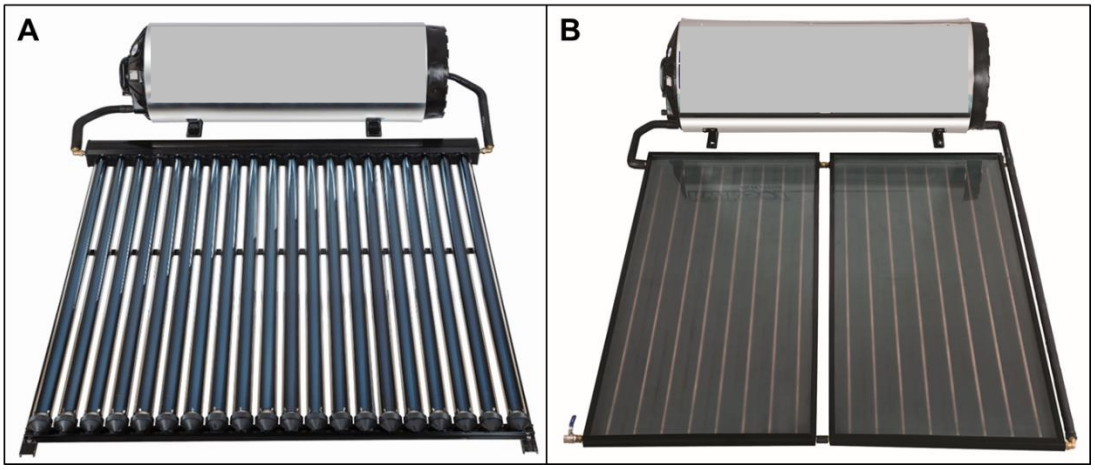

Fig. 1.4. The two types of SOPAS systems that are commercially available: **(A)** Direct SOPAS System; **(B)** Indirect SOPAS System (adopted from Saving Energy Solutions, 2016).

Ciochetti and Metcalf (1984) suggested that pasteurization would eliminate bacteria, rotaviruses and enteroviruses commonly found in contaminated water. Research conducted by Spinks et al. (2003; 2006) and Despins et al. (2009) indicated that the storage of rainwater at temperatures between 55 – 65 °C was required for the thermal inactivation of heat-resistant bacterial species including *Enterococcus* spp., *Escherichia* spp., *Salmonella* spp., *Aeromonas* spp., *Pseudomonas* spp., *Shigella* spp., *Klebsiella* spp. and *Vibrio* spp. Spinks et al. (2006) then concluded that the optimal temperature required for hot water systems to effectively remove bacteria was 60 °C. This temperature is in agreement with that suggested earlier by Ciochetti and Metcalf (1984) and Safapour and Metcalf (1999). Feachem et al. (1983) reported that the time required to pasteurize water decreases as the temperature increases. For example, for every 10 °C increase in temperature in excess of 50 °C, the time required to pasteurize water will decrease approximately by a factor of 10.

A disadvantage of SOPAS is that it does not improve the chemical quality of treated water (Islam & Johnston, 2006). In a previous study conducted by Dobrowsky et al. (2015), various metals were found to leach from a stainless steel SOPAS apparatus and thus influence the chemical quality of the water. It was therefore suggested that to prevent leaching, the storage tank of the pasteurization system should be manufactured from an alternative material such as a high grade polyethylene. In the same study, SOPAS was earmarked as a possible solution for

the improvement of the microbial quality of harvested rainwater. This was associated with SOPAS at temperatures in excess of 72 °C that reduced the levels of indicator bacteria (including heterotrophic bacteria, *E. coli* and total coliforms) to values below the detection limit. However, when utilising the PCR technique, certain pathogenic microorganisms were detected at temperatures exceeding the recommended pasteurization temperature of 72 °C. *Legionella* spp. and *Pseudomonas* spp. were also detected in pasteurized water treated at 91 °C, *Yersinia* spp. at 81 °C and *Klebsiella* spp. at 74 °C (Dobrowsky et al. 2015). In a study conducted by Lombard et al. (2013) the presence of adenovirus was detected in pasteurized water which had been treated at temperatures of 85 – 90 °C, by using PCR. However, a major disadvantage of using the PCR technique is that viability cannot be evaluated due to the persistence of deoxyribonucleic acid (DNA) after cell death (Masters et al. 1994). These findings remain a cause for concern as these organisms are pathogens and their viability must be confirmed.

1.5 Viability detection systems

Research has demonstrated that pathogenic microorganisms commonly occur in various environmental water samples including harvested rainwater. The detection of these organisms is crucial in order to monitor whether the water is safe to use for potable and domestic purposes. Traditionally, culture-based methods have been used to monitor the quality of harvested rainwater but it is well documented that certain microorganisms at times lose their ability to be cultured on artificial media. However, these microbes remain viable and can regain their ability to grow once conditions become favourable (Oliver, 2000; Murga et al. 2001; Hwang et al. 2006; García et al. 2007; Dusserre et al. 2008). Of concern is that these viable but non-culturable cells may pose a major health risk if they are ingested by humans (De Man et al. 2014). Furthermore, traditional culture methods are often time-consuming. For these reasons, most laboratories have introduced the use of molecular-based techniques that enable the rapid, sensitive and specific detection of organisms in both clinical and environmental samples (CDC, 2013). However, merely detecting the presence of a pathogenic microorganism, using only molecular-based techniques in a water sample following disinfection treatment, will not provide an accurate indication as to the potential health risks associated with using the water. This is because some of these methods detect non-viable microorganisms which do not pose a threat to the consumer, as only the viable portion of microbial contaminants pose a significant health risk. Researchers have therefore suggested targeting three properties of bacterial viability viz. metabolic activity/responsiveness, detection of nucleic acids and cellular integrity (Keer & Birch, 2003) (**Fig. 1.5**).

The detection of intact nucleic acid sequences (such as DNA), using the PCR technique, was initially used as an indicator of cell viability as it was assumed that DNA would be degraded in a dead cell more rapidly than other cellular components (Jamil et al. 1993). McCarty and Atlas

(1993) then reported that the detection of longer intact DNA sequences correlated more closely with viability than shorter sequences. Furthermore, Moore et al. (2001) stated that the consistent detection of nucleic acid sequences from pathogenic bacteria in an environment can indicate potential health risks as the detected organisms would likely be viable as they are either consistently introduced to the environment or are able to proliferate. However, despite the advantages provided by the use of PCR, many challenges remain. The presence of possible PCR inhibitors in environmental water samples will prevent the amplification of the target DNA and subsequently lead to false negative results (Wang & Levin, 2006). In addition, cell viability cannot be evaluated using the PCR technique as DNA may persist after cell death (Masters et al. 1994).

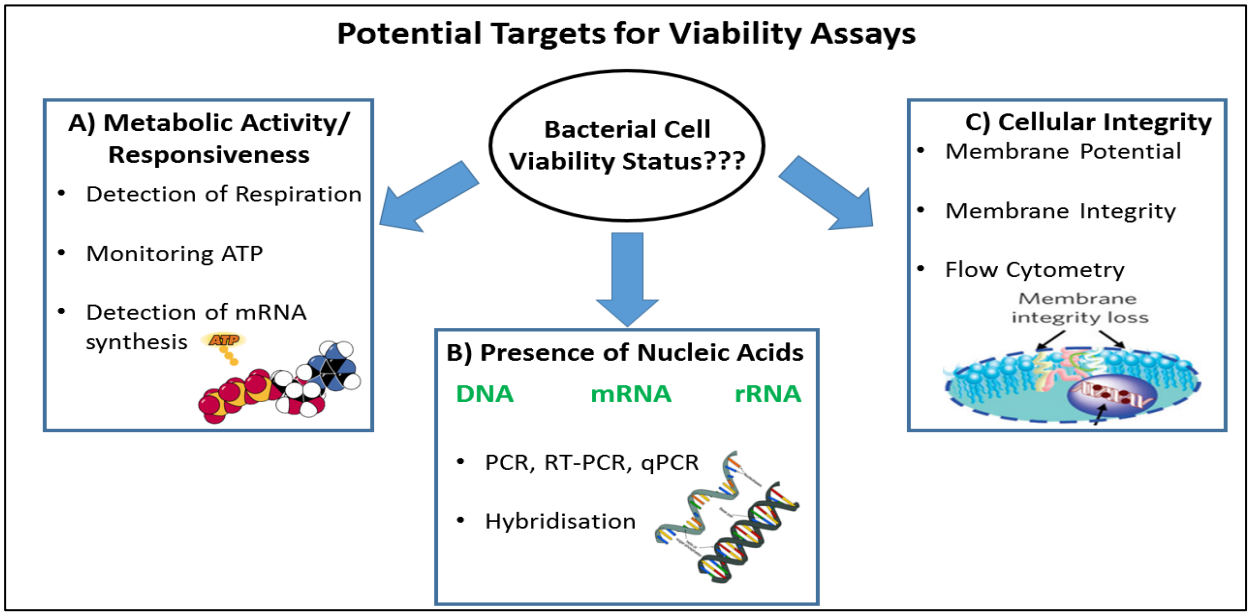

Fig. 1.5. The range of approaches used for the assessment of bacterial viability: **(A)** metabolic activity/responsiveness; **(B)** presence of nucleic acids; **(C)** cellular integrity (adapted from Keer & Birch, 2003).

In order to overcome these limitations various strategies have been introduced and used. The use of internal amplification controls during the PCR allows for the detection of possible PCR inhibitors (Ahmed et al. 2008; Fittipaldi et al. 2012). It was also suggested that changing the amplification target of PCR from DNA to ribonucleic acid (RNA) would provide the best option for indicating the presence of viable cells as the presence of mRNA indicates the presence of metabolically active cells (McKillip et al. 1999; Villarino et al. 2000; Bleve et al. 2003; Keer & Birch, 2003; Morin et al. 2004). However, the use of mRNA as the target, detected by using reverse transcription PCR (RT-PCR), also has limitations as bacterial mRNA degrades rapidly. It is also notable that mRNA expression levels are dependent on the physiological status of the cell and subsequently mRNA from viable dormant or extremely slow-growing microorganisms may be at levels below the detection limit of the PCR (Nocker et al. 2006). McKillip et al. (1998)

and Sheridan et al. (1998) also reported difficulty in the reproducibility of results when using mRNA to determine viable and dead cell numbers. As a result, scientists have investigated the use of nucleic acid binding dyes (viability-qPCR) and enzymatic treatments (DNase enzyme-based assay). These techniques would then exclude extracellular DNA as well as DNA from dead microorganisms and subsequently detect DNA only from viable microorganisms.

Cellular integrity can also be used to distinguish between viable and dead bacteria as it is assumed that viable cells will have an intact membrane that cannot be penetrated by selected staining compounds whereas dead cells are considered to have compromised permeable membranes (Stiefel et al. 2015). Flow cytometry coupled with staining kits is one of the most commonly used molecular techniques that targets cellular integrity (Hoefel et al. 2003; Stiefel et al. 2015). Flow cytometry in combination with the LIVE/DEAD® Baclight™ Bacterial Viability and Counting Kit (Invitrogen™) is used to distinguish live from dead cells based on the fluorescence of propidium iodide (PI) and SYTO 9. Propidium iodide is a red-fluorescent nucleic acid stain which only penetrates cells with disrupted membranes (dead cells). As PI enters the dead cells it randomly binds to the DNA present in the cell causing an increased red fluorescence which can be monitored by a flow cytometer. The SYTO 9 is a green-fluorescent nucleic acid stain which has the ability to enter both membrane-compromised (damaged cells) and intact (viable) cells and binds to the DNA present in the cell, causing green fluorescence. However, PI exhibits a stronger affinity for the binding of DNA and will supress the binding of SYTO 9 to DNA. Thus viable cells will stain green, while non-viable cells will stain red (Stiefel et al. 2015). Although this method can provide an accurate indication of the proportion of live to dead cells present in a sample, it cannot indicate whether cells of a specific genus or species are viable or dead when analysing a mixed culture. Thus, environmental samples with mixed bacterial populations become more complicated to analyse when compared with monospecific pure culture samples.

Scenarios could however occur where cells maintain membrane integrity but are metabolically inactive (Stiefel et al. 2015). Thus another target for viability assays includes assessing the metabolic activity or responsiveness of the cell as this is a definitive indication of whether the cell is viable. This can be achieved by monitoring mRNA synthesis as mRNA will only be produced by metabolically active cells. However, as mentioned previously, researchers have identified limitations associated with this approach such as the physiological status of the cell, which will directly influence mRNA expression levels. An improved approach to monitoring the metabolic activity of a cell is to monitor adenosine triphosphate (ATP) levels (energy levels), or the formation of metabolites (indicating an active metabolism). A readily used assay based on metabolic activity or responsiveness that allows for the monitoring of the viable portion of microbial contaminants in water samples following treatment, is the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI, USA). The BacTiter-Glo™ Microbial Cell Viability Assay, DNase enzyme-based assay and viability-qPCR will be discussed below, as these

methods target the proposed three indicators of bacterial viability referred to above and were subsequently employed in the current study (Chapter 3).

1.5.1 Viability-qPCR using EMA and PMA

In order to overcome the various disadvantages of using molecular-based techniques that target only one aspect of viability, scientists have started utilising methods that target multiple indicators of viability. For example, in order to overcome the shortcomings of conventional PCR and RT-PCR methods, viability-qPCR was developed. This latter method combines the detection of nucleic acids with cellular integrity and allows for a viability assessment of a specific organism present in a mixed-culture, as primers specific to the target organism are utilised.

Quantitative PCR has successfully been utilised to quantify possible microbial contaminants in environmental water samples (Behets et al. 2007; Yaradou et al. 2007; Dusserre et al. 2008). The term viability-qPCR then refers to the quantitative PCR (qPCR) analysis of a sample that has been pre-treated with a nucleic acid-binding dye (referred to as a viability dye). Such dyes are ethidium monoazide bromide (EMA) or propidium monoazide (PMA) (Fittipaldi et al. 2012). The distinction between viable and non-viable cells for both viability dyes is based on membrane integrity as the dyes are membrane-impermeant (Delgado-Viscogliosi et al. 2009). The viability dyes selectively penetrate cells possessing damaged membranes and covalently bind to DNA after photoactivation (activation of a chemical reaction by light). The binding of the dye to the DNA prevents the PCR amplification of the DNA and thereby leads to a strong signal reduction during qPCR. Simultaneously when the binding of the dye to the DNA occurs, any unbound excess dye reacts with water molecules and in so doing prevents the reaction of the excess dye with DNA extracted from intact cells during the subsequent DNA extraction procedures (Nocker & Camper, 2009). Intact membranes of viable cells then prevent the viability dyes from entering the cell and therefore the DNA from these cells can be amplified and quantified (Fittipaldi et al. 2012).

The mechanism by which the viability dyes prevent the amplification of the bound DNA is not fully understood and many theories have been proposed (Fittipaldi et al. 2012). After the dye has penetrated damaged cell membranes, upon photo-activation, it is covalently cross-linked to the DNA. This binding is thought to inhibit the amplification of the DNA by rendering the DNA insoluble thereby allowing for its removal along with other cell debris in the subsequent DNA extraction (Nocker & Camper, 2006). It is also possible that the binding of EMA to DNA cleaves the chromosomal DNA of non-viable bacteria thus preventing amplification (Soejima et al. 2007).

In order for the viability dyes to be effective, they should thus lead to the exclusion of DNA signals from membrane-compromised cells and simultaneously not affect the DNA signals from

cells with intact membranes. Various methods have therefore been used in order to assess the effectiveness of both viability dyes (EMA and PMA) for dead and live cells, with regard to specificity of dye penetration and possible dye toxicity. These methods include quantitative PCR analysis (Vesper et al. 2008; Yañez et al. 2011), fluorescence microscopy (Nocker et al. 2006; Flekna et al. 2007; Nam et al. 2011) and cytotoxicity assays (Rueckert et al. 2005; Flekna et al. 2007; Soejima et al. 2007). Using these methods, it has been demonstrated that both EMA and PMA can be used effectively as viability dyes, but each has specific advantages and disadvantages (Fittipaldi et al. 2012). Ethidium monoazide bromide is more efficient at DNA signal suppression than is PMA; however it has been proposed that EMA is able to penetrate live cells with intact membranes (Fittipaldi et al. 2012). Propidium monoazide (PMA) is considered to be membrane-impermeant and is therefore considered to be more effective in distinguishing live from dead cells. Conversely, studies have shown that PMA does not always adequately penetrate cells with compromised membranes and the number of dead cells in a sample could therefore be underestimated (Fittipaldi et al. 2012). To overcome these limitations, certain experimental parameters need to be considered when optimising the assay for use with a specific sample.

Research has shown that EMA is able to penetrate the membranes of non-viable cells at dye concentrations lower than those required for PMA. Using EMA at these lower concentrations will in turn also limit the ability of EMA to enter viable cells. In addition, incubation time and temperature play a role in the uptake of EMA by live cells as temperature affects membrane permeability. It is therefore recommended that the incubation step of the procedure is performed on ice instead of at ambient temperature and only for a brief time period (Fittipaldi et al. 2012). Conversely, limitations associated with the use of PMA can be overcome by using higher dye concentrations and increasing incubation temperature in order for PMA to readily enter dead cells. Higher dye concentrations can be tolerated as PMA is less cytotoxic than EMA. The use of membrane-destabilising agents as well as targeting longer DNA sequences for amplification have also been shown to increase the exclusion of dead cell signals (Fittipaldi et al. 2012).

It is important to note that the use of viability-qPCR provides an indication of cell viability based on both membrane integrity and the detection of nucleic acids. There are thus certain limitations in using this method as it cannot be applied to monitor viability following biocidal treatments or other treatments that do not damage the membrane sufficiently (Nocker et al. 2006). Nevertheless, when implemented correctly this method provides valuable information and it has successfully been used to differentiate between live and dead cells of bacteria, fungi, protozoa and yeasts (Rueckert et al. 2005; Wang & Levin, 2006; Pan & Breidt, 2007; Pisz et al. 2007; Soejima et al. 2007; Vesper et al. 2008; Chang et al. 2009; Delgado-Viscogliosi et al. 2009; Rawsthorne et al. 2009; Agusti et al. 2010; Fittipaldi et al. 2011; 2012; Reyneke et al. 2016).

1.5.2 DNase enzyme-based assay

Enzyme-based viability assays involve treating a sample with nucleases such as DNase, followed by the inactivation of the nuclease. The DNA of undamaged bacteria/viruses is extracted and eventually quantified using qPCR. The DNase enzyme-based assay thus combines the detection of nucleic acids with cellular integrity in order to provide an indication of cell viability. Similar to viability-qPCR, the assay allows for the viability assessment of a specific microorganism present in a mixed-culture as primers specific to the target organism of choice are utilised. A brief time exposure of live bacterial cells to enzymes, such as DNase I and trypsin, has little effect on the morphology, function or viability of the cells as the membrane (bacteria) or capsid (viruses) has the ability to protect the organism from proteases and nucleases (Girones et al. 2010; Viancelli et al. 2012; Fongaro et al. 2013). However, cells with damaged membranes will be digested by these enzymes to the extent that they are excluded from measurement (Darzynkiewics et al. 1994). DNase I is an endonuclease that is able to digest both single- and double-stranded DNA by hydrolysing phosphodiester bonds. This procedure removes all DNA from cells with compromised membranes (dead cells) from the sample, leaving only the intact cells (viable) to be analysed and quantified during qPCR. Since elimination of the dead cells with enzyme digestion has been shown to be selective, the method is commonly applied when studying the viable portion of biofilm communities, as well as to remove necrotic or apoptotic cells from sample material (Darzynkiewics et al. 1994). In addition, this method has been used to detect viable *Lactobacillus acidophilus, E. coli* and infective adenoviruses present in environmental water samples (Fong et al. 2008; Viancelli et al. 2012; Fongaro et al. 2013; Shakeri et al. 2014).

1.5.3 BacTiter-Glo™ Microbial Cell Viability Assay

The BacTiter-Glo™ Microbial Cell Viability Assay is an affordable and effective assay that is used to monitor for the presence of viable microbial cells (Deininger & Lee, 2006; Berney et al. 2008). The assay determines the number of viable microbial cells by monitoring the presence of ATP, which is an indicator of the presence of metabolically active cells (Berney et al. 2008). Briefly, the BacTiter-Glo™ Reagent is added to a sample, which causes cell lysis and the production of a luminescent signal which can be measured with a luminometer. The luminescent signal produced is proportional to the amount of ATP present, which is directly proportional to the number of viable cells in the sample (Berney et al. 2008; Promega, 2013). The assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and a proprietary buffer formulation for extracting ATP from bacteria. However, the assay has also been shown to detect a variety of yeast and fungi (Promega, 2013). The advantages the assay provides over other viability detection systems include; simplicity (reduction in the number of protocol and handling steps), time-effectiveness (results

can be obtained in 5 min), increased sensitivity (as few as 10 bacterial cells in a sample can be detected) and the use of a stable luminescent signal (half-life of more than 30 min) (Promega, 2013). As a result, the assay is commonly used for monitoring antimicrobial activity or bacterial growth. Research has also shown that cultivation-independent viability indicators, such as the BacTiter-Glo™ Microbial Cell Viability Assay are useful parameters for the rapid monitoring of water treatment efficiencies and could therefore aid in monitoring overall water quality (Berney et al. 2008; Reyneke et al. 2016). However, it is important to note that the assay will provide an overall indication of viability in the sample, as ATP is produced by all metabolically active cells and is therefore not specific for the detection of a certain organism. In a study conducted by Berney et al. (2008) viability results indicated that the measurement of total ATP values correlated positively with results obtained from the measurement of esterase activity and the high nucleic acid (HNA) bacterial fraction in various water samples. Heterotrophic plate count values however exhibited a weak correlation with all the other tested viability parameters. During a study conducted by Reyneke et al. (2016), the BacTiter-Glo™ Microbial Cell Viability Assay was used to monitor ATP levels present in rainwater samples before and after SOPAS. Results indicated a mean decrease of >99% in ATP (relative light units/100 µL sample) following pasteurization at temperatures in excess of 71.5 °C.

1.6 Description of the study site

Due to increased urbanisation, it has been reported that approximately 1.2 million households currently reside in 2 700 informal settlements dispersed across South Africa (Bennett & Fieuw, 2012). The Enkanini informal settlement (**Fig. 1.6**) was established during 2006 when families started relocating from neighbouring Kayamandi to open municipal ground as they could not afford housing in Kayamandi (Wessels & Swilling, 2015). Subsequently individuals arriving primarily from the rural Eastern Cape regions and seeking employment in the Western Cape region, inhabited this area as well. During 2006 an eviction court order for Enkanini residents was issued, but to date it has not been implemented (Tavener-Smith, 2012). The settlement is therefore the largest illegal informal settlement in Stellenbosch. Although the establishment and history of Enkanini has been associated with violent protests and an unwillingness to work with certain non-profit organisations and projects associated with the Stellenbosch Municipality, attitudes in recent years have changed primarily as a result of the work performed by the Stellenbosch Sustainability Institute in Enkanini (Keller & Swilling, 2012; Mollatt & Swilling, 2014; Von der Heyde & Swilling, 2014; Wessels & Swilling, 2015).

Fig. 1.6. Aerial image of Enkanini informal settlement located in Stellenbosch (Western Cape, South Africa) (GPS Coordinates: 33°54' 28.42"S 18°25' 03.56"E).

Inhabitants of Enkanini informal settlement have thus been involved in various Sustainability Institute projects since 2011. The projects focussed principally on the implementation of alternative technologies in order to improve the quality of life of the individuals living in the settlement (Keller & Swilling, 2012; Mollatt & Swilling, 2014; Von der Heyde & Swilling, 2014; Wessels & Swilling, 2015). These factors indicate a possible favourable attitude towards an alternative technology such as rainwater harvesting and also compliance, without fear of vandalism, as the community takes ownership of these technologies. The collaboration between individuals living in Enkanini and the Sustainability Institute also resulted in the establishment of the Enkanini Research Centre (Wessels & Swilling, 2015).

The Enkanini informal settlement was therefore selected as the study site for the installation and use of small- and large-scale SOPAS rainwater harvesting treatment systems, as only 32 communal water supply taps are located throughout Enkanini, which supply water to the estimated 4 450 residents (139 individuals per municipal tap) (Community Organisation Resource Centre, 2012). However, other studies have suggested that the number of Enkanini inhabitants is greatly underestimated and the residents themselves estimate the number to be between 8 000 and 10 000 individuals (Tavener-Smith, 2012; Wessels & Swilling, 2015). In either case, the situation clearly contravenes South African design guidelines which state that a maximum of 100 people should be served per standpipe (DWAF, 2004). Additionally, results from a social perception study completed as part of WRC Project K5/2368//3 (2016) in the Enkanini informal settlement, indicated that 61% of the respondents were familiar with the concept of rainwater harvesting. Moreover, 67% of the respondents were favourably inclined

towards using rainwater for their daily needs, 77% indicated that they would use the rainwater for bathing and cleaning their houses, 65% stated that they would use the rainwater for cooking purposes and 46% would use the rainwater for potable or drinking purposes.

1.7 Project aims

In order to alleviate the demand on municipal water supplies and centralised water collection systems, rainwater harvesting has been earmarked by the South African government as an additional water source to provide clean and potable water directly to households (Mwenge Kahinda et al. 2007; Li et al. 2010). However, research has indicated that numerous chemical and microbial contaminants are associated with stored tank water sources, including rainwater, and these potentially pose a serious health risk to the consumer (Simmons et al. 2001; Ahmed et al. 2008; 2011; Helmreich & Horn, 2009; Li et al. 2010). Pre-treatment methods should thus be introduced to ensure that the rainwater is safe to utilise for all daily water requirements.

Although various treatment technologies have been developed, research has shown that SODIS and SOPAS are effective treatment options to treat contaminated water (Spinks et al. 2003; 2006; Islam & Johnston, 2006; Despins et al. 2009; Helmreich & Horn, 2009; Li et al. 2010; McGuigan et al. 2012). Solar pasteurization effectively treats larger volumes of water and is not influenced by factors such as turbidity and pH, which could influence other water treatment systems. However, bacteria and viruses occurring at temperatures exceeding the recommended pasteurization temperature (72 °C) have previously been detected with the use of PCR assays in rainwater sources (Lombard et al. 2013; Dobrowsky et al. 2015). As referred to in the foregoing, the PCR technique does not indicate viability of contaminants and culture-based methods are ineffective for the culturing of viable but non-culturable microorganisms. Therefore, it is essential that reliable viability detection systems are employed to assess water safety.

In order to achieve this, researchers have suggested targeting three aspects of bacterial viability viz. cell metabolic activity or responsiveness, the presence of nucleic acids and cell membrane integrity (Keer & Birch, 2003). The BacTiter-Glo™ Microbial Cell Viability Assay and DNase enzyme-based assay have been used successfully to monitor the viable portion of microbial contaminants found in water sources (Berney et al. 2008; Viancelli et al. 2012; Fongaro et al. 2013; Reyneke et al. 2016). Viability-qPCR has also permitted researchers to overcome certain limitations when using molecular based methods and allows for the rapid, sensitive and specific detection of viable microbial cells in samples (Fittipaldi et al. 2012; Reyneke et al. 2016).

The primary aim of the current study was thus to construct and monitor the operational sustainability of small- and large-scale domestic rainwater harvesting SOPAS systems in Enkanini informal settlement (Stellenbosch, South Africa). These systems were introduced to

alleviate the pressures on the existing communal standpipe systems and to provide the inhabitants of the informal settlement with an alternative water source. In order to accomplish these aims, the following objectives were completed:

Objective 1 (Chapter 2): Operational sustainability and efficacy of small- and large-scale solar pasteurization treatment systems installed in Enkanini informal settlement (Stellenbosch) for the treatment of rainwater:

- Unpasteurized and pasteurized rainwater samples were routinely collected from two small-scale and one large-scale rainwater harvesting SOPAS systems installed in the Enkanini informal settlement (Stellenbosch). On all sampling occasions, unpasteurized samples were collected directly from the respective rainwater tanks, while pasteurized samples were collected directly from the two small-scale SOPAS storage tanks and from the solar manifold system and holding tank of the large-scale system. During the high rainfall periods, rainwater samples were collected one to four days after a rain event from the large-scale and small scale-systems (pasteurized rainwater samples collected at temperatures exceeding 50 °C). Thereafter during the low rainfall periods, samples were collected every three weeks. It should be noted that samples were only collected/processed if the pasteurization temperature was above 50 °C. Samples were thus collected at the temperature range of 50 to 59 °C, 60 to 69 °C, 70 to 79 °C and 80 °C and above. The temperature and pH of the rainwater samples were measured on site during all sampling occasions. Rainfall and temperature patterns were obtained from the South African Weather Services, while solar radiation (ultraviolet readings) data were provided by the Stellenbosch Weather Services, Faculty of Engineering, Stellenbosch University.
- The chemical quality of the unpasteurized and pasteurized rainwater samples was determined by monitoring cation and anion concentrations.
- For microbial analysis before and after SOPAS, the enumeration of traditional indicator bacteria, *E. coli*, total and faecal coliforms, enterococci and heterotrophic bacteria, was performed.
- In order to determine the length of time the pasteurized rainwater could be stored before microbial re-growth occurred, pasteurized rainwater samples were stored at ambient temperature out of direct light and the heterotrophic plate count was determined every two days over a period not exceeding two weeks.
- For the detection of a library of selected pathogenic bacterial genera (including *Aeromonas* spp., *Bacillus* spp., *Enterococcus* spp., *Klebsiella* spp., *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Staphylococcus* spp., *Streptomyces* spp. and *Yersinia* spp.) and one DNA virus (adenovirus), commonly

associated with rainwater, conventional PCRs were optimised and then applied to the unpasteurized and pasteurized rainwater samples.

- Following the completion of the conventional PCR assays, the most readily detected bacterial genera were quantified using qPCR assays.
- The BacTiter-Glo™ Microbial Cell Viability Assay was then used to monitor for the presence of metabolically active cells in the unpasteurized and pasteurized rainwater samples and thus provide an overall indication of viability.
- The operational sustainability of the systems was also determined by calculating the volume of water that the systems could produce at a specific pasteurization temperature. Additionally, the pasteurized rainwater usage and municipal tap water usage of the participating households were monitored throughout the sampling period.

Objective 2 (Chapter 3): Molecular-based viability versus metabolic responsiveness assays for the accurate determination of microbial cell viability:

Research has demonstrated that there is a need for assays that allow for the accurate determination of viable cells in environmental samples. The objective of the current chapter was thus to assess molecular-based viability assays (EMA-qPCR, PMA-qPCR and DNase treatment in combination with qPCR) and a metabolic responsiveness assay (BacTiter-Glo™ Microbial Cell Viability Assay) for their ability to accurately identify the presence of viable cells in water samples that had been spiked with a test organism (*Legionella* spp., *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp. and *Enterococcus* spp.). For this, a heat treated sample (representing a sample containing both viable and dead cells), an autoclaved sample (negative control – only dead cells) and an untreated sample (positive control – only viable cells) of each of the respective organisms was included. As varying concentrations of the nucleic acid binding dyes, EMA and PMA, have been reported in literature and as research has shown that EMA and PMA have different membrane permeability potentials depending on the target organisms' cell wall/membrane composition, optimal concentrations of EMA and PMA were also assessed in the current study for the detection of the test organisms.

- The test organisms utilised for the optimisation of the viability assays on the "spiked" water samples included three Gram-negative (*Legionella pneumophila* ATCC 33152, *Pseudomonas aeruginosa* ATCC27853 and *Salmonella enterica* serovar Typhimurium ATCC 14028) and two Gram-positive [*Staphylococcus aureus* ATCC 25925 and *Enterococcus faecalis* (sequence verified clinical isolate)] organisms.
- The test organisms were each spiked into three sterile 500 mL water aliquots, respectively. The first 500 mL water aliquot for each organism was heat treated at 70 °C for 15 min (representing a sample containing both viable and dead cells), the second

500 mL water aliquot was autoclaved (negative control – only dead cells), while the last 500 mL aliquot remained untreated (positive control – only viable cells).

- The heat treated (viable and dead), autoclaved (dead) and untreated (viable) water samples for each respective organism were then analysed using culturing analysis. This was performed as a control to enumerate the viable and culturable organisms present in each sample. Subsequently, the BacTiter-Glo™ Microbial Cell Viability Assay was applied, which enabled the monitoring of adenosine triphosphate as an indication of the presence of metabolically active cells. The samples for each of the respective organisms were then analysed using EMA-qPCR and PMA-qPCR. For EMA-qPCR and PMA-qPCR, various concentrations of EMA (6 µM, 12.5 µM, 25 µM, 35 µM and 50 µM) and PMA (25 µM, 50 µM and 100 µM) were analysed. The concentration ranges of both EMA and PMA that were applied to the samples were selected based on concentrations that had been reported in literature for the respective test organisms. Lastly, the DNase enzyme-based assay was used to analyse the samples.
- The results obtained using culture-based analysis, the molecular-based viability assays (EMA-qPCR, PMA-qPCR and DNase treatment in combination with qPCR) and metabolic responsiveness assay (BacTiter-Glo™ Microbial Cell Viability Assay) were then compared to determine which assay was the most reliable in detecting viable cells.

1.8 References

Abbott, S.E., Douwes, J. & Caughley, B.P. 2006. *A survey of the microbiological quality of roof-collected rainwater of private dwellings in New Zealand. Rainwater and urban design*. 2007(1):9-16. Available: http://search.informit.com.au/documentSummary;dn=885521567814046;res=IELENG [2015, November 10].

Abdulla, F.A. & Al-Shareef, A.W. 2009. Roof rainwater harvesting systems for household water supply in Jordan. *Desalination*. 243(1-3):195-207.

Abraham, J.P., Plourde, B.D. & Minkowycz, W.J. 2015. Continuous flow solar thermal pasteurization of drinking water: Methods, devices, microbiology, and analysis. *Renewable Energy*. 81:795-803.

Agusti, G., Codony, F., Fittipaldi, M., Adrados, B. & Morató, J. 2010. Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. *Helicobacter*. 15(5):473–476.

Ahmed, W., Huygens, F., Goonetilleke, A. & Gardner, T. 2008. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. *Applied and Environmental Microbiology*. 74(17):5490-5496.

Ahmed, W., Vieritz, A., Goonetilleke, A. & Gardner, T. 2010a. Health risk from the use of roof-harvested rainwater in Southeast Queensland, Australia, as potable or nonpotable water, determined using quantitative microbial risk assessment. *Applied and Environmental Microbiology*. 76(22):7382-7391.

Ahmed, W., Goonetilleke, A. & Gardner, T. 2010b. Implications of faecal indicator bacteria for the microbiological assessment of roof-harvested rainwater quality in Southeast Queensland, Australia. *Canadian Journal of Microbiology*. 56(6):471–479.

Ahmed, W., Gardner, T. & Toze, S. 2011. Microbiological quality of roof-harvested rainwater and health risks: A review. *Journal of Environmental Quality*. 40(1):1-9.

Albrechtsen, H.J. 2002. Microbiological investigations of rainwater and graywater collected for toilet flushing. *Water Science and Technology*. 46(6-7):311-316.

Allegra, S., Berger, F., Berthelot, P., Grattard, F., Pozzetto, B. & Riffard, S. 2008. Use of flow cytometry to monitor *Legionella* viability. *Applied and Environmental Microbiology*. 74(24):7813-7816.

Allegra, S., Grattard, F., Girardot, F., Riffard, S., Pozetto, B. & Berthelot, P. 2011. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. *Applied and Environmental Microbiology*. 77(4):1268-1275.

Amin, M.T. & Han, M.Y. 2009. Roof-harvested rainwater for potable purposes: application of solar disinfection (SODIS) and limitations. *Water Science and Technology*. 60(2):419–431.

An, H.R., Mainelis, G. & White, L. 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples.*Atmospheric Environment*. 40(40):7924 –7939.

Areerachakul, N., Kitiphatmontree, M., Kandasamy, J., Kus, B., Duangduen, C., Pivsa-Art, S. & Vigneswaran, S. 2009. Submerged membrane system with biofilter as a treatment to rainwater. *Water, Air and Soil Pollution*. 9(5-6):431–438.

Ashbolt, NJ., Grabow, WOK., Snozzi, M. 2001. Indicators of microbial water quality. In: Fewtrell L, Bartram J, eds. Water quality—Guidelines, standards and health: Assessment of risk and risk management for water-related infectious disease. London, IWA Publishing, pp. 289–315 (WHO Water Series).

Bauer, H., Giebl, H., Hitzenberger, R., Kasper-Giebl, A., Reischl, G., Zibuschka, F. & Puxbaum, H. 2003. Airborne bacteria as cloud condensation nuclei. *Journal of Geophysical Research*. 108(D21):4658-4665.

Behets, J., Declerck, P., Delaedt, Y., Creemers, B. & Ollevier, F. 2007. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. *Journal of Microbiological Methods*. 68(1):137–144.

Bennett, J. & Fieuw, W. 2012. *ISN Gauteng implements CUFF projects*. SDI, South Africa Alliance, October 2012. Available: http://sasdialliance.org.za/isn-gauteng-cuff-projects/ [2016, January 20].

Benson, R.F. & Fields, B.S. 1998. Classification of the genus *Legionella*. *Seminars in Respiratory Infection*. 13(2):90-99.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H-A., Egli, T. & Hammes, F. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Research*. 42(14):4010-4018.

Bleve, G., Rizzotti, L., Dellaglio, F. & Torriani, S. 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. *Applied and Environmental Microbiology*. 69(7):4116–4122.

Bojer, M.S., Struve, C., Ingmer, H., Hansen, D.S. & Krogfelt, K.A. 2010. Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. *PLoS ONE*. 5(11):e15467. DOI:10.1371/journal.pone.0015467.

Boyle, M.A., Sichel, C., Fernández-Ibánez, P., Arias-Quiroz, G.B., Iriarte-Puna, M. & McGuigan, K.G. 2008. Identifying the bactericidal limits of Solar Disinfection (SODIS) of water under real sunlight conditions. *Applied and Environmental Microbiology*. 74(10):2997–3001.

Bruins, H.J., Evenari, M. & Nessler, U. 1986. Rainwater harvesting agriculture for food production in arid zones: the challenge of the African famine. *Applied Geography*. 6(1):13-32.

Burch, J.D. & Thomas, K.E. 1998. Water disinfection for developing countries and potential for solar thermal pasteurization. *Solar Energy*. 64(1):87-97.

Cabral, J. 2010. Water microbiology. Bacterial pathogens and water. *International Journal of Environmental Research and Public Health*. 7(10):3657-3703.

Campisano, A. & Modica, C. 2012. Optimal sizing of storage tanks for domestic rainwater harvesting in Sicily. *Resources, Conservation and Recycling*. 63:9-16.

Caslake, L.F., Connolly, D.J., Menon, V., Duncanson, C.M., Rojas, R. & Tavakoli, J. 2004. Disinfection of contaminated water by using solar irradiation. *Applied Environmental Microbiology*. 70(2):1145-1150.

Centers for Disease Control and Prevention (CDC). 2013. *Legionella (Legionnaires' disease and Pontiac Fever)*. Available: http://www.cdc.gov/legionella/clinicians.html#footnotes [2014, November 3].

Chang, M., McBroom, M.W. & Beasley, R.S. 2004. Roofing as a source of non-point water pollution. *Journal of Environmental Management*. 73(4):307-315.

Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F. & Watanabe, H. 2009. Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. *Applied and Environmental Microbiology*. 75(1):147-153.

Chapman, H., Cartwright, T., Huston, R. & O'Toole, J. 2008. *Water quality and health risks from urban rainwater tanks.* (Research Report 42). Adelaide, Australia: Cooperative Research Centre for Water Quality and Treatment.

Cheyne, B.M., Van Dyke, M.I., Anderson, W.B. & Huck, P.M. 2010. The detection of *Yersinia enterocolitica* in surface water by quantitative PCR amplification of the *ail* and *yadA* genes. *Journal of Water and Health*. 8(3):487-499.

Ciochetti, D.A. & Metcalf, R.H. 1984. Pasteurization of naturally contaminated water with solar energy. *Applied Environmental Microbiology*. 47(2):223-228.

Community Organisation Resource Centre. 2012. *Enkanini (Kayamandi) household enumeration report*. Available: http://sasdialliance.org.za/wpcontent/uploads/docs/reports/Enumerations/Enkanini%20Final%20Report.pdf [2015, June 20].

Coutinho, H.D., Falcao-Silva, V.S. & Goncalves, G.F. 2008. Pulmonary bacterial pathogens in cystic fibrosis patients and antibiotic therapy: A tool for the health workers. *International Archives of Medicine*. 1(24):1-7.

Darzynkiewics, Z., Robinson, J.P. & Crissman, H.A. 1994. *Methods in cell biology, flow cytometry.* Part A, Vol 41. Rev. 2nd ed. California, USA: Academic Press Inc.

Deininger, R.A. & Lee, J. 2006. Modern tools and methods of water treatment for improving living standards. In: *Proceedings of the NATO Advanced Research Workshop on Modern Tools and Methods of Water Treatment for Improving Living Standards Dnepropetrovsk, Ukraine*. A. Omelchenko, A.A. Pivovarov & W.J. Swindall, Eds. 19–22 November 2003. Springer, Dordrecht, Netherlands. 71–78.

Dejung, S., Fuentes, I., Almanza, G., Jarro, R., Navarro, L., Arias, G., Urquieta, E., Torrico, A., Fenandez, W., Iriarte, M., Birrer, C., Stahel, W.A., Wegelin, M. 2007. Effect of solar water disinfection (SODIS) on model microorganisms under improved and field SODIS conditions. *Journal of Water Supply: Research and Technology – AQUA*. 56(4):245–256.

De Kwaadsteniet, M., Dobrowsky, P.H., Van Deventer, A., Khan, W. & Cloete, T.E. 2013. Domestic rainwater harvesting: Microbial and chemical water quality and point-of-use treatment systems. *Water, Air and Soil Pollution*. 224(7):1-19.

Delgado-Viscogliosi, P., Solignac, L. & Delattre, J-M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. *Applied and Environmental Microbiology*. 75(11):3502-3512.

De Man, H., Bouwknegt, M., Van Heijnsbergen, E., Leenen, E.J.T.M., Van Knapen, F. & De Roda Husman, A.M. 2014. Health risk assessment for splash parks that use rainwater as source water. *Water Research*. 54:254-261.

Department of Water Affairs and Forestry (DWAF). 1996. *South African Water Quality Guidelines* 2nd ed. Volume 1: Domestic Water Use. Pretoria, South Africa: CSIR Environmental Services. ISBN 0-7988-5338-7.

Department of Water Affairs and Forestry (DWAF). 2004. *Technical guidelines for the development of water and sanitation infrastructure.* 2nd ed. Available: www.dwaf.gov.za [2014, May 17].

Department of Water Affairs (DWA). 2009. *Water for growth and development framework.* Available: http://www.dwa.gov.za/wfgd/documents/wfgd_frameworkv7.pdf [2014, April 22].

Department of Water Affairs (DWA). 2012. *Department of Water Affairs Annual report 2011/2012*. Available: https://www.dwa.gov.za/documents/AnnualReports/DWA%20AR%202011-12%20Reduced.pdf. [2014, April 22].

Department of Water Affairs (DWA). 2013. *Department of Water Affairs Annual report 2012/2013*. Available: http://www.gov.za/documents/download.php?f=200726. [2014, April 20].

Despins, C., Farahbakhsh, K. & Leidl, C. 2009. Assessment of rainwater quality from rainwater harvesting systems in Ontario, Canada. *Journal of Water Supply: Research and Technology – AQUA*. 58(2):117–134.

De Villiers, A. 2011. Comparative analysis of innovative technology and conventional technology in low income building. *The South African Housing Foundation International Conference.* 11 -14 September 2011. Cape Town, South Africa.

Dobrowsky, P.H., Cloete, T.E. & Khan, W. 2014. Quality assessment of domestic harvested rainwater in the peri-urban region of Kleinmond, Western Cape and the optimisation of point-of-use treatment systems. M.Sc. Thesis. University of Stellenbosch.

Dobrowsky, P.H., Carstens, M., De Villiers, J., Cloete, T.E. & Khan, W. 2015. Efficiency of a closed-coupled solar pasteurization system in treating roof harvested rainwater. *Science of the Total Environment*. 536:206-214.

Dunlop, P.S.M., Byrne, J.A., Manga, N. & Eggins, B.R. 2002. The photocatalytic removal of bacterial pollutants from drinking water. *Journal of Photochemistry and Photobiology A: Chemistry*. 148(1-3):355–363.

Dusserre, E., Ginevra, C., Hallier-Soulier, S., Vandenesch, F., Festoc, G., Etienne, J., Jarraud, S. & Molmeret, M. 2008. A PCR-based method for monitoring *Legionella pneumophila* in water samples detects viable but noncultivable legionellae that can recover their cultivability. *Applied and Environmental Microbiology*. 74(15):4817–4824.

Edberg, S.C., Rice, E.W., Karlin, R.J. & Allen, M.J. 2000. *Escherichia coli*: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology*. 88(29):106-116.

Eisen, R.J. & Gage, K.L. 2008. Adaptive strategies of *Yersinia pestis* to persist during inter-epizootic and epizootic periods. *Veterinary Research*. 40(2):1-14. DOI: http://dx.doi.org/10.1051/vetres:2008039.

Ekström, S., Noziere, B., Hultberg, M., Alsberg, T., Magner, J., Nilsson, E.D. & Artaxo, P. 2010. A possible role of ground-based microorganisms on cloud formation in the atmosphere. *Biogeosciences*. 7(1):387–394.

Environment Agency. 2002. *The urban environment in England and Wales - A detailed assessment.* Environmental Agency Report. Bristol, England.

Evans, C.A., Coombes, P.J. & Dunstan, R.H. 2006. Wind, rain and bacteria: The effect of weather on the microbial composition of roof-harvested rainwater. *Water Research*. 40(1):37–44.

Feachem, R.E., Bradley, D.J., Garelick, H. & Mara, D.D. 1983. *Sanitation and disease: Health aspects of excreta and wastewater management.* New York, USA: Wiley.

Fernández-Ibánez, P., Sichel, C., Polo-López, I., De Cara-García, M. & Tello, J.C. 2009. Photocatalytic disinfection of natural well water contaminated with *Fusarium solani* using TiO_2 slurry in solar CPC photo-reactors. *Catalysis Today*. 144(1-2):62–68.

Fewster, E., Mol, A. & Wiessent-Brandsma, C. 2004. The long term sustainability of household bio-sand filtration. *Proceedings of the 30th WEDC International Conference.* 30 October 2004. Vientiane, Laos.

Fields, B.S. 1996. The molecular ecology of legionellae. *Trends in Microbiology*. 4(7):286–290.

Fields, B.S., Benson, R.F. & Besser, R.E. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical Microbiology Reviews*. 15(3):506-526.

Fisher, M.B., Iriarte, M. & Nelson, K.L. 2012. Solar water disinfection (SODIS) of *Escherichia coli*, *Enterococcus* spp., and MS2 coliphage: effects of additives and alternative container materials. *Water Research*. 46(6):1745–1754.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K. & Morato, J. 2011. Viable real-time PCR in environmental samples: can all data be interpreted directly? *Microbial Ecology*. 61(1):7–12.

Fittipaldi, M., Nocker, A. & Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods*. 91(2):276-289.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S. & Hein, I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Research in Microbiology*. 158(5):405–412.

Fluri, T.P. 2009. The potential of concentrating solar power in South Africa. *Energy Policy*. 37(12):5075-5080.

Fong, H.C., Lin, T.P. & Wan, V.L. 2008. Development of molecular method for detection of viable *Escherichia coli* in environmental waters. M.Sc. Thesis. University of Malaysia, Sabah.

Fongaro, G., Nascimento, M.A., Rigotto, C., Ritterbusch, G., Da Silva, A.D.A., Esteves, P.A. & Barardi, C.R.M. 2013. Evaluation and molecular characterization of human adenovirus in drinking water supplies: viral integrity and viability assays. *Journal of Virology*. 10(1):166-174.

Food and Agriculture Organization (FAO) of the United Nations Corporate Document Repository. 1985. Chapter 4 - Rainfall and Evapotranspiration. In *Irrigation Water Management: Training Manual No. 1 - Introduction to Irrigation.* Available: http://www.fao.org/docrep/r4082e/r4082e05.htm. [2015, May 20].

Franklin, L.J., Fielding, J.E., Gregory, J., Gullan, L., Lightfood, D., Poznaski, S.Y. & Vally, H. 2009. An outbreak of *Salmonella typhimurium* 9 at a school camp linked to contamination of rainwater tanks. *Epidemiology Infection*. 137(3):434–440.

Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar, R.G., Harris, J., Mallison, G.F., Martin, S.M., McDade, J.E., Shepard, C.C., Brachman, P.S. 1977. Legionnaires' disease: description of an epidemic of pneumonia. *New England Journal of Medicine*. 297(22):1189–1197.

García, M.T., Jones, S., Pelaz, C., Millar, R.D. & Abu Kwaik, Y. 2007. *Acanthamoeba polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after disinfection. *Environmental Microbiology*. 9(5):1267–1277.

Gikas, G.D. & Tsihrintzis, V.A. 2012. Assessment of water quality of first-flush roof runoff and harvested rainwater. *Journal of Hydrology*. 466-467:115-126.

Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., Corrêa, A.A., Hundesa, A., Carratala, A., Bofill-Mas, S. 2010. Molecular detection of pathogens in water – The pros and cons of molecular techniques. *Water Research*. 44(15):4325–4339.

Glick, T.H., Gregg, M.B., Berman, B., Mallison, G., Rhodes, W.W. & Kassanoff, I. 1978. Pontiac fever. An epidemic of unknown etiology in a health department. International clinical and epidemiologic aspects*. American Journal Epidemiology*. 107(2):149–160.

Global Development Research Center (GDRC). 2015*. Rainwater harvesting and utilisation.* Available: http://www.gdrc.org/uem/water/rainwater/rainwaterguide.pdf [2015, May 20].

Gofti, L., Zmirou, D., Murandi, F.S., Hartemann, P. & Poleton, J.L. 1999. Waterborne microbiological risk assessment: a state of the art and perspectives.*Revue d'Epidemiologie et de Sante´ Publique*. 47(1):61–75.

Gordon, G., Adam, L. & Bubnis, B. 1995. Minimizing chlorate ion formation. *Journal of the American Water Works Association*. 87(6):97–106.

Gould, J. 1999. Contributions relating to rainwater harvesting – Assessment of Water Supply Options (Thematic Review IV.3). In *Dams and Development, A New Framework for Decision Making*. The Report of the World Commission on Dams. Earthscan Publications Ltd, London and Sterling, VA. Available: http://www.unep.org/dams/WCD/report/WCD_DAMS%20report.pdf [2016, August 22].

Gould, J. & Nissen-Petersen, E. 1999. *Rainwater Catchment Systems for Domestic Supply: Design, Construction and Implementation*. London, UK: Intermediate Technology Publications.

Goyer, R.A. 1993. Lead toxicity: current concerns. *Environmental Health Perspectives*. 100:177–187.

Griffin, D.W., Gibson, C.J., Lipp, E.K., Riley, K., Paul, J.H. & Rose, J.B. 1999. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. *Applied and Environmental Microbiology*. 65(9):4118–4125.

Gwenzi, W., Dunjana, N., Pisa, C., Tauro, T. & Nyamadzawo, G. 2015. Water quality and public health risks associated with roof rainwater harvesting systems for potable supply: Review and perspectives. *Sustainability of Water Quality and Ecology*. 6:107-118.

Handia, L., Tembo, J.M. & Mwiindwa, C. 2003. Potential of rainwater harvesting in urban Zambia. *Physics and Chemistry of the Earth*. 28(20-27):893–896.

Harding, A.S. & Schwab, K.J. 2012. Using limes and synthetic psoralens to enhance solar disinfection of water (SODIS): a laboratory evaluation with norovirus, *Escherichia coli*, and MS2. *American Journal of Tropical Medicine and Hygiene*. 86(4):566–572.

Hartung, H. 2006. Local financing mechanisms for roof-water harvesting in Uganda. *Waterlines*. 24(4):8–11.

Hartung, H. 2007. Rainwater utilisation in Africa, some experiences. *Proceedings of the 1st International Rainwater Leadership Workshop. International Water Association Rainwater Harvesting and Management Specialist Group (IWA WHM SG)*. Seoul National University Brain Korea 21 Sustainable Infrastructure Research group (SNU BK21 SIR group), United Nations Environment Programme (UNEP), Seoul, Korea.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R. & Rose, J.B. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Applied and Environmental Microbiology*. 71(6):3163–3170.

Hauser, A. & Ozer, E.A. 2011. *Pseudomonas aeruginosa. Nature Reviews: Microbiology*. 9(3):1-2.

Helmreich, B. & Horn, H. 2009. Opportunities in rainwater harvesting. *Desalination*. 248(1):118-124.

Heyworth, J.S., Glonek, G., Maynard, E.J., Baghurst, P.A. & Finlay-Jones, J. 2006. Consumption of untreated tank rainwater and gastroenteritis among young children in South Australia. *International Journal of Epidemiology*. 35(4):1051-1058.

Hörman, A., Rimhannen-Finne, R., Maunula, L., Von Bonsdorff, C.H., Torvela, N., Heikinheimo, A. & Hänninen, M.L. 2004. *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000–2001. *Applied Environmental Microbiology*. 70(1):87–95.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S. & Saint, C.P. 2003. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *Journal of Microbiological Methods*. 55(3):585-597.

Huang, X., Qu, Y., Cid, C.A., Finke, C., Hoffmann, M.R., Lim, K. & Jiang, S.C. 2016. Electrochemical disinfection of toilet wastewater using wastewater electrolysis cell. *Water Research*. 164-172.

Huston, R., Chan, Y.C., Gardner, T., Shaw, G. & Chapman, H. 2009. Characterisation of atmospheric deposition as a source of contaminants in urban rainwater tanks. *Water Research*. 43(6):1630-1640.

Huston, R., Chan, Y.C., Chapman, H., Gardner, T. & Shaw, G. 2012. Source apportionment of heavy metals and ionic contaminants in rainwater tanks in a subtropical urban area in Australia. *Water Research*. 46(4):1121-1132.

Hwang, M.G., Katayama, H. & Ohgaki, S. 2006. Effect of intracellular resuscitation of *Legionella pneumophila* in *Acanthamoeba polyphage* cells on the antimicrobial properties of silver and copper. *Environmental Science and Technology*. 40(23):7434–7439.

Ibánez, A.J., Litter, I.M. & Pizarro, A.R. 2003. Photocatalytic bactericidal effect of TiO_2 on *Enterobacter cloacae* comparative study with other Gram negative bacteria. *Journal of Photochemistry and Photobiology A: Chemistry*. 157(1):81–85.

Islam, M.F. & Johnston, R.B. 2006. Household pasteurization of drinking-water: The *Chulli* water-treatment system.*Journal of Health, Population and Nutrition*. 24(3):356-362.

Jamil, S., Keer, J.T., Lucas, S.B., Dockrell, H.M., Chiang, T.J., Hussain, R. & Stoker, N.G. 1993. Use of polymerase chain reaction to assess efficacy of leprosy chemotherapy. *Lancet*. 342(8866):264-268.

Jones, K. 1997. Strategies for survival. In *Coliforms and E. coli. Problem or solution?* D. Kay & C. Fricker, Eds. Cambridge, UK: The Royal Society of Chemistry.

Jørgensen, S.B., Bojer, M.S., Boll, E.J., Martin, Y., Helmersen, K., Skogstad, M. & Struve, C. 2016. Heat-resistant, extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in endoscope-mediated outbreak. *The Journal of Hospital Infection*. 93(1):57-62.

Kaushik, R., Balasubramanian, R. & De La Cruz, A.A. 2012. Influence of air quality on the composition of microbial pathogens in fresh rainwater. *Applied and Environmental Microbiology*. 78(8):2813-2818.

Keer, J.T. & Birch, L. 2003. Molecular methods for the assessment of bacterial viability. *Journal of Microbiological Methods*. 53(2):175-183.

Kehoe, S.C., Joyce, T.M., Ibrahim, P., Gillespie, J.B., Shahar, R.A. & McGuigan, K.G. 2001. Effect of agitation, turbidity, aluminium foil reflectors and container volume on the inactivation efficiency of batch-process solar disinfectors. *Water Research*. 35(4):1061–1065.

Keller, A. & Swilling, M. 2012. Conceptualising a sustainable energy solution for *in situ* informal settlement upgrading. M.Sc. Thesis. University of Stellenbosch.

Korzeniewska, E., Filipkowska, Z., Gotkowska-Płachta, A., Janczukowicz, W. & Rutkowski, B. 2008. Bacteriological pollution of the atmospheric air at the municipal and dairy wastewater treatment plant area and in its surroundings. *Archives of Environmental Protection*. 34(4):13–23.

Krishna, H.J., Brown, C., Gerston, J. & Colley, S. 2005. *The Texas manual on rainwater harvesting.* 3rd ed. Austin, Texas, USA: Water Development Board. Available: http://www.twdb.texas.gov/publications/brochures/conservation/doc/RainwaterHarvestingManual.pdf [2016, May 30].

Langeland, G. 1983. *Yersinia enterocolitica and Yersinia enterocolitica*-like bacteria in drinking water and sewage sludge. *Acta Pathologica Microbiologica Scandinavica Series B*. 91(3):179-185.

Lemarchand, K. & Lebaron, P. 2003. Occurrence of *Salmonella* spp. and *Cryptosporidium* spp. in a French coastal watershed: Relationship with fecal indicators. *FEMS Microbiology Letters*. 218(1):203–209.

Li, Z., Boyle, F. & Reynolds, A. 2010. Rainwater harvesting and greywater treatment systems for domestic application in Ireland. *Desalination*. 260(1):1-8.

Li, H., Xin, H. & Li, S.F.Y. 2015. Multiplex PMA-qPCR assay with internal amplification control for simultaneous detection of viable *Legionella pneumophila*, *Salmonella typhimurium* and *Staphylococcus aureus* in environmental waters. Environmental Science and Technology 49:14249-14256.

Liu, D., Sansalone, J.J. & Cartledge, F.K. 2005. Comparison of absorptive filter media for treatment of metals in runoff. *Journal of Environmental Engineering*. 131(8):1178–1186.

Lombard, M., De Kwaadsteniet, M., Khan, S. & Khan, W. 2013. Comparison of treatment methods for the removal of Adeno- and Rotavirus from rain- and river water sources. B.Sc. Hons. Thesis. University of Stellenbosch.

Lonnen, J., Kilvington, S., Kehoe, S.C., Al-Touati, F. & McGuigan, K.G. 2005. Solar and photocatalytic disinfection of protozoan, fungal and bacterial microbes in drinking water. *Water Research*. 39(5):877–883.

Lye, D.J. 2002. Health risks associated with consumption of untreated water from household roof catchment systems. *Journal of the American Water Resources Association*. 38(5):1301-1306.

MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B.L. 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *The New England Journal of Medicine*. 331(3):161–167.

Malema, S., Abia, L.K.A., Mwenge Kahinda, J. & Ubomba-Jaswa, E. 2016. Gaining a better understanding of the factors that influence the quality of harvested rainwater in South Africa – a review. *WISA 2016 Biennial conference.* 16 - 19 May 2016. Durban, South Africa.

Malherbe, J. & Olivier, C. 2012. *An overview of the 2011/2012 summer season.* Available: http://www.grainsa.co.za/an-overview-of-the-2011/2012-summer-season [2016, May 22].

Marston, B.J., Lipman, H.B. & Breiman, R.F. 1994. Surveillance for legionnaires' disease. Risk factors for morbidity and mortality. *Archives of Internal Medicine*. 154(21):2417–2422.

Martin-Dominguez, A., Alarcón Herrera, M.T., Martin-Dominguez, I.R. & Gonzalez-Herrera, A. 2005. Efficiency in the disinfection of water for human consumption in rural communities using solar radiation. *Solar Energy*. 78(1):31–40.

Martinson, D.B. & Thomas, T.H. 2005. Quantifying the first-flush phenomenon. *Proceedings of the 12th IRCSA Conference.* November 2005. New Delhi, India. Available:

http://greywateraction.org/wp-content/uploads/2014/11/quantifying-first-flush-phenomenon.pdf [2015, May 22].

Masters, C.I., Shallcross, J.A. & Mackey, B.M. 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. *Journal of Applied Bacteriology*. 77(1):73–79.

McCarty, S.C. & Atlas, R.M. 1993. Effect of amplicon size on PCR detection of bacteria exposed to chlorine. *Genome Research*. 3(3):181–185.

McGuigan, K.G., Conroy, R.M., Mosler, H., Du Preez, M., Ubomba-Jaswa, E. & Fernandez-Ibañez, P. 2012. Solar water disinfection (SODIS): A review from bench-top to roof-top. *Journal of Hazardous Materials*. 235-236:29-46.

McKillip, J.L., Jaykus, L.A. & Drake, M.1998. rRNA stability in heat killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*. 64(11):4264–4268.

McKillip, J.L., Jaykus, L.A. & Drake, M. 1999. Nucleic acid persistence in heat-killed *Escherichia coli* O157:H7 from contaminated skim milk. *Journal of Food Protection*. 62(8):839–844.

Merritt, A., Miles, R. & Bates, J. 1999. An outbreak of *Campylobacter enteritis* on an island resort, north Queensland. *Communicable Diseases Intelligence*. 23(8):215–219.

Milne, R. 2007. *Legionella, legionnaires disease explanation.* Enviropaedia – Rethinking Reality, Ecosafe. Available: http://www.enviropaedia.com/topic/default.php?topic_id=151 [2014, April 19].

Mollatt, M.C. & Swilling, M. 2014. Ecological food sense: Connections between food waste flows and food production in Enkanini informal settlement, Stellenbosch. M.Sc. Thesis. University of Stellenbosch.

Moore, A.C., Herwaldt, B.L., Craun, G.F., Calderon, R.L., Highsmith, A.K. & Juranek, D.D. 1994. Waterborne disease in the United States, 1991 and 1992. *Journal – American Water Works Association*. 86(2):87– 99.

Moore, J., Caldwell, P. & Millar, B. 2001. Molecular detection of *Campylobacter* spp. in drinking, recreational and environmental water supplies. *International Journal of Hygiene and Environmental Health*. 204(2):185–189.

Morin, N.J., Gong, Z. & Li, X.F. 2004. Reverse transcription-multiplex PCR assay for simultaneous detection of *Escherichia coli* O157: H7, *Vibrio cholerae* O1, and *Salmonella typhi*. *Clinical Chemistry*. 50(11):2037–2044.

Morrow, A.C., Dunstan, R.H. & Coombes, P.J. 2010. Elemental composition at different points of the rainwater harvesting system. *Science of the Total Environment*. 408(20):4542-4548.

Mosler, H.J., Kraemer, S.M. & Johnston, R.B. 2013. Achieving long-term use of solar water disinfection in Zimbabwe. *Public Health*. 127(1):92-98.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. & Donlan, R.M. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. *Microbiology*. 147(11):3121-3126.

Mwabi, J.K., Adeyemo, F.E., Mahlangu, T.O., Mamba, B.B., Brouckaert, B.M., Swartz, C.D., Offringa, G., Mpenyana-Monyatsi, L., Momba, M.N.B. 2011. Household water treatment systems: A solution to the production of safe drinking water by the low-income communities of Southern Africa. *Physics and Chemistry of the Earth*. 36(14-15):1120-1128.

Mwenge Kahinda, J., Taigbenu, A.E. & Boroto, R.J. 2007. Domestic rainwater harvesting to improve water supply in rural South Africa. *Physics and Chemistry of the Earth*. 32(15-18):1050–1057.

Mwenge Kahinda, J., Taigbenu, A.E. & Boroto, R.J. 2010. Domestic rainwater harvesting as an adaptation measure to climate change in South Africa. *Physics and Chemistry of the Earth*. 35(13-14):742-751.

Mwenge Kahinda, J. & Taigbenu, A.E. 2011. Rainwater harvesting in South Africa: Challenges and opportunities. *Physics and Chemistry of the Earth*. 36(14):968-976.

Nam, S., Kwon, S., Kim, M.J., Chae, J.C., Jae Maeng, P., Park, J.G. & Lee, G.C. 2011. Selective detection of viable *Helicobacter pylori* using ethidium monoazide or propidium monoazide in combination with real-time polymerase chain reaction. *Microbiology and Immunology*. 55(12):841–846.

NHMRC and NRMMC (ADWG). 2011. *Australian drinking water guidelines Paper 6 National Water Quality Management Strategy*. National Health and Medical Research Council, National Resource Management Ministerial Council Commonwealth of Australia, Canberra.

Nieuwoudt, M.N. & Mathews, E.H. 2005. A mobile solar water heater for rural housing in Southern Africa. *Building and Environment*. 40(9):1217–1234.

Noble, R.T., Moore, D.F., Leecaster, M.K., McGee, C.D. & Weisberg, S.B. 2003. Comparison of total coliform, fecal coliform, and enterococcus bacterial indicator response for ocean recreational water quality testing. *Water Research*. 37(7):1637-1643.

Nocker, A. & Camper, A.K. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Applied and Environmental Microbiology*. 72(3):1997–2004.

Nocker, A., Cheung, C.Y. & Camper, A.K. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*. 67(2):310–320.

Nocker, A. & Camper, A.K. 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. *FEMS Microbiology Letters*. 291(2):137–142.

Oliver, J.D. 2000. The public health significance of viable but nonculturable bacteria. In *Nonculturable microorganisms in the environment*. R. Colwell & D. J. Grimes, Eds. Washington, DC, USA: ASM Press. 277-300.

Özen, A.I. & Ussery, D.W. 2012. Defining the *Pseudomonas* genus: Where do we draw the line with *Azobacter*? *Microbial Ecology*. 63(2):239-248.

Pan, Y. & Breidt, F. 2007. Enumeration of viable *Listeria monocytogenes* cells by real time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. *Applied and Environmental Microbiology*. 73(24):8028–8031.

Paspaltsis, K., Lagoudaki, R., Grigoriadis, N., Poulios, I. & Sklaviadis, T. 2006. Titanium dioxide photocatalytic inactivation of prions. *Journal of General Virology*. 87(10):3125–3130.

Payment, P., Richardson, L., Siemiatycki, J., Dewar, R., Edwardes, M. & Franco, E. 1991. A randomized trial to evaluate the risk of gastrointestinal disease due to consumption of drinking water meeting current microbiological standards. *American Journal of Public Health*. 81(6):703–708.

Perry, R.D. & Fetherston, J.D. 1997. *Yersinia pestis* – Etiologic agent of plague. *Clinical Microbiology Reviews*. 10(1):35-66.

Peters, A.J., Weidner, K.L. & Howley, C.L. 2008. The chemical water quality in roof-harvested water cisterns in Bermuda. *Journal of Water Supply: Research and* Technology – *AQUA*. 57(3):153-163.

Peters-Varbanets, M., Zurbrügg, C., Swartz, C. & Pronk, W. 2009. Decentralized systems for potable water and the potential of membrane technology. *Water Research*. 43(2):245–265.

Pina, S., Puig, M., Lucena, F., Jofre, J. & Girones, R. 1998. Viral pollution in the environment and shellfish: human adenovirus detection by PCR as an index of human viruses. *Applied and Environmental Microbiology*. 64(9):3376-3382.

Pisz, J.M., Lawrence, J.R., Schafer, A.N. & Siciliano, S.D. 2007. Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. *Journal of Microbiological Methods*. 71(3):312–318.

Pitkänen, T., Paakkari, P., Miettinen, I.T., Heinonen-Tanski, H., Paulin, L. & Hänninen, M.L. 2007. Comparison of media for enumeration of coliform bacteria and *Escherichia coli* in non-disinfected water. *Journal of Microbiological Methods*. 68(3):522-529.

Promega, 2013. *BacTiter-Glo™ microbial cell viability assay manual.* Available: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/101/BacTiter-Glo%20Microbial%20Cell%20Viability%20Assay%20Protocol.pdf. [2014, April 23].

Rao, M., Knight, S. & Samarth, A. 2010. Environmental sustainability, climate change and health: reflections on the Millennium Development Goals. *South African Health Review.* 111-122.

Rawsthorne, H., Dock, C.N. & Jaykus, L.A. 2009. PCR-based method using propidium monoazide to distinguish viable from non-viable *Bacillus subtilis* spores. *Applied and Environmental Microbiology*. 75(9):2936–2939.

Reyneke, B., Dobrowsky, P.H., Ndlovu, T., Khan, S. & Khan, W. 2016. EMA-qPCR to monitor the efficiency of a closed-coupled solar pasteurization system in reducing *Legionella* contamination of roof-harvested rainwater. *Science of the Total Environment*. 553:662-670.

Rompré, A., Servais, P., Baudart, J., De-Roubin, M.R. & Laurent, P. 2002. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. *Journal of Microbiological Methods*. 49(1):31-54.

Rueckert, A., Ronimus, R.S. & Morgan, H.W. 2005. Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus. Journal of Applied Microbiology*. 99(5):1246–1255.

Safapour, N. & Metcalf, R.H. 1999. Enhancement of solar water pasteurization with reflectors. *Applied and Environmental Microbiology*. 65(2):859-861.

Saitoh, T.S. & El-Ghetany, H.H. 2002. A pilot solar water disinfecting system: performance analysis and testing. *Solar Energy*. 72(3):261–269.

Sakamoto, R., Ohno, A., Nakahara, T., Satomura, K., Iwanaga, S., Kouyama, Y., Kura, F., Kato, N., Matsubayashi, K., Okumiya, K., Yamaguchi, K. 2009. *Legionella pneumophila* in rainwater on roads. *Emerging Infectious Diseases*. 15(8):1295-1297.

Saving Energy Solutions. 2016. *Direct and indirect solar pasteurization systems.* Available: http://savingenergy.co.za/solar-geyser/ [2016, January 20].

Sazakli, E., Alexopoulos, A. & Leotsinidis, M. 2007. Rainwater harvesting, quality assessment and utilization in Kefalonia Island, Greece. *Water Research*. 41(9):2039-2047.

Schets, F.M., Italiaander, R., Van Den Berg, H.H.J.L. & De Roda Husman, A.M. 2010. Rainwater harvesting: quality assessment and utilization in the Netherlands. *Journal of Water and Health*. 8(2):224-235.

Schlech, W.F., Gorman, G.W., Payne, M.C. & Broome, C.V. 1985. Legionnaires' disease in Caribbean: An outbreak associated with a resort hotel. *Archives of Internal Medicine*. 145(11):2076–2079.

Servais, P., Billen, G. & Bouillot, P. 1994. Biological colonization of granular activated carbon filters in drinking-water treatment. *Journal of Environmental Engineering*. 120(4):888-899.

Seven, O., Dindar, B., Aydemir, S., Metin, D., Ozinel, M.A. & Icli, S. 2004. Solar photocatalytic disinfection of a group of bacteria and fungi aqueous suspensions with TiO_2, ZnO and Sahara desert dust. *Journal of Photochemistry and Photobiology A: Chemistry*. 165(1-3):103–107.

Shakeri, M., Shahidi, F., Mortazavi, A., Bahrami, A.R. & Nassiri, M.R. 2014. Application of PCR technique in combination with DNAse treatment for detection of viable *Lactobacillus acidophilus* bacteria. *Journal of Food Quality*. 37:291–295.

Sheridan, G.E., Masters, C.I., Shallcross, J.A. & MacKey, B.M. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Applied and Environmental Microbiology*. 64(4):1313–1318.

Silby, M.W., Winstanley, C., Godfrey, S.A., Levy, S.B. & Jackson, R.W. 2011. *Pseudomonas* genomes: Diverse and adaptable. *FEMS Microbiology Reviews*. 35(4):652–680.

Simmons, G., Hope, V., Lewis, G., Whitmore, J. & Wanzhen, G. 2001. Contamination of potable roof-collected rainwater in Auckland, New Zealand. *Water Research*. 35(6):1518–1524.

Simmons, G., Jusry, S., Thornley, C., Harte, D., Mohiuddin, J. & Taylor, M. 2008. A Legionnaires' disease outbreak: A water blaster and roof-collected rainwater systems. *Water Research*. 42(6):1449–1458.

Sinton, L.W., Hall, C.H., Lynch, P.A. & Davies-Colley, R.J. 2002. Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. *Applied and Environmental Microbiology*. 68(3):1122–1131.

Sobsey, M.D. 1989. Inactivation of health-related microorganisms in water by disinfection processes. *Water Science and Technology*. 21(3):179–195.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A. & Yoshida, S. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. *Microbiology and Immunology*. 51(8):763–775.

Solargis, 2016. *SolarGIS © 2016 GeoModel Solar*. Available: http://solargis.info/doc/free-solar-radiation-maps-DNI [2016, May 3].

Sommer, B., Marino, A., Solarte, Y., Salas, M.L., Dierolf, C., Valiente, C., Mora, D., Rechsteiner, R., Setter, P., Wirojanagud, W., Ajarmeh, H., Al-Hassan, A., Wegelin, M. 1997. SODIS – an emerging water treatment process. *Journal of Water Supply: Research and Technology – AQUA*. 46(3):127–137.

South African Bureau of Standards (SABS). 2005. South African National Standards (SANS) 241: In *Drinking water quality management guide for water services authorities*. 6th ed. Annexure 1. ISBN 0-626-17752-9.

Spinks, A.T., Coombes, P., Dunstan, R.H. & Kuczera, G. 2003. Water quality treatment processes in domestic rainwater harvesting systems. *Proceedings of the 28th International Hydrology and Water Resources Symposium*. 10-13 November 2003. Wollongong, Australia.

Spinks, A.T., Dunstan, R.H., Harrison, T., Coombes, P. & Kuczera, G. 2006. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. *Water Research*. 40(6):1326–1332.

Statistics South Africa. 2010. *In-depth analysis of the general household survey, Water and Sanitation 2002-2010.* Report No. 03-18-02 (2002-2010). GHS Series Volume III. Pretoria: Statistics South Africa: 1-37.

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. & Ren, Q. 2015. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiology*. 15(1):36-45.

Stortz, G. & Zheng, M. 2000. Oxidative stress. In *Bacterial Stress*. G. Stortz & R. Hennge-Aronis, Eds. Washington DC, USA: ASM Press: 47-59.

Stout, J.E., Rihs, J.D. & Yu, V.L. 2003. *Legionella*. In: *Manual of clinical microbiology.* Rev. 8th ed. P.R. Murray, Ed. Washington, DC., USA: American Society for Microbiology Press: 809–823.

Strauss, A., Dobrowsky, P.H., Ndlovu, T., Reyneke, B., & Khan W. 2016. Comparative analysis of solar pasteurization versus solar disinfection for the treatment of harvested rainwater. *BMC Microbiology*. 16:289-305.

Sturm, M., Zimmermann, M., Schütz, K., Urban, W. & Hartung, H. 2009. Rainwater harvesting as an alternative water resource in rural sites in central northern Namibia. *Physics and Chemistry of the Earth*. 34(13-16):776-785.

Tavener-Smith, L. 2012. Housing: The challenge of informal settlements. In *Sustainable Stellenbosch – opening dialogues*. M. Swilling, B. Sebitosi & R. Loots, Eds. Stellenbosch, South Africa: SUN MeDIA: 68-83.

Turkum, A., Pekey, H., Pekey, B. & Tuncel, G. 2008. Investigating relationships between aerosol and rainwater compositions at different locations in Turkey. *Atmospheric Research*. 89(4):315–323.

Uba, B.N. & Aghogho, O. 2000. Rainwater quality from different roof catchments in the Port Harcourt District, Rivers State, Nigeria. *Journal of Water Supply: Research and Technology – AQUA*. 49:281–288.

UNAIDS., 2013. *Global Report: UNAIDS report on the global AIDS epidemic 2013*. WHO Library Cataloguing-in-Publication Data. ISBN 978-92-9253-032-7. Available: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf [2014, April 17].

UNICEF & WHO. 2009. *Diarhhoea: Why children are still dying and what can be done.* WHO library Cataloguing-in-Publication Data. Available: http://whqlibdoc.who.int/publications/2009/9789241598415_eng.pdf [2014, April 17].

United Nations MDG Report, 2015. *The Millenium Development Goals report*. Available: http://www.undp.org/content/dam/undp/library/MDG/english/UNDP_MDG_Report_2015.pdf [2015, December 11].

Vasudevan, P. & Pathak, N. 2000. *Water quality in domestic roofwater harvesting systems* (*DRWH*). (Report C3 Water Quality in DRWH). Available:

http://www.watersanitationhygiene.org/References/EH_KEY_REFERENCES/WATER/Rainwater%20Harvesting/Water%20Quality%20of%20Rainwater%20Harvesting.pdf [2016, August 23].

Venkobachar, C., Leela, L. & Prabhakara, R. 1977. Mechanism of disinfection: Effect of chlorine on cell membrane functions. *Water Research*. 11(8):727-729.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S. & Vesper, A. 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). *Journal of Microbiological Methods*. 72(2):180–184.

Viancelli, A., Garcia, L.A.T., Kunz, A., Steinmetz, R., Esteves, P.A. & Barardi, C.R.M. 2012. Detection of circoviruses and porcine adenoviruses in water samples collected from swine manure treatment systems. *Research in Veterinary Science*. 93(1):538-543.

Vidal, A. & Diaz, A.I. 2000. High-performance, low-cost solar collectors for disinfection of contaminated water. *Water Environment Research*. 72(3):271–276.

Villarino, A., Bouvet, O.M., Regnault, B., Martin-Delautre, S. & Grimont, P.A.D. 2000. Exploring the frontier between life and death in *Escherichia coli*: evaluation of different viability markers in live and heat- or UV-killed cells. *Research in Microbiology*. 151(9):755– 768.

Von der Heyde, V.S. & Swilling, M. 2014. Towards a sustainable incremental waste management system in Enkanini: A transdisciplinary case study. M.Sc. Thesis. University of Stellenbosch.

Wang, S. & Levin, R.E. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *Journal of Microbiological Methods*. 64(1):1–8.

Water Research Commission (WRC). 2016. *Design, construction and monitoring of sustainable domestic rainwater harvesting solar pasteurization treatment systems in Enkanini informal settlement, Stellenbosch* (WRC Project K5/2368//3 Report) Pretoria, South Africa.

Wegelin, M., Canonica, S., Meschner, K., Tleischmann, T., Pasaro, F. & Metzler, A. 1994. Solar water disinfection: scope of the process and analysis of radiation experiments. *Journal of Water Supply: Research and Technology – AQUA*. 43(3):154–169.

Wessels, B.S. & Swilling, M. 2015. Turning points: Exploring power transitions in an incremental upgrading process in Enkanini, Stellenbosch. M.Sc. Thesis. University of Stellenbosch.

World Health Organisation (WHO). 2003*. Heterotrophic plate counts and drinking-water safety – the significance of HPCs for water quality and human health.* London, United Kingdom: IWA Publishing. ISBN 1-84339-025-6.

World Health Organisation (WHO). 2011. *Guidelines for drinking-water quality.* Rev. 4th ed. World Health Organisation. Geneva, Switzerland: WHO Press. ISBN: 978-92-4- 154815-1.

Wilson, D.A., Yen-Lieberman, B., Reischi, U., Gordon, S.M. & Procop, G.W. 2003. Detection of *Legionella pneumophila* by real-time PCR for the *mip* gene. *Journal of Clinical Microbiology*. 41(7):3327–3330.

Yañez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L. & Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. *Journal of Microbiological Methods*. 85(2):124–130.

Yaradou, D.F., Hallier-Soulier, S., Moreau, S., Poty, F., Hillion, Y., Reyrolle, M., Andre´, J., Festoc, G., Delabre, K., Vandenesch, F., Etienne, J., Jarraud, S. 2007. Integrated real-time PCR for detection and monitoring of *Legionella pneumophila* in water systems. *Applied and Environmental Microbiology*. 73(5):1452–1456.

Zhu, K., Zhang, L., Hart, W., Liu, M. & Chen, H. 2004. Quality issues in harvested rainwater in arid and semi-arid Loess Plateau of Northern China. *Journal of Arid Environments*. 57(4):487–505.

Chapter 2:

(Chapter 2 is compiled in the format of the Science of the Total Environment journal and UK spelling is employed)

Operational sustainability and efficacy of small- and large-scale solar pasteurization treatment systems installed in Enkanini informal settlement (Stellenbosch) for the treatment of rainwater

B. Reyneke[1], P.H. Dobrowsky[1], T. Ndlovu[1], S. Khan[2] and W. Khan[1]*

[1] Department of Microbiology, Faculty of Science, Stellenbosch University, Private Bag X1, Stellenbosch, 7602, South Africa.

[2] Faculty of Health and Applied Sciences, Namibia University of Science and Technology, 13 Storch Street, Private Bag 13388, Windhoek, Namibia.

Abstract

The aim of the current study was to monitor the operational sustainability and efficacy of two small-scale (Sites 1 and 2) and one large-scale (Site 3) solar pasteurization (SOPAS) systems, installed in Enkanini informal settlement (South Africa), to treat harvested rainwater. Indicator organisms, cations and anions were routinely monitored for in the tank water before and after SOPAS treatment using conventional water quality monitoring techniques, while molecular analysis was used to identify a range of pathogens frequently detected in rainwater sources. Anions and cations analysed for in the untreated and SOPAS treated rainwater for both the small- and large-scale systems were within national and international drinking water guidelines. However, depending on the roof catchment system utilised, zinc concentrations at Sites 1 and 2 (mean 3919 µg/L before pasteurization, mean 3964 µg/L after pasteurization) and arsenic concentrations at Site 3 (mean 18.69 µg/L before pasteurization, mean 18.30 µg/L after pasteurization) contravened the respective drinking water guidelines. Additionally, the total coliform, *E. coli* and faecal coliform counts exceeded the drinking water guideline limits in 100%, 55% and 36% of the unpasteurized tank water samples collected from the small-scale systems (Sites 1 and 2) and 100%, 100% and 50% of the unpasteurized tank water samples collected from the large-scale system (Site 3), respectively. However, total coliforms, *E. coli* and faecal coliforms were effectively reduced to below the detection limit (< 1 CFU/100 mL) following SOPAS treatment above 53 °C for the small-scale systems (Sites 1 and 2) and above 55 °C for the large-scale system (Site 3). Results indicated that a minimum SOPAS temperature of 66 °C (small scale systems) and 71 °C (large scale system) was required to reduce the levels of heterotrophic bacteria in the unpasteurized tank water samples to within drinking water standards. However, results from the BacTiter-Glo™ Microbial Cell Viability Assay indicated that viable microbial cells may be present in the samples following SOPAS. Moreover, molecular analysis indicated that *Legionella* spp. was the most prevalent pathogen in both the unpasteurized (100%) and pasteurized (91%) tank water samples from the small-scale systems and the unpasteurized (83%) and stored pasteurized (100%) tank water samples from the large-scale system. Quantitative PCR analysis confirmed that *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp. were still detected following SOPAS treatment for both the small- and large-scale systems. Analysis of the operational sustainability of the systems indicated that minimal maintenance after installation was required and the small-scale systems were efficient in providing the inhabitants with an alternative domestic water source. Additionally, as warm water was being produced by the small-scale systems at Site 1 and 2, the households were using less paraffin/gas for the heating of water used for domestic purposes.

Keywords: Rainwater harvesting; solar pasteurization; microbial and chemical quality; microbial pathogens; informal settlements

2.1 Introduction

Population growth, urbanisation and climate change (prolonged and increased droughts) have placed severe pressure on existing centralised governmental water supplies (Spinks et al., 2006; Da Silva et al., 2016). Domestic rainwater harvesting has therefore been earmarked by many international governmental authorities as an additional decentralised water source which could provide clean and potable water directly to the consumer (Helmreich and Horn, 2009; Lee et al., 2010; Li et al., 2010; Mwenge Kahinda et al., 2010; Ahmed et al., 2011; Gwenzi et al., 2015). In addition, efforts by the South African government have focused on promoting the use of domestic rainwater harvesting systems in South Africa (particularly in rural communities and urban informal settlements), as the lack of infrastructure in ever-expanding urban informal settlements often results in an inadequate water supply (Statistics South Africa, 2010; Department of Water Affairs, 2013). Subsequently, through initiatives from the Department of Water Affairs and the Department of Science and Technology, rainwater harvesting tanks have been implemented in all nine provinces of South Africa with approximately 70 000 households using rainwater as their primary water source (Malema et al., 2016).

However, numerous research groups have detected chemical pollutants, such as heavy metals and pesticides (Peters et al., 2008; Morrow et al., 2010; Huston et al., 2012) and microbial contaminants including the commonly described traditional faecal indicators, various pathogenic bacterial and protozoan species, in rainwater (Ahmed et al., 2008, 2011; Li et al., 2010; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014). Factors such as the type, quality and cleanliness of the catchment area (roof surface), geographical location (level of atmospheric pollution) of the tank and storage conditions (e.g. sealed lid), may all negatively affect the quality of stored rainwater. Research has subsequently demonstrated that harvested rainwater may not be safe to use for potable purposes (Lye, 2002; Chang et al., 2004; Evans et al., 2006; Abdulla and Al-Shareef, 2009; Huston et al., 2012). In addition, while chemical contaminants have not been directly associated with incidences of disease, sporadic outbreaks of infection associated with microbial pathogens have been linked to the utilisation of rainwater sources for potable and domestic purposes (Merritt et al., 1999; Simmons et al., 2008; Franklin et al., 2009).

In order to ensure that harvested rainwater is safe to utilise for all daily water requirements, treatment technologies, such as chlorination, filtration, solar disinfection (SODIS) and solar pasteurization (SOPAS), have been investigated for their ability to treat harvested rainwater to within drinking water standards (Chapman et al., 2008; McGuigan et al., 2012; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2015a, 2015b). While the use of chemical disinfectants (such as chlorine) are effective in treating water and preventing microbial regrowth, determining the chemical concentration required to effectively treat the rainwater in rural areas and urban informal settlements may be problematic, as the optimum concentration is dependent on the

microbial community and levels of organic matter present in the harvested rainwater (Feachem et al., 1983; Servais et al., 1994). Additionally, filtration systems are effective in reducing both microbial and chemical contaminants in water sources (De Kwaadsteniet et al., 2013). However, the prolonged treatment time and small volume of water that can be treated, essentially hampers its successful implementation as a water treatment method in urban informal settlements and rural communities. In addition, while SODIS is considered an inexpensive and simple system which utilises the synergistic effects of ultraviolet (UV) light and heat to inactivate microbial contaminants, research has indicated that certain microorganisms, specifically endospore forming bacteria, can survive SODIS treatment (Helmreich and Horn, 2009; Li et al., 2010; McGuigan et al., 2012). Physico-chemical properties of the water such as turbidity, can then affect SODIS by shielding microorganisms from UV-radiation, may react with chemical disinfectants such as chlorine and may also clog filtration systems (Servais et al., 1994; Wegelin et al., 1994; Burch and Thomas, 1998; McGuigan et al., 2012).

Solar pasteurization has been identified as a reliable cost-effective method for the treatment of large volumes of water and the removal of microbial pathogens is independent of parameters such as, turbidity and pH (Burch and Thomas, 1998; Abraham et al., 2015; Dobrowsky et al., 2015a). In a study conducted by Ciochetti and Metcalf (1984), it was reported that pasteurization could eliminate bacteria, rotaviruses and enteroviruses commonly found in contaminated water. Additionally, the storage of rainwater at temperatures between 55 °C to 65 °C has been shown to thermally inactivate heat-resistant bacterial species including *Aeromonas* spp., *Enterococcus* spp., *Escherichia* spp., *Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. (Spinks et al., 2003; 2006). Moreover, in developing countries, such as South Africa, an extremely high potential for solar power generation exists as a result of the high solar irradiation that is observed across the country annually, with an average direct normal solar irradiance of 7.0 kWh/m^2/d reported (Fluri, 2009). This, coupled with an average annual rainfall of 464 mm, makes rainwater harvesting SOPAS systems a viable solution in providing an alternative water source in South Africa.

Pilot-scale studies conducted by our research group then indicated that the level of indicator organisms [including heterotrophic bacteria, *Escherichia coli* (*E. coli*) and total coliforms] was reduced to below the detection limit by SOPAS at temperatures higher than 72 °C (Dobrowsky et al., 2015a). However, increased concentrations of aluminium (Al), lead (Pb), nickel (Ni) and iron (Fe) were observed in the harvested rainwater samples at levels exceeding various national and international drinking water guidelines [Department of Water Affairs and Forestry (DWAF, 1996); South African National Standards (SANS) 241 (South African Bureau of Standards (SABS), 2005); Australian Drinking Water Guidelines (ADWG) (NHMRC and NRMMC, 2011); World Health Organisation (WHO, 2011)], following SOPAS treatment. The authors hypothesised that these metals were leaching from the stainless steel storage tank of the

Apollo™ SOPAS system utilised in the study and recommended that a SOPAS system with a high grade polyethylene storage tank be utilised for the treatment of harvested rainwater (Dobrowsky et al., 2015a). In the current study a new Phungamanzi™ SOPAS system, which was designed and manufactured in South Africa and which consists of a 125 L high grade polyethylene storage tank, was utilised for the SOPAS of harvested rainwater. Two small-scale Phungamanzi™ SOPAS systems and one large-scale SOPAS manifold system were thus installed in Enkanini informal settlement (Stellenbosch) for the treatment of harvested rainwater. This pilot-scale study was launched in Enkanini as currently 32 communal standpipes service a community consisting of approximately 8 000 inhabitants (Tavener-Smith, 2012; Wessels and Swilling, 2015). Solar pasteurized rainwater could thus serve as a supplementary or additional water source however, the on-site operational sustainability of the systems had to be determined as numerous factors including treatment time, water turbidity, ease of use and system maintenance may influence the effectiveness of a particular water treatment system, in effectively providing a supplementary water source (Mwabi et al., 2011; McGuigan et al., 2012; De Kwaadsteniet et al., 2013). Moreover, in order to determine if the treated harvested rainwater may be used for potable purposes, the quality of the water needs to be assessed by comparing it to national and international water quality guidelines that specify microbial and chemical parameters (Ahmed et al., 2011).

The primary aim of the current study was thus to monitor the small- and large-scale rainwater harvesting SOPAS treatment systems installed in Enkanini informal settlement for their efficiency in providing the community with a safe alternative water source. To achieve this aim, the microbial quality of the harvested rainwater was monitored by enumerating traditional indicator organisms (*E. coli*, total coliforms, faecal coliforms, enterococci and heterotrophic bacteria) using culture-based techniques, while conventional polymerase chain reaction (PCR) assays were utilised to screen for a library of pathogens (including *Legionella* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Klebsiella* spp., amongst others) generally associated with harvested rainwater. Quantitative PCR (qPCR) assays were then optimised and utilised to quantify the most readily detected pathogens in the harvested rainwater samples. Additionally, the BacTiter-Glo™ Microbial Cell Viability Assay was utilised to monitor the efficiency of the SOPAS treatments systems in reducing the level of metabolically active cells in the treated rainwater samples. The chemical quality of the harvested rainwater before and after SOPAS treatment was also assessed by conducting cation and anion analysis. The operational sustainability of the systems was monitored to determine whether the systems are efficient in providing a sufficient volume of water to the users and whether the system components are durable for long term use.

2.2 Materials and methods

2.2.1 Sample site and description of SOPAS systems

Two small-scale (Phungamanzi™ system) SOPAS systems and one large-scale (Crest EVT collector system) SOPAS system were installed in Enkanini informal settlement, Stellenbosch, South Africa (GPS Coordinates: 33°54' 28.42"S 18°25' 03.56"E) in August 2015 (**Appendix A Fig.A1**). A detailed description of the site selection and on-site construction of the rainwater harvesting SOPAS systems is outlined in **Appendix A**. Briefly explained, the first small-scale SOPAS system was installed next to a house in Enkanini (Site 1) (**Fig. 2.1A**; **Appendix A Fig. A2**) and the second system was installed at a local church (Site 2) (**Fig. 2.1B**; **Appendix A Fig. A3**). A large-scale SOPAS system was then installed at the Enkanini Research Centre (ERC) (**Fig. 2.2A**; **Appendix A Fig. A4**). The differences between the small- and large-scale SOPAS systems are the respective sizes of the storage tanks containing the treated water and the mechanism by which the water is treated (small-scale system: direct SOPAS treatment; large-scale system: indirect SOPAS treatment).

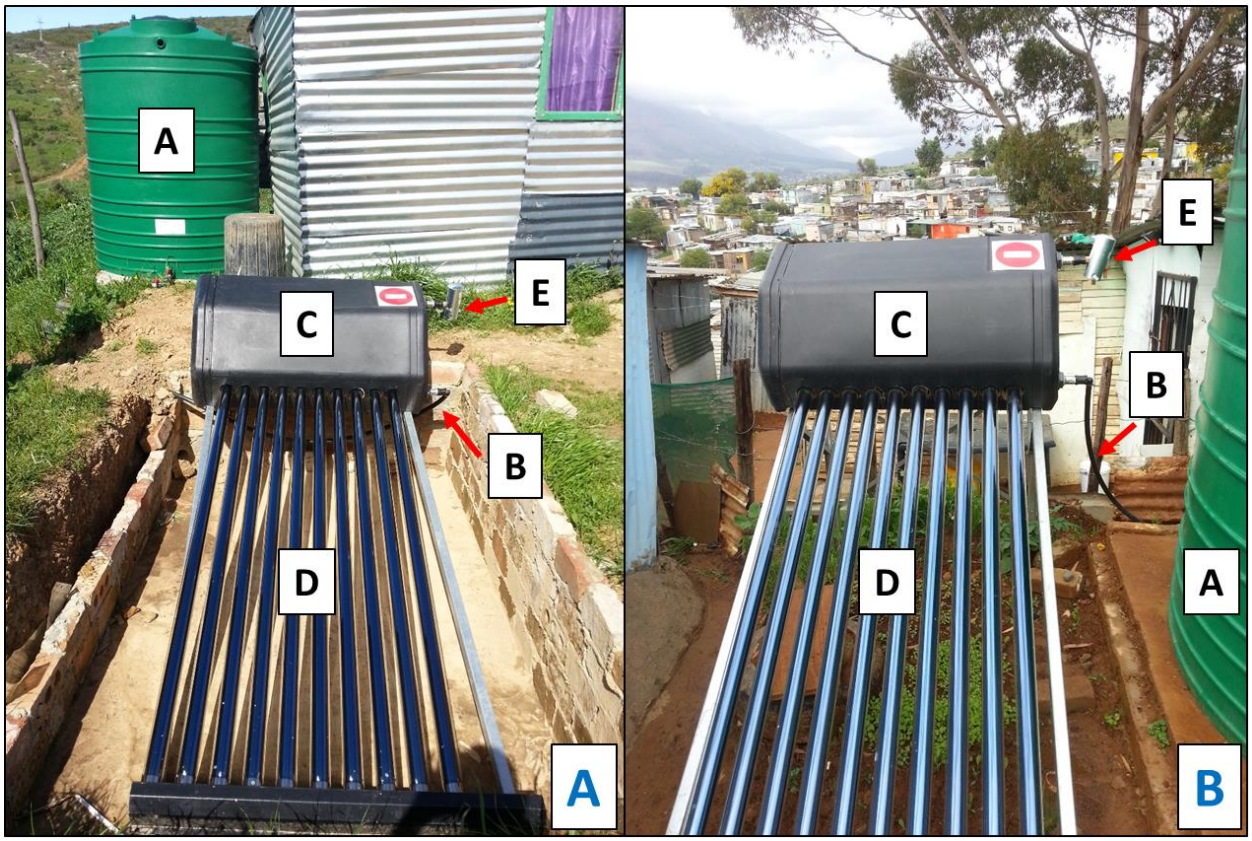

Fig. 2.1. (A) Small-scale SOPAS system installed at Site 1. **(B)** Small-scale SOPAS system installed at Site 2. The labelled system components are: (A) rainwater harvesting tank, (B) pipe connecting rainwater tank to SOPAS system, (C) storage tank of SOPAS system, (D) high borosilicate glass tubes, (E) outlet tap.

For Site 1, a 2 500 L polyethylene rainwater harvesting (RWH) tank was installed next to the house in Enkanini and a small-scale SOPAS system was connected to the RWH tank at an angle which allowed for the passive flow of harvested rainwater (hereafter referred to as tank water) from the RWH tank into the SOPAS system (**Fig. 2.1A**; **Appendix A Fig.A2**). The sampling site (Site 1) was located on the periphery of the Enkanini informal settlement, alongside an open field. No trees or other obstructions covered the zinc sheeting catchment area (roof area of 15.27 m^2) of the house. For Site 2, a 5 000 L polyethylene RWH tank was installed next to the local church in Enkanini. A small-scale SOPAS system was then connected to the RWH tank at an angle that also allowed for the passive movement of tank water into the SOPAS system (**Fig. 2.1B**; **Appendix A Fig. A3**). The sampling site (Site 2) was surrounded by trees, however, no branches obstructed the zinc sheeting catchment area (roof area of 55.9 m^2) of the church. Additionally, sampling Site 2 was surrounded by dirt roads that were continuously used throughout the day by the inhabitants of the informal settlement. Dust particles from the dirt road are thus continuously dispersed throughout the day and it is important to note that water used for domestic purposes (laundry, washing, cleaning) is often discarded down the slope of the road (Enkanini is situated on a steep hill).

The passive flow of water from the respective rainwater tanks into the SOPAS systems, located at Sites 1 and 2 (**Fig. 2.1A** and **Fig. 2.1B**) was as follows; cold water flowed from the rainwater tanks (A) through pipes (B) into the high grade polyethylene storage tanks (C) of the SOPAS systems, which have a 125 L storage capacity. The water then moved through the high borosilicate glass cylinders (D) lined with black paint that enabled them to capture heat (the black coating, on the inner tube of the borosilicate glass cylinders, absorbs solar energy and transfers it to the water, effectively heating it). Due to the thermo-siphoning effect, the water moved to the top of the glass tubes and into the storage tanks (C) as it was heated. The taps (E), connected to the main storage tanks, could then be used to collect the pasteurized tank water.

For Site 3, a 5 000 L polyethylene RWH tank was installed next to the ERC. A large-scale solar manifold SOPAS system was then connected to the RWH tank, while an additional 1 500 L RWH tank was connected to the outlet of the solar manifold SOPAS system (1 500 L storage tank) (**Fig. 2.2A**; **Appendix A Fig. A4**). No obstructions were observed that covered the catchment area (88.5 m^2); however, it should be noted that the zinc sheeting roofing material of the ERC was painted and photovoltaic solar panels were installed on the roof (catchment area) to provide the ERC with electricity. Additionally, sampling Site 3 was also surrounded by dirt roads that were continuously used throughout the day by the inhabitants of the informal settlement.

For the large-scale system (Site 3), tank water was treated in the following manner (**Fig. 2.2A** and **Fig. 2.2B**); cold water flowed from the rainwater tank (A) through a pipe (B) into the holding tank (C) of the solar manifold SOPAS system. Inside the high borosilicate glass cylinders (D) are copper rods filled with a heat transfer liquid (**Fig. 2.2B**). As the high borosilicate glass cylinders absorbed ultraviolet (UV) light, the copper rods were heated and the liquid contained in them started to move up the rod, effectively heating it. Once the copper rods had been heated (can reach 250 °C), the top part of the copper rods [which enter the holding tank (C) of the SOPAS manifold system], transferred heat to the water contained inside the holding tank effectively heating the tank water (**Fig. 2.2A** and **Fig. 2.2B**). Once the water had been heated to above 75 °C, a thermostatic release valve opened and the water flowed into the separate 1 500 L storage tank (E) (**Fig. 2.2A**).

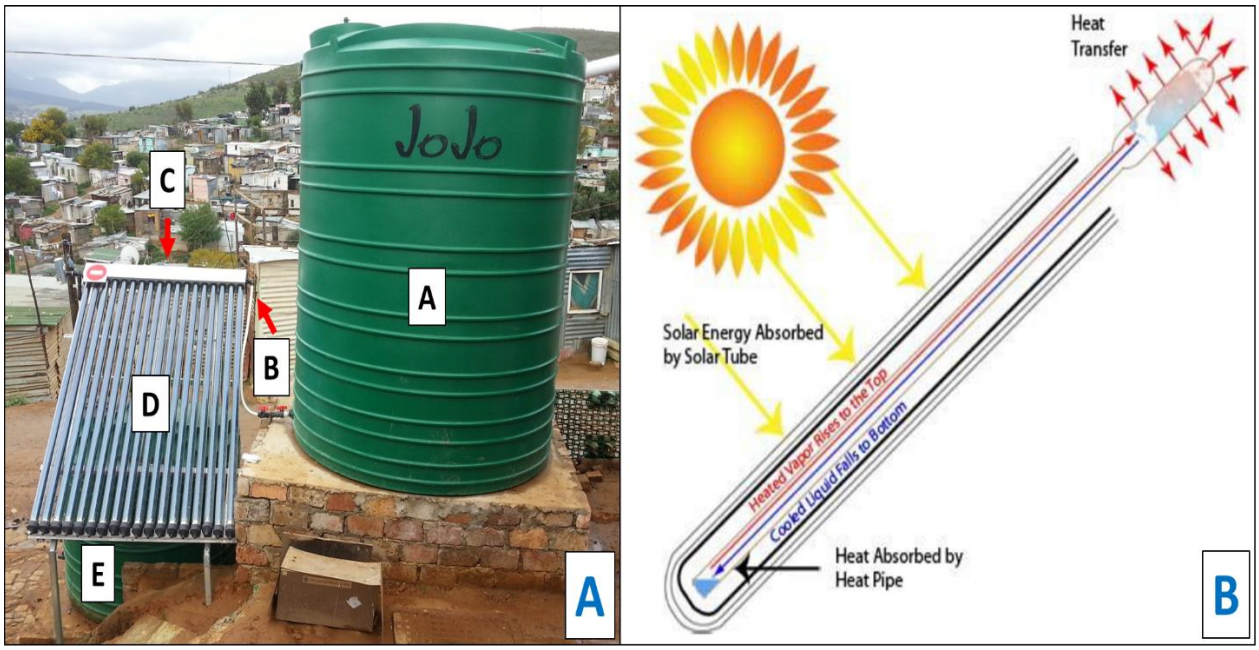

Fig. 2.2. (A) Large-scale SOPAS system located at the ERC with labelled system components. **(B)** Diagram illustrating the working mechanism of the copper heat pipes inside the high borosilicate glass tubes (adopted from International Technology Sourcing Solar, 2016).

2.2.2 Sample collection

For the microbial and chemical analysis of the two small-scale systems installed at Sites 1 and 2 (**Fig. 2.1A** and **Fig. 2.1B**), a 5 L sample was collected directly from the rainwater tank (A) (before pasteurization) and the outlet tap of the SOPAS system (E) (after pasteurization), respectively. For both systems, pasteurized tank water samples were collected at temperatures exceeding 50 °C [Site 1 - 52 °C, 60 °C, 67 °C, 72 °C, 73 °C, 75 °C (duplicate samples collected on different sampling occasions) and 85 °C; Site 2 – 53 °C, 58 °C and 66 °C]. For the microbial and chemical analysis of the large-scale system at Site 3 (**Fig. 2.2A**), a 5 L sample was collected directly from the rainwater tank (A) (before pasteurization) and the 1 500 L storage

tank (E) (stored pasteurized), while a 2 L sample was collected directly from the solar system manifold (C) (after pasteurization). It is important to note that the pasteurized tank water does not remain heated after it exits the solar system manifold (C) into the 1 500 L storage tank (E). Thus for the large-scale system, samples were collected based on the temperature of the pasteurized tank water in the solar system manifold (C), which were also above 50 °C (55 °C, 61 °C, 66 °C, 71 °C, 72 °C and 79 °C). For all three systems installed at Sites 1 to 3, samples were collected from October 2015 to October 2016 [Site 1 (n = 8); Site 2 (n = 3); Site 3 (n = 6)].

The temperature and pH of all water samples were measured at the sampling site using a hand-held mercury thermometer and pH meter (Martini Instruments, Rocky Mount, USA). Rainfall and solar radiation (UV readings) data were obtained from the Stellenbosch Weather Services, Engineering Faculty (Stellenbosch Weather, 2016), while ambient temperature patterns were obtained from the South African Weather Services (Pretoria, South Africa).

2.2.3 Chemical analysis

The chemical quality of the tank water samples, before and after pasteurization, was determined by monitoring cation and anion concentrations. Representative samples of a specific temperature range were analysed [Site 1 (52 °C, 67 °C, 75 °C and 85 °C); Site 2 (58 °C and 66 °C); Site 3 (55 °C, 66 °C and 79 °C)]. For the determination of the metal concentrations and sample collection, Falcon™ 50 mL high-clarity polypropylene tubes with polyethylene caps, were pre-treated with 1% nitric acid before sampling. The concentrations of 25 metals including boron (B), copper (Cu), vanadium (V), chromium (Cr) and manganese (Mn), amongst others, were then determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) and nitric acid digestion according to Saleh et al. (2000). All samples were analysed for the presence of metals at the Central Analytical Facility (CAF), Stellenbosch University. The total water hardness of the tank water samples was then calculated using the concentrations of calcium (Ca) and magnesium (Mg) as indicated in **Eq. 1** and were compared to the water hardness quality range as described by Kunin (1972).

$$[\text{Total hardness} = 2.497 \times (\text{mg Ca}) + 4.118 \times (\text{mg Mg})] \quad (\mathbf{1})$$

Anion concentrations were analysed by PathCare Reference Laboratory (PathCare Park, Cape Town, South Africa). All anions including, chloride, fluoride, nitrates, nitrites and phosphate were measured utilising a Thermo Scientific Gallery™ Automated Photometric Analyser.

2.2.4 Culturing of indicator organisms

The microbial quality of the tank water samples collected from the small-scale systems (Sites 1 and 2) were determined by monitoring all unpasteurized and pasteurized tank water samples, while unpasteurized, pasteurized and stored pasteurized (1 500 L storage tank) tank water

samples were monitored for the large-scale system (Site 3). Total coliforms (TC) and *E. coli* were enumerated simultaneously by filtering a total volume of 100 mL (undiluted, 10^{-1} and 10^{-2}) 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 placed onto Membrane Lactose Glucuronide Agar (MLGA) (Oxoid, Hampshire, England) and were incubated at 35 ± 2 °C for 18 - 24 hrs (U.S. Environmental Protection Agency, 2009). In order to enumerate enterococci, 100 µL of an undiluted sample was spread plated onto Slanetz and Bartley Agar (Oxoid), with the plates incubated for 44 – 48 hrs at 36 ± 2 °C. In order to enumerate faecal coliforms (FC), 100 µL of an undiluted sample was spread plated onto m-FC Agar (Biolab, Merck, Wadeville, South Africa), with the plates incubated for 44 – 48 hrs at 35 ± 2 °C. For the enumeration of the heterotrophic plate count (HPC), a serial dilution (10^{-1}–10^{-3}) was prepared for each sample and by use of the spread plate method 100 µL of an undiluted sample and each dilution (10^{-1}–10^{-3}) was plated onto R2A agar (BD Difco, New Jersey, USA), with the plates incubated at 37 °C for up to four days. All analyses were performed in duplicate.

The regrowth of bacteria in the pasteurized tank water samples (Sites 1 and 2 – samples collected directly from SOPAS system; Site 3 – samples collected directly from solar manifold) where HPC were reduced to below the detection limit [BDL; < 1 colony forming units (CFU)/100 mL], were monitored for two weeks after sampling. This was performed in order to determine how long the pasteurized tank water could be stored after collection. Briefly, 20 mL of each pasteurized sample was stored in a sterile McCartney bottle at room temperature and 100 µL of the treated tank water was spread plated onto R2A agar (BD Difco) every second day, for a period of two weeks. The plates were then incubated at 37 °C for up to four days.

2.2.5 BacTiter-Glo™ Microbial Cell Viability Assay for the detection of viable cells in pasteurized and unpasteurized tank water samples

For each sampling event, an unpasteurized and pasteurized tank water sample was collected from the small-scale systems, while an unpasteurized, pasteurized and stored pasteurized tank water sample was collected at the large-scale system. The BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI, USA) was then used to monitor for the presence of viable microbial cells in samples collected before and after SOPAS treatment by detecting the presence of adenosine triphosphate (ATP), as an indicator of metabolically active cells (Deininger and Lee, 2006; Berney et al., 2008). The BacTiter-Glo™ Buffer and Substrate were mixed (now called BacTiter-Glo™ Reagent) and equilibrated for approximately 10 hrs at room temperature, to ensure that all ATP was hydrolysed (“burned off”) (Berney et al., 2008). Duplicate before and after pasteurization tank water and control samples (sterile milliQ water) were analysed, per sampling event as follows; 100 µL of each sample and an equal volume of

the BacTiter Glo™ Reagent was mixed and loaded in duplicate into a 96-well White Cliniplate (Thermo Scientific Fisher, Finland). The addition of the BacTiter-Glo™ Reagent to a sample causes cell lysis and the production of a luminescent signal which was measured with a Veritas™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). All sample analyses were conducted in triplicate.

2.2.6 Isolation and identification of organisms proliferating in the large-scale SOPAS system storage tank (1 500 L)

In order to determine which organisms were proliferating in the large-scale system storage tank (1 500 L storage tank), morphologically distinct colonies were selected from the R2A plates used for the enumeration of the HPC (section 2.2.4) and were isolated by re-streaking the selected colonies onto nutrient agar (Biolab, Merck, Kenilworth, USA). After obtaining pure cultures, the isolates were inoculated into 5 mL nutrient broth (Biolab, Merck) and were cultured overnight at 37 °C. Deoxyribonucleic acid (DNA) extractions were then performed using the boiling method as previously described by Ndlovu et al. (2015). Briefly explained, 1 mL aliquots of the overnight broth cultures were centrifuged at 10 000 × g for 10 min. After centrifugation, the supernatant was discarded and the pellet was re-suspended in 100 µL sterile milliQ water. The samples were then boiled in a water bath for 15 min at 95 °C, whereafter the samples were cooled on ice for 10 min. The samples were centrifuged at 10 000 × g for 5 min and the supernatant was used for the 16S rRNA PCR analysis. The primer pair fDD2 (5′ - CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG - 3′) and rPP2 (5′ - CCAAGCTTCTAG ACGGITACCTTGTTACGACTT - 3′) was used to amplify a 1 600 bp region of the 16S rRNA gene (Rawlings, 1995).

The PCR mixture consisted of a final volume of 25 µL and contained 5 µL Green GoTaq® Flexi buffer (1X final concentration; Promega), 2 µL $MgCl_2$ (2.0 mM; Promega), 0.25 µL of a dNTP mix (0.1 mM; Thermo Scientific, Hudson, NH, USA), 1.25 µL of the respective forward and reverse PCR primers (0.5 µM), 0.15 µL (1.5 U) GoTaq® Flexi DNA polymerase (Promega) and 2.5 µL template DNA, per respective isolate. Amplification was performed using an initial denaturation step of 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C for 1.5 min, with a final elongation at 72 °C for 5 min, on a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Sterile distilled H_2O was used as a negative control, while genomic DNA extracted from *Legionella pneumophila* ATCC 33152 was used as positive control.

All PCR products were analysed by agarose gel electrophoresis in 0.8% agarose (SeaKem® LE Agarose; Lonza, Rockland, ME, USA) containing 0.5 µg/mL ethidium bromide after electrophoresis at 80 volts for 60 min with the use of 1X Tris/Borate/EDTA (TBE) buffer. Once

the size of the PCR products had been confirmed, representative PCR products were purified and concentrated using the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA) as per manufacturer's instructions. The cleaned PCR products were sent to CAF at Stellenbosch University for sequencing performed in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems®, Foster City, USA). The chromatograms of each sequence were examined using FinchTV version 1.4.0 software and were aligned using DNAman™ version 4.1.2.1 software. The sequence identification was completed using the National Center for Biotechnology Information (NCBI). The Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, was used to find the closest match of local similarity between the isolates and the sequence data available on the international databases in GenBank, EMBL, DDBJ and PDB (Altschul et al., 1990). The sequences of representative isolates that showed > 97% similarity (< 3% diversity) to organisms on the database were recorded.

2.2.7 Extraction of total genomic DNA from tank water samples

Total genomic DNA extractions were performed for each of the samples collected before and after pasteurization as outlined in Dobrowsky et al. (2015b). Briefly explained, 2 mL of 1 M calcium chloride ($CaCl_2$; Biolab, Merck) and 2 mL of 1 M di-sodium hydrogen orthophosphate (Na_2HPO_4; Saarchem, Durban, South Africa) was added to a 1 L water sample (for each sampling event an unpasteurized and pasteurized sample was analysed for the small-scale systems, while an unpasteurized, pasteurized and stored pasteurized sample was analysed for the large-scale system). The mixtures were stirred for 5 min using a magnetic stirrer to allow for flocculation, whereafter the samples were filtered through a 47 mm, 0.45 µm pore size non-charged mixed-ester membrane filter (Whatman GmbH, Dassel, Germany) at a flow rate of approximately ≥ 65 mL/min/cm^2 at 0.7 bar (70 kPa). Each membrane filter was then transferred to a 9 cm petri dish containing 2 mL of 0.3 M citrate buffer (pH 3.5) and was allowed to soak for 3 min. The membrane filters were discarded and the remaining citrate buffer solution was transferred to a 2 mL microcentrifuge tube. The 2 mL microcentrifuge tubes were centrifuged (16 000 × g, 5 min) and the supernatant was discarded. Following centrifugation, the resulting pellets were re-suspended in 200 µL phosphate-buffered saline [PBS; pH 7.4, 8 g/L sodium chloride (NaCl; Saarchem), 0.2 g/L potassium chloride (KCl; Saarchem), 1.42 g/L Na_2HPO_4 (Saarchem), 0.24 g/L potassium di-hydrogen orthophosphate (KH_2PO_4; Saarchem)]. The DNA extractions were then performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland), according to the manufacturer's instructions. The presence of DNA was confirmed by visualisation on a 0.8% agarose gel (SeaKem® LE Agarose; Lonza) stained with 0.5 µg/mL ethidium bromide after gel electrophoresis at 80 volts for 60 min with the use of 1X TBE buffer. Additionally, the DNA concentration of the samples

was determined using a NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA).

2.2.8 Genus-specific PCRs for indigenous rainwater pathogens

Primers and PCR conditions as outlined in **Table 2.1** were utilised in the current study for the identification of bacterial and viral pathogens (and opportunistic pathogens) commonly associated with rainwater.

Each PCR mixture was performed in a final volume of 25 µL in a T100™ Thermal Cycler (Bio-Rad Laboratories). For the detection of *Aeromonas* spp., *Shigella* spp. and *Salmonella* spp., the PCR mix consisted of 5 µL Green GoTaq® Flexi buffer (1X final concentration; Promega), 2 µL $MgCl_2$ (2.0 mM; Promega), 0.25 µL of a dNTP mix (0.1 mM; Thermo Scientific), 0.25 µL of the respective forward and reverse PCR primers (0.1 µM; **Table 2.1**), 0.15 µL of GoTaq® Flexi DNA polymerase (1.5 U, Promega) and 2.5 µL of template DNA. For *Streptomyces* spp., the same PCR mixture was used, with the exception that 0.5 µL of the respective forward and reverse PCR primers (0.2 µM) were added (**Table 2.1**). For *Klebsiella* spp., *Serratia* spp., *Yersinia* spp. and adenovirus, the same PCR mixture was used, with the exception that 0.75 µL of the respective forward and reverse PCR primers (0.3 µM) were added (**Table 2.1**). In addition, for *Pseudomonas* spp., *Legionella* spp., *Enterococcus* spp. and *Staphylococcus* spp., the same reaction mixture was used; however, 1.0 µL (*Pseudomonas* spp.), 1.25 µL (*Legionella* spp. and *Enterococcus* spp.) and 2.5 µL (*Staphylococcus* spp.) of each PCR primer (0.4 µM, 0.5 µM and 1.0 µM final concentrations) was used, respectively (**Table 2.1**). Lastly, for the detection of *Bacillus* spp., the same PCR mix was used, with the exception that 0.5 µL of the dNTP mix (0.2 mM) and 2.0 µL volumes of the respective forward and reverse PCR primers (0.8 µM) were added (**Table 2.1**).

For each PCR, sterile distilled H_2O was used as a negative control, while genomic DNA extracted from ATCC and sequence verified environmental and clinical strains was used as positive controls. The following strains were cultured as positive controls: *Legionella pneumophila* ATCC 33152, *Shigella sonnei* ATCC 25931, *Salmonella enterica* serovar Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Aeromonas hydrophila* (environmental strain), *Klebsiella pneumoniae* ATCC 13385, *Serratia marcescens* ATCC 13880, *Enterococcus faecalis* (clinical isolate), *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 25925, *Streptomyces* spp. ATCC 25607 and *Yersinia enterocolitica* ATCC 27729. An adenovirus positive control DNA obtained from Coris BioConcept (Belgium) was also included.

Table 2.1: Primers and PCR cycling parameters for the detection of indigenous rainwater pathogens.

Organism	Primers	Primer sequence (5'-3')	*PCR Cycling Parameters	Gene (bp)	References
Aeromonas spp.	Aero-F	TGTCGGSGATGACATGGAYGTG	2 min at 95 °C; 35 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2.5 min	*Aerolysin* (720)	Kong et al., 2002
	Aero-R	CCAGTTCCAGTCCCACCACTTCA			
Bacillus spp.	p-gyrAF	CAGTCAGGAAATGCGTACGTCCTT	4 min at 94 °C; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min	*Gyrase A* (928)	Rooney et al., 2009
	p-gyrAR	CAAGGTAATGCTCCAGGCATTGCT			
Enterococcus spp.	ECST784F	AGAAATTCCAAACGAACTTG	5 min at 95 °C; 50 cycles of 95 °C for 15 s, 60 °C for 1 min, 72 °C for 20 s	23S rRNA (75)	Frahm and Obst, 2003
	ENC854R	CAGTGCTCTACCTCCATCATT			
Klebsiella spp.	gyrA-A	CGCGTACTATACGCCATGAACGTA	3 min at 95 °C; 35 cycles of 94 °C for 1 min, 50 °C for 30 s, 72 °C for 30 s	*Gyrase A* (383)	Brisse and Verhoef, 2001
	gyrA-C	ACCGTTGATCACTTCGGTCAGG			
Legionella spp.	JFP	AGGGTTGATAGGTTAAGAGC	5 min at 95 °C; 40 cycles of 94 °C for 1 min, 57 °C for 1.5 min, 72 °C for 1 min	16S rRNA (386)	Jonas et al., 1995
	JRP	CCAACAGCTAGTTGACATCG			
Pseudomonas spp.	PA-GS-F	GACGGGTGAGTAATGCCTA	2 min at 95 °C; 25 cycles of 94 °C for 20 s, 54 °C for 20 s, 72 °C for 40 s	16S rRNA (618)	Spilker et al., 2004
	PA-GS-R	CACTGGTGTTCCTTCCTATA			
Salmonella spp.	IpaB-F	GGACTTTTTAAAAGCGGCGG	2 min at 95 °C; 35 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2.5 min	*IpaB* (314)	Kong et al., 2002
	IpaB-R	GCCTCTCCCAGAGCCGTCTGG			

* A final elongation step of 10 min at 72 °C was included for each PCR assay

Table 2.1 (continued): Primers and PCR cycling parameters for the detection of indigenous rainwater pathogens.

Organism	Primers	Primer sequence (5'-3')	*PCR Cycling Parameters	Gene (bp)	References
Serratia spp.	Fpfs1	CCGGCATCGGCAAAGTCT	5 min at 94 °C; 30 cycles of 94 °C for 45 s, 55 °C for 30 s, 72 °C for 15 s	*pfs* (193)	Zhu et al., 2008
	Rpfs2	ATCTGGCCCGGCTCGTAGCC			
Shigella spp.	IpaH-F	CCTTGACCGCCTTTCCGATA	2 min at 95 °C; 35 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2.5 min	*IpaH* (606)	Kong et al., 2002
	IpaH-R	CAGCCACCCTCTGAGGTACT			
Staphylococcus spp.	PanStaphF	CAATGCCACAAACTCG	5 min at 95 °C; 45 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s	*tuf* (462)	Sakai et al., 2004
	PanStaphR	GCTTCAGCGTAGTCTA			
Streptomyces spp.	StrepB	ACAAGCCCTGGAAACGGGGT	5 min at 98 °C; 30 cycles of 95 °C for 45 S, 54 °C for 40S, 72 °C for 2 min	16S rRNA (519)	Rintala et al., 2001
	StrepE	CACCAGGAATTCCGATCT			
Yersinia spp.	227Fmod	GTCTGGGCTTTGCTGGTC	5 min at 95 °C; 40 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 15 s	*ompF* (428 - 465)	Stenkova et al., 2008
	669R	GCGTCGTATTTAGCACCAACG			
Adenovirus	AQ1	GCCACGGTGGGGTTTCTAAACTT	2 min at 95 °C; 35 cycles of 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min	*Hexon* (110)	Heim et al., 2003; Rohayem et al., 2004
	AQ2	GCCCCAGTGGTCTTACATGCACATC			

* A final elongation step of 10 min at 72 °C was included for each PCR assay

All PCR products were analysed by agarose gel electrophoresis in 1.2% agarose (SeaKem® LE Agarose; Lonza) containing 0.5 µg/mL ethidium bromide in 1X TBE buffer. Once the size of the PCR products had been confirmed, representative PCR products were purified and concentrated and sent for sequencing as outlined in section 2.2.6.

2.2.9 Quantitative PCR parameters for the most readily detected indigenous rainwater pathogens

In order to quantify the most readily detected indigenous rainwater pathogens identified during conventional PCR analysis, qPCR was conducted. All qPCR assays were performed using a LightCycler® 96 (Roche Diagnostics) instrument with the primers as outlined in **Table 2.2** (*Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp.) and **Table 2.1** (*Staphylococcus* spp.). For all qPCR assays, the reaction mixture (final volume of 20 µL) consisted of 10 µL FastStart Essential DNA Green Master Mix (1X), 5 µL template DNA and 0.4 µL of each primer (0.2 µM). All samples were diluted (10-fold) prior to analysis with the respective qPCR assays (minimise PCR inhibitors) and all DNA samples were analysed in duplicate. Quantitative PCR analysis for the tank water samples collected from the small-scale systems was only conducted for the water samples collected from Site 1, as the samples had a broader temperature range as compared to Site 2, while all the water samples collected from Site 3 were included in the analysis (large-scale system).

For the quantification of *Legionella* spp., the primers LegF and LegR were utilised to amplify the 23S rRNA gene according to Herpers et al. (2003). The amplification conditions for the quantification of *Legionella* spp. were as follows: 95 °C (10 min) followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 11 s.

For the quantification of *Pseudomonas* spp., the primers PS1 and PS2 were utilised to amplify the *oprI* gene according to Bergmark et al. (2012). The amplification conditions for the quantification of *Pseudomonas* spp. were as follows: 95 °C (10 min) followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s.

For the quantification of *Salmonella* spp., the primers rpoD-20-F and rpoD-20-R were utilised to amplify the *rpoD* gene according to Barbau-Piednoir et al. (2013). For this qPCR assay, 0.5 µL of the forward primer (0.25 µM) and 2 µL of the reverse primer (1 µM) was used. The amplification conditions for the quantification of *Salmonella* spp. were as follows: 95 °C (10 min) followed by 45 cycles of denaturation at 95 °C for 15 s and a combined annealing and extension step at 60 °C for 60 s.

For the quantification of *Staphylococcus* spp., the primers PanStaphF and PanStaphR were utilised to amplify the *tuf* gene according to Sakai et al. (2004) (**Table 2.1**). The amplification

conditions for the quantification of *Staphylococcus* spp. were as follows: 95 °C (10 min) followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s and extension at 72 °C for 30 s.

Melt curve analysis was included for all of the SYBR green real-time PCR assays in order to verify the 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. To generate a standard curve for the quantification of *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp., the target genes (*Legionella* spp. = 23S rRNA; *Pseudomonas* spp. = *oprI*; *Salmonella* spp. = *rpoD*; *Staphylococcus* spp. = *tuf*) were first amplified by using the respective primers in conventional PCR assays on DNA extracted from *Legionella pneumophila* ATCC 33152, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 25925, respectively. For the amplification of the 23S rRNA gene from *Legionella* spp., used for the standard curve, in a total volume of 25 µL the conventional PCR mixtures consisted of 5 µL Green GoTaq® Flexi buffer (1X final concentration; Promega), 2 µL $MgCl_2$ (2.0 mM; Promega), 0.25 µL of a dNTP mix (0.1 mM; Thermo Scientific), 1 µL of the respective forward and reverse PCR primers (0.4 µM; **Table 2.2**), 0.15 µL of GoTaq® Flexi DNA polymerase (1.5 U, Promega) and 2.5 µL of template DNA. Similarly, for the amplification of the *oprI* gene from *Pseudomonas* spp., the *rpoD* gene from *Salmonella* spp. and the *tuf* gene from *Staphylococcus* spp., used for the standard curve, 0.5 µL (0.2 µM), 0.625 µL (0.25 µM) and 2.5 µL (1.0 µM), of the respective forward and reverse PCR primers were added to the PCR mixtures (outlined above), respectively.

Table 2.2: Primers for the amplification of the 23S rRNA (*Legionella* spp.), *oprI* (*Pseudomonas* spp.) and *rpoD* (*Salmonella* spp.) genes utilised for qPCR.

Organism	Primers	Primer sequence (5'-3')	Gene (bp)	References
Legionella spp.	LegF	CTAATTGGCTGATTGTCTTGAC	23S rRNA (259)	Herpers et al., 2003
	LegR	CAATCGGAGTTCTTCGTG		
Pseudomonas spp.	PS1	ATGAACAACGTTCTGAAATTC	*oprI* (249)	Bergmark et al., 2012
	PS2	CTGCGGCTGGCTTTTTCCAG		
Salmonella spp.	rpoD-20-F	ACATGGGTATTCAGGTAATGGAAGA	*rpoD* (75)	Barbau-Piednoir et al., 2013
	rpoD-20-R	CRGTGCTGGTGGTATTTTCA		

The obtained PCR products were cleaned and concentrated as described in section 2.2.6. Following DNA concentration determination using a NanoDrop® ND-1000 (Nanodrop

Technologies Inc.), the DNA concentration and gene product size were used to calculate the dilution required to obtain a final DNA concentration of 10^9 gene copies/µL (Dobrowsky et al., 2016). Serial 10-fold dilutions (10^9 to 10^0) of the PCR products (*Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp.) were then prepared in order to generate a standard curve, with a concentration of 1.00×10^9 gene copies/µL for the dilution with the highest copy number and a concentration of 1.00×10^0 gene copies/µL for the dilution with the lowest copy number. The lower limit of detection (LLOD) for all qPCR assays was determined as the lowest number of gene copies consistently detected within the respective standard curves. Representative PCR products of each of the qPCR assays for each organism were cleaned and concentrated and were sequenced as described in section 2.2.6.

2.2.10 Operational sustainability of the small-scale and large-scale SOPAS systems

2.2.10.1 Volume of pasteurized tank water produced by the SOPAS systems

In order to determine whether the small-scale and large-scale SOPAS systems would be able to produce an adequate volume of water for the households to use, the volume of pasteurized tank water (m = kg/h) that could theoretically be produced by the SOPAS systems was calculated as previously described by Klein (1975). For the two small-scale SOPAS systems (Sites 1 and 2) the volume of pasteurized tank water that could be produced was calculated based on the temperatures of the collected unpasteurized and pasteurized tank water samples. As the treated tank water of the large-scale SOPAS system (Site 3) was released through a thermostatic release valve, the temperature of the unpasteurized tank water samples and 75 °C (opening temperature of the thermostatic release valve) was used to calculate the volume of pasteurized tank water that could be produced by the system. In the calculation the useful heat gain (Q) is dependent on the mass flow rate (m), specific heat (c_p) and a change in temperature from the inlet (T_i) to the outlet (T_o) of the storage tank (**Eq. 2a** and **2b**).

$$[Q = mc_p(T_o - T_i)] \quad (\mathbf{2a})$$

$$[m = Q/c_p(T_o - T_i) \quad (\mathbf{2b})$$

2.2.10.2 Monitoring the water usage by the households utilising the SOPAS systems

In order to monitor the water usage for each of the ten participating households, a co-researcher on the project located in Enkanini was provided with a log book for each site (**Appendix A**). The co-researcher visited the participating households every Friday of the trial period (September 2015 to September 2016) and posed the following two questions (answers recorded in the log books):

1. How much water was collected from the solar systems per day (how many times per day using the 20 L water container provided)?
2. How much water did you collect from the standpipe/tap systems per day (how many times using another container)?

2.2.11 Bacterial removal efficiency of the SOPAS systems

The bacterial removal efficiency of the SOPAS systems was measured by comparing the bacterial counts obtained from the tank water samples collected before pasteurization and the bacterial counts obtained from tank water samples collected after pasteurization. The log reduction was calculated using **Eq. 3** and the percentage reduction was calculated using **Eq. 4** (Brözel and Cloete, 1991).

$$[\text{Log reduction} = (\text{Log}_{10} \text{ bacterial count}_{\text{ before pasteurization}} - \text{Log}_{10} \text{ bacterial count}_{\text{ after pasteurization}})] \quad (\mathbf{3})$$

$$[\text{Percentage reduction} = 100 - \frac{\text{Survivor count}}{\text{Initial count}} \times 100] \quad (\mathbf{4})$$

2.2.12 Statistical analysis

The data obtained from the microbial and chemical analysis and the temperature of the collected tank water samples (before pasteurization and after pasteurization), was assessed using the statistical software package Statistica™ Ver. 12.6 (Stat Soft Inc., Tulsa, OK, USA). The means of duplicate replicates were used during data analysis. Analysis was performed to determine whether the data differed significantly following pasteurization using the parametric Paired t-test and then confirmed using the non-parametric Wilcoxon test. In order to determine whether there was a significant correlation between rainfall data and the presence of the indicator organisms, parametric Pearson correlations were performed. In all hypothesis tests, a significance level of 5% ($p < 0.05$) was used as standard (Dunn and Clark, 1974).

2.3 Results

2.3.1 Physico-chemical properties

2.3.1.1 Small-scale SOPAS systems (Sites 1 and 2)

The sampling dates and corresponding pasteurized (after treatment) and unpasteurized (before treatment) tank water temperatures and pH values for the two small-scale systems, are outlined in **Table 2.3**. For Site 1, an overall mean pH of 7.15 and 7.68 were obtained for the pasteurized (n = 8) and unpasteurized (n = 8) tank water samples, respectively. Correspondingly, an overall mean pH of 7.50 was obtained for all pasteurized (n = 3) tank water samples collected at Site 2, while a mean pH of 7.67 was measured for all unpasteurized tank water samples (n = 3)

(**Table 2.3**). For Site 1, the temperature of the unpasteurized tank water samples collected directly from the rainwater harvesting tank ranged from the lowest temperature of 20.8 °C (26/08/2016) to the highest temperature of 25.9 °C (04/11/2015). The temperature of the pasteurized tank water samples then ranged from the lowest temperature of 52.0 °C (08/04/2016) to the highest temperature of 85.0 °C (26/10/2016). For Site 2, the temperature of the unpasteurized tank water samples collected directly from the rainwater harvesting tank ranged from the lowest temperature of 20.7 °C (07/10/2015) to the highest temperature of 24.5 °C (04/11/2015). The temperature of the pasteurized tank water samples ranged from the lowest temperature of 53.0 °C (20/10/2015) to the highest temperature of 66.0 °C (04/11/2015).

Table 2.3: Temperatures and pH values of the pasteurized and unpasteurized tank water samples collected from the two small-scale SOPAS systems in Enkanini.

Sampling Date	Location	Temperature of Pasteurized Water (°C)	pH of Pasteurized Water	Temperature of Unpasteurized Water (°C)	pH of Unpasteurized Water
22/10/2015	Site 1	75.0	7.8	24.6	8.4
04/11/2015	Site 1	75.0	7.6	25.9	7.9
08/04/2016	Site 1	52.0	6.6	21.0	7.1
26/08/2016	Site 1	60.0	6.5	20.8	6.7
14/09/2016	Site 1	67.0	7.1	22.5	7.1
03/10/2016	Site 1	72.0	7.1	23.2	8.4
04/10/2016	Site 1	73.0	7.2	25.2	8.0
26/10/2016	Site 1	85.0	7.3	24.2	7.8
07/10/2015	Site 2	58.0	7.7	20.7	8.3
20/10/2015	Site 2	53.0	8.4	23.9	8.0
04/11/2015	Site 2	66.0	6.4	24.5	6.7

The daily rainfall and ambient temperatures recorded throughout the 2015/2016 research period as well as the sampling sessions for each site are depicted in **Fig. 2.3**. A total rainfall of 61.0 mm was recorded during October 2015 to February 2016 (low rainfall period), while 84.7 mm was recorded during March to May 2016 (medium rainfall period). The rainfall then increased to 276.4 mm during June to September 2016 (high rainfall period) and decreased again to 16.5 mm during October 2016 (low rainfall period).

2.3.1.2 Large-scale SOPAS system (Site 3)

The sampling dates and corresponding unpasteurized (before treatment), pasteurized (during treatment) and stored pasteurized (after treatment) tank water temperatures and pH values, for the large-scale system located at Site 3 (ERC), are outlined in **Table 2.4**.

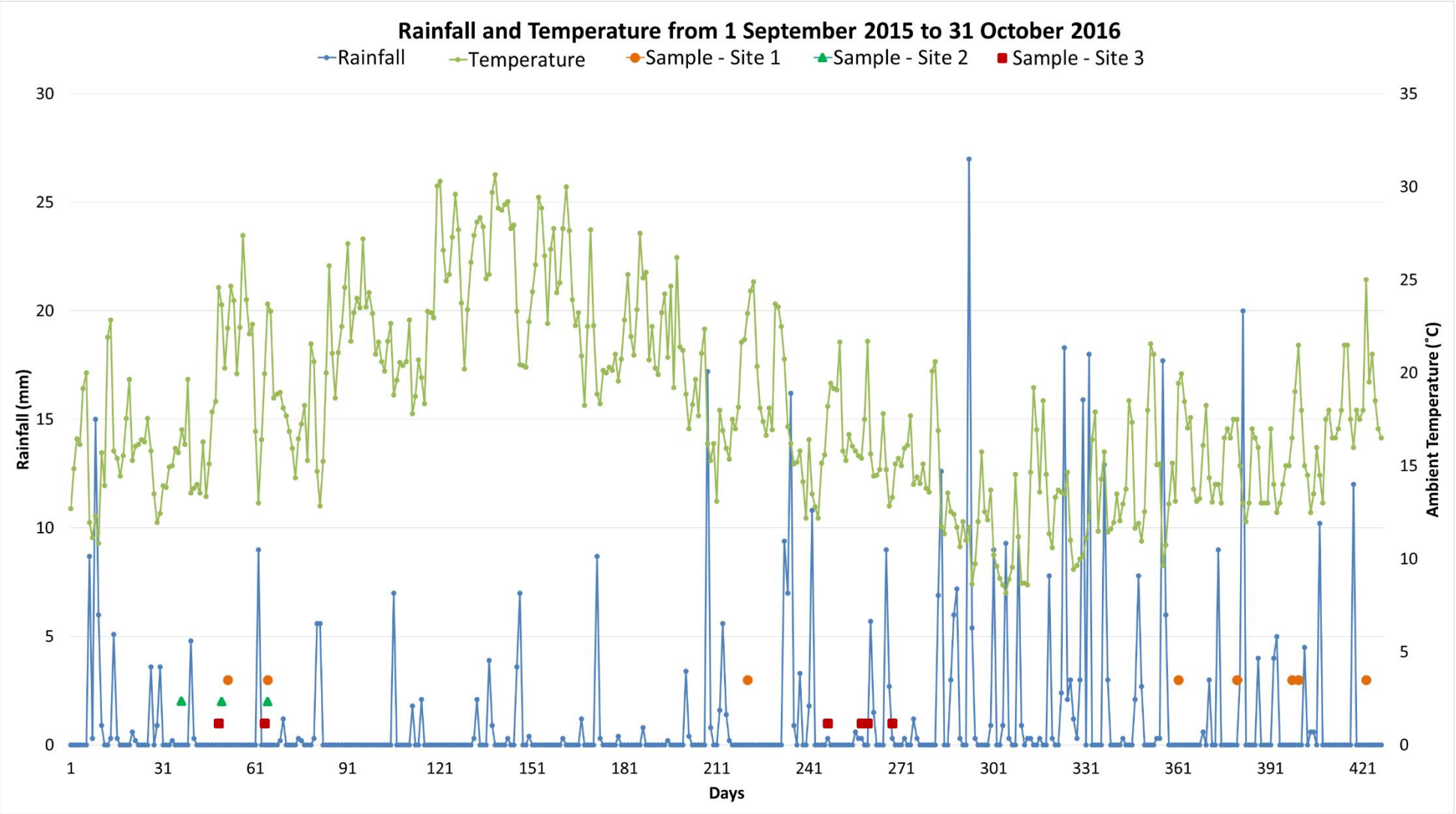

Fig. 2.3. The daily rainfall and ambient temperatures recorded for each day during the 2015/2016 sampling period. The sampling sessions for each of the three sites are also indicated.

An overall mean pH of 7.05 and 7.10 was obtained for all pasteurized (n = 6) and stored pasteurized (n = 6) tank water samples, respectively, while a mean pH of 6.95 was measured for all unpasteurized (n = 6) tank water samples. The temperatures of the unpasteurized tank water samples collected directly from the rainwater harvesting tank at Site 3 then ranged from the lowest temperature of 15.5 °C (26/05/2016) to the highest temperature of 25.7 °C (20/10/2015). The temperature of the pasteurized tank water samples ranged from the lowest temperature of 55 °C (05/05/2016) to the highest temperature of 79.0 °C (04/11/2015). Additionally, the temperature of the stored pasteurized tank water samples ranged from the lowest temperature of 18.7 °C (16/05/2016) to the highest temperature of 33.8 °C (04/11/2015).

Table 2.4: Temperature and pH values of the pasteurized, unpasteurized and stored pasteurized tank water samples collected from the large-scale system in Enkanini.

Sampling Date	Temperature of Pasteurized Water (°C)	pH of Pasteurized Water	Temperature of Unpasteurized Water (°C)	pH of Unpasteurized Water	Temperature of Stored Pasteurized Water (°C)	pH of Stored Pasteurized Water
20/10/2015	66.0	8.2	25.7	8.0	33.7	8.2
04/11/2015	79.0	6.8	24.2	6.7	33.8	6.6
05/05/2016	55.0	7.4	20.1	7.3	24.5	7.5
16/05/2016	72.0	6.9	19.4	6.8	18.7	7.3
17/05/2016	61.0	6.5	17.5	6.6	24.5	6.5
26/05/2016	71.0	6.5	15.5	6.3	19.7	6.5

2.3.2 Chemical analysis

The results for the anion analyses of the small-scale SOPAS systems (Sites 1 and 2) are represented in **Table 2.5**. For both small-scale systems, all anions were within the drinking water guidelines according to DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011). No significant difference in anion concentrations was observed following SOPAS treatment for both small-scale systems (Site 1: p = 0.21; Site 2: p = 0.11) at all temperatures analysed (Site 1: 52 °C, 67 °C, 75 °C and 85 °C; Site 2: 58 °C and 66 °C). Additionally, no significant difference (p = 0.35) in anion concentrations was observed between the tank water samples collected from Sites 1 and 2, respectively. For example, for Site 1, mean sulphate (as SO_4) concentrations of 4.70 mg/L and 4.71 mg/L, were obtained before and after pasteurization, respectively. Similarly, for Site 2, mean SO_4 concentrations of 4.39 mg/L and 4.18 mg/L, were obtained before and after pasteurization, respectively. Overall, the SO_4 concentrations recorded at the two sites (small-scale) ranged from 3 to 7.15 mg/L before SOPAS and 3 to 7.2 mg/L after SOPAS.

Table 2.5: Anion concentrations obtained from the unpasteurized and pasteurized tank water samples collected from the small-scale SOPAS systems (Sites 1 and 2; n = 12) compared to the concentrations as stipulated by the respective drinking water guidelines.

Anions (mg/L)	Site 1								Site 2				Drinking Water Guidelines			
	Before 52 °C	After 52 °C	Before 67 °C	After 67 °C	Before 75 °C	After 75 °C	Before 85 °C	After 85 °C	Before 58 °C	After 58 °C	Before 66 °C	After 66 °C	SANS 241	DWAF	ADWG	WHO
Sulphate as SO_4	7.15	7.2	3	3	4.68	4.64	4	4	4.05	3.86	4.73	4.5	200	100	250	-
Chloride as Cl^-	9	11	6	6	7	6	6	6	6	6	7	6	400	200	250	-
Nitrite as NO_2	0.01	0.02	0.01	0.01	0.02	0.03	0.02	0.02	0.01	0.03	0.02	0.02	10	6	50	50
Nitrate as NO_3	1.59	1.56	0.39	0.60	1.07	1.05	0.63	0.67	0.93	0.36	0.97	0.96	10	6	50	50
Phosphate as PO_4	0.31	0.43	0.01	0.02	0.12	0.12	0.02	0.01	0.03	0.03	0.03	0.03	-	-	-	-
Fluoride as F	0.06	0.08	BDL*	BDL*	0.06	0.05	BDL*	BDL*	0.07	0.07	0.07	0.07	1	1	1.5	1.5

***BDL = Below detection limit**

Table 2.6: Anion concentrations obtained from the unpasteurized and stored pasteurized tank water samples collected from the large-scale SOPAS system (Site 3; n = 6) compared to the concentrations as stipulated by the respective drinking water guidelines.

Anions (mg/L)	Site 3						Drinking Water Guidelines			
	Before 55 °C	After 55 °C	Before 66 °C	After 66 °C	Before 79 °C	After 79 °C	SANS 241	DWAF	ADWG	WHO
Sulphate as SO_4	4.13	5.08	3.79	3.72	4.14	4	200	100	250	-
Chloride as Cl^-	7	9	6	6	7	7	400	200	250	-
Nitrite as NO_2	0.01	0.02	0.01	0.02	0.02	0.02	10	6	50	50
Nitrate as NO_3	0.92	1.17	1.21	1.11	1.19	1.19	10	6	50	50
Phosphate as PO_4	0.09	0.06	0.06	0.06	0.06	0.03	-	-	-	-
Fluoride as F	0.07	0.1	0.03	0.05	0.05	0.05	1	1	1.5	1.5

The results for the anion analyses of the large-scale SOPAS system (Site 3) are represented in **Table 2.6**. Similar to the results obtained for the small-scale systems, all anions were within the drinking water guidelines according to DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011). No significant difference (p = 0.19) in anion concentrations was observed when comparing the unpasteurized to stored pasteurized tank water samples and no significant difference (p = 0.26) in anion concentrations was observed when comparing the large-scale SOPAS system to the two small-scale SOPAS systems. For example, chloride (Cl^-) concentrations recorded for samples collected from the large–scale system ranged from 6 to 7 mg/L (mean 6.67 mg/L) before pasteurization and from 6 to 9 mg/L (mean 7.33 mg/L) after pasteurization (stored pasteurized samples) (**Table 2.6**). Similarly, the Cl^- concentrations recorded in the samples collected from the small-scale systems (**Table 2.5**) ranged from 6 to 9 mg/L before SOPAS (mean 7.0 mg/L) and 6 to 11 mg/L after SOPAS (mean 7.25 mg/L).

Cation concentration analysis was conducted by CAF at Stellenbosch University. For both small-scale SOPAS systems (Sites 1 and 2) (**Table 2.7**; representative cations presented), the results indicated that all cations were within the drinking water guidelines according to DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011), with the exception of zinc (Zn) which exceeded the DWAF (1996) and ADWG (NHMRC and NRMMC, 2011) limit of 3 000 µg/L and the 5 000 µg/L limit as stipulated by SANS 241 (SABS, 2005), in four of the six sampling sessions analysed (Site 1 – 52 °C and 75 °C; Site 2 – 58 °C and 66 °C). However, for both small-scale systems no significant difference (Site 1: p = 0.21; Site 2: p = 0.76) in Zn concentrations was observed following SOPAS treatment. For example, for Site 1 the overall mean Zn concentration increased from 3 602 µg/L before pasteurization to 3 692 µg/L after pasteurization treatment (52 °C, 67 °C, 75 °C and 85 °C). For Site 2 the overall mean Zn concentration then decreased from 4 552 µg/L before pasteurization to 4 509 µg/L after pasteurization treatment at 58 °C and 66 °C (**Table 2.7**).

While still within the drinking water guidelines, notable increases in Ni, Al, Cu and Pb were observed following SOPAS treatment at both Sites 1 and 2 (**Table 2.7**). For Site 1 the mean concentrations of Ni, Al, Cu and Pb in the collected samples, increased from 0.34 µg/L, 7.82 µg/L, 4.84 µg/L and 0.16 µg/L before pasteurization, to 3.82 µg/L, 13.52 µg/L, 21.24 µg/L and 2.03 µg/L after pasteurization, respectively. Similarly, for Site 2, the mean concentrations of Ni, Al, Cu and Pb in the collected samples increased from 0.25 µg/L, 2.06 µg/L, 0.15 µg/L and 0.06 µg/L before pasteurization, to 4.38 µg/L, 3.80 µg/L, 0.73 µg/L and 0.38 µg/L after pasteurization, respectively.

Table 2.7: Cation concentrations obtained from tank water samples collected from the small-scale SOPAS systems (Sites 1 and 2) compared to the recommended concentrations as stipulated by the respective drinking water guidelines.

Cation	Site 1						Site 2						Drinking Water Guidelines			
	Before 52 °C	After 52 °C	Before 67 °C	After 67 °C	Before 75 °C	After 75 °C	Before 85 °C	After 85 °C	Before 58 °C	After 58 °C	Before 66 °C	After 66 °C	SANS 241	DWAF	ADWG	WHO
Calcium as Ca (mg/L)	1.51	1.68	0.53	0.68	0.93	0.89	0.63	0.62	1.29	1.23	1.98	1.90	150	200	200	-
Potassium as K (mg/L)	0.53	0.55	0.22	0.19	0.32	0.32	0.20	0.19	0.25	0.27	0.32	0.31	50	50	-	-
Magnesium as Mg (mg/L)	0.58	0.59	0.34	0.31	0.38	0.37	0.35	0.35	0.32	0.31	0.41	0.40	70	30	-	-
Sodium as Na (mg/L)	3.96	4.12	2.67	2.43	2.55	2.53	2.48	2.51	2.25	2.17	2.53	2.50	200	100	180	-
Silicon as Si (mg/L)	0.16	0.57	0.05	0.06	0.07	0.13	0.06	0.11	0.10	0.11	0.15	0.18	-	-	-	-
Aluminium as Al (µg/L)	22.78	14.85	2.23	12.30	0.06	0.94	6.22	25.98	0.61	5.34	3.51	2.26	300	150	100	100
Boron as B (µg/L)	ND*	ND*	ND*	ND*	10.15	12.66	6.94	8.04	ND*	ND*	2.80	4.12	-	-	4000	2400
Iron as Fe (µg/L)	13.84	1.92	0.90	1.23	< 0.5	< 0.5	2.88	< 0.5	<0.19	0.56	< 0.5	5.52	200	100	300	-
Nickel as Ni (µg/L)	0.58	4.04	0.21	0.90	0.37	9.74	0.21	0.61	0.28	2.65	0.22	6.10	150	-	20	70
Copper as Cu (µg/L)	18.13	57.12	0.15	9.08	0.52	13.21	0.57	5.57	<0.07	0.36	0.22	1.10	1000	1000	2000	2000
Zinc as Zn (µg/L)	**5098**	**5350**	2459	2491	**3993**	**4069**	2861	2859	**4394**	**4239**	**4710**	**4778**	5000	3000	3000	-
Arsenic as As (µg/L)	0.82	1.62	0.42	0.66	0.91	1.12	0.46	0.65	0.47	0.44	0.49	0.46	10	10	10	10
Selenium as Se (µg/L)	0.16	0.19	0.07	0.13	0.10	0.17	0.14	0.18	0.12	0.16	0.15	0.07	20	20	10	40
Lead as Pb (µg/L)	0.21	0.48	0.21	3.24	0.22	3.44	0.02	0.94	0.09	0.13	0.03	0.63	20	10	10	10

*ND = Not determined; values indicated by bold text are exceeding guidelines.

The total hardness of the tank water samples collected before and after SOPAS from the small-scale systems was then determined (using concentrations of Ca and Mg) as total hardness is an indicator of the corrosive capabilities of the water which may affect the plumbing installations used in the SOPAS system (DWAF, 1996) (results not shown). The calculated total hardness for the unpasteurized tank water samples is then expressed as a $CaCO_3$ equivalent in mg/L.

For tank water samples collected at Site 1, total hardness was recorded as 6.15 mg/L, 2.72 mg/L, 3.89 mg/L and 3.01 mg/L before pasteurization while total hardness of the corresponding pasteurized tank water samples was recorded at 6.62 mg/L (52 °C), 2.97 mg/L (67 °C), 3.75 mg/L (75 °C) and 2.99 mg/L (85 °C), respectively. The total hardness of the tank water samples collected from Site 2 was also calculated. Following pasteurization at 58 °C and 66 °C, the total hardness decreased from 4.54 mg/L and 6.63 mg/L to 4.35 mg/L and 6.39 mg/L, respectively. As the total hardness values were below 50 mg/L, the water was described as soft (DWAF, 1996).

For the large-scale SOPAS system (Site 3), all cations were within the drinking water guidelines according to DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011), with the exception of arsenic (As) which exceeded all four drinking water guidelines' limit of 10 µg/L (**Table 2.8**; representative cations presented). However, no significant difference (p = 0.18) in As concentration was observed in the unpasteurized and stored pasteurized tank water samples. For Site 3 the overall mean As concentration of 18.69 µg/L before pasteurization then decreased to 18.30 µg/L in the stored pasteurized samples.

Similar to the results obtained for the small-scale SOPAS systems, notable increases in Ni, Cu and Pb were also observed following SOPAS treatment using the large-scale system (**Table 2.8**). The overall mean concentrations of Ni (0.34 µg/L), Cu (8.43 µg/L) and Pb (0.44 µg/L) measured before pasteurization, increased to 0.57 µg/L, 407.45 µg/L and 6.77 µg/L in the stored pasteurized samples, respectively. However, in contrast to the results obtained for the small-scale systems, a decrease in the overall mean concentration of Al was observed following SOPAS treatment (large-scale system) as the overall mean Al concentration before SOPAS decreased from 11.69 µg/L to 4.89 µg/L, in the stored pasteurized samples.

The total hardness of the tank water samples collected before and after SOPAS with the large-scale system were calculated by comparing the unpasteurized to the stored pasteurized tank water samples (results not shown). Before SOPAS (corresponding SOPAS samples of 55 °C, 66 °C and 79 °C), the total hardness fluctuated from 7.12 mg/L, 7.96 mg/L and 7.49 mg/L, to 9.91 mg/L, 7.84 mg/L and 6.55 mg/L following pasteurization, respectively. The tank water collected from Site 3 can therefore also be described as soft (< 50 mg/L).

Table 2.8: Cation concentrations obtained from the unpasteurized and stored pasteurized tank water samples collected from the large-scale SOPAS system (Site 3) compared to the recommended concentrations as stipulated by the respective drinking water guidelines.

Cation	Site 3						Drinking Water Guidelines			
	Before 55 °C	After 55 °C	Before 66 °C	After 66 °C	Before 79 °C	After 79 °C	SANS 241	DWAF	ADWG	WHO
Calcium as Ca (mg/L)	2.06	3.03	2.43	2.38	2.19	1.93	150	200	200	-
Potassium as K (mg/L)	0.37	0.58	0.44	0.46	0.43	0.44	50	50	-	-
Magnesium as Mg (mg/L)	0.48	0.57	0.46	0.46	0.49	0.42	70	30	-	-
Sodium as Na (mg/L)	3.40	3.57	2.63	2.68	4.13	2.43	200	100	180	-
Silicon as Si (mg/L)	0.21	0.32	0.21	0.20	0.20	0.17	-	-	-	-
Aluminium as Al (µg/L)	6.26	3.19	12.28	6.94	16.54	4.56	300	150	100	100
Boron as B (µg/L)	ND*	ND*	16.59	16.64	16.71	16.80	-	-	4000	2400
Iron as Fe (µg/L)	0.56	1.84	< 0.5	< 0.5	< 0.5	< 0.5	200	100	300	-
Nickel as Ni (µg/L)	0.27	0.57	0.38	0.60	0.36	0.55	150	-	20	70
Copper as Cu (µg/L)	2.03	317.39	11.73	435.85	11.52	469.11	1000	1000	2000	2000
Zinc as Zn (µg/L)	375.1	437.4	365.99	497.79	352.84	493.45	5000	3000	3000	-
Arsenic as As (µg/L)	**19.11**	**18.75**	**17.87**	**17.80**	**19.09**	**18.36**	10	10	10	10
Selenium as Se (µg/L)	0.17	0.28	0.15	0.11	0.11	0.11	20	20	10	40
Lead as Pb (µg/L)	0.09	2.65	0.51	8.25	0.73	9.40	20	10	10	10

*ND = Not determined; values indicated by bold text are exceeding guidelines.

2.3.3 Indicator bacteria detected in unpasteurized and pasteurized tank water samples

2.3.3.1 Small-scale SOPAS systems (Sites 1 and 2)

Unpasteurized (before treatment) and pasteurized (after treatment) tank water samples were collected from the small-scale SOPAS systems located at Sites 1 and 2, respectively. Total coliforms and *E. coli* (**Table 2.9**), as well as heterotrophic bacteria (**Fig. 2.4**), faecal coliforms and enterococci were enumerated in all collected samples, with the results being compared to various national and international drinking water guidelines to determine if the water could be used for potable purposes. Additionally, for the samples where no heterotrophic bacteria were detected after pasteurization, samples were stored at room temperature and the heterotrophic bacteria were enumerated every second day for a two week period in order to determine how long the treated water could be stored, before the regrowth of heterotrophic bacteria was observed.

For Site 1, a mean total coliform count of 1.4×10^3 CFU/100 mL before pasteurization was reduced to 2.9×10^2 CFU/100 mL after pasteurization (0.68 log reduction) at 52 °C (**Table 2.9**). A mean total coliform count of 6.6×10^3 CFU/100 mL before pasteurization was then reduced to BDL (< 1 CFU/100 mL) after pasteurization at 60 °C and 67 °C (3.82 log reduction), respectively. On average a 4 log reduction in total coliforms was observed for the four samples pasteurized at 72 °C to 75 °C (**Table 2.9**) as the mean total coliform counts were reduced from 1.2×10^4 CFU/100 mL before pasteurization to BDL (< 1 CFU/100 mL) after pasteurization. Additionally, a mean total coliform count of 2.3×10^3 CFU/100 mL before pasteurization was reduced to BDL (< 1 CFU/100 mL) after pasteurization at 85 °C.

Table 2.9: Enumeration of total coliforms and *E. coli* before and after SOPAS treatment for tank water samples collected from Sites 1 and 2.

Pasteurization Temp °C (Location)	Indicator	Unpasteurized Sample (CFU/100 mL)	Pasteurized Sample (CFU/100 mL)	Log Reduction	% Reduction
52 °C (Site 1)	TC	1.4×10^3	2.9×10^2	0.68	79
	E. coli	1.0×10^1	BDL*	1	>99
60 °C (Site 1)	TC	3.1×10^3	BDL*	3.49	>99
	E. coli	3	BDL*	NA*	NA*
67 °C (Site 1)	TC	1.0×10^4	BDL*	4	>99
	E. coli	1	BDL*	NA*	NA*
72 °C (Site 1)	TC	9.9×10^3	BDL*	3.99	>99
	E. coli	1.3×10^1	BDL*	1.11	>99
73 °C (Site 1)	TC	1.4×10^4	BDL*	4.15	>99
	E. coli	1.8×10^1	BDL*	1.26	>99
75 °C (1) (Site 1)	TC	1.1×10^4	BDL*	4.04	>99
	E. coli	BDL*	BDL*	NA*	NA*
75 °C (2) (Site 1)	TC	1.2×10^4	BDL*	4.08	>99
	E. coli	BDL*	BDL*	NA*	NA*
85 °C (Site 1)	TC	2.3×10^3	BDL*	3.36	>99
	E. coli	3	BDL*	NA*	NA*
53 °C (Site 2)	TC	1.2×10^3	BDL*	3	>99
	E. coli	BDL*	BDL*	NA*	NA*
58 °C (Site 2)	TC	1.2×10^3	BDL*	3	>99
	E. coli	BDL*	BDL*	NA*	NA*
66 °C (Site 2)	TC	6.3×10^2	BDL*	2	>99
	E. coli	BDL*	BDL*	NA*	NA*

*BDL = Below the detection limit / *NA = Not applicable

All the unpasteurized tank water samples collected from Site 1 exceeded the DWAF (1996) (< 5 CFU/100 mL) and SANS 241 (< 10 CFU/100 mL) (SABS, 2005) drinking water standards

for total coliforms. However, pasteurization of the tank water samples at 60 °C and above effectively reduced the total coliforms to BDL (< 1 CFU/100 mL) and subsequently to within the DWAF (1996) and SANS 241 (SABS, 2005) limits. The drinking water standards, according to DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011) also stipulate that there should be no *E. coli* or faecal coliforms (< 1 CFU/100 mL) present in water if it is to be used for potable purposes. For Site 1, *E. coli* (mean 8 CFU/100 mL) was detected in 75% (n = 6) of the unpasteurized tank water samples (unpasteurized samples corresponding to the 52 °C, 60 °C, 67 °C, 72 °C, 73 °C and 85 °C samples). However, the *E. coli* counts were subsequently reduced to BDL (< 1 CFU/100 mL) and were within the drinking water standards following SOPAS at the respective temperatures (**Table 2.9**). Additionally, faecal coliforms (mean 2.2×10^3 CFU/100 mL) were detected in the three (38%) unpasteurized samples collected for the corresponding 75 °C samples (1 and 2) and the corresponding 73 °C sample, but were also reduced to BDL (< 1 CFU/100 mL) and were within drinking water standards [DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011)] after pasteurization (results not shown). All the tank water samples collected before and after pasteurization from Site 1 were within the < 1 CFU/100 mL enterococci guideline limit as stipulated by ADWG (NHMRC and NRMMC, 2011), as no enterococci were detected in any of the samples.

Similar to the results obtained for Site 1, on average a 3 log reduction in total coliforms was observed in the pasteurized samples collected from Site 2, as the mean total coliform counts were reduced from 1.0×10^3 CFU/100 mL before pasteurization to BDL (< 1 CFU/100 mL) after pasteurization for all temperatures analysed. Total coliform counts in the unpasteurized samples thus exceeded the DWAF (1996) (< 5 CFU/100 mL) and SANS 241 (< 10 CFU/100 mL) (SABS, 2005) drinking water standards, while the total coliforms in the pasteurized tank water samples were reduced to within the respective standards for all temperatures analysed at Site 2. For Site 2, no *E. coli* or enterococci were detected in any of the unpasteurized and pasteurized samples collected. Faecal coliforms were only detected in one sample (unpasteurized sample collected before pasteurization at 53 °C) at 1.0×10^3 CFU/100 mL, but was subsequently reduced to BDL (< 1 CFU/100 mL) after pasteurization at 53 °C (results not shown) and was subsequently within the respective drinking water guideline limits of < 1 CFU/100 mL [DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011)].

Results for the enumeration of the HPC at Sites 1 and 2 are depicted in **Fig. 2.4**. According to the DWAF (1996) drinking water guidelines, HPC should not exceed 1.0×10^4 CFU/100 mL as this increases the likelihood of microbial infection related to the use of the water source. For Site 1 the HPC before pasteurization was reduced from 4.1×10^6 CFU/100 mL to 7.0×10^5 CFU/100 mL following pasteurization at 52 °C (0.77 log reduction). Additionally, a 2.44 log reduction was recorded following pasteurization at 60 °C, with the HPC decreasing from

6.4 × 10^6 CFU/100 mL to 2.3 × 10^4 CFU/100 mL. Thus both the 52 °C and 60 °C pasteurized samples exceeded the DWAF (1996) limit of 1.0 × 10^4 CFU/100 mL HPC. However, for the remaining samples [67 °C, 72 °C, 73 °C, 75 °C (1 and 2) and 85 °C] the HPC was reduced to within the DWAF drinking water standard following SOPAS treatment. The HPC count was reduced by 6.28 logs after pasteurization treatment at 67 °C [1.9 × 10^6 CFU/100 mL before pasteurization to BDL (< 1 CFU/100 mL) after pasteurization]. For the samples collected from 72 °C to 75 °C, a mean 6.92 log reduction was recorded as the HPC was reduced from a mean of 8.3 × 10^6 CFU/100 mL to BDL (< 1 CFU/100 mL) after pasteurization [72 °C, 73 °C, 75 °C (1 and 2)]. Pasteurization at 85 °C resulted in a 6.43 log reduction in the HPC, as the HPC before pasteurization (2.7 × 10^6 CFU/100 mL) was reduced to BDL (< 1 CFU/100 mL) after pasteurization (**Fig. 2.4**).

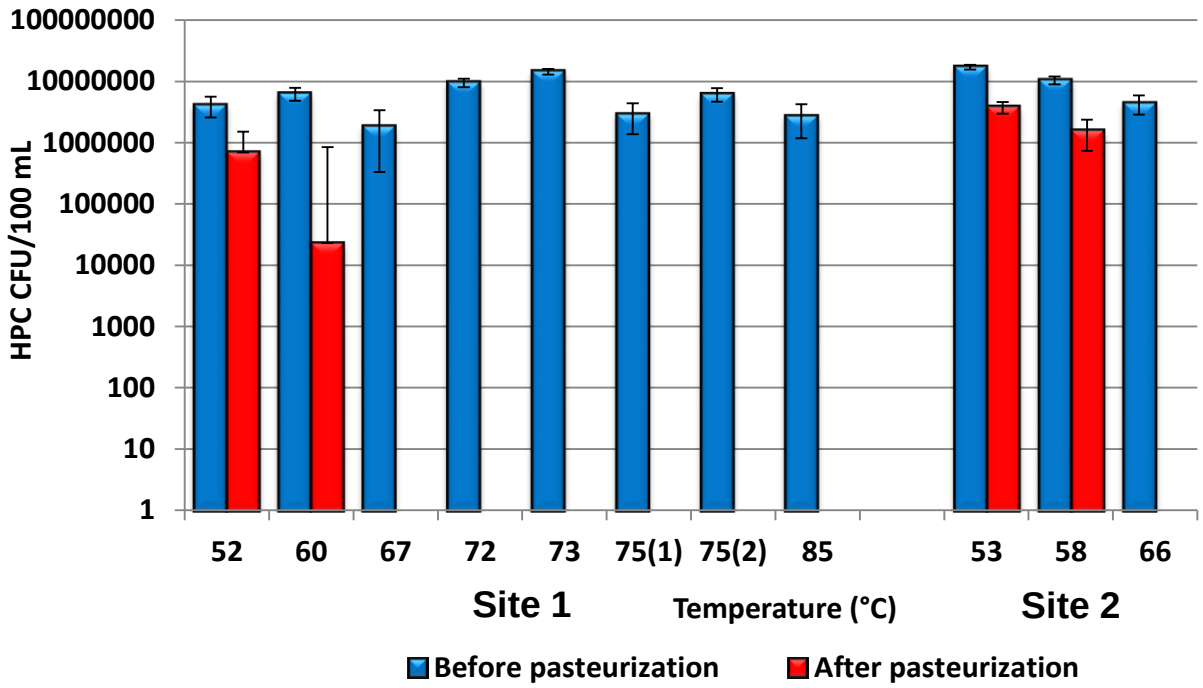

Fig. 2.4. Heterotrophic plate counts (HPC) obtained before and after SOPAS for the samples collected at Sites 1 and 2.

For Site 2, a mean decrease of 0.71 logs in HPC was then recorded for the samples collected at 53 °C and 58 °C following pasteurization (mean of 1.4 × 10^7 CFU/100 mL before pasteurization reduced to a mean of 2.7 × 10^6 CFU/100 mL after pasteurization); however, the HPC in both samples still exceeded the DWAF (1996) drinking water limit of 1.0 × 10^4 CFU/100 mL. In comparison, the HPC for Site 2 was reduced from 4.4 × 10^6 CFU/100 mL to BDL (< 1 CFU/100 mL) after pasteurization at 66 °C (6.64 log reduction) and was subsequently within the DWAF (1996) drinking water limit.

The 67 °C, 72 °C, 73 °C, both 75 °C (1 and 2) and the 85 °C pasteurization samples collected from Site 1 and the 66 °C pasteurization sample collected from Site 2 were subsequently

monitored for a two week period in order to determine how long the treated tank water could be stored. The results then indicated that the water could be stored for up to six days after treatment at 66 °C and 67 °C and up to two weeks following treatment above 72 °C, before microbial regrowth occurred (results not shown). In addition, no significant correlation was observed between the microbiological quality of the unpasteurized tank water collected from Sites 1 and 2 and rainfall data over the sampling period [total coliforms (r = - 0.11; p = 0.74), *E.coli* (r = - 0.16; p = 0.62) and HPC (r = - 0.37; p = 0.26)].

2.3.3.2 Large-scale SOPAS system (Site 3)

In order to determine whether the water produced by the large-scale SOPAS system (Site 3) meets the microbiological quality as stipulated by the respective drinking water guidelines (DWAF, 1996; SABS, 2005; NHMRC and NRMMC, 2011; WHO, 2011), unpasteurized (before treatment), pasteurized (directly from solar manifold system) and stored pasteurized tank water samples (after treatment collected from 1 500 L storage tank) were collected and analysed for the presence of *E. coli*, total coliforms, enterococci, faecal coliforms and heterotrophic bacteria. While the total coliform (mean 3.2 × 10^3 CFU/100 mL) and *E. coli* (mean 8 CFU/100 mL) counts in all unpasteurized tank water samples exceeded the respective drinking water guideline limits (**Table 2.10**), on average a > 3 log reduction (total coliforms) and > 1 log reduction (*E. coli*) was recorded following pasteurization of the tank water samples at all temperatures analysed (55 °C, 61 °C, 66 °C, 71 °C, 72 °C and 79 °C; collected from manifold system). Additionally, total coliforms and *E.coli* were BDL (< 1 CFU/100 mL) in the stored pasteurized tank water samples (1 500 L tank).

Table 2.10: Enumeration of total coliforms and *E. coli* in the unpasteurized, pasteurized and stored pasteurized tank water samples collected from Site 3.

Pasteurization Temp (°C)	Indicator	Unpasteurized Sample (CFU/100 mL)	Pasteurized Sample (CFU/100 mL)	Stored Pasteurized Sample (CFU/100mL)	Log Reduction	% Reduction
55 °C	TC	3.9 × 10^3	BDL*	BDL*	3.59	>99
	E. coli	2.2 × 10^1	BDL*	BDL*	1.34	>99
61 °C	TC	1.6 × 10^3	BDL*	BDL*	3.20	>99
	E. coli	1	BDL*	BDL*	NA*	NA*
66 °C	TC	3.5 × 10^3	BDL*	BDL*	3.54	>99
	E. coli	4	BDL*	BDL*	NA*	NA*
71 °C	TC	7.0 × 10^3	BDL*	BDL*	3.84	>99
	E. coli	1.7 × 10^1	BDL*	BDL*	1.23	>99
72 °C	TC	2.1 × 10^3	BDL*	BDL*	3.32	>99
	E. coli	2	BDL*	BDL*	NA*	NA*
79 °C	TC	1.1 × 10^3	BDL*	BDL*	3.04	>99
	E. coli	4	BDL*	BDL*	NA*	NA*

*BDL = Below the detection limit / *NA = Not applicable

No enterococci were detected in the unpasteurized, pasteurized or stored pasteurized tank water samples (results not shown). In addition, faecal coliforms were only detected in 50% (n = 3) of the unpasteurized samples [unpasteurized samples corresponding to 55 °C, 66 °C and 79 °C samples (collected from solar manifold)] with a mean of 10 CFU/mL recorded, but were also reduced to BDL (< 1 CFU/100 mL) and were within the drinking water standards for all temperatures analysed, as no faecal coliforms were detected in any of the pasteurized or stored pasteurized samples (results not shown).

Results for the HPC analysis of samples collected at Site 3 during the sampling period are depicted in **Fig. 2.5**. For the sample collected at 55 °C the HPC was reduced from 4.3×10^6 CFU/100 mL before pasteurization to 9.0×10^5 CFU/100 mL after pasteurization, however, the HPC then increased to 1.6×10^6 CFU/100 mL in the 1 500 L storage tank sample. A similar trend was observed for the samples collected at the 61 °C to 66 °C temperature range, where the HPC decreased from a mean of 5.4×10^6 CFU/100 mL before pasteurization to a mean of 2.2×10^6 CFU/100 mL after pasteurization and then subsequently increased in the 1 500 L storage tank (mean 6.9×10^6 CFU/100 mL).

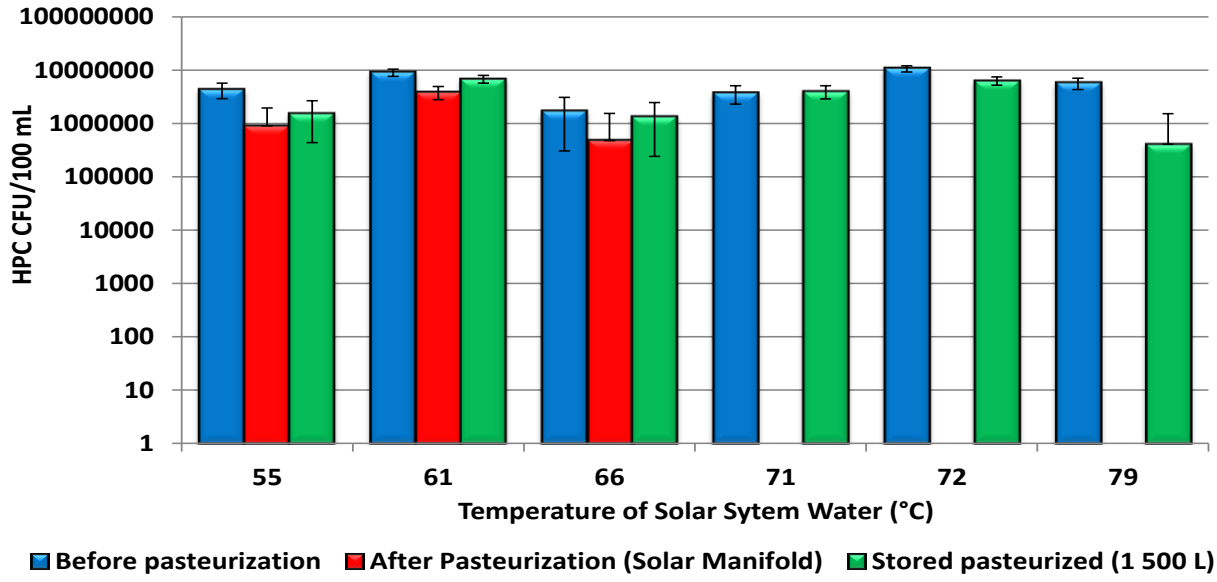

Fig. 2.5. Heterotrophic plate counts (HPC) obtained from the large-scale SOPAS system (Site 3). Samples were collected from three different points along the system: Unpasteurized (Rainwater tank), Pasteurized (Solar Manifold System) and Stored Pasteurized (1 500 L Storage tank) tank water.

Moreover, while the HPC decreased from a mean of 6.7×10^6 CFU/100 mL to BDL (< 1 CFU/100 mL) and was within drinking water standards after pasteurization for all the temperatures above 71 °C, regrowth of the heterotrophic bacteria was again noted in the 1 500 L storage tank (mean of 3.6×10^6 CFU/100 mL). All the unpasteurized and stored pasteurized (1 500 L storage tank) tank water samples collected from Site 3 thus exceeded the

DWAF (1996) limit of 1.0 × 10^4 CFU/100 mL. Results then indicated that the pasteurized tank water samples collected directly from the solar manifold at 71 °C and above were within the DWAF (1996) standard.

Similar to the results obtained at Sites 1 and 2, no significant correlation was observed between the microbiological quality of the unpasteurized tank water collected from Site 3 and rainfall data over the sampling period [total coliforms (r = 0.40; p = 0.43), *E. coli* (r = 0.76; p = 0.08) and HPC (r = -0.21; p = 0.69)]. Pasteurized tank water samples collected from the solar manifold system, where the HPC were recorded at BDL (> 1 CFU/100 mL), were then stored at room temperature in order to determine how long the treated tank water could be stored before microbial regrowth occurred. For the large-scale SOPAS system, the results obtained for the pasteurized tank water samples collected directly from the solar manifold system at 71 °C and above, indicated a > 99% reduction in heterotrophic bacteria as counts were reduced to BDL (< 1 CFU/100 mL). Culturing analysis then indicated that the water could be stored for up to two weeks after treatment at 71 °C, before microbial regrowth occurred (results not shown).

In order to determine which organisms were proliferating in the 1 500 L storage tank, morphologically distinct colonies were isolated from R2A plates and were identified using 16S rRNA PCR as outlined in section 2.2.6. The representative isolates identified included: *Flectobacillus* spp. (GenBank accession no. KJ190182.1), *Acidovorax* spp. (GenBank accession no. KU233259.1), *Pseudomonas* spp. (GenBank accession no. DQ884361.1), *Dechlorosoma* spp. (GenBank accession no. AY171616.1), *Bordetella* spp. (GenBank accession no. KT274791.1), *Novosphingobium* spp. (GenBank accession no. D84598.2) and *Sphingomonas* spp. (GenBank accession no. KF441634.1).

2.3.4 Presence of metabolically active cells in the unpasteurized and pasteurized tank water samples

2.3.4.1 Small-scale SOPAS systems (Sites 1 and 2)

The BacTiter-Glo™ Microbial Cell Viability Assay was utilised as a presence/absence test, in order to determine whether viable (metabolically active) cells were present in any of the tank water samples collected before and after pasteurization. The assay measures the total ATP in the water sample, as was thus not specific for the detection of a target organism. Thus an overall indication of viability in the tank water samples was obtained. Duplicate unpasteurized and pasteurized tank water and background controls (sterile RNAse/DNAse free water) were measured for each sampling event. A triplicate technical repeat was also performed. The mean luminescent signals (RLU) obtained for the background control samples were subtracted from the mean before and after pasteurization luminescent signals (RLU) for each specific temperature (Reyneke et al., 2016). The results (mean luminescent signal obtained before and

after pasteurization) obtained for the small-scale SOPAS systems using the BacTiter-Glo™ Microbial Cell Viability Assay are depicted in **Fig. 2.6**. Results obtained for the small-scale systems indicate that there was a significant reduction ($p = 0.00003$) in ATP (indicator of metabolically active cells) following SOPAS.

For the small-scale SOPAS system located at Site 1, the mean luminescent signal decreased from 2.32×10^4 RLU/100 µL before pasteurization to 2.84×10^3 RLU/100 µL after pasteurization at 52 °C (0.91 log reduction). Similarly, the mean luminescent signal decreased from 3.99×10^4 RLU/100 µL before pasteurization to 2.94×10^3 RLU/100 µL after pasteurization (1.14 log reduction) and from 1.56×10^4 RLU/100 µL before pasteurization to 1.68×10^3 RLU/100 µL after pasteurization (0.95 log reduction), for the samples treated at 60 °C and 67 °C, respectively.

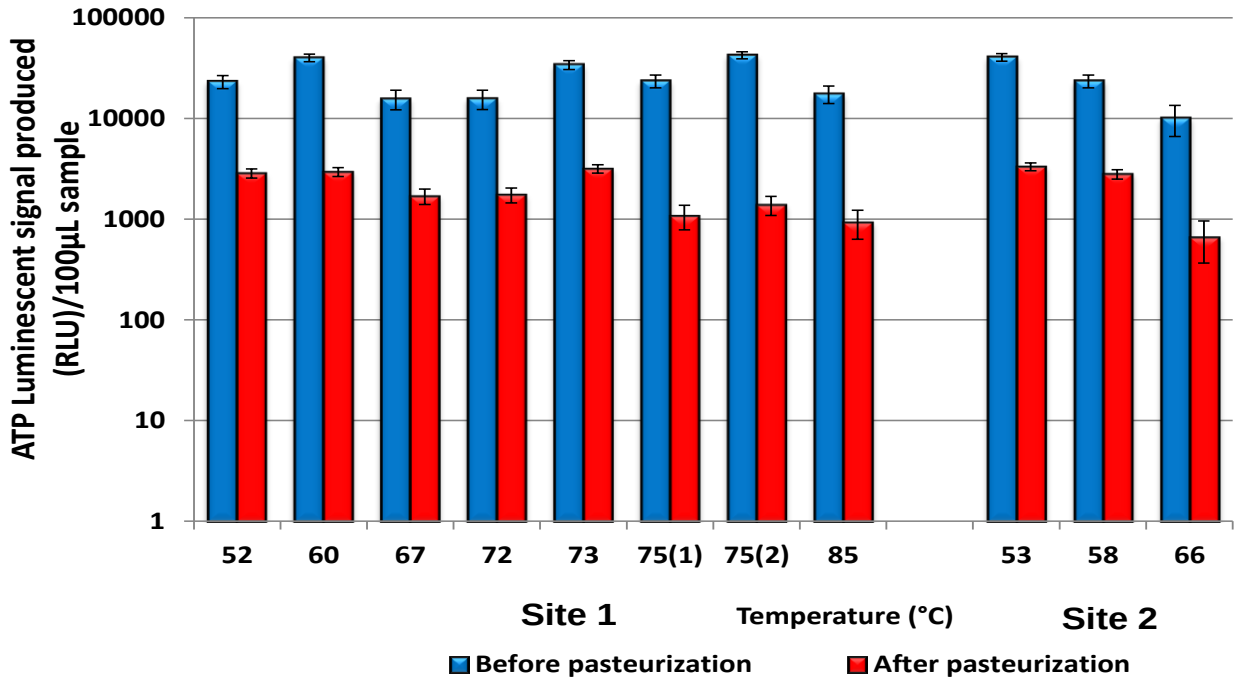

Fig. 2.6. Mean relative light units (RLU) detected before and after SOPAS in the tank water samples collected from the small-scale SOPAS systems (Sites 1 and 2).

For the samples collected at 72 °C to 75 °C, the mean luminescent signal decreased from 2.89×10^4 RLU/100 µL before pasteurization to 1.84×10^3 RLU/100 µL after pasteurization (1.21 log reduction). Additionally, a mean luminescent signal of 1.74×10^4 RLU/100 µL before pasteurization was reduced to 9.26×10^2 RLU/100 µL, following SOPAS at 85 °C (1.28 log reduction). Similarly, for Site 2, following SOPAS treatment at 53 °C and 58 °C, a decrease of 0.99 log was observed as the mean RLU decreased from 3.2×10^4 RLU/100 µL before pasteurization to 3.0×10^3 RLU/100 µL after pasteurization. For the sample collected at 66 °C, the mean RLU/100 µL decreased from 1.1×10^4 to 6.6×10^2 (1.18 log reduction) after SOPAS. However, results indicate that viable cells are still present in all of the pasteurized tank water

samples collected from the two small-scale systems, including the samples where culturing analysis indicated that the HPC was BDL [Site 1 – 67 °C, 72 °C, 73 °C, 75 °C (1 and 2) and 85 °C; Site 2 – 66 °C].

2.3.4.2 Large-scale SOPAS system (Site 3)

Correspondingly, the BacTiter-Glo™ Microbial Cell Viability Assay (Promega) was utilised as a presence/absence test, to determine whether viable cells were present in any of the tank water samples collected from the large-scale SOPAS system. Duplicate unpasteurized, pasteurized and stored pasteurized tank water and background controls (sterile RNAse/DNAse free water) were measured for each sample. The results obtained for the large-scale SOPAS system using the BacTiter-Glo™ Microbial Cell Viability Assay are depicted in **Fig. 2.7**.

Results obtained for the large-scale system indicated that there was a significant reduction (p = 0.0013) in viable cells following SOPAS. For the water sample collected at 55 °C, the mean luminescent signal decreased from 6.3 × 10^4 RLU/100 µL in the unpasteurized sample to 7.0 × 10^3 RLU/100 µL in the pasteurized sample collected directly from the manifold system (0.95 log reduction). However, an increase in RLU was then observed in the stored pasteurized sample, as a value of 3.1 × 10^4 RLU/100 µL was obtained (overall decrease of 0.31 log in comparison to the unpasteurized sample).

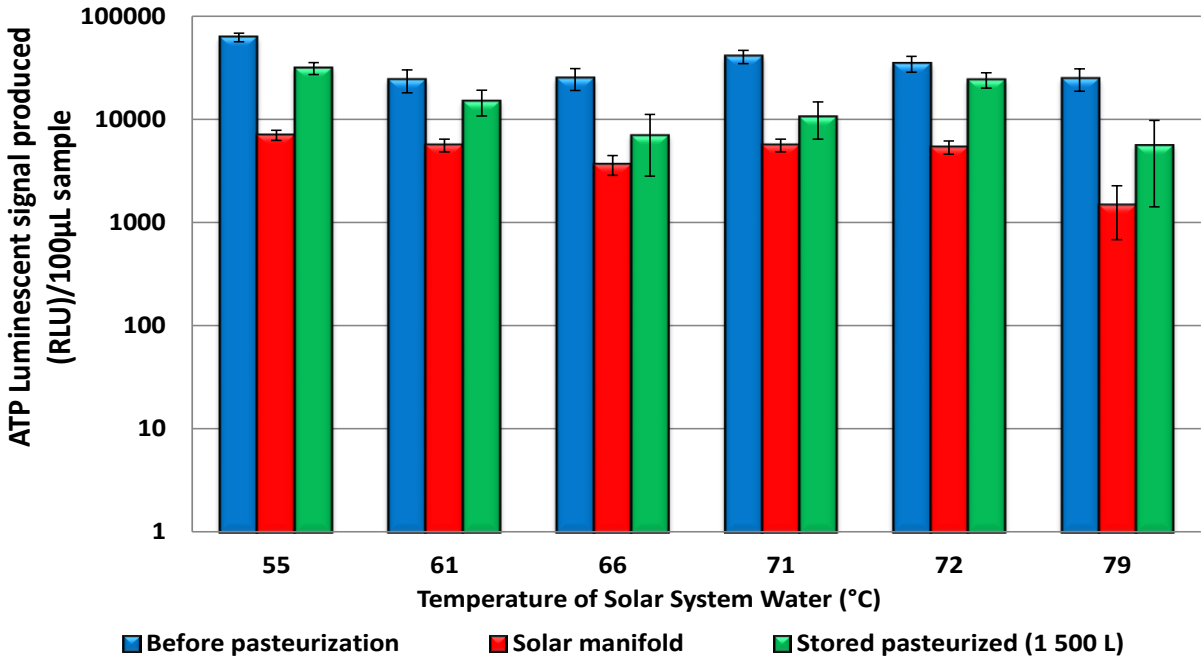

Fig. 2.7. Mean relative light units (RLU) detected in the unpasteurized and corresponding pasteurized (collected directly from solar manifold system) and stored pasteurized (collected from 1 500 L storage tank) tank water samples.

For the samples collected at 61 °C and 66 °C, the mean luminescent signal decreased from 2.5×10^4 RLU/100 µL in the unpasteurized sample to 4.6×10^3 RLU/100 µL in the pasteurized sample (0.74 log reduction). An increase in the mean RLU was again observed in the stored pasteurized sample (1.1×10^4 RLU/100 µL) (overall decrease of 0.36 log in comparison to the unpasteurized sample). Finally, for the temperature range 71 °C to 79 °C, a mean decrease in the luminescent signal of 3.3×10^4 RLU/100 µL in the unpasteurized sample, to 4.2×10^3 RLU/100 µL in the pasteurized sample, was observed (0.89 log reduction). However, as was observed for the samples collected at 55 °C, 61 °C and 66 °C, an increase in the mean luminescent signal was observed for the stored pasteurized samples (1.3×10^4 RLU/100 µL) (overall decrease of 0.40 log in comparison to the unpasteurized sample). These BacTiter-Glo™ Microbial Cell Viability Assay results corresponded to the results obtained during the culturing analysis, where an increase in viable cells following pasteurization in the solar manifold system was observed after the water enters and is stored in the 1 500 L tank. Thus while a significant decrease in RLU was observed during SOPAS (0.85 log reduction overall), microbial cells could proliferate in the storage tank of the large-scale system.

2.3.5 Genus-specific PCRs for identification of indigenous rainwater pathogens

2.3.5.1 Small-scale SOPAS systems (Sites 1 and 2)

Genus-specific PCR assays (detection of pathogens and opportunistic pathogens) were performed on all unpasteurized and pasteurized tank water samples collected from the small-scale SOPAS systems located at Sites 1 and 2, respectively (**Fig. 2.8**, **Fig. 2.9** and **Appendix B Tables B1** and **B2**). For Site 1, *Aeromonas* spp. and *Shigella* spp. were not detected in any of the unpasteurized or pasteurized tank water samples (results not shown), while *Bacillus* spp. (GenBank accession no. KJ534453.1) were detected in 25% (n = 2) of the unpasteurized and 13% (n = 1; 60 °C) of the pasteurized tank water samples (**Fig. 2.8**). Similarly, *Enterococcus* spp. (GenBank accession no. CP014529.1) and *Yersinia* spp. (GenBank accession no. CP016945.1) were detected in 25% (n = 2) of the unpasteurized tank water samples, but were not detected in any tank water samples following pasteurization treatment.

Salmonella spp. (GenBank accession no. CP009565.1) and adenovirus (GenBank accession no. K01264.1; JN381195.1) were then only detected in 50% (n = 4) and 38% (n = 3) of the unpasteurized tank water samples, respectively, but were also absent in the pasteurized tank water samples (**Fig. 2.8**). In contrast, *Klebsiella* spp. (GenBank accession no. AF303617.1) and *Streptomyces* spp. (GenBank accession no. KP082881.1) were both detected in 75% (n = 6) of the unpasteurized tank water samples and in 25% (n = 2; *Klebsiella* spp., 72 °C and 73 °C; *Streptomyces* spp., 60 °C and 72 °C) of the pasteurized tank water samples. Similarly, *Serratia* spp. (GenBank accession no. CP005927.1) were detected in 63% (n = 5) of the

unpasteurized tank water samples, whereafter their detection in the pasteurized samples decreased to 38% (n = 3; 52 °C, 67 °C and 72 °C).

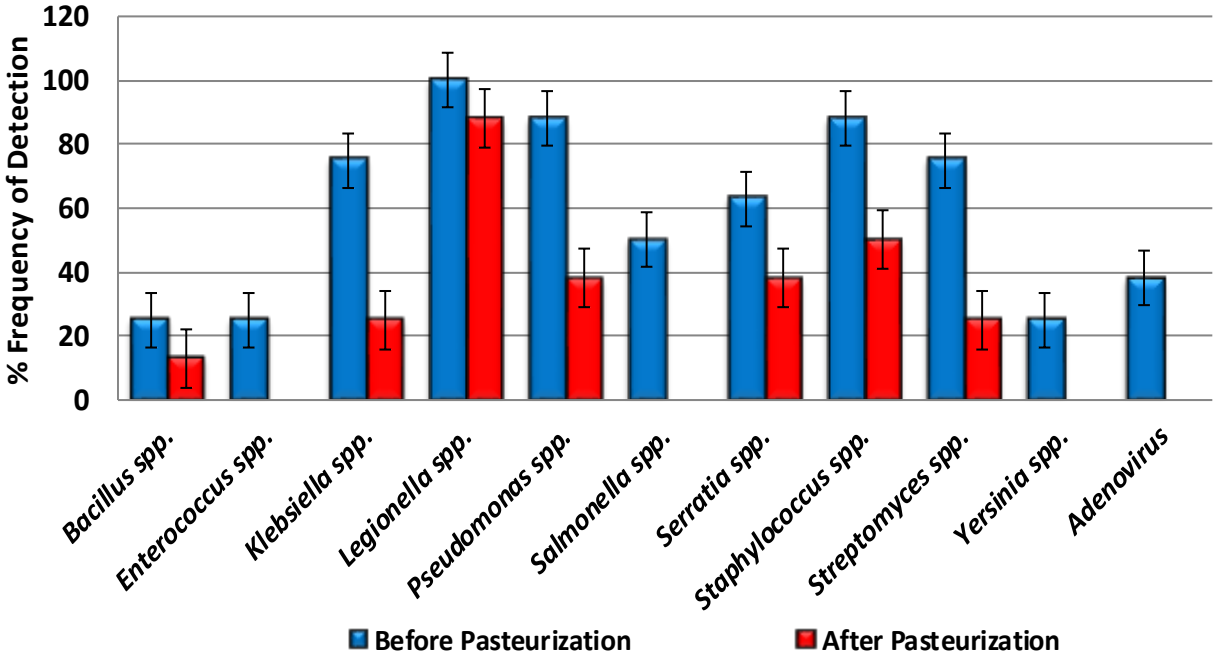

Fig. 2.8. Frequency of detection (%) for the indigenous rainwater pathogens detected before pasteurization (n = 8) and after pasteurization (n = 8) from the small-scale SOPAS system located at Site 1.

Both *Pseudomonas* spp. (GenBank accession no. LC130640.1; KU321261.1) and *Staphylococcus* spp. (GenBank accession no. CP009623.1) were detected in 88% (n = 7) of the unpasteurized tank water samples, however, *Pseudomonas* spp. were only detected in 38% (n = 3; 60 °C, 72 °C and 75 °C) of the pasteurized tank water samples, while *Staphylococcus* spp. were detected in 50% (n = 4; 52 °C, 72 °C, 73 °C and 75 °C) of the pasteurized tank water samples. For both these organisms the highest pasteurization temperature of detection was recorded as 75 °C. *Legionella* spp. (GenBank accession no. LC094348.1; AB933899.1) were the most prevalent opportunistic pathogens as 100% (n = 8) of the unpasteurized tank water samples tested positive for *Legionella* spp., while 88% (n = 7; 52 °C, 60 °C, 67 °C, 72 °C, 73 °C, 75 °C and 85 °C) of the pasteurized tank water samples tested positive for the presence of *Legionella* spp.

For Site 2, *Aeromonas* spp. and *Shigella* spp., *Bacillus* spp., *Enterococcus* spp., *Serratia* spp. and *Yersinia* spp. were not detected in any of the unpasteurized or pasteurized tank water samples (results not shown). *Klebsiella* spp. and adenovirus were detected in 33% (n = 1) of the unpasteurized tank water samples (**Fig. 2.9**). However, *Klebsiella* spp. were not detected in any pasteurized tank water samples, while adenovirus was detected in one of the pasteurized tank water samples (58 °C).

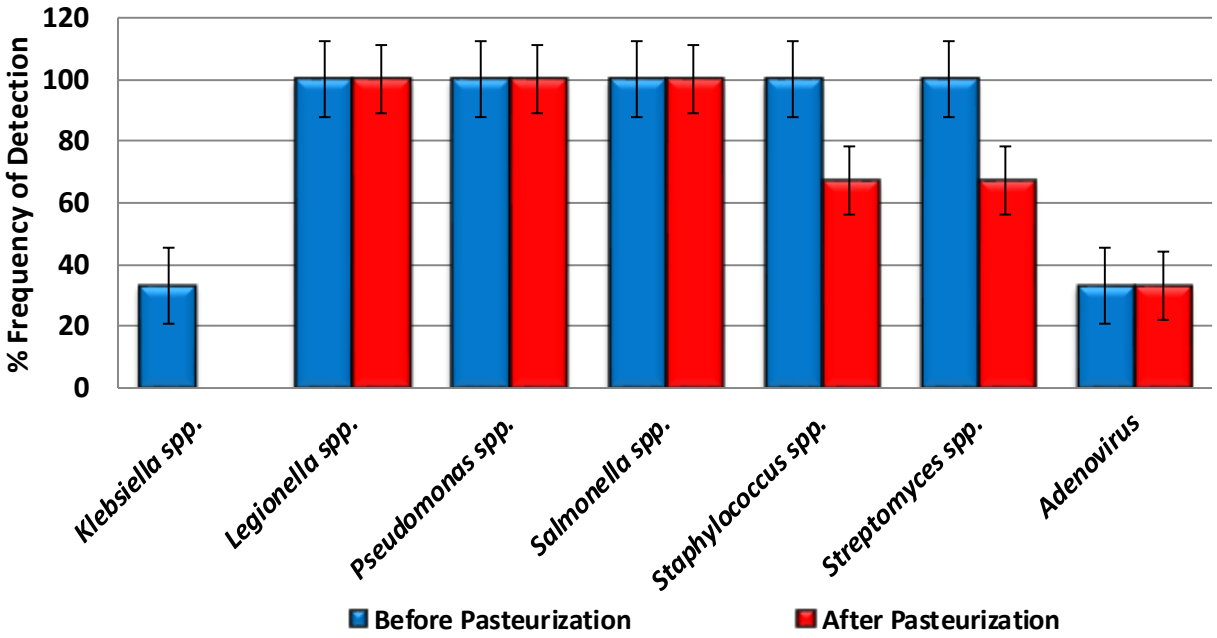

Fig. 2.9. Frequency of detection (%) for the indigenous rainwater pathogens before pasteurization (n = 3) and after pasteurization (n = 3) from the small-scale SOPAS system located at Site 2.

Additionally, while *Staphylococcus* spp. and *Streptomyces* spp. were detected in all unpasteurized tank water samples (n = 3), they were only detected in 67% (n = 2; 53 °C and 66 °C; 53 °C and 58 °C, respectively) of the pasteurized tank water samples, respectively. In contrast, *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp., were detected in all unpasteurized and pasteurized tank water samples (n = 3), respectively (**Fig. 2.9**).

2.3.5.2 Large-scale SOPAS system (Site 3)

Genus-specific PCR assays were also performed on unpasteurized and stored pasteurized tank water samples collected from the large-scale SOPAS system located at Site 3 (**Appendix B Table B3**). The PCR assays were utilised to screen for opportunistic bacterial and viral pathogens considered indigenous to rainwater sources. The frequency of detection for all the selected organisms screened for in the tank water samples are represented in **Fig 2.10**.

Aeromonas spp., *Shigella* spp. and *Yersinia* spp. were not detected in any of the unpasteurized (n = 6) or stored pasteurized (n = 6) tank water samples (results not shown), while *Enterococcus* spp. were only detected in 17% (n = 1) of the unpasteurized tank water samples (**Fig 2.10**). In contrast, *Streptomyces* spp. and adenovirus were both detected in one unpasteurized tank water sample (17%) and in 33% (n = 2) and 50% (n = 3) of the stored pasteurized tank water samples, respectively. *Bacillus* spp. were detected in 50% (n = 3) of the unpasteurized and stored pasteurized tank water samples, respectively, while *Salmonella* spp. were only detected in 33% (n = 2) and 17% (n = 1) of the unpasteurized and stored pasteurized

tank water samples, respectively. As indicated in **Fig. 2.10**, the frequency of detection of *Klebsiella* spp. decreased from 83% (n = 5) to 50% (n = 3), for *Pseudomonas* spp. from 67% (n = 4) to 33% (n = 2), while a decrease from 67% (n = 4) to 50% (n = 3) was observed for *Serratia* spp., when comparing the unpasteurized to the stored pasteurized tank water samples.

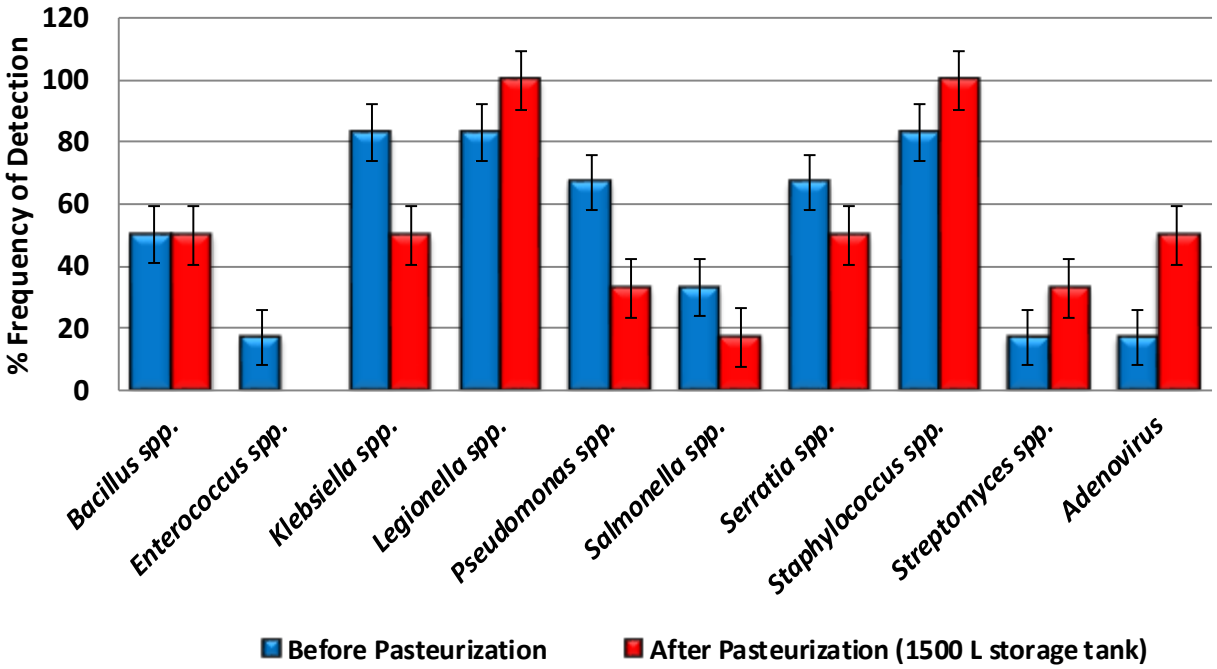

Fig. 2.10. Frequency of detection (%) for the indigenous rainwater pathogens detected before pasteurization (n = 6) and after pasteurization (n = 6) (1 500 L storage tank) from the large-scale SOPAS system.

Overall, for the large-scale system, *Legionella* spp. and *Staphylococcus* spp. were the most prevalent bacteria, as they were detected in 83% of the unpasteurized tank water samples and 100% of the stored pasteurized tank water samples, respectively.

2.3.6 Quantitative PCR analysis of the prevalent indigenous rainwater pathogens

Based on the results obtained during the conventional PCR analysis of the tank water samples collected from Sites 1, 2 and 3, *Legionella* spp., *Staphylococcus* spp., *Pseudomonas* spp. and *Salmonella* spp. were identified as the most readily detected indigenous rainwater pathogens and were thus quantified using qPCR. However, during the *Staphylococcus* spp. qPCR analysis, after numerous optimisation attempts, multiple melting peaks were obtained in a single sample for the melt curve analysis, which indicated that non-specific binding of the primers occurred. The accurate quantification of *Staphylococcus spp*. could thus not be performed and subsequently the gene copies for only *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp. were quantified for the small-scale SOPAS system located at Site 1 (broader temperature range was obtained at Site 1 as compared to Site 2) and the large-scale SOPAS system located at Site 3.

The qPCR assays for the quantification of *Legionella* spp. in both the small-scale and large-scale system had an average amplification efficiency (E) of 93% (1.86) and a correlation coefficient (R^2) of 0.99, while the lower limit of detection (LLOD) was determined to be 8 to 12 gene copies/µL. For the quantification of *Pseudomonas* spp. in both the small-scale and large-scale systems, the qPCR assays had an average amplification efficiency (E) of 92% (1.84) and a correlation coefficient (R^2) of 0.99, while the LLOD was determined to be 5 to 11 gene copies/µL. Lastly, for the quantification of *Salmonella* spp. in both the small-scale and large-scale system, the qPCR assays had an average amplification efficiency (E) of 97% (1.94) and a correlation coefficient (R^2) of 0.99, while the LLOD was determined to be 6 to 9 gene copies/µL.

2.3.6.1 Small-scale SOPAS system (Site 1)

The qPCR analysis for *Legionella* spp. indicated that there was a significant ($p = 0.034$) reduction in 23S rRNA gene copies/mL following SOPAS treatment with the small-scale system (**Fig. 2.11A**). For the sample collected at 52 °C, the gene copies/mL decreased by 84.5% from 3.97×10^6 gene copies/mL before pasteurization to 6.06×10^5 gene copies/mL after pasteurization (0.81 log reduction). When comparing the before and after pasteurization samples collected at 60 °C and 67 °C, the mean gene copies/mL decreased from 8.01×10^5 gene copies/mL before pasteurization to 4.21×10^5 gene copies/mL after pasteurization (47.5%; 0.28 log reduction). For the samples collected after pasteurization at 72 °C to 75 °C [72 °C, 73 °C and 75 °C (1 and 2)], a mean decrease of 92.6% was observed in the gene copies/mL, as 2.87×10^6 gene copies/mL detected before pasteurization decreased to 2.14×10^5 gene copies/mL after pasteurization (1.13 log reduction). For the sample collected at 85 °C, the highest reduction (96.5%) was observed as the gene copies/mL decreased from 5.80×10^6 gene copies/mL before pasteurization to 2.03×10^5 gene copies/mL after pasteurization (1.46 log reduction).

Similarly, the qPCR analysis for *Pseudomonas* spp. indicated that there was a reduction ($p = 0.058$) in *oprI* gene copies/mL following SOPAS treatment using the small-scale system (**Fig. 2.11B**). For the sample collected at 52 °C, the gene copies/mL decreased by 64.5% from 2.26×10^6 gene copies/mL before pasteurization to 7.95×10^5 gene copies/mL after pasteurization (0.45 log reduction). Similarly, the mean gene copies/mL decreased from 8.15×10^6 gene copies/mL before pasteurization to 3.12×10^6 gene copies/mL after pasteurization at 60 °C to 67 °C (61.98%; 0.42 log reduction). For the samples collected after pasteurization at 72 °C to 75 °C [72 °C, 73 °C and 75 °C (1 and 2)], a 97.3% decrease in the mean gene copies/mL was observed, as 3.32×10^7 gene copies/mL were detected before pasteurization, which decreased to 8.92×10^5 gene copies/mL after pasteurization (1.57 log reduction).

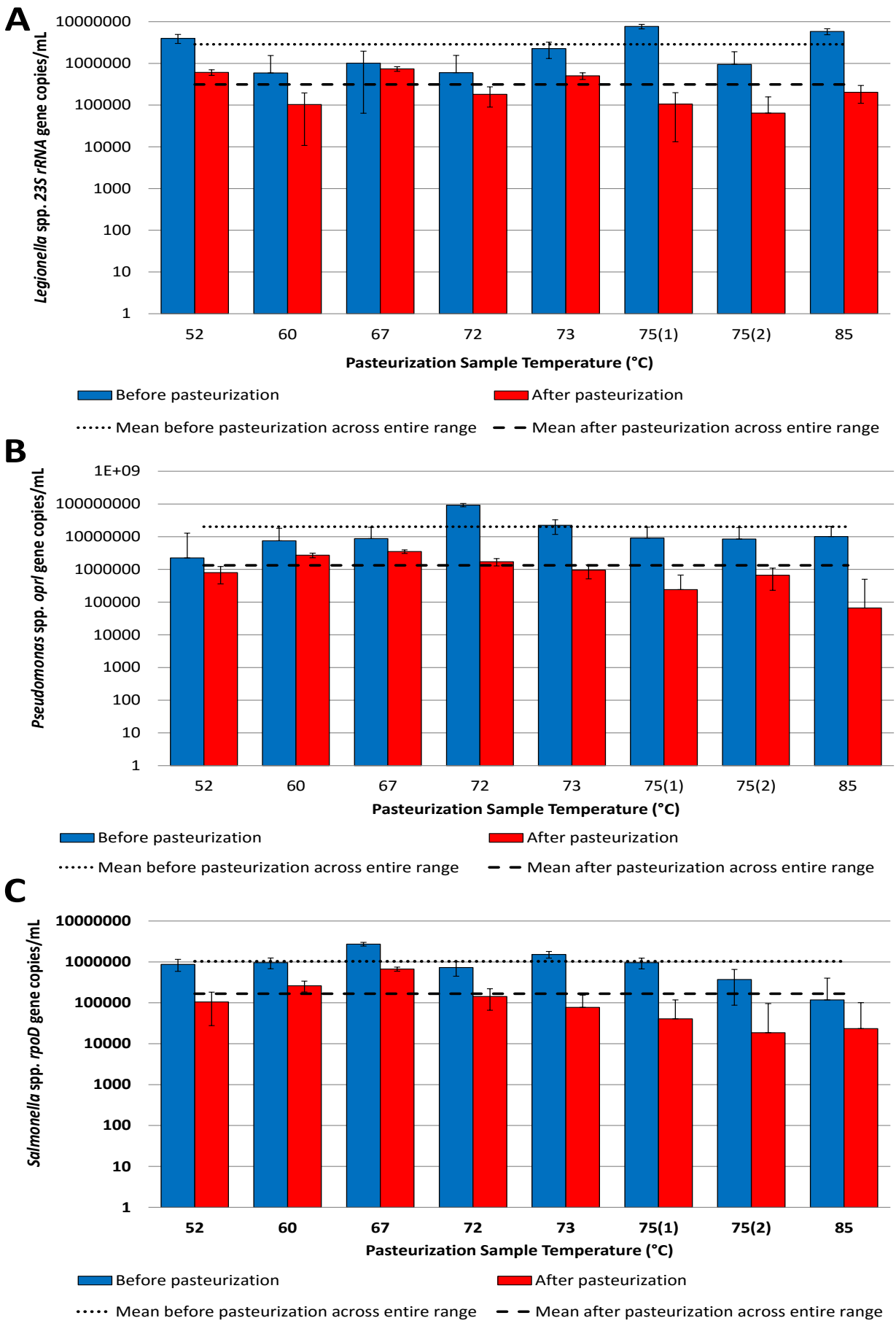

Fig. 2.11. Quantification of *Legionella* spp. **(A)**, *Pseudomonas* spp. **(B)** and *Salmonella* spp. **(C)** in the tank water samples collected from the small-scale system at Site 1.

Similar to the results obtained for the *Legionella* spp. gene copies/mL analysis, after pasteurization at 85 °C, the highest reduction of 99.3% in *Pseudomonas* spp. gene copies/mL was observed. For the sample collected at 85 °C, the gene copies/mL decreased from 1.01×10^7 gene copies/mL before pasteurization to 6.62×10^4 gene copies/mL after pasteurization (2.18 log reduction).

The qPCR analysis for *Salmonella* spp. indicated that there was a significant reduction ($p = 0.0057$) in *rpoD* gene copies/mL following SOPAS treatment using the small-scale system (**Fig. 2.11C**). For the sample collected at 52 °C, the gene copies/mL decreased by 87.98%, as 8.71×10^5 gene copies/mL were detected before pasteurization, which was reduced to 1.05×10^5 gene copies/mL after pasteurization (0.92 log decrease). When comparing the before and after pasteurization samples collected at 60 °C and 67 °C, a 74.9% decrease in the mean gene copies/mL was observed as the gene copies/mL decreased from 1.84×10^6 gene copies/mL before pasteurization to 4.66×10^5 gene copies/mL after pasteurization (0.60 log decrease). For the samples collected after pasteurization at 72 °C to 75 °C [72 °C, 73 °C and 75 °C (1 and 2)], the highest reduction of 92.23% was recorded as the mean gene copies/mL then decreased from 8.93×10^5 gene copies/mL before pasteurization to 6.97×10^4 gene copies/mL after pasteurization (1.11 log decrease). A 0.70 log decrease was observed following SOPAS at 85 °C, as the gene copies/mL decreased from 1.18×10^5 gene copies/mL before pasteurization to 2.34×10^4 gene copies/mL after pasteurization (80% decrease).

2.3.6.2 Large-scale SOPAS system (Site 3)

For the samples collected from the large-scale system, the qPCR analysis for *Legionella* spp. indicated that there was an overall reduction ($p = 0.297$) in *Legionella* spp. following SOPAS treatment (sample collected from the solar manifold), however an increase ($p = 0.781$) in *Legionella* spp. was observed in the tank water samples collected from the 1 500 L storage tank (**Fig. 2.12A**). For the sample collected at 55 °C, the gene copies/mL decreased by 0.51 log as 4.05×10^6 gene copies/mL detected before pasteurization decreased to 1.26×10^6 gene copies/mL after pasteurization (69.1% decrease). An 18.72% increase in gene copies/mL was then noted in the stored pasteurized sample, as the gene copies/mL increased to 1.54×10^6 gene copies/mL (0.09 log increase). For the samples collected after pasteurization at 61 °C to 66 °C, the mean gene copies/mL decreased by 86.2% from 1.38×10^5 gene copies/mL before pasteurization to 1.90×10^4 gene copies/mL after pasteurization (0.86 log decrease). The detected gene copies/mL then increased by 93.5% to 2.96×10^5 gene copies/mL in the stored pasteurized sample (1.19 log increase).

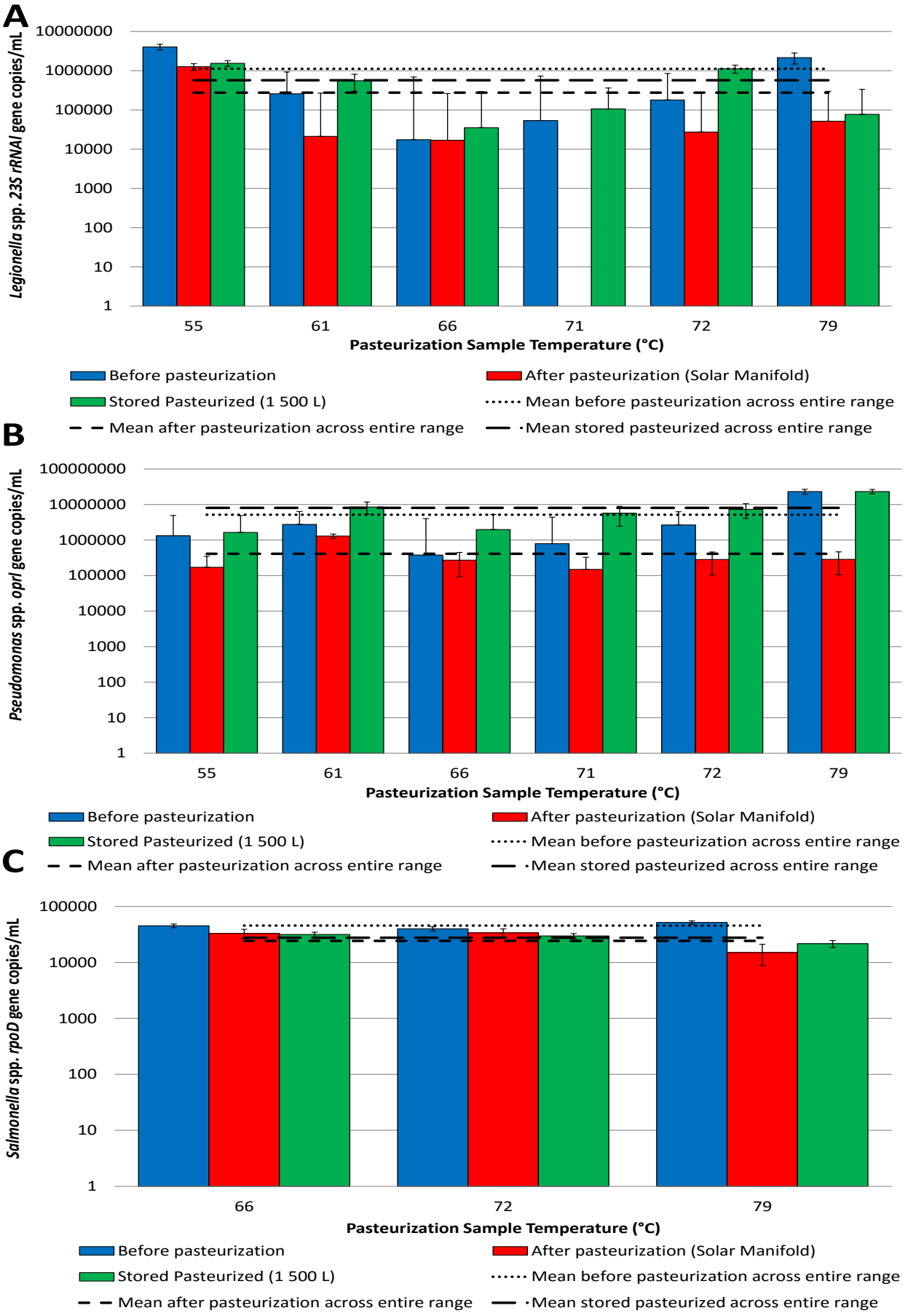

Fig. 2.12. Quantification of *Legionella* spp. **(A)**, *Pseudomonas* spp. **(B)** and *Salmonella* spp. **(C)** in the tank water samples collected from the large-scale system at Site 3.

However, for the sample collected at 71 °C, the gene copies/mL decreased by > 99.99%, from 5.38 × 10^4 gene copies/mL before pasteurization to below the LLOD (< 8 to 12 gene copies/µL) after pasteurization (4.73 log reduction). However, the detected 23S rRNA gene copies then increased by > 99.99% to 1.06 × 10^5 gene copies/mL in the stored pasteurized sample (5.02 log increase).

For the sample collected after pasteurization at 72 °C the mean gene copies/mL decreased from 1.80 × 10^5 gene copies/mL before pasteurization to 2.74 × 10^4 gene copies/mL (84.8%; 0.82 log decrease) after pasteurization and then increased by 97.6% to 1.13 × 10^6 gene copies/mL in the stored pasteurized sample (1.62 log increase). For the sample collected after pasteurization at 79 °C the mean gene copies/mL decreased from 2.15 × 10^6 gene copies/mL before pasteurization to 5.18 × 10^4 gene copies/mL (97.5%; 1.61 log decrease) after pasteurization and then increased by 32.3% to 7.72 × 10^4 gene copies/mL in the stored pasteurized sample (0.17 log increase).

The qPCR analysis of the samples collected from the large-scale system indicated that there was a decrease (p = 0.246) in *Pseudomonas* spp. following SOPAS treatment (sample collected from the solar manifold), however an increase (p = 0.639) in *Pseudomonas* spp. was observed in the tank water samples collected from the 1 500 L storage tank (**Fig. 2.12B**). An 87.1% decrease in the gene copies/mL was observed as the gene copies decreased from 1.32 × 10^6 gene copies/mL before pasteurization to 1.72 × 10^5 gene copies/mL after pasteurization at 55 °C (0.89 log decrease). The gene copies/mL for *Pseudomonas* spp. then increased by 89.3% to 1.64 × 10^6 gene copies/mL in the stored pasteurized sample (0.97 log increase). Additionally, at pasteurization temperatures of 61 °C to 66 °C, the mean gene copies/mL decreased by 49.9%, from 1.57 × 10^6 gene copies/mL before pasteurization to 7.84 × 10^5 gene copies/mL after pasteurization (0.30 log decrease), while an 85.2% increase to 5.26 × 10^6 gene copies/mL was then noted in the stored pasteurized sample (0.83 log increase).

For the sample collected at 71 °C, an 80.9% decrease was observed, as the gene copies/mL decreased from 7.88 × 10^5 gene copies/mL before pasteurization to 1.49 × 10^5 gene copies/mL after pasteurization (0.72 log decrease). A 1.58 log increase (97.4%) in gene copies was then recorded for the stored pasteurized sample (5.68 × 10^6 gene copies/mL). For the samples collected after pasteurization at 72 °C and 79 °C, the mean gene copies/mL decreased from 1.29 × 10^7 gene copies/mL before pasteurization to 2.84 × 10^5 gene copies/mL after pasteurization (97.8%; 1.66 log decrease). However, the detected gene copies/mL then increased by 98.1% to 1.52 × 10^7 gene copies/mL in the stored pasteurized sample (1.73 log increase).

The qPCR analysis of the tank water samples collected from the large-scale system corresponded to the results obtained during the conventional PCR analysis where *Salmonella* spp. were only sporadically detected in the tank water samples collected from the large-scale system. Results from the qPCR analysis indicated that *Salmonella* spp. were below the LLOD (6 to 9 gene copies/µL) in the unpasteurized and pasteurized (samples collected directly from the solar manifold) tank water samples collected for the 55 °C, 61 °C and 71 °C pasteurization temperatures. However, *Salmonella* spp. were detected in the corresponding stored pasteurized samples at a mean concentration of 5.4×10^4 gene copies/mL (results not shown). For the remaining samples (66 °C, 72 °C and 79 °C) the results obtained indicated that there was a reduction ($p = 0.190$) in *rpoD* gene copies/mL following SOPAS treatment (sample collected from the solar manifold), however, the concentration of *Salmonella* spp. remained comparable in the tank water samples collected from the 1 500 L storage tank ($p = 0.921$) (**Fig. 2.12C**). For the samples collected at 66 °C, 72 °C and 79 °C, the gene copies/mL decreased by 27.6%, 14.9% and 71.2%, respectively, as the detected gene copies decreased from 4.51×10^4 gene copies/mL, 3.98×10^4 gene copies/mL and 5.17×10^4 gene copies/mL before pasteurization to 3.30×10^4 gene copies/mL, 3.38×10^4 gene copies/mL and 1.50×10^4 gene copies/mL after pasteurization, respectively (0.14, 0.07 and 0.54 log decrease, respectively). However, *Salmonella* spp. were detected in all the corresponding stored pasteurized samples at a mean concentration of 3.2×10^4 gene copies/mL.

2.3.7 Operational sustainability of the small-scale and large-scale SOPAS systems

2.3.7.1 Volume of pasteurized tank water produced by the SOPAS systems

In order to determine the volume of pasteurized tank water that could be produced by the small-scale systems located at Sites 1 and 2, **Eq. 2b** was utilised. The energy output of one evacuated tube was 62 W (personal communication with manufacturer) and with 10 evacuated tubes being used in a system, the total thermal energy (Q) was calculated as 620 W which was converted to 2232 kJ/h (3.6 MJ = 1 kWh). Using the specific heat (c_p) of water (4.2 J/g °C), the volume of pasteurized tank water produced (m) could be calculated by inserting the unpasteurized (input) and pasteurized (output) temperatures of the collected tank water samples (as indicated in **Table 2.3**) into **Eq. 2b**. Using an average of 6 hrs sunshine per day, 1000 W/m^2 solar irradiance and a 30% solar energy conversion rate, 17.14 kg/h pasteurized tank water could be produced at 52 °C for the small-scale system located at Site 1. For the 60 °C and 67 °C tank water samples collected at Site 1, a mean of 12.75 kg/h pasteurized tank water could be produced. The volume of pasteurized tank water that could be produced by the system at Site 1 then decreased to 10.84 kg/h for the 72 °C to 75 °C temperature range. A volume of 8.74 kg/h could then be produced at 85 °C. Similarly for Site 2, 16.25 kg/h

pasteurized water could be produced for the 53 °C to 58 °C temperature range, while 12.81 kg/h pasteurized water could be produced for the 66 °C pasteurization temperature.

Additionally, it was determined that due to the design of the small-scale system (outlet tap located near the top of the SOPAS storage tank and inlet point located at the bottom of the SOPAS storage tank), 8 L of pasteurized tank water could be collected from the system before unpasteurized tank water would start entering the small-scale SOPAS system. However, it was also determined that while unpasteurized tank water would start entering the system, the temperature of the pasteurized tank water would not decrease immediately. By draining a small-scale Phungamanzi™ SOPAS system that had been installed on Welgevallen Experimental Farm, Stellenbosch University, South Africa (GPS Coordinates: 33°56' 36.19"S, 18°52' 06.08"E), it was determined that 124 L, 136 L, and 132 L of hot water could be collected from the system at starting temperatures of 90 °C, 81 °C and 73 °C, before the temperature of the rainwater would decrease to 66 °C. Additionally, it was determined that the water inside the system will increase from 20 °C to above 70 °C after a cumulative 8 hrs sunshine exposure; however, this time period will also be dependent on climate conditions and the initial temperature inside the small-scale SOPAS storage tank.

For the large-scale system (Site 3), the average total thermal energy (Q) for a solar manifold system installed in the Western Cape was reported to be 6480 kJ/h (personal communication with manufacturer). Using the specific heat (c_p) of water (4.2 J/g °C), the volume of pasteurized tank water produced (m) could be calculated by inserting the unpasteurized (input) temperatures of the collected tank water samples (as indicated in **Table 2.4**) into **Eq. 2b** and using 75 °C (temperature at which the thermostatic release valve is set to open) as the output temperature. The mean volume of pasteurized tank water that could be produced by the system at an output temperature of 75 °C was calculated to be 30.04 kg/h.

2.3.7.2 Monitoring the water usage by the households utilising the SOPAS systems

In order to monitor the water usage for each of the ten participating households, a co-researcher on the project (inhabitant of Enkanini informal settlement) was provided with a log book for each site (**Appendix A**). The co-researcher visited the participating households every Friday from September 2015 to September 2016 and enquired how much water the household was collecting from the SOPAS systems per day and how much water was being collected from the municipal standpipes per day. The answers were recorded directly into the log books and a detailed summary of the results is provided in **Appendix A**.

For the small-scale SOPAS system located at Site 1, three households (House 1, 2 and 3) had access to the system. Monitoring of the pasteurized tank water usage indicated that 93 L/week, 78 L/week and 96 L/week, of pasteurized tank water was being used by House 1, 2 and 3,

respectively, during the weeks when pasteurized tank water was available (**Appendix A Table A4**). Over the same time period, 218 L/week, 246 L/week and 260 L/week of municipal tap water was being used by House 1, 2 and 3, respectively. However, in the weeks when pasteurized tank water was not available [October 2015 (pressure release pipe needed to be inserted into the system), December 2015 to January 2016 (rainwater harvesting tank started to empty and borosilicate glass tube broke)], the households' weekly municipal tap water usage increased to 385 L/week, 410 L/week and 430 L/week, for House 1, 2 and 3, respectively.

For the small-scale SOPAS system located at Site 2, three households (Church 1, 2 and 3) had access to the system. Monitoring of the pasteurized tank water usage indicated that 97 L/week, 112 L/week and 127 L/week, of pasteurized tank water was being used by Church 1, 2 and 3, respectively (**Appendix A Table A5**). Over the same time period, 13 L/week, 183 L/week and 226 L/week of municipal tap water was being used by Church 1, 2 and 3, respectively. However, in the weeks when less pasteurized tank water was available for the households to use [December 2015 to January 2016 (outlet tap of SOPAS system needed to be replaced and rainwater harvesting tank started to empty), May 2016 (rainwater harvesting tank started to empty), June 2016 (borosilicate glass tube broke)], Church 1, 2 and 3 used 48 L/week, 37 L/week and 48 L/week, of the pasteurized tank water, respectively, while the households' weekly municipal tap water usage increased to 16 L/week, 291 L/week and 438 L/week, for Church 1, 2 and 3, respectively.

For the large-scale SOPAS system located at Site 3, four households (ERC 1, 2, 3 and 4) had access to the system. Monitoring of the pasteurized tank water usage indicated that 146 L/week, 123 L/week, 130 L/week and 85 L/week, of pasteurized tank water was being used by ERC 1, 2, 3 and 4, respectively, in the weeks when pasteurized tank water was available (**Appendix A Table A6**). Over the same time period, 271 L/week, 187 L/week, 202 L/week and 108 L/week of municipal tap water was being used by ERC 1, 2, 3 and 4, respectively. However, during May 2016 to August 2016 when less pasteurized tank water was being used [ERC 1 = 39 L/week, ERC 2 = 84 L/week, ERC 3 = not determined (relocated from Enkanini) and ERC 4 = 20 L/week], the households' weekly municipal tap water usage increased to 476 L/week, 295 L/week and 115 L/week, for ERC 1, 2 and 4, respectively. It is however important to note that the pasteurized water does not remain heated inside the 1 500 L storage tank of the large-scale system.

Upon completion of a social perception survey [by members from our research group as part of the Water Research Commission (WRC) project no. K5/2368//3], titled: "*Social perception of implementing a pilot Domestic Rainwater Harvesting (DRWH) Multi-Tank station in Enkanini, Stellenbosch*", results indicated that 61% of the respondents were familiar with the concept of rainwater harvesting. In addition, many of the respondents (67%) were favourably inclined

towards using rainwater for their daily needs, with 77% indicating that they would use the water for bathing and cleaning their house, while 65% would use the water for cooking and 46% would use the water for potable or drinking purposes. Most of the respondents (67%) also indicated that they perceive rainwater to be safe to drink. However, during the workshop (WRC Project K5/2368//3, 2016) that was conducted after the systems had been installed, it was stressed that the water should not be used for drinking purposes. Personal communication with the households utilising the systems then revealed that the households were predominantly using the pasteurized tank water samples (Sites 1 and 2) and stored pasteurized tank water samples (Site 3) for laundry, bathing and cleaning of their homes. Additionally, it was reported by a household using the small-scale system located at Site 1, that the household was using less paraffin/gas to heat water for use in domestic activities, as the water was already hot upon collection from the SOPAS system.

In total, one borosilicate glass tube needed to be replaced at each of the Sites (1, 2 and 3), respectively, and the outlet tap of the SOPAS system at Site 2 was replaced during January 2016. However, the replacement of the borosilicate glass tubes and the outlet tap did not require specialised equipment and could be completed within 10 min. In addition, the assistance of the manufacturer was required to install the pressure release pipes for both the small-scale systems located at Sites 1 and 2 (**Appendix A**). With smaller roof catchment areas compared to Site 3, results indicated that the small-scale systems located at Sites 1 and 2 were able to provide water to the households until January 2016, whereafter the systems could be utilised after March 2016 (rainfall started). In comparison, the large-scale system was able to provide water to the households until the beginning of March 2016.

2.4 Discussion

The results obtained for the physico-chemical (pH) analysis of the unpasteurized and pasteurized tank water samples collected from Sites 1 and 2 (pH range of 6.5 to 8.4 and 6.7 to 8.4, respectively) and the unpasteurized, pasteurized and stored pasteurized tank water samples collected from Site 3 (pH range of 6.3 to 8.2), indicated that the pH of the tank water samples, fell within the target quality range (6.0 to 9.0) as stipulated by DWAF (1996). In a study conducted by Owusu-Boateng and Gadogbe (2015), it was reported that rainwater had an average pH of 5.94, which increased to 7.11 after contact with the catchment area and then decreased to 6.8 after storage in the rainwater harvesting tank. The results obtained for the analysis of the pH of the tank water samples collected from all three sites, thus correspond to results reported in literature (Jawad Al Obaidy and Joshi, 2006; Diwakar et al., 2008; Islam et al., 2010; Lee et al., 2010; Owusu-Boateng and Gadogbe, 2015). The pH of rainwater is an important consideration of water quality, as Diwakar et al. (2008) and Abbasi and Abbasi (2011)

stated that a pH of less than 7 could lead to the corrosion of metal surfaces which could result in the subsequent leaching of toxic metals such as Pb, Zn and Cu from the roofing material.

In the current study, all anion concentrations were within drinking water standards following SOPAS for both the small- and large-scale systems, which correspond to results reported in literature, where it was shown that the anion levels in tank water samples were generally within drinking water guidelines (Lee et al., 2010; Dobrowsky et al., 2015a; Reyneke et al., 2016). Moreover, the results correspond to previous studies where it was indicated that SOPAS does not adversely affect anion concentrations (Dobrowsky et al., 2015a; Reyneke et al., 2016; Strauss et al., 2016). For example, overall, the SO_4 concentrations recorded at Sites 1 and 2 ranged from 3 to 7.15 mg/L before SOPAS and 3 to 7.2 mg/L after SOPAS, which corresponded to the 2.4 to 6.2 mg/L SO_4 concentration range reported for harvested rainwater by Lee et al. (2010). In the same study it was reported that pure rainwater had Cl^- concentrations ranging from 1.1 to 10 mg/L, while rainwater collected from a harvesting tank had Cl^- concentrations ranging from 5 to 18 mg/L, which then corresponded to the mean Cl^- concentrations reported for all three sites in the current study. The concentration of Cl^- is an important consideration when monitoring water quality as Cl^- concentrations > 50 mg/L may lead to the increased corrosion of metals, including iron, while concentrations > 2 000 mg/L may be detrimental to human health (DWAF, 1996). In addition, anions such as SO_4, have predominantly been shown to affect the organoleptic properties of water and may cause gastroenteritis in humans at concentrations > 200 mg/L (DWAF, 1996). It has also been demonstrated that the Cl^- to SO_4 mass ratio $[Cl^-]/[SO_4]$ needs to be monitored, as a ratio above 0.5 may lead to the increased leaching of Pb, which has a lower recommended health guideline limit (10 µg/L), as compared to Cl^- and SO_4 (Nguyen et al., 2010). As anion concentrations, including SO_4 and Cl^-, are predominantly dependent on environmental conditions (DWAF, 1996), it is important to establish whether these anions are present at levels exceeding the recommended guidelines as additional treatment methods may be required to ensure that the harvested rainwater is safe to use for potable purposes.

However, of concern in the current study, was the low fluoride (as F) concentrations observed for all three systems before and after pasteurization. For Site 1, F concentrations ranged from BDL to 0.08 mg/L. For Site 2, the mean concentrations recorded before and after pasteurization were 0.07 mg/L, while a mean concentration of 0.05 mg/L and 0.07 mg/L was recorded for the unpasteurized and stored pasteurized tank water samples obtained for the large-scale SOPAS system (Site 3). Although it has been questioned whether fluoride is essential for human health, research has shown that fluoride at concentrations of 0.7 to 1.2 mg/L can prevent dental caries and may help in the prevention and treatment of osteoporosis (U.S. Department of Health and Human Services, 2015). Thus, fluoridation, which is the controlled addition of fluoride to public

water supplies, has become common practise. If the pasteurized tank water is to be used as a primary drinking water source, fluoridation of the tank water may thus be required.

While all the cations analysed were within drinking water standards, for Sites 1 and 2, 67% of the unpasteurized (n = 8) and pasteurized (n = 8) tank water samples exceeded the Zn limit of 3 000 µg/L as stipulated by DWAF (1996) and ADWG (NHMRC and NRMMC, 2011), while the tank water pasteurized at 52 °C (Site 1) and the corresponding unpasteurized sample exceeded the 5 000 µg/L Zn SANS 241 limit (SABS, 2005). It is hypothesised that the high Zn concentrations observed in both small-scale systems can be attributed to the galvanised Zn sheeting, which was used as the catchment roofing material at the respective sites (Sites 1 and 2). Metal roofing materials have been shown to be a major contributor of metal ion contamination, as the acidity of rainwater (pH 5.0 – 5.6) in combination with the exposure of the roof surface to the sun, could facilitate the leaching of metals from the roofing material (Chang et al., 2004). Additionally, metal-based roofs generally have a protective coating of zinc or a zinc-aluminium alloy applied, which protects it from corrosion (Berdahl et al., 2008). Handia et al. (2003) also observed that harvested rainwater collected from roofs constructed from galvanised Zn sheets contained higher Zn concentrations (0.14 to 3.16 mg/L) than that of rainwater harvested from asbestos cement roofs (< 0.001 to 0.025 mg/L). Moreover, it has been reported that Zn concentrations may range from 0.1 to 10 mg/L in roof runoff water samples, depending on the type of Zn roofing material used as varying concentrations have been reported in rainwater for galvanised Zn, pure Zn, painted Zn or coated Zn roofing materials (Heijerick et al., 2002).

Cations analysed for the large-scale system were also within drinking water standards, however the levels of As (which is highly toxic) in both the unpasteurized and stored pasteurized tank water samples collected from the large-scale SOPAS system (Site 3) exceeded the respective drinking water guidelines limits of 10 µg/L (DWAF, 1996; SABS, 2005; NHMRC and NRMMC, 2011; WHO, 2011). As no significant difference (p = 0.18) in As concentrations were recorded in the untreated and stored pasteurized water, the elevated levels of As are not as a result of the SOPAS system or process and the As must thus have been introduced into the system via the catchment area. Although the catchment system located at Site 3 is also constructed from galvanised Zn sheeting, it was observed that the entire roofing surface was painted. However, no records exist of which paint was utilised on the roofing material (personal communication). It has however been reported that certain paints or coatings may contain As and it is thus hypothesised that the high levels of As may be attributed to the paint utilised on the roofing material (Kopylov et al., 2007; Anomaly, 2009). The detection of As is of major concern, as while As can be excreted by the body, it is a slow process and this toxicant can thus easily accumulate (DWAF, 1996). As indicated in the DWAF (1996) drinking water guidelines, As may be removed from water by filtration, therefore the installation of an appropriate filter to the outlet

tap of the 1 500 L storage tank may decrease the As levels to within drinking water standards. It should be noted that based on the As concentrations detected, the tank water from the large-scale system (Site 3) cannot be used for drinking purposes. However, the water can be used for irrigation purposes as the water conforms to the guidelines as stipulated by the DWAF (1996) agricultural irrigation use guidelines, where the As concentration limit is < 0.1 mg/L.

Additionally, it is hypothesised that the increased concentrations of Ni, Cu and Pb observed for the small-scale systems (Sites 1 and 2) and the large-scale system (Site 3) following SOPAS treatment, may be due to the leaching of these metals from the SOPAS system components. Although the small-scale SOPAS systems used in the current study have storage tanks constructed from a high grade polyethylene (compared to the more readily used stainless steel storage tanks), Pb is utilised to treat polyethylene surfaces during the manufacturing process, as a UV and heat stabiliser and Pb could thus have leached from the surface of the storage tank into the rainwater (Strauss et al., 2016). Additionally, SABS approved Ni coated dezincification resistant (DZR) brass (62% Cu) connector points were utilised to construct the small- and large-scale SOPAS systems. Nickel and Cu could thus have leached from these connectors during exposure to high temperatures in the SOPAS system (Strauss et al., 2016). However, it is important to note that although increased concentrations of Ni, Cu and Pb were observed following SOPAS treatment, the concentrations were still within the respective drinking water guidelines (DWAF, 1996; SABS, 2005; NHMRC and NRMMC, 2011; WHO, 2011). In studies conducted by Dobrowsky et al. (2016) and Reyneke et al. (2016), where a SOPAS system with a stainless steel storage tank was investigated, Al, Pb, Ni and Fe were detected at levels exceeding various national and international drinking water guidelines, following SOPAS. The results obtained in the current study therefore suggest that using a high grade polyethylene storage tank may result in decreased leaching of metals such as Pb, Ni and Fe.

Calculation of the total water hardness revealed that the unpasteurized and pasteurized (Sites 1 and 2) and unpasteurized and stored pasteurized (Site 3) tank water samples collected from all three sites could be described as “soft” (equivalent to < 50 mg $CaCO_3$). The total water hardness is an important consideration when monitoring water quality during disinfection treatments. Excessive total water hardness (> 200 mg $CaCO_3$) can result in scaling (deposition of hard mineral coatings or corrosion deposits) in plumbing and heating systems and as a result may decrease the effectiveness of the systems (DWAF, 1996). Conversely, water that is classified as “soft” may lead to corrosive water qualities and may affect metals such as Cu plumbing components by facilitating the leaching of metals (DWAF, 1996). The increased corrosive water qualities associated with soft water, may then have contributed to the leaching (corrosion) of metals, which may in turn have contributed to the increased Cu concentrations observed in the tank water samples following SOPAS.

It has been well documented that numerous pathogens and opportunistic pathogens are associated with stored rainwater and researchers have linked incidences of disease to the consumption of this water source (Hoque et al., 2003; Simmons et al., 2008; Abbasi and Abbasi, 2011). Moreover, it has been shown that the levels of microbial contaminants may fluctuate as a result of seasonal variation, the system location and the surrounding environment (Owusu-Boateng and Gadogbe, 2015). It is therefore essential to monitor the microbial quality of harvested rainwater before it can be used for potable purposes; however, no guidelines for rainwater quality have been formulated. Studies investigating the microbial quality of harvested rainwater thus use various national and international drinking water guidelines as a reference (Pitkänen et al., 2007; De Kwaadsteniet et al., 2013). These guidelines require the monitoring of multiple indicator bacteria such as enterococci, *E. coli*, faecal coliforms and total coliforms (DWAF, 1996; SABS, 2005; NHMRC and NRMMC, 2011; WHO, 2011). The presence of total coliforms indicate that the water source may be prone to contamination by other harmful organisms, while *E. coli*, enterococci and faecal coliforms serve as an indication of faecal contamination of the water source (Owusu-Boateng and Gadogbe, 2015). Monitoring for the removal of these organisms thus serves as an indication of the general water disinfection effectiveness of the SOPAS treatment systems (WHO, 2003, 2011).

The results obtained during the current study corresponded to the results reported in literature, where *E. coli*, total coliforms and faecal coliforms were detected in harvested rainwater at levels exceeding the various drinking water guidelines (Ahmed et al., 2009; Radaidah et al., 2009; Dobrowsky et al., 2014). It should however be noted that for the small-scale and large-scale systems (Sites 1, 2 and 3), with the exception of the 52 °C sample collected at Site 1, all total coliforms, *E. coli* and faecal coliforms enumerated after pasteurization treatment, were reduced to BDL (< 1 CFU/100 mL) and were subsequently within the drinking water guidelines (DWAF, 1996; SABS, 2005; NHMRC and NRMMC, 2011; WHO, 2011). Results for the enumeration of the heterotrophic bacteria then indicated that a pasteurization temperature of at least 66 °C for the small-scale systems and 71 °C for the large-scale system was required for the water to meet the DWAF (1996) drinking water guideline limit of < 1.0×10^4 CFU/100 mL. Similarly, Spinks et al. (2003, 2006) and Despins et al. (2009) reported that temperatures between 55 °C and 65 °C are required for the thermal inactivation of heat-resistant bacterial species. In a study conducted by Coombes et al. (2000), indicator bacteria (total coliforms, faecal coliforms and heterotrophic bacteria) counts were monitored in rainwater collected directly from rainwater harvesting tanks and water collected from hot water systems connected to the rainwater harvesting tanks. The results also indicated that temperatures between 55 °C and 63 °C were sufficient in reducing indicator counts to within the ADWG (NHMRC and NRMMC, 2011). The results obtained in the current study therefore correspond to results obtained in literature (Coombes et al., 2000; Spinks et al., 2003, 2006; Despins et al., 2009; Dobrowsky et al., 2015a;

Strauss et al., 2016), where it has been demonstrated that temperatures ranging from 55 °C to 72 °C have the ability to reduce microbial contamination in rainwater to within drinking water standards (DWAF, 1996; SABS, 2005, NHMRC and NRMMC, 2011, WHO, 2011).

Although results for the large-scale SOPAS rainwater harvesting system (Site 3) indicated that the water collected directly from the solar system manifold above 71 °C was within the drinking water standards, the analyses of the stored pasteurized water collected from the 1 500 L storage tank exceeded the DWAF (1996) recommended drinking water guideline limit for heterotrophic bacteria (< 1.0×10^4 CFU/100 mL). Based on the microbial analysis, the water from the large-scale SOPAS system storage tank should therefore be used for predominantly domestic purposes (such as cooking, cleaning, laundry, etc.) and irrigational purposes, as the DWAF (1996) domestic (excluding drinking), recreational and agricultural (irrigation) guidelines do not stipulate values for heterotrophic bacteria. Based on the results obtained it was then hypothesized that the microbial regrowth observed in the 1 500 L storage tank was most likely due to the large-scale system design. Firstly, the storage tank is not insulated, thus the water does not stay heated after it exits the solar system manifold. Secondly, the outlet tap connected to the storage tank is not located at the bottom of the tank, but approximately five centimetres from the bottom and therefore approximately 78 L of water can accumulate at the bottom of the tank and stagnate. Lastly, dust particles can enter the 1 500 L storage tank through either the storage tank lid or the overflow opening located on the side of the tank and subsequently re-contaminate the treated water. In contrast, the water collected from the small-scale systems remains heated in the 125 L storage tank and due to the closed-system design, dust particles and debris cannot enter the system.

Conventional culture-based analysis of a water sample is however, not sufficient to identify all the bacteria that may be present in a water sample or adequately describe changes in the microbial community following disinfection treatment (Signor and Ashbolt, 2006; Vital et al., 2012). Additionally, it is possible for bacteria to enter a viable but non-culturable state during exposure to a disinfection treatment and they may therefore not be detected during culturing analysis, but may still pose a significant health risk (Ahmed et al., 2008; Dusserre et al., 2008; Li et al., 2015). The use of cultivation-independent viability indicators, such as the BacTiter-Glo™ Microbial Cell Viability Assay, are thus increasingly being investigated for their ability to monitor water quality (Deininger and Lee, 2006; Berney et al., 2008; Vital et al., 2012; Nescerecka et al., 2016; Reyneke et al., 2016). Results from the BacTiter-Glo™ Microbial Cell Viability Assay then indicated that there was an overall significant decrease (p = 0. 00003) in RLU/100 µL (ATP) in all the pasteurized tank water samples in comparison to the unpasteurized tank water RLU/100 µL values for samples collected from the small-scale SOPAS systems (Sites 1 and 2). However, results indicated that viable (metabolically active) cells were still present at the highest temperatures analysed for the two small-scale systems [66 °C (Site 2) and 85 °C (Site

1), respectively]. This may then elucidate the microbial regrowth observed in the 67 °C (Site 1) and 66 °C (Site 2) samples after 6 days, during the storage culturing analysis. Additionally, the results for the BacTiter-Glo™ Microbial Cell Viability Assay indicated that for the temperature range of 55 °C to 79 °C, an 85.87% (0.85 log) reduction in RLU/100 µL was observed following SOPAS treatment with the large-scale SOPAS system (Site 3). However, corresponding to the results obtained during the culturing analysis of the HPC, an increase in metabolically active cells following pasteurization in the solar manifold system was observed when the water enters the 1 500 L storage tank as an overall percentage reduction of 55.33% (0.35 log) was recorded (in comparison to the unpasteurized sample). The BacTiter-Glo™ Microbial Cell Viability Assay is commonly used for measuring antimicrobial activity or bacterial growth however, research has demonstrated that the assay is useful for the rapid monitoring of water treatment efficiencies and could therefore aid in monitoring overall water quality (Berney et al., 2008; Reyneke et al., 2016). This was confirmed by Berney et al. (2008), where the total ATP values correlated with the results obtained for esterase activity and the high nucleic acid (HNA) bacterial fraction in various water samples, while HPC values only showed a weak correlation with all the other tested viability parameters. Additionally, Reyneke et al. (2016) used the BacTiter-Glo™ Microbial Cell Viability Assay to monitor ATP levels present in tank water samples collected before and after SOPAS, where a mean decrease of > 99% in ATP (RLU/100 µL sample) was recorded following pasteurization above 71.5 °C. In a study conducted by Vital et al. (2012), the efficiencies of two drinking water treatment and distribution plants were monitored using culture-based analysis, flow cytometry and the BacTiter-Glo™ Microbial Cell Viability Assay. Results indicated that depending on the cultivation method utilised, culturing analysis was only able to detect 0.04% to 5.99% of intact cells detected by flow cytometry, while the BacTiter-Glo™ Microbial Cell Viability Assay results correlated with flow cytometry. It was therefore concluded that flow cytometry in combination with the BacTiter-Glo™ Microbial Cell Viability Assay allows for the comprehensive analysis of the water treatment process in comparison to culture-based techniques.

Based on the results obtained for the HPC analysis of the 1500 L storage tank (large-scale system), various bacterial genera were proliferating with numerous opportunistic pathogens identified. The isolates identified included: *Flectobacillus* spp., *Acidovorax* spp., *Pseudomonas* spp., *Dechlorosoma* spp., *Bordetella* spp., *Novosphingobium* spp. and *Sphingomonas* spp. *Pseudomonas* spp. are found ubiquitously in nature (both water and soil environments) and have previously been detected in stored and freshly captured rainwater (Kaushik et al., 2012; Özen and Ussery, 2012; Strauss et al., 2016). *Pseudomonas aeruginosa* (*P. aeruginosa*) poses the greatest risk as it has been known to cause pneumonia, keratitis, as well as burn wound, gastrointestinal and urinary tract infections (Coutinho et al., 2008; Silby et al., 2011). Immunocompromised individuals, such as HIV and tuberculosis infected individuals

and cystic fibrosis patients, are also at the greatest risk of infection (Coutinho et al., 2008; Silby et al., 2011). *Bordetella* spp. have been isolated from a broad range of hosts, including cats, dogs and humans and have been shown to cause respiratory infections in humans, sheep, birds and poultry (Le Coustumier et al., 2004). All *Bordetella* spp. are obligate pathogens of their host organisms, with the exception of *Bordetella petrii* which may survive in a wide variety of different ecological niches. *Sphingomonas* spp. and *Novosphingobium* spp. have also been isolated from aquatic and soil environments (Ryan and Adley, 2010). These bacteria are able to survive in low nutrient environments and some species have been shown to cause nosocomial infections (most notably *Sphingomonas paucimobilis*) (Ryan and Adley, 2010). *Dechlorosoma* spp. are readily found in the soil environment and have been used to remove perchlorate in contaminated waters (Achenbach et al., 2001; Xu et al., 2004), while *Flectobacillus* spp. are common to freshwater aquatic systems and causes disease (flectobacillosis) in freshwater fish (Sheu et al., 2009; Adikesavalu et al., 2015). *Acidovorax* spp. are also ubiquitously distributed in nature having been isolated from soil and aquatic environments and cause bacterial fruit and leaf blotch (Shetty et al., 2005). The preliminary identification of the organisms proliferating in the 1 500 L storage tank thus revealed that a broad range of organisms are present in the stored pasteurized water, which were not part of the library of opportunistic rainwater pathogens (commonly associated with rainwater sources), screened for in the current study (section 2.2.8). The results obtained thus indicate that numerous other pathogens and opportunistic pathogens may be present and persist in the rainwater systems. Techniques such as automated rRNA intergenic spacer analysis (ARISA) (Machado and Bordalo, 2014) or high throughput next generation sequencing platforms (Tan et al., 2015) may be applied to comprehensively identify a broader range of organisms that may be present in untreated and treated rainwater. The application of these techniques may then contribute to the development of bio-indicators and identification of microbial source tracking markers that could be used to provide new insights into the monitoring of water samples (Tan et al., 2015). The use of molecular-based detection methods are also important with regards to the investigation of water treatment systems, as the identification of organisms that are capable of surviving the disinfection treatment and the elucidation of the survival mechanisms of the pathogens may lead to improvements in the design of water disinfection treatment systems.

As research has indicated that a poor correlation exists between the presence of indicator and pathogenic organisms in water sources (Harwood et al., 2005; Ahmed et al., 2008), genus-specific PCR assays were utilised to screen for opportunistic pathogens commonly associated with rainwater. The genus-specific PCR assays then indicated that for the small-scale SOPAS systems located at Sites 1 and 2, *Legionella* spp., *Staphylococcus* spp., *Pseudomonas* spp. and *Salmonella* spp. were the most prevalent organisms in the unpasteurized and pasteurized tank water samples. Similarly, *Legionella* spp. and *Staphylococcus* spp., were the indigenous

bacteria most readily detected in the water samples analysed for the large-scale SOPAS system (Site 3). Quantitative PCR assays were then used to quantify *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp., in the small-scale SOPAS system located at Site 1 and the large-scale SOPAS system located at Site 3. However, during qPCR analysis for *Staphylococcus* spp. multiple melting peaks were obtained per sample during the melt curve analysis, which invalidated the quantification of *Staphylococcus* spp. Quantitative PCR analysis then indicated that while a significant mean decrease in the detection of *Legionella* spp. (89.0%), *Pseudomonas* spp. (93.4%) and *Salmonella* spp. (83.8%) was observed following SOPAS treatment (all temperatures analysed) using the small-scale system located at Site 1, these bacteria persisted in the treated rainwater. Moreover, results from the large-scale system indicated that while *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp., were reduced by a mean of 79.4%, 92.1% and 41%, when comparing the unpasteurized to the pasteurized (solar manifold samples) tank water samples, an increase in *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp. gene copies was observed in the 1 500 L storage tank. The detection of specifically *Legionella* spp., *Pseudomonas* spp., and *Salmonella* spp. in the treated rainwater is of concern as these organisms are considered opportunistic pathogens that pose a serious health risk to especially immunocompromised individuals (Fields et al., 2002; Hall-Stoodley and Stoodley, 2005; Hauser and Ozer, 2011). However, it is important to note that conventional PCR and qPCR cannot provide an indication of the viability of the detected organisms as DNA may persist in the environment after cell death (Masters, 1994). However, Moore et al. (2001) stated that the consistent detection of nucleic acid sequences from pathogenic bacteria in an environment can indicate potential health risks, as the detected organisms would likely be viable as they are either consistently introduced into the environment or they are able to proliferate.

It is thus hypothesised that the pathogenic and opportunistic pathogens detected by conventional PCR and qPCR in the untreated and treated rainwater may be continuously introduced into the rainwater harvesting systems in two ways. Firstly, it has been proposed that bioaerosol particles (suspension of airborne particles that contain living organisms) may contaminate rainwater during a rainfall event by acting as cloud condensation nuclei which enable the transfer of microbial pathogens into rainwater through cloud droplets as the cloud and rain droplets traverse the atmosphere (Bauer et al., 2003; An et al., 2006; Turkum et al., 2008; Ekström et al., 2010; Kaushik et al., 2012). This proposal was then supported by the detection of *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp. in bioaerosol particles (Woo et al., 2013; Wei et al., 2016) and the detection of *E. coli*, *P. aeruginosa* and *Klebsiella pneumoniae* in freshly captured rainwater (Bauer et al., 2003; Korzeniewska et al., 2008; Ekström et al., 2010; Kaushik et al., 2012). Secondly, it has been shown that organic debris and faecal matter (which may contain microbial pathogens) deposited

on rooftops, will be washed into the rainwater harvesting tanks during a rainfall event and thereby continuously contaminate the harvested rainwater (Ahmed et al., 2008; Gwenzi et al., 2015; Waso et al., 2016). Moreover, research has demonstrated that certain pathogenic microorganisms are able to survive disinfection treatments, such as SOPAS, as a result of heat shock proteins (Fields et al., 2002; Bojer et al., 2010; Jørgensen et al., 2016), their ability to form and proliferate within biofilms (Murga et al., 2001; Hauser and Ozer, 2011; Jørgensen et al., 2016), ability to form survival structures such as endospores or cysts (Jones, 1997; Stortz and Zheng, 2000) and finally, certain bacteria such as *Legionella* spp. and *Pseudomonas* spp., can survive as intracellular parasites of protozoa (Fields et al., 2002). Additionally, it has been reported that bacteria are able to undergo an adaptive response and build-up resistance to an environmental stressor such as high temperatures, if constantly exposed to the same stress condition (Wesche et al., 2009).

The results obtained during the current study for all three sites, thus correspond to the results obtained in Dobrowsky et al. (2015a), where *Legionella* spp., *Pseudomonas* spp. and *Klebsiella* spp., were detected in tank water samples after pasteurization treatment above 73 °C. Additionally, in a study conducted by Dobrowsky et al. (2016), the gene copies/mL of the protozoa, *Naegleria fowleri* (5 log) and *Vermamoeba vermiformis* (3 log) were reduced to below the lower limit of detection at temperatures of 68 to 93 °C and 74 °C to 93 °C, respectively. However, *Legionella* spp. and *Acanthamoeba* spp. were detected in all tank water samples following SOPAS (68 °C to 93 °C). It is however, crucial that the viability of the pathogenic and opportunistic pathogens detected (conventional PCR and qPCR) be confirmed in future studies in order to determine whethere the treated water is safe to utlise for potable purposes. Molecular-based viability indicators such as viability-qPCR [Ethidium monoazide bromide (EMA)-qPCR or Propidium monoazide (PMA)-qPCR], could then be utilised to detect intact cells, as the indication of viability is based on membrane integrity and the detection of nucleic acids. For example, using the EMA-qPCR technique, Reyneke et al. (2016) and Strauss et al. (2016) were able to demonstrate that intact cells of *Legionella* spp. and *Pseudomonas* spp. were present following SOPAS treatment above 90 °C. Additionally, as indicated above, Dobrowsky et al. (2016) detected intact cells of *Legionella* spp. and *Acanthamoeba* spp. (utilising EMA-qPCR) following SOPAS treatment above 93 °C. Due to the health risks associated with the presence of pathogenic microorganisms in water sources, quantitative microbial risk assessment (QMRA) studies also need to be conducted in order to determine the acceptable exposure levels to these pathogens when using the water for drinking and domestic purposes.

An interesting observation was the low detection frequency of *Pseudomonas* spp. (33%; n = 2) in the stored pasteurized tank water samples collected from the 1 500 L storage tank using conventional PCR, as *Pseudomonas* spp. was one of the heterotrophic bacteria isolates

identified, using 16S rRNA PCR analysis, proliferating in the storage tank (section 2.3.3.2). It should however be noted that the 16S rRNA PCR analysis of the isolates persisting in the 1 500 L storage tank was conducted as a preliminary identification and thus isolates were solely selected based on colony morphology. Frequency of detection was not considered and the 16S rRNA PCR analysis of isolates was subsequently not performed for each sampling event. Furthermore, Dwidjosiswojo et al. (2011) demonstrated that the presence of Cu at concentrations found in water distribution systems (0.021 – 0.066 mg/L) induces a loss of culturability and cytotoxicity in *P. aeruginosa*. The mean Cu concentration (0.452 mg/L) of the stored pasteurized tank water samples (section 2.3.2) thus exceed the limits analysed by Dwidjosiswojo et al. (2011) and therefore could have influenced the detection of *Pseudomonas* spp. in the stored pasteurized tank water (1 500 L storage tank). Additionally, Soni et al. (2014) demonstrated that certain metals, including Cu, are toxic towards *P. aeruginosa.* This hypothesis is further supported as *Pseudomonas* spp. was one of the most readily detected organisms for both Sites 1 and 2, where lower mean Cu concentrations of 0.004 mg/L and 0.016 mg/L, was observed in the unpasteurized and pasteurized tank water samples, respectively.

As mentioned in **Appendix A**, following the installation of the small-scale and large-scale SOPAS systems, a workshop was conducted in Enkanini informal settlement with representatives of each of the ten participating households utilising the systems. The aim of the workshop was to provide the end users with information on the concept of rainwater harvesting, the general quality of rainwater and the maintenance of the SOPAS systems. Calculation of the theoretical volume of water that could be produced by the small-scale (SOPAS) system located at Site 1, then indicated that 17.14 kg/h, 12.75 kg/h, 10.84 kg/h and 8.74 kg/h, could be produced at the pasteurization temperatures of 52 °C, 60 °C to 67 °C, 72 °C to 75 °C and 85 °C, respectively. Similarly, for Site 2 at 53 °C to 58 °C and 66 °C, 16.25 kg/h and 12.81 kg/h pasteurized tank water could be produced. The results thus indicate that at a lower pasteurization temperature a larger volume of water could be produced. As culturing analysis indicated that a minimum temperature of 66 °C is required to reduce the indicators to within drinking water standards, theoretically 12.81 kg/h pasteurized tank water could be produced at this temperature. For the large-scale system (Site 3), the mean volume of pasteurized tank water that could be produced by the system at an output temperature of 75°C was calculated to be 30.04 kg/h. This increased capability to produce pasteurized tank water in comparison to the small-scale systems may be attributed to the increased total thermal energy (Q) reported for the system (6480 kJ/h). Therefore, depending on the household size, it would be possible for the small- and large-scale systems to provide a household with a supplementary water source, as the United Nations (2010) recommended that individuals living in Sub-Saharan Africa require 25 L/person per day. However, it is important to note that the actual volume of water that could

be produced by the system will vary from day to day depending on climate conditions. Additionally, the monitoring of water usage by the end-users demonstrated that both the small-scale and large-scale SOPAS systems are effective in providing individuals residing in informal settlements with an alternative/supplementary water source, as municipal tap water usage only increased during the months when less pasteurized tank water was available (**Appendix A**). Moreover, as the small-scale SOPAS systems provide warm water, the households using the system reported using less paraffin/gas to heat water for domestic purposes (personal communication).

2.5 Conclusion

The chemical and microbial quality of water produced by two small-scale and one large-scale SOPAS systems were assessed in order to determine whether these decentralised systems may be viable solutions in providing inhabitants of urban informal settlements with an alternative water source. Results indicated that cations including Zn (Sites 1 and 2) and As (Site 3) were detected at levels exceeding the recommended drinking water guidelines in the unpasteurized and pasteurized (Sites 1 and 2) and stored pasteurized (Site 3) tank water samples. Research should therefore be conducted to determine the effect of roofing materials generally utilised in informal settlements as well as paints or other materials (rubber coating) commonly used to cover roofing materials, on the chemical quality of harvested rainwater. A reduction in the level of the chemical contaminants detected may then be achieved by combining the current SOPAS technology installed in Enkanini with filtration systems. The efficacy of different cost-effective filtration systems will however, also need to be investigated in future studies.

Based on the culturing analysis, the small- and large-scale SOPAS systems were effectively able to reduce the level of indicator organisms detected to within drinking water standards, with a minimum pasteurization temperature of 66 °C required for the small-scale systems and a minimum pasteurization temperature of 71 °C required for the large-scale system (samples collected from solar manifold system). However, of concern was the increase in the heterotrophic bacteria count recorded in the 1 500 L storage tank of the large-scale system following SOPAS treatment. It is that highly recommended that the large-scale system is re-designed in order to prevent contamination of the pasteurized tank water. A suggestion would be to insulate the storage tank to allow it to retain heat and in so doing possibly limit bacterial re-growth. In addition, repositioning the outlet tap of the tank to prevent the stagnation of water and sealing the overflow valve and lid so that dust particles cannot enter storage tank should also be considered. Furthermore, while it is recommended that the treated water be used on the same day of collection, based on the results obtained the end-users could effectively store the treated tank water in a closed-container for up to 6 days after collection. Although the results of the genus specific PCRs indicated that a pasteurization temperature of 85 °C is required to

reduce the majority of indigenous rainwater pathogens to BDL, qPCR analysis revealed that gene copies of *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp. were detected following SOPAS treatment. However, as PCR assays cannot assess viability, future studies should assess the viability of the readily detected opportunistic pathogens, including *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp., following water disinfection treatments. The use of molecular-based viability assays would not only provide a better indication as to the treatment efficacy of the disinfection treatment, but will also provide an indication of the health-risks associated with using the water. Moreover, QMRA studies are required to determine whether the treated rainwater is safe to use for drinking and other domestic purposes.

Results from the operational sustainability monitoring then indicated that rainwater harvesting SOPAS systems were effective in providing the inhabitants with an alternative water source, However, it is important to note that the inhabitants of Enkanini are predominantly from rural Eastern Cape regions, where rainwater harvesting is practised. Furthermore, Enkanini has been used to launch various Stellenbosch Sustainability Institute projects related to the use of alternative technologies. It should therefore be determined whether rainwater harvesting SOPAS systems will also be accepted in other communities where rainwater harvesting is less known and the community has not previously been exposed to the use of alternative technologies. Overall, however, the results obtained demonstrates that decentralised rainwater harvesting SOPAS treatment systems (specifically the small-scale systems), may be a viable solution in providing the inhabitants of urban informal settlements with an alternative water source.

2.6 References

Abbasi, T. & Abbasi, S.A. 2011. Sources of pollution in rooftop rainwater harvesting systems and their control. Crit. Rev. Env. Sci. Tec. 41:2097-2167.

Abdulla, F.A. & Al-Shareef, A.W. 2009. Roof rainwater harvesting systems for household water supply in Jordan. Desalination 243:195-207.

Abraham, J.P., Plourde, B.D. & Minkowycz, W.J. 2015. Continuous flow solar thermal pasteurization of drinking water: Methods, devices, microbiology, and analysis. Renew. Energ. 81:795-803.

Achenbach, L.A., Michaelidou, U., Bruce, R.A., Fryman, J. & Coates, J.D. 2001. *Dechloromonas agitate* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. Int. J. Syst. Evol. Micr. 51:527-533.

Adikesavalu, H., Patra, A., Banerjee, S., Sarkar, A. & Abraham, T.J. 2015. Phenotypic and molecular characterization and pathology of *Flectobacillus roseus* causing flectobacillosis in captive held carp *Labeo rohita* (Ham.) fingerlings. Aquaculture 439:60-65.

Ahmed, W., Huygens, F., Goonetilleke, A. & Gardner, T. 2008. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. Appl. Environ. Microb. 74:5490-5496.

Ahmed, W., Vieritz, A., Gardner, T. & Goonetilleke, A. 2009. Microbial risks from rainwater tanks in southeast Queensland. Water 36:80–85.

Ahmed, W., Gardner, T. & Toze, S. 2011. Microbiological quality of roof-harvested rainwater and health risks: A Review. J. Environ. Qual. 40:13-21.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

An, H.R., Mainelis, G. & White, L. 2006. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. Atmos. Environ. 40:7924 –7939.

Anomaly, J. 2009. Harm to others: the social cost of antibiotics in agriculture. J. Agric. Environ. Ethics 22:423-435.

Barbau-Piednoir, E., Bertrand, S., Mahillon, J., Roosens, N.H. & Botteldoorn, N. 2013. SYBR® Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels. Appl. Microbiol. Biotechnol. 97:9811-9824.

Bauer, H., Giebl, H., Hitzenberger, R., Kasper-Giebl, A., Reischl, G., Zibuschka, F. & Puxbaum, H. 2003. Airborne bacteria as cloud condensation nuclei. J. Geophys. Res. 108:4658-4665.

Berdahl, P., Akbari, H., Levinson, R. & Miller, W.A. 2008. Weathering of roofing materials – an overview. Constr. Build. Mater. 22:423-433.

Bergmark, L., Poulsen, P.H.B., Abu Al-Soud, W., Norman, A., Hansen, L.H. & Sørensen, S.J. 2012. Assessment of the specificity of *Burkholderia* and *Pseudomonas* qPCR assays for detection of these genera in soil using 454 pyrosequencing. FEMS Microbiol. Let. 333:77-84.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H-A., Egli, T. & Hammes, F. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res. 42: 4010-4018.

Bojer, M.S., Struve, C., Ingmer, H., Hansen, D.S. & Krogfelt, K.A. 2010. Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. Plos One. 5:e15467. DOI:10.1371/journal.pone.0015467.

Brisse, S. & Verhoef, J. 2001. Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC gene sequencing and automated ribotyping. Int. J. Syst. Evol. Micr. 51:915-924.

Brözel, V.S. & Cloete, T.E. 1991. Resistance of bacteria from cooling waters to bactericides. J. Ind. Microbiol. 8:273-276.

Burch, J.D. & Thomas, K.E. 1998. Water disinfection for developing countries and potential for solar thermal pasteurization. Sol. Energy 64:87-97.

Chang, M., McBroom, M.W. & Beasley, R.S. 2004. Roofing as a source of non-point water pollution. J. Environ. Manage. 73:307-315.

Chapman, H., Cartwright, T., Huston, R. & O'Toole, J. 2008. Water quality and health risks from urban rainwater tanks. Research Report 42, Cooperative Research Centre for Water Quality and Treatment.

Ciochetti, D.A. & Metcalf, R.H. 1984. Pasteurization of naturally contaminated water with solar energy. Appl. Environ. Microb. 47:223-228.

Coombes, P.J., Argue, J.R. & Kuczera, G. 2000 Figtree Place: a case study in water sensitive urban development (WSUD). Urban Water 1:335-343.

Coutinho, H.D., Falcao-Silva, V.S. & Goncalves, G.F. 2008. Pulmonary bacterial pathogens in cystic fibrosis patients and antibiotic therapy: A tool for the health workers. Int. Arch. Med. 1:1-7.

Da Silva, G.C., Tiba, C. & Calazans, G.M.T. 2016. Solar pasteurizer for the microbiological decontamination of water. Renew. Energ. 87:711-719.

Deininger, R.A. & Lee, J. 2006. Modern tools and methods of water treatment for improving living standards. In: Omelchenko, A., Pivovarov, A. A., Swindall, W. J. (Eds.), Proceedings of the NATO Advanced Research Workshop on Modern Tools and Methods of Water Treatment for Improving Living Standards Dnepropetrovsk, Ukraine 19–22 November 2003. Springer, Netherlands, pp. 71–78.

De Kwaadsteniet, M., Dobrowsky, P.H., Van Deventer, A., Khan, W. & Cloete, T.E. 2013 Domestic rainwater harvesting: Microbial and chemical water quality and point-of-use treatment systems. Water Air Soil Poll. 224:1-19.

Department of Water Affairs and Forestry (DWAF). 1996. South African Water Quality Guidelines 2nd Edition, Volume 1: Domestic Water Use. Pretoria: CSIR Environmental Services.

Department of Water Affairs (DWA). 2013. Department of Water Affairs Annual report 2012/2013. Available: http://www.gov.za/documents/download.php?f=200726. Accessed [20 April 2014].

Despins, C., Farahbakhsh, K. & Leidl, C. 2009. Assessment of rainwater quality from rainwater harvesting systems in Ontario, Canada. J. Water Supply Res. T. 58:117–134.

Diwakar, J., Yami, K.D. & Prasai, T. 2008. Assessment of drinking water of Bhaktapur municipality area in pre-monsoon season. Scientific World 6:94-98.

Dobrowsky, P.H., Cloete, T.E. & Khan, W. 2014. Quality assessment of domestic harvested rainwater in the peri-urban region of Kleinmond, Western Cape and the optimisation of point-of-use treatment systems. M.Sc. Thesis. University of Stellenbosch.

Dobrowsky, P.H., Carstens, M., De Villiers, J., Cloete, T.E. & Khan, W. 2015a. Efficiency of a closed-coupled solar pasteurization system in treating roof harvested rainwater. Sci. Total Environ. 536:206-214.

Dobrowsky, P.H., Lombard, M., Cloete, W.J., Saayman, M., Cloete, T.E., Carstens, M., Khan, S. & Khan, W. 2015b. Efficiency of microfiltration systems for the removal of bacterial and viral contaminants from surface and rainwater. Water Air Soil Pollut. 226:1-14.

Dobrowsky, P.H., Khan, S., Cloete, T.E. & Khan, W. 2016. Molecular detection of *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* as vectors for *Legionella* spp. in untreated and solar pasteurized harvested rainwater. Parasit. Vectors 9:539-552.

Dunn, O.J. & Clark, V.A. 1974. Applied Statistics: Analysis of variance and regression, Second ed. John Wiley, and Sons, London, UK.

Dusserre, E., Ginevra, C., Hallier-Soulier, S., Vandenesch, F., Festoc, G., Etienne, J., Jarraud, S. & Molmeret, M. 2008. A PCR-based method for monitoring *Legionella pneumophila* in water samples detects viable but noncultivable legionellae that can recover their cultivability. Appl. Environ. Microbiol. 74:4817–4824.

Dwidjosiswojo, Z., Richard, J., Moritz, M.M., Dopp, E., Flemminga, H.C. & Wingender, J. 2011. Influence of copper ions on the viability and cytotoxicity of *Pseudomonas aeruginosa* under conditions relevant to drinking water environments. Int. J. Hyg. Envir. Heal. 214:485-492.

Ekström, S., Noziere, B., Hultberg, M., Alsberg, T., Magner, J., Nilsson, E.D. & Artaxo, P. 2010. A possible role of ground-based microorganisms on cloud formation in the atmosphere. Biogeosciences 7:387–394.

Evans, C.A., Coombes, P.J. & Dunstan, R.H. 2006. Wind, rain and bacteria: The effect of weather on the microbial composition of roof-harvested rainwater. Water Res. 40:37–44.

Feachem, R.E., Bradley, D.J., Garelick, H. & Mara, D.D. 1983. Sanitation and disease: Health aspects of excreta and wastewater management. New York, USA: Wiley.

Fields, B.S., Benson, R.F. & Besser, R.E. 2002. *Legionella* and Legionnaires' Disease: 25 Years of Investigation. Clin. Microbiol. Rev. 15:506-526.

Fluri, T.P. 2009. The potential of concentrating solar power in South Africa. Energ. Policy 37:5075-5080.

Frahm, E. & Obst, U. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Microbiol. Meth. 52:123-131.

Franklin, L.J., Fielding, J.E., Gregory, J., Gullan, L., Lightfood, D., Poznaski, S.Y. & Vally, H. 2009. An outbreak of *Salmonella Typhimurium* 9 at a school camp linked to contamination of rainwater tanks. Epidemiol. Infect. 137:434–440.

Gwenzi, W., Dunjana, N., Pisa, C., Tauro, T. & Nyamadzawo, G. 2015. Water quality and public health risks associated with roof rainwater harvesting systems for potable supply: Review and perspectives. Sustain Water Qual Ecol. 6:107-118.

Hall-Stoodley, L. & Stoodley, P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 13:7-10.

Handia, L., Tembo, J.M. & Mwiindwa, C. 2003. Potential of rainwater harvesting in urban Zambia. Phys. Chem. Earth PT A/B/C 28:893-896.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R. & Rose, J.B. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Environ. Microbiol. 71:3163–3170.

Hauser, A. & Ozer, E.A. 2011. *Pseudomonas aeruginosa*. Nature Rev. Microbiol. 9:1-2.

Heijerick, D.G., Janssen, C.R., Karlen, C., Wallinder, I.O. & Leygraf, C. 2002. Bioavailability of zinc in runoff water from roofing materials. Chemosphere 47:1073-1080.

Heim, A., Ebnet, C., Harste, G., Akerblom, P.P. 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J. Med. Virol. 70:228-239.

Helmreich, B. & Horn, H. 2009. Opportunities in rainwater harvesting. Desalination 248:118-124.

Herpers, B.L., De Jongh, B.M., Van Der Zwaluw, K. & Van Hannen, E.J. 2003. Real-time PCR assay targets the 23S-5S spacer for direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila*. J. Clin. Microbiol. 41:4815-4816.

Hoque, M., Hope, V., Scragg, R. & Kjellström, T. 2003. Children at risk of giardiasis in Auckland: A case–control analysis. Epidemiol. Infect. 131:655–662.

Huston, R., Chan, Y.C, Chapman, H., Gardner, T. & Shaw, G. 2012. Source apportionment of heavy metals and ionic contaminants in rainwater tanks in a subtropical urban area in Australia. Water Res. 46: 1121–1132.

International Technology Sourcing Solar. 2016. How do evacuated tubes work? Available: http://www.itssolar.co.za/its-downloads. Accessed [20 May 2016].

Islam, M.M., Chou, F.N.F., Kabir, M.R. & Liaw, C.H. 2010. Rainwater: A potential alternative source for scarce safe drinking and arsenic contaminated water in Bangladesh. Water Resour. Manag. 24:3987-4008.

Jawad Al Obaidya, A.H.M. & Joshi, H. 2006. Chemical composition of rainwater in a tropical urban area of northern India. Atmos. Environ. 40:6886-6891.

Jonas, D., Rosenbaum, A., Weyrich, S., Bhakdi, S. 1995. Enzyme-linked immunoassay for detection of PCR-amplified DNA of *Legionellae* in bronchoalveolar fluid. J. Clin. Microbiol. 33:1247-1252.

Jones, K. 1997. Strategies for survival. In Coliforms and *E. coli*. Problem or solution? D. Kay & C. Fricker, Eds. Cambridge, UK: The Royal Society of Chemistry.

Jørgensen, S.B., Bojer, M.S., Boll, E.J., Martin, Y., Helmersen, K., Skogstad, M. & Struve, C. 2016. Heat-resistant, extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in endoscope-mediated outbreak. J. Hosp. Infect. 93:57-62.

Kaushik, R., Balasubramanian, R. & De La Cruz, A.A. 2012. Influence of air quality on the composition of microbial pathogens in fresh rainwater. Appl. Environ. Microb. 78:2813-2818.

Klein, S.A. 1975. Calculation of flat-plate collector loss coefficients. Sol. Energy 17:79-80.

Kong, R.Y.C., Lee, S.K.Y., Law, T.W.F, Law, S.H.W. & Wu, R.S.S. 2002. Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. Water Res. 36:2802-2812.

Kopylov, N.I., Kaplin, Y.M., Litinov, V.P. & Kaminskii, Y.D. 2007. Large-scale use of arsenic in the production of antifouling coatings. Theor. Found. Chem. Eng. 41:780-785.

Korzeniewska, E., Filipkowska, Z., Gotkowska-Płachta, A., Janczukowicz, W. & Rutkowski, B. 2008. Bacteriological pollution of the atmospheric air at the municipal and dairy wastewater treatment plant area and in its surroundings. Arch. Environ. Prot. 34:13–23.

Kunin, R. 1972. Water Softening by Ion Exchange, Proceedings Fourteenth Water Quality Conference, University of Illinois, Urbana, Il.

Le Coustumier, A., Njamkepo, E., Cattoir, V., Guillot, S. & Guiso, N. 2011. *Bordetella petrii* infection with long-lasting persistence in humans. Emerg. Infect. Dis. 17:612-618.

Lee, J.Y., Yang, J.S., Han, M. & Choi, J. 2010. Comparison of the microbiological and chemical characterization of harvested rainwater and reservoir water as alternative water resources. Sci. Total Environ. 408:896-905.

Li, Z., Boyle, F. & Reynolds, A. 2010. Rainwater harvesting and greywater treatment systems for domestic application in Ireland. Desalination 260:1-8.

Li, H., Xin, H. & Li, S.F.Y. 2015. Multiplex PMA-qPCR assay with internal amplification control for silmultaneous detection of viable *Legionella pneumophila*, *Salmonella typhimurium* and *Staphylococcus aureus* in environmental waters. Environ. Sci. Technol. 49:14249-14256.

Lye, D.J. 2002. Health risks associated with consumption of untreated water from household roof catchment systems. J. Am. Water Resour. As. 38:1301-1306.

Machado, A. & Bordalo, A.A. 2014. Analysis of the bacterial community composition in acidic well water used for drinking in Guinea-Bissau, West Africa. J. Environ. Sci. 26:1605-1614.

Malema, S., Abia, L.K.A., Mwenge Kahinda, J. & Ubomba-Jaswa, E. 2016. Gaining a better understanding of the factors that influence the quality of harvested rainwater in South Africa – a review. Paper presented at the WISA 2016 Biennial conference. 16th -19th May 2016, Durban, South Africa.

Masters, C.I., Shallcross, J.A. & Mackey, B.M. 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. J. Appl. Bacteriol. 77:73–79.

McGuigan, K.G., Conroy, R.M., Mosler, H., Du Preez, M., Ubomba-Jaswa, E. & Fernandez-Ibañez, P. 2012. Solar water disinfection (SODIS): A review from bench-top to roof-top. J. Hazard. Mater. 235-236:29-46.

Merritt, A., Miles, R. & Bates, J. 1999. An outbreak of *Campylobacter enteritis* on an island resort, north Queensland. Commun. Dis. Intell. 23:215–219.

Moore, J., Caldwell, P. & Millar, B. 2001. Molecular detection of *Campylobacter* spp. in drinking, recreational and environmental water supplies. Int. J. Hyg. Envir. Heal. 204:185–189.

Morrow, A.C., Dunstan, R.H. & Coombes, P.J. 2010. Elemental composition at different points of the rainwater harvesting system. Sci. Total Environ. 408:4542-4548.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. & Donlan, R.M. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology 147:3121-3126.

Mwabi, J.K., Adeyemo, F.E., Mahlangu, T.O., Mamba, B.B., Brouckaert, B.M., Swartz, C.D., Offringa, G., Mpenyana-Monyatsi, L. & Momba, M.N.B. 2011. Household water treatment systems: A solution to the production of safe drinking water by the low-income communities of Southern Africa. Phys. Chem. Earth PT A/B/C 36:1120-1128.

Mwenge Kahinda, J., Taigbenu, A.E. & Boroto, R.J. 2010. Domestic rainwater harvesting as an adaptation measure to climate change in South Africa. Phys. Chem. Earth PT A/B/C 35:742-751.

Nescerecka, A., Juhna, T. & Hammes, F. 2016. Behaviour and stability of adenosine triphosphate (ATP) during chlorine disinfection. Water Res. 101:490-497.

Ndlovu, T., Le Roux, M., Khan, W. & Khan, S. 2015. Co-detection of virulent *Escherichia coli* genes in surface water sources. Plos One 10: e0116808. Doi:10.137/journal.pone.0116805.

Nguyen, C., Stone, K., Clark, B. & Edwards, M. 2010. Impact of chloride: Sulfate mass ration (CSMR) changes on lead leaching in potable water. Water Research Foundation. Available: http://www.waterrf.org/publicreportlibrary/4088.pdf [Accessed 7 December 2016].

NHMRC and NRMMC. 2011. Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy. National Health and Medical Research Council, National Resource Management Ministerial Council Commonwealth of Australia, Canberra.

Owusu-Boateng, G. &, Gadogbe, M.K. 2015. Domestic Rainwater Harvesting in a Water-Stressed Community and Variation in Rainwater Quality from Source to Storage. Consilience 14:225-243.

Özen, A.L. & Ussery, D.W. 2012. Defining the *Pseudomonas* genus: Where do we draw the line with Azobacter? Microbial Ecol. 63:239-248.

Peters, A.J., Weidner, K.L., Howley, C.L. 2008. The chemical water quality in roof-harvested water cisterns in Bermuda. J. Water Supply Res. T. 57:153-163.

Pitkänen, T., Paakkari, P., Miettinen, I.T., Heinonen-Tanski, H., Paulin, L. & Hänninen, M.L. 2007. Comparison of media for enumeration of coliform bacteria and *Escherichia coli* in non-disinfected water. J. Microbiol Meth. 68:522-529.

Radaidah, J., Al-Zboon, K., Al-Harahsheh, A. & Al-Adamat, R. 2009. Quality assessment of harvested rainwater for domestic uses. Jordan J. Earth Environ. Sci. 2:26–31.

Rawlings, D.E. 1995. Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferooxidans, Thiobacillus thiooxidans, Leptospirillum ferooxidans* strains in leaching environments. In: Jerez CA, Vargas T, Toledo H and Wiertz JV (eds.) Biohydrometallurgical Processing, University of Chile Press, Santiago, 9-17.

Reyneke, B., Dobrowsky, P.H., Ndlovu, T., Khan, S. & Khan, W. 2016. EMA-qPCR to monitor the efficiency of a closed-coupled solar pasteurization system in reducing *Legionella* contamination of roof-harvested rainwater. Sci. Total Environ. 553:662-670.

Rintala, H., Nevalainen, A., Ronka, E. & Suutari, M. 2001. PCR primers targeting the 16S rRNA gene for the specific detection of streptomycetes. Mol. Cell. Probe. 15:337-347.

Rohayem, J., Berger, S., Juretzek, T., Herchenröder, O., Mogel, M., Poppe, M., Henker, J. & Rethwilm, A. 2004. A simple and rapid single-step multiplex RT-PCR to detect Norovirus, Astrovirus and Adenovirus in clinical stool samples. J. Virol. Methods 118:49-59.

Rooney, A.P., Price, N.J.P., Ehrhardt, C., Swezey, J.L. & Bannan, J.D. 2009. Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. Int. J. Syst. Evol. Micr. 59:2429-2436.

Ryan, M.P. & Adley, C.C. 2010. *Sphingomonas paucimobilis:* a persistent Gram-negative nosocomial infectious organism. J. Hosp. Infect. 75:153-157.

Sakai, H., Procop, G.W., Kobayashi, N., Togawa, D., Wilson, D.A., Borden, L., Krebs, V. & Bauer, T.W. 2004. Simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. J. Clin. Microbiol. 42:5739-5744.

Saleh, M.A., Ewane, E., Jones, J. & Wilson, B.L. 2000. Monitoring Wadi El Raiyan lakes of the Egyptian desert for inorganic pollutants by ion-selective electrodes, ion chromatography and inductively coupled plasma spectroscopy. Ecotox. Environ. Safe. 45:310-316.

Servais, P., Billen, G. & Bouillot, P. 1994. Biological colonization of granular activated carbon filters in drinking-water treatment. J. Environ. Eng. 120:888-899.

Shetty, A., Barnes, R.A., Healy, B., Groves, P. 2005. A case of sepsis caused by *Acidovorax*. J. Infection 51:171-172.

Sheu, S., Chiu, T.F., Cho, N., Chou, J., Sheu, D., Arun, A.B., Young, C., Chen, C.A., Wang, J. & Chen, W. 2009. *Flectobacillus roseus* sp. nov., isolated from freshwater in Taiwan. Int. J. Syst. Evol. Micr. 59:2546-2551.

Signor, R.S. & Ashbolt, N.J. 2006. Pathogen monitoring offers questionable protection against drinking-water risks: a QMRA (quantitative microbial risk analysis) approach to assess management strategies. Water Sci. Technol. 54:261-268.

Silby, M.W., Winstanley, C., Godfrey, S.A., Levy, S.B., & Jackson, R.W. 2011. *Pseudomonas* genomes: Diverse and adaptable. FEMS Microbiol. Rev. 35:652–680.

Simmons, G., Jusry, S., Thornley, C., Harte, D., Mohiuddin, J. & Taylor, M. 2008. A Legionnaires' disease outbreak: A water blaster and roof-collected rainwater systems. Water Res. 42:1449–1458.

Soni, R.K., Acharya, P.B. & Modi, H.A. 2014. Effect of some metals on growth of *Pseudomonas aeruginosa* arsks20 and its decolorization ability of Reactive red 35. Int. J. Curr. Microbiol. Appl. Sci. 3:411-419.

South African Bureau of Standards (SABS). 2005. South African National Standards (SANS) 241: Drinking Water Quality Management Guide for Water Services Authorities. Sixth edition. Annexure 1. ISBN 0-626-17752-9.

Spilker, T., Coenye, T., Vandamme, P. & Lipuma, J.J. 2004. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. J. Clin. Microbiol. 42:2074-2079.

Spinks, A.T., Coombes, P., Dunstan, R.H. & Kuczera, G. 2003. Water quality treatment processes in domestic rainwater harvesting systems. 28th International Hydrology and Water Resources Symposium, Wollongong, Australia.

Spinks, A.T., Dunstan, R.H., Harrison, T., Coombes, P. & Kuczera, G. 2006. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Res. 40:1326–1332.

Statistics South Africa. 2010. In-depth analysis of the general household survey, Water and Sanitation 2002-2010. Report No. 03-18-02 (2002-2010). GHS Series Volume III. Pretoria: Statistics South Africa: 1-37.

Stellenbosch Weather. 2016. Available: http://weather.sun.ac.za/. Accessed [9 September 2016].

Stenkova, A.M., Isaeva, M.P. & Rasskazov, V.A. 2008. Development of a multiplex PCR procedure for detection of *Yersinia* genus with identification of pathogenic species (*Y. pestis, Y. pseudotuberculosis and Y. enterocolitica*). Mol. Gen. Microbiol. Virol. 23:119-125.

Stortz, G. & Zheng, M. 2000. Oxidative stress. In Bacterial Stress. G. Stortz & R. Hennge-Aronis, Eds. Washington DC, USA: ASM Press: 47-59.

Strauss, A., Dobrowsky. P.H., Ndlovu, T., Reyneke, B. & Khan, W. 2016. Comparative analysis of solar pasteurization versus solar disinfection for the treatment of harvested rainwater. BMC Microbiol. 16:289-305.

Tan, B., Ng, C., Nshimyimana, J.P., Loh, L.L., Gin, K.Y.H. & Thompson, J.R. 2015. Next-generation sequencing (NGS) for assessment of microbial water quality:current progress, challenges, and future opportunities. Front. Microbiol. 6:1-20.

Tavener-Smith, L. 2012. Housing: The challenge of informal settlements. In Sustainable Stellenbosch – opening dialogues. M. Swilling, B. Sebitosi & R. Loots, Eds. Stellenbosch, South Africa: SUN MeDIA: 68-83.

Turkum, A., Pekey, H., Pekey, B. & Tuncel, G. 2008. Investigating relationships between aerosol and rainwater compositions at different locations in Turkey. Atmos. Res. 89:315–323

United Nations. 2010. The human right to water and sanitation. Available: http://www.un.org/waterforlifedecade/pdf/human_right_to_water_and_sanitation_media_brief.pdf. Accessed [28 October 2014].

U.S. Department of Health and Human Services. 2015. U.S. Public Health Service Recommendation for Fluoride Concentration in Drinking Water for the Prevention of Dental Caries. Available: http://www.publichealthreports.org/documents/PHS_2015_Fluoride_Guidelines.pdf. Accessed [16 June 2016].

U.S. Environmental Protection Agency. 2009. Analytical methods approved for drinking water compliance monitoring under the total coliform rule – National Primary Drinking Water Regulations. U.S. Environmental Protection Agency Office of Water, Washington, DC.

Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L. & Hammes, F. 2012. Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Res. 46:4665-4675.

Water Research Commission (WRC), Project No. K5/2368//3. (2016). *Design, Construction and Monitoring of Sustainable Domestic Rainwater Harvesting Treatment Systems in Enkanini Informal Settlement, Stellenbosch*. Report to the Water Research Commission, Project No. K5/2368 by Department of Microbiology, Stellenbosch University. Stellenbosch, South Africa.

Waso, M., Ndlovu, T., Dobrowsky, P.H., Khan, S. & Khan, W. 2016. Presence of microbial and chemical source tracking markers in roof-harvested rainwater and catchment systems for the detection of fecal contamination. Environ. Sci. Pollut. R. 23:16987-17001.

Wegelin, M., Canonica, S., Meschner, K., Tleischmann, T., Pasaro, F., Metzler, A. 1994. Solar water disinfection: scope of the process and analysis of radiation experiments. J. Water Supply Res. T. 43:154–169.

Wei, K., Zou, Z., Zheng, Y., Li, J., Shen, F., Wu, C., Wu, Y., Hu, M. & Yao, M. 2016. Ambient bioaerosol particle dynamics observed during haze and sunny days in Beijing. Sci. Total Environ. 550:751-759.

Wesche, A.M., Gurtler, J.B., Marks, B.P. & Ryser, E.T. 2009. Stress, sub-lethal injury, resuscitation and virulence of bacterial foodborne pathogens. J. Food Prot. 5:926-1138.

Wessels, B.S. & Swilling, M. 2015. Turning points: Exploring power transitions in an incremental upgrading process in Enkanini, Stellenbosch. Unpublished Masters dissertation. Stellenbosch: University of Stellenbosch.

Woo, A.C., Manreetpal, S.B., Chan, Y., Lau, M.C.Y., Leung, F.C.C., Scott, J.A., Vrijmoed, L.L.P., Zawar-Reza, P. & Pointing, S.B. 2013. Temporal variation in airborne microbial populations and microbially-derived allergens in a tropical urban landscape. Atmos Enviorn. 74:291-300.

World Health Organisation (WHO). 2003. Heterotrophic plate counts and drinking-water safety – the significance of HPCs for water quality and human health. London, United Kingdom: IWA Publishing. ISBN 1-84339-025-6.

World Health Organisation (WHO). 2011. Guidelines for drinking-water quality. Fourth Edition. World Health Organisation, Geneva, Switzerland.

Xu, J., Trimble, J.J., Steinberg, L. & Logan, B.E. 2004 Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA. Water Res. 38:673-680.

Zhu, H., Sun, S-J., & Dang, H-Y. 2008. PCR detection of *Serratia* spp. using primers targeting *pfs* and *luxS* genes involved in AI-2 dependent quorum sensing. Curr. Microbiol. 57: 326-330.

Chapter 3:

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

Molecular-based viability assays versus metabolic responsiveness assays for the accurate determination of microbial cell viability

B. Reyneke[1], S. Khan[2] and W. Khan[1]*

[1] Department of Microbiology, Faculty of Science, Stellenbosch University, Private Bag X1, Stellenbosch, 7602, South Africa.

[2] Faculty of Health and Applied Sciences, Namibia University of Science and Technology, 13 Storch Street, Private Bag 13388, Windhoek, Namibia.

Abstract

The primary aim of the current study was to assess and compare molecular-based viability assays [ethidium monoazide bromide (EMA) quantitative polymerase chain reaction (qPCR), propidium monoazide (PMA)-qPCR and DNase treatment in combination with qPCR] and a metabolic responsiveness assay (BacTiter-Glo™ Microbial Cell Viability Assay) with culture based analysis for the accurate determination of microbial cell viability. Additionally, varying EMA and PMA concentrations were analysed in order to determine which dye and concentration allowed for the optimal identification of viable cells. Viable, heat treated (70 °C for 15 min) and autoclaved *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcocus aureus* and *Enterococcus faecalis* were utilised in the respective viability assays. For the analysis of the viable samples, based on the concentration of the respective viability dyes utilised, variable log reductions were recorded for both EMA (log reductions ranging from 0.01 to 2.71) and PMA (log reductions ranging from 0.06 to 1.02) in comparison to the no viability treatment control. Similarly, while the log reductions for the different concentrations of EMA and PMA were greater for the heat treated samples, variable log reductions of 0.27 to 2.85 and 0.62 to 2.46 were recorded for EMA and PMA, respectively. Based on the results obtained, 6 µM EMA and 50 µM PMA were then identified as the optimal dye concentrations as low log reductions were recorded (viable and heat treated samples) in comparison to the no viability treatment control. Comparison of the 6 µM EMA and 50 µM PMA results to the DNase treatment [log reductions ranging from 0.06 to 0.82 (viable) and 0.70 to 2.91 (heat treated)] results indicated that for the analysis of most of the test organisms (viable and heat treated), the 6 µM EMA concentration was comparable to either the 50 µM PMA or the DNase treatment. Moreover, the results for the culturing analysis [colony forming units (CFU)] of the viable *S. typhimurium* as well as the viable and heat treated samples of *L. pneumophila* and *P. aeruginosa* were comparable to the gene copies detected using molecular-based viability assays. However, the CFU in the heat treated samples of *S. typhimurium* were significantly lower than the gene copies detected using DNase in combination with qPCR, with no gene copies or CFU detected in the heat treated samples of *S. aureus* and *E. faecalis*. In contrast, while adenosine triphosphate (ATP) was detected in all the viable and heat treated samples for the respective test organisms, ATP was also detected in all the autoclaved control samples. It was thus concluded that molecular-based assays may be used to supplement culture based analysis for the comprehensive identification of the viable microbial population in water sources.

Keywords: Viability assays; ethidium monoazide bromide; propidium monoazide; DNase treatment

3.1 Introduction

Traditionally water quality is monitored by screening for the presence of indicator organisms using culture based methods. These techniques usually allow for the reliable detection of microorganisms in environmental water samples and selective media specific for the enumeration and identification of pathogenic strains may also be utilised (Delgado-Viscogliosi et al., 2009; Li et al., 2015). Culture-based analysis is however, often labour-intensive and may require approximately 24 hrs or more, for definite results to be obtained (Lemarchand et al., 2005). In addition, these methods introduce a bias towards viable and culturable cells and exclude the microorganisms that are classified as viable but not culturable (VBNC). This is a serious limitation as these VBNC organisms may regain their ability to grow and proliferate once conditions become favourable (Oliver, 2000; Murga et al., 2001; Hwang et al., 2006; García et al., 2007; Dusserre et al., 2008). Molecular-based techniques such as the polymerase chain reaction (PCR) are thus increasingly being used for the rapid and specific detection of pathogenic microorganisms in both clinical and environmental samples (Shannon et al., 2007).

Molecular-based techniques targeting nucleic acids overcome the major drawbacks associated with using culturing techniques by detecting specific pathogenic microorganisms as well as organisms present in a VBNC state within an environmental sample (Shannon et al., 2007; Delgado-Viscogliosi et al., 2009; Barbau-Piednoir et al., 2014; Li et al., 2015). Recent improvements in PCR-based technologies also allow for the simultaneous detection of multiple target organisms or genes (e.g. multiplex PCR) in a single assay (Girones et al., 2010). Additionally, the use of quantitative PCR (qPCR) allows for the successful quantification of pathogenic microorganisms in environmental samples that may be present at very low concentrations or below the detection limit of culturing analysis or conventional PCR (Guy et al., 2009). Loge et al. (2002) then utilised PCR analysis to detect the pathogenic microorganisms, *Salmonella* spp., *Shigella* spp., *Giardia lamblia* and *Cryptosporidium parvum*, as part of a risk-based analysis in water samples collected from stormwater drains and adjacent pavement surfaces. However, a limitation noted during the study was the presence of PCR inhibitors in the environmental samples that negatively influenced the detection of the organisms. Similarly, using conventional PCR, Dobrowsky et al. (2015a) detected the pathogenic microorganisms *Klebsiella* spp., *Legionella* spp., *Pseudomonas* spp. and *Yersinia* spp., in rainwater pasteurized at temperatures above 74 °C. A major disadvantage of the PCR technique however, is its inability to distinguish live from dead cells, as research has demonstrated that deoxyribonucleic acid (DNA) may persist after cell death (Masters et al., 1994; Wolffs et al., 2005).

Merely detecting the presence of pathogenic microorganisms in a water sample using molecular-based analysis will thus not provide an accurate indication as to the potential health risks associated with the use of the specific water source, as only the viable microbial contaminants pose a significant health risk to the end-user. It is therefore essential that the

accurate determination and quantification of viable microbial contaminants in water sources need to be monitored. The definition of what constitutes a “viable bacterial cell” is however a contentious issue as the standard methods utilised, usually monitor for bacterial growth and culturability on an appropriate medium [able to produce colony forming units (CFU)] (Trevors, 2012; Barbau-Piednoir et al., 2014). However, as culturability is not the sole indicator of the viability of an organism, it has been suggested that metabolic activity/responsiveness, the detection of nucleic acids and cellular integrity, be utilised to assess the true viability of microorganisms (Keer and Birch, 2003; Nocker and Camper, 2009).

Initially, it was suggested that the detection of messenger ribonucleic acids (mRNA) would provide the most direct molecular-based detection of viable cells as it would combine the detection of nucleic acids with metabolic activity/responsiveness (Keer and Birch, 2003). However, it has been reported that mRNA persistence will depend on the target gene and the conditions during which the target organism was inactivated (Girones et al., 2010). For example, in a study conducted by Sheridan et al. (1998), the viability of *Escherichia coli* (*E. coli*) was assessed following disinfection treatment (heat treatment vs ethanol disinfection), by detecting the expression of the *groEL*, *rpoH* and *tufA* genes. While the researchers were able to assess viability, results indicated that depending on the disinfection treatment utilised, mRNA persisted for an extended time period in *E. coli* cells exposed to ethanol disinfection. The authors then concluded that the mRNA degraded at a faster rate in the *E. coli* after heat treatment compared to ethanol disinfection, where the targeted mRNA was observed to be more stable (degradation was slower). Additionally, it was shown that the mRNA levels decreased with time, which influenced the accurate detection of mRNA in the samples. It has also been demonstrated that a specific disinfection treatment may produce contradictory results when analysing different mRNA targets, as chlorine treatment of *E. coli* resulted in the disintegration of *cplB* mRNA, while the *vacA* mRNA from *Heliobacter pylori* was unaffected (Cenciarini-Borde et al., 2009). Other limitations identified include the fact that mRNA expression levels are dependent on the physiological status of the cell and subsequently mRNA from viable dormant or slow-growing microorganisms may be present at levels below the detection limit of the reverse-transcriptase PCR (RT-PCR) (Nocker et al., 2006; Girones et al., 2010). Due to the limitations associated with using mRNA as an indicator of viability, it has been suggested that mRNA detection methods only be used to assess viability in organisms subjected to extreme lethal treatments where the targeted mRNA will be influenced (Cenciarini-Borde et al., 2009).

In order to overcome these limitations, the use of nucleic acid binding dyes in combination with qPCR have been investigated (Nocker and Camper, 2006; Vesper et al., 2008; Yáñez et al., 2011; Fittipaldi et al., 2012). Ethidium monoazide bromide qPCR (EMA-qPCR) and propidium monoazide qPCR (PMA-qPCR) are assays that provide an indication of viability based on membrane integrity and the detection of nucleic acids. For both techniques, the sample which

will be analysed is pre-treated with a nucleic acid binding dye (such as EMA or PMA), which is considered to be membrane impermeable. During the treatment process, upon photoactivation, the dyes are able to bind to extracellular DNA or DNA from cells with compromised cell walls/membranes. This effectively removes the bound DNA from the sample as it cannot be amplified during the subsequent qPCR. These assays have therefore successfully been used to differentiate between intact and dead cells however, varying optimal dye concentrations have been reported in literature for specific organisms (Fittipaldi et al., 2012). This is mainly due to the membrane permeability potential of each dye as well as the target organism. While comparing the efficacy of EMA and PMA for the detection of viable *Legionella* spp., Chang et al. (2010) reported that both dyes are effectively able to differentiate between viable and dead cells of this organism, however a 4-fold higher concentration of PMA (200 µM) was required to detect viable cells. In contrast, Yáñez et al. (2011) reported that a 50 µM PMA concentration was sufficient for the detection of viable *Legionella* spp. In contrast, Reyneke et al. (2016) and Strauss et al. (2016) utilised 6 µM EMA, as a pre-treatment to qPCR analysis, to successfully detect viable *Legionella* spp. and *Pseudomonas* spp. in solar pasteurized rainwater samples. Based on these analyses, it is therefore essential that the dye concentration is optimised before it is applied to environmental samples, as the target organism and sample matrix need to be considered (Fittipaldi et al., 2011).

The use of nuclease treatments, to remove extracellular DNA or DNA from cells with compromised cell walls/membranes, was also initially applied to assess mammalian cell viability using flow cytometric analysis (Frankfurt, 1983; Darzynkiewics et al., 1994). DNase I is an endonuclease that digests single- and double-stranded DNA by hydrolysing phosphodiester bonds. Similar to the EMA-qPCR and PMA-qPCR principle, this technique will thus provide an indication of viability based on membrane integrity and the detection of nucleic acids. Moreover, exposure of live bacterial cells or infective viruses to enzymes, such as DNase I, has a limited effect on the morphology, function or viability of the cells, as the membrane (bacteria) or capsid (viruses) has the ability to protect the organism from nucleases. DNase treatment has thus been utilised to detect viable food-borne pathogens (Nogva et al., 2000; Mukhopadhyay and Mukhopadhyay, 2002; Rueckert et al., 2005; Do et al., 2009; Shakeri et al., 2014) and infective viruses in water samples (Girones et al., 2010; Viancelli et al., 2012; Fongaro et al., 2013). In a study conducted by Rueckert et al. (2005), EMA-qPCR and DNase treatment were investigated, respectively, for their ability to differentiate between viable and dead thermophilic bacilli in factory milk powder samples. Results then indicated that DNase treatment qPCR analysis was more effective for the detection of viable thermophilic bacilli as the high EMA concentrations utilised (10 and 100 µg/mL) were cytotoxic to these cells.

Metabolic activity or responsiveness may then serve as a more direct approach for the assessment of bacterial cell viability (Pyle et al., 1995; Lee and Deininger, 2001; Keer and

Birch, 2003). This may be achieved by monitoring the utilisation of substrates (substrate responsiveness) or production of vital cellular components (metabolic/respiratory activity) by the target organism (Keer and Birch, 2003). Using esterase substrates, such as carboxyfluorescein diacetate or Chemchrome V6, researchers have been able to monitor for esterase activity as an indication of viable bacterial cells (Delgado-Viscogliosi et al., 2005; Wang et al., 2010). These esterase substrates are membrane permeable and fluoresce upon activation by intracellular esterases, which are produced by metabolically active cells. Another assay that has been used for the detection of viable microbial cells is the BacTiter-Glo™ Microbial Cell Viability Assay (BacTiter ATP assay; Promega, Madison, WI, USA). The assay allows for the determination of viable microbial cells by monitoring for the presence of adenosine triphosphate (ATP). The BacTiter ATP assay has also successfully been used to detect the presence of viable microbial cells in water samples and has been shown to correlate positively with other viability indicators, including esterase activity and a high nucleic acid (HNA) bacterial fraction (Deininger and Lee, 2006; Berney et al., 2008). In a study conducted by Bosshard et al. (2008), the susceptibility of *Salmonella typhimurium* and *Shigella flexerni* to solar disinfection was investigated using the BacTiter ATP assay. Results from the study indicated that the ATP content of the cells were reduced by > 95% in comparison to the control sample following treatment. Additionally, while investigating the efficiency of solar pasteurization to reduce the contamination of *Legionella* spp. in tank water samples, results from the BacTiter ATP assay indicated that while solar pasteurization above 71.5 °C resulted in a mean ATP decrease of > 99%, viable cells were still present in the tank water (Reyneke et al., 2016).

The primary aim of the current study was thus to compare molecular-based viability assays to a metabolic responsiveness assay for the accurate determination of microbial cell viability. In order to achieve this aim, the molecular-based viability assays EMA-qPCR, PMA-qPCR and DNase treatment in combination with qPCR and the metabolic responsiveness BacTiter ATP assay, were compared to culture based analysis for their ability to accurately determine cell viability in water samples inoculated with a bacterial monoculture. Three Gram-negative (*Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp.) and two Gram-positive (*Staphylococcus* spp. and *Enterococcus* spp.) bacteria commonly associated with water sources were selected as test organisms. Various EMA and PMA dye concentrations were also analysed, based on a range of concentrations that have been reported in literature in order to determine which dye is optimal and at what concentration (Nocker et al., 2006; Soejima et al., 2007; Kobayashi et al., 2008; Chen and Chang, 2009; Delgado-Viscogliosi et al., 2009; Yáñez et al., 2011; Tavernier and Coenye, 2015).

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

Legionella pneumophila (*L. pneumophila*) ATCC 33152, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) ATCC 14028 and *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853 were used as test bacteria representing Gram-negative pathogens commonly associated with water sources. *Staphylococcus aureus* (*S. aureus*) ATCC 25925 and *Enterococcus faecalis* (*E. faecalis*) were utilised as test bacteria representing Gram-positive pathogens commonly associated with water sources. All strains were obtained from Microbiologics® (St Cloud, MN, USA), with the exception of *E. faecalis* which was a sequence verified clinical isolate (sequence similarity to GenBank accession no. CP008816.1).

Legionella pneumophila was cultured at ± 35 °C for 4 days on buffered charcoal yeast extract (BCYE) agar (Oxoid, LTD, Hampshire, England) supplemented with *Legionella* BCYE growth supplement [buffer/potassium hydroxide (10 g/L), ferric pyrophosphate (0.25 g/L), alpha-ketoglutarate (1.0 g/L) and L-cysteine HCL (0.4 g/L); Oxoid]. *Enterococcus faecalis* was cultured on Slanetz and Bartley agar (Oxoid) at 37 °C for 24 – 48 hrs, while *S. aureus*, *P. aeruginosa* and *S. typhimurium* were grown at 37 °C for 24 hrs using Nutrient agar (Biolab, Merck, Wadeville, South Africa).

For the optimisation experiments (spiked water sample experiments; section 3.2.2), pure cultures of *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. faecalis* were inoculated into 10 mL sterile Luria Bertani broth (Biolab, Merck), respectively and were incubated overnight at 37 °C on a test tube rotator (New Brunswick, NY, USA). *Legionella pneumophila* was inoculated into Lennox Broth [10 g/L Tryptone (Biolab, Merck), 5 g/L Yeast Extract (Biolab, Merck), 5 g/L sodium chloride (NaCl; Saarchem, Durban, South Africa)] supplemented with *Legionella* BCYE growth supplement (Oxoid) and was incubated at 37 °C for 48 hrs on a test tube rotator (New Brunswick).

3.2.2 Preparation of the viable, heat treated and dead spiked water samples

For the respective viability assay analyses (EMA-qPCR, PMA-qPCR, DNase enzyme assay and BacTiter ATP assay), three 500 mL aliquots of sterile distilled water were spiked with 2 mL (A_{600}~0.65) of either the *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* or *E. faecalis* pure cultures (section 3.2.1), respectively. One of the 500 mL aliquots was heat treated at 70 °C for 15 min in a recirculating water bath (Memmert, GmbH, Schwabach, Germany). In addition, one 500 mL aliquot was autoclaved (negative control, presumed to contain dead cells), while the other 500 mL aliquot remained untreated (positive control, presumed to contain viable cells). A heat treated, autoclaved and viable sample, for each test organism, was thus utilised for all viability assay analysis.

3.2.3 Culturing analysis

The spread plate technique was used to enumerate *L. pneumophila*, *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. faecalis*, respectively, in the spiked distilled water samples [untreated (viable) positive control, autoclaved (dead) negative control and heat treated samples], directly after spiking the viable sample, after heat treatment for the heat treated sample and after the dead sample had been autoclaved, respectively. All experimental samples were serially diluted (10^{-1} to 10^{-5}) and 100 µL of the undiluted sample and serial dilutions were spread plated onto the corresponding media used for the culturing of the respective organisms (section 3.2.1), in duplicate. The agar plates used for the culturing of *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis* were then incubated at 37 °C for 24 hrs, while the agar plates used for the culturing of *L. pneumophila* were incubated at 37 °C for 72 hrs. Following the incubation period, the CFU/mL were enumerated (range of 25 to 250 CFU), whereafter the log reduction of the respective organisms in the heat treated sample was calculated using **Eq. 1** (Brözel and Cloete, 1991).

$$[\text{Log reduction} = (\text{Log}_{10}\ \text{bacterial count}_{\ \text{before treatment}} - \text{Log}_{10}\ \text{bacterial count}_{\ \text{after treatment}})] \quad (1)$$

3.2.4 BacTiter-Glo™ Microbial Cell Viability Assay (BacTiter ATP assay)

The BacTiter ATP assay was used to determine the presence of metabolically active cells in the spiked water samples [viable, heat treated and dead (autoclaved)] for each of the respective test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*) by monitoring for the presence of ATP. The BacTiter-Glo™ Buffer and Substrate were mixed (designated BacTiter-Glo™ Reagent) and equilibrated for approximately 10 hrs at room temperature to ensure that all ATP was hydrolysed ("burned off") (Berney et al., 2008). All samples were analysed in duplicate by loading 100 µL of the respective spiked water samples and an equal volume of the BacTiter-Glo™ Reagent into a 96-well White Cliniplate (Thermo Scientific Fisher, Finland). Additionally, sterile milliQ water was analysed as a negative control in order to obtain the "background" threshold value that was subtracted from the values obtained when analysing the samples. The luminescence of the samples was then measured in triplicate, using a Veritas™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA).

3.2.5 Concentration of the spiked water samples

The remaining volume (± 495 mL) of the respective spiked water samples [viable, heat treated and dead (autoclaved)], for each of the test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*), was then concentrated as previously described by Dobrowsky et al. (2015b). Briefly, 1 mL of 1 M calcium chloride ($CaCl_2$; Biolab, Merck) and 1 mL of 1 M di-sodium hydrogen orthophosphate (Na_2HPO_4, Saarchem) was added to each spiked

water sample. The mixtures were stirred for 5 min using a magnetic stirrer to allow for flocculation, whereafter the samples were filtered through a 47 mm, 0.45 µm pore size non-charged mixed-ester membrane filter (Whatman GmbH, Germany) at a flow rate of approximately ≥ 65 ml/min/cm^2 at 0.7 bar (70 kPa). Each membrane filter was then transferred to a 9 cm petri dish containing 2 mL of 0.3 M citrate buffer [pH 3.5, 0.1 M citric acid monohydrate ($C_6H_8O_7.H_2O$; Saarchem), 0.1 M tri-sodium citrate dehydrate ($C_6H_5O_7Na_3.H_2O$; Saarchem)] and were allowed to soak for 3 min. The membrane filters were discarded and each 2 mL concentrate was divided into 10 aliquots of 200 µL, respectively. Subsequently, for each test organism and experimental analysis [viable, heat treated and dead (autoclaved)], five aliquots were subjected to EMA treatment (6 µM, 12.5 µM, 25 µM, 35 µM and 50 µM; section 3.2.6), three aliquots were subjected to PMA treatment (25 µM, 50 µM and 100 µM; section 3.2.7), one sample was subjected to DNase treatment (section 3.2.8) and the remaining sample was left untreated (hereafter referred to as the "no viability treatment control").

3.2.6 Ethidium monoazide bromide treatment

Ethidium monoazide bromide was obtained from Biotium (Hayward, CA, USA) and was dissolved in 20% dimethyl sulfoxide (Merck, Darmstadt, Germany) in the absence of light (EMA is light sensitive) to obtain a 5 mg/mL stock solution (Delgado-Viscogliosi et al., 2009). Aliquots of 20 µL stock solution were then stored at -20 °C in microcentrifuge tubes covered in foil. To determine the optimal EMA concentration that would allow for the suppression of DNA from dead cells without affecting viable cells, 200 µL concentrated spiked water samples [viable, heat treated and dead (autoclaved)] of the respective test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*), prepared as described in section 3.2.5, were treated with EMA to obtain a final concentration of 6 µM, 12.5 µM, 25 µM, 35 µM and 50 µM (Soejima et al., 2007; 2008;Kobayashi et al., 2008; Chen and Chang, 2009; Delgado-Viscogliosi et al., 2009; Fittipaldi et al., 2012). Following the addition of EMA, the respective samples, were vortexed vigorously and incubated in the dark on ice for 10 min, followed by a 15 min halogen light (500 W; Eurolux, South Africa) exposure at a distance of 20 cm (Delgado-Viscogliosi et al., 2009). The halogen light exposure aids in cross-linking the EMA to the DNA and was performed while keeping the sample tubes horizontally on ice to avoid over-heating. The EMA treated samples were then washed with 1 mL of 0.85% NaCl (Saarchem) followed by centrifugation (16 000 × g, 5 min). The supernatant was discarded and the resulting pellet was re-suspended in 200 µL phosphate-buffered saline [PBS; pH 7.4, 8 g/L NaCl (Saarchem), 0.2 g/L potassium chloride (KCl; Saarchem), 1.42 g/L Na_2HPO_4 (Saarchem), 0.24 g/L potassium di-hydrogen orthophosphate (KH_2PO_4; Saarchem)], which is the first step in the DNA extraction procedure using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) (section 3.2.9).

3.2.7 Propidium monoazide treatment

Propidium monoazide (Biotium) was dissolved in sterile milliQ water according to the manufacturer's instructions in the absence of light (PMA is light sensitive) in order to obtain a 20 mM stock solution. Aliquots of 20 µL stock solution were stored at -20°C in microcentrifuge tubes covered in foil. To determine the optimal PMA concentration that would allow for the suppression of DNA from dead cells without affecting viable cells, 200 µL concentrated spiked water samples [viable, heat treated and dead (autoclaved)] of the respective test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*) were treated with PMA to obtain a final concentration of 25 µM, 50 µM and 100 µM (Nocker et al., 2006; Yáñez et al., 2011; Tavernier and Coenye, 2015). Following the addition of PMA, the respective samples were vortexed vigorously and incubated in the dark at room temperature for 10 min, followed by a 5 min halogen light (500 W; Eurolux, South Africa) exposure at a distance of 20 cm. Similar to the principle for EMA analysis, the halogen light exposure aided in cross-linking the PMA to the DNA and was performed while keeping the sample tubes horizontally on ice to avoid over-heating. The PMA treated samples were then centrifuged at 10 000 × g for 5 min. The supernatant was discarded and the resulting pellet was re-suspended in 200 µL PBS.

3.2.8 DNase treatment

Based on information provided in previous studies (Viancelli et al., 2012; Fongaro et al., 2013), 200 µL concentrated spiked water samples [viable, heat treated and dead (autoclaved)] of the respective test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*) were treated with DNase I (Thermo Fisher Scientific, Waltham, MA) at a final concentration of 5 U/mL. In addition, 10X reaction buffer [100 mM Tris-HCl (pH 7.5), 25 mM magnesium chloride ($MgCl_2$; Saarchem), 1 mM $CaCl_2$ (Biolab, Merck)] was added to each sample to obtain a 1X final concentration (Viancelli et al., 2012; Fongaro et al., 2013). The samples were then incubated at 37 °C for 60 min using a Accublock™ digital dry bath (Labnet International Inc., Woodbridge, NJ, USA). Following the incubation step, the DNase I enzyme was inactivated by adding 25 mM ethylenediaminetetraacetic acid (EDTA; Saarchem) to the sample. The samples were subsequently incubated at 65 °C for 10 min in a recirculating water bath (Memmert, GmbH). The DNase treated samples were then centrifuged at 10 000 × g for 5 min. The supernatant was discarded and the resulting pellet was re-suspended in 200 µL PBS.

3.2.9 DNA extractions following EMA, PMA and DNase treatment

Deoxyribonucleic acid was extracted from each of the samples subjected to the respective molecular viability treatments using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) according to the manufacturer's instructions.

Additionally, for the generation of the standard curve used during the qPCR analysis (section 3.2.10), positive control DNA was extracted from 2 mL pure culture aliquots of the respective organisms (section 3.2.1). Briefly explained, the 2 mL pure culture aliquots were centrifuged at 3 000 × g for 10 min. The supernatant was discarded and the resulting pellet was re-suspended in 200 µL PBS, whereafter DNA extraction was completed using the High Pure PCR Template Preparation Kit (Roche Diagnostics), according to the manufacturer's instructions.

The presence of DNA was confirmed by visualisation on a 0.8% agarose gel stained with 0.5 µg/mL ethidium bromide after gel electrophoresis at 80 volts for 60 min using 1X Tris/Borate/EDTA (TBE) buffer. Additionally, the DNA concentrations of the samples were determined using a NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA), prior to qPCR analysis.

3.2.10 Quantitative PCR analysis

In order to determine whether the viability treatments allowed for the accurate quantification of the viable cells in the respective samples, qPCR analysis was conducted as the final step of EMA-qPCR, PMA-qPCR and DNase enzyme assay on the viable, heat treated and dead (autoclaved) samples, for each of the five test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*). All qPCR analyses were performed using a LightCycler® 96 (Roche) instrument with the primers and cycling parameters as outlined in **Table 3.1**. For all qPCR assays (performed in duplicate), the reaction mixture (final volume of 20 µL) consisted of 10 µL FastStart Essential DNA Green Master Mix (1X), 5 µL template DNA and 0.4 µL of each primer (0.2 µM), with the exception of the *S. typhimurium* qPCR assay where 0.5 µL of the forward primer (0.25 µM) and 2 µL of the reverse primer (1 µM) was used. All DNA samples were diluted (10-fold) prior to analysis with the respective qPCR assays (minimise PCR inhibitors).

Melt curve analysis was included for all of the SYBR® Green real-time PCR assays in order to verify the 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. To generate a standard curve for the quantification of *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*, conventional PCR was performed on the positive control DNA extracted in section 3.2.9.

Table 3.1: Primers and PCR cycling parameters (conventional and qPCR) for the detection of the test organisms.

Organism	Primers	Primer sequence (5'-3')	*Cycling Parameters		Gene (bp)	References
			Conventional PCR	Quantitative PCR		
Legionella spp.	LegF	CTAATTGGCTGATTGTCTTGAC	5 min at 95 °C; 35 cycles of 94 °C for 1 min, 60 °C for 1.5 min, 72 °C for 1 min	10 min at 95 °C; 50 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 11 s	23S rRNA (259)	Herpers et al., 2003
	LegR	CAATCGGAGTTCTTCGTG				
Pseudomonas spp.	PS1	ATGAACAACGTTCTGAAATTC	5 min at 95 °C; 50 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s	10 min at 95 °C; 50 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s	*oprI* (249)	Bergmark et al., 2012
	PS2	CTGCGGCTGGCTTTTTCCAG				
Salmonella spp.	rpoD-20-F	ACATGGGTATTCAGGTAATGGAAGA	5 min at 95 °C; 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s	10 min at 95 °C; 45 cycles of 95 °C for 15 s, **60 °C for 1 min	*rpoD* (75)	Barbau-Piednoir et al., 2013
	rpoD-20-R	CRGTGCTGGTGGTATTTTCA				
Staphylococcus spp.	PanStaphF	CAATGCCACAAACTCG	5 min at 95 °C; 45 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s	10 min at 95 °C; 45 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s	*tuf* (475)	Sakai et al., 2004
	PanStaphR	GCTTCAGCGTAGTCTA				
Enterococcus spp.	ECST784F	AGAAATTCCAAACGAACTTG	5 min at 95 °C; 50 cycles of 95 °C for 15 s, 60 °C for 1 min, 72 °C for 20 s	10 min at 95 °C; 50 cycles of 95 °C for 15 s, **60 °C for 1 min	23S rRNA (75)	Frahm and Obst, 2003
	ENC854R	CAGTGCTCTACCTCCATCATT				

* A final elongation step of 10 min at 72 °C was included for each conventional PCR assay; ** combined annealing and elongation step

Each PCR mixture was performed in a final volume of 25 µL, using the primers and conventional PCR cycling parameters as outlined in **Table 3.1**, on a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). For the detection of *L. pneumophila*, the PCR mix consisted of 5 µL Green GoTaq® Flexi buffer (1X final concentration; Promega), 2 µL $MgCl_2$ (2.0 mM; Promega), 0.25 µL of a dNTP mix (0.1 mM; Thermo Scientific), 1 µL of the respective forward and reverse PCR primers (0.4 µM), 0.15 µL of GoTaq® Flexi DNA polymerase (1.5 U, Promega) and 2.5 µL of template DNA.

For *P. aeruginosa*, *S. typhimurium*, *E. faecalis* and *S. aureus* the same reaction mixture was used, however, 0.5 µL (0.2 µM), 0.625 µL (0.25 µM), 1.25 µL (0.5 µM) and 2.5 µL (1.0 µM), of each PCR primer was used, respectively. For each PCR assay, sterile distilled H_2O was used as a negative control. All PCR products were analysed by agarose gel electrophoresis at 80 V for 1 hour 20 min in 1.5% agarose (Bio-Rad Laboratories, USA) containing 0.5 µg/mL ethidium bromide in 1X TBE buffer.

The PCR products were then cleaned and concentrated using the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA). Following DNA concentration determination using a NanoDrop® ND-1000 (Nanodrop Technologies Inc.), the DNA concentration and gene product size were used to calculate the dilution required to obtain a final DNA concentration of 10^9 gene copies/µL (Dobrowsky et al., 2016). Serial 10-fold dilutions (10^9 to 10^0) of the PCR products were then prepared in order to generate a standard curve, with a concentration of 1.00×10^9 gene copies/µL for the dilution with the highest copy number and a concentration of 1.00×10^0 gene copies/µL for the dilution with the lowest copy number. The lower limit of detection (LLOD) for all qPCR assays was determined as the lowest number of genome copies consistently detected.

The conventional 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 purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research) and were sent to the Central Analytical Facility (Stellenbosch University) for sequencing performed in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems®, Foster City, USA). The chromatograms of each sequence were examined using FinchTV version 1.4.0 and sequence identification was completed using the National Centre for Biotechnology Information (NCBI). The Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, was used to find the closest match of local similarity between the samples and the sequence data available on the international databases in GenBank, EMBL, DDBJ and PDB (Altschul et al., 1990). The sequences of representative isolates that showed > 97% similarity (< 3% diversity) to organisms on the database were recorded.

3.3 Results

3.3.1 EMA-qPCR and PMA-qPCR analysis

A viable, heat treated and dead (autoclaved) sample of *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis* was analysed, respectively, in order to determine which concentration of EMA (6 µM, 12.5 µM, 25 µM, 35 µM and 50 µM) or PMA (25 µM, 50 µM and 100 µM) allows for the optimal detection of intact (and possibly viable) cells (**Fig. 3.1**).

The effect of the various EMA concentrations analysed on viable *L. pneumophila* during EMA-qPCR are depicted in **Fig. 3.1A**. All qPCR assays for the detection of the 23S rRNA gene from intact *L. pneumophila* following viability treatments (EMA, PMA and DNase treatment, respectively) had an average amplification efficiency (E) of 96.5% (1.93) and a correlation coefficient (R^2) of 0.99. For the autoclaved sample (dead control) analysed at the respective EMA concentrations, the gene copies were reduced to below the LLOD which ranged from 7 to 13 gene copies/µL (results not shown). Compared to the no viability treatment control of *L. pneumophila,* where 1.56×10^6 gene copies/µL were obtained, 5.92×10^5 gene copies/µL of viable *L. pneumophila* were recorded in the sample treated with 6 µM EMA concentration. The 23S rRNA gene copies of *L. pneumophila* were thus only reduced by 0.42 log. In contrast, as the EMA concentration increased (12.5 µM, 25 µM, 35 µM and 50 µM), the log decrease in the 23S rRNA gene copies of *L. pneumophila,* in comparison to the no viability treatment control increased. For example, the highest log reduction was observed for the viable *L. pneumophila* sample treated at 35 µM EMA, where 3.05×10^3 gene copies/µL were detected (2.71 log decrease) (**Fig. 3.1A**). The results for the various EMA concentrations analysed on heat treated *L. pneumophila* are depicted in **Fig. 3.1B**. For the heat treated *L. pneumophila* no viability treatment control sample, 8.51×10^4 gene copies/µL were obtained, while 4.52×10^4 gene copies/µL were detected in the 6 µM EMA heat treated sample. The 23S rRNA gene copies were thus reduced by 0.27 log in the 6 µM EMA heat treated sample. Similar to the results obtained for the viable *L. pneumophila* sample (**Fig. 3.1A**), an increase in EMA dye concentration again resulted in a greater log reduction of 23S rRNA gene copies in the *L. pneumophila* heat treated samples, with a 2.85 log reduction observed when 35 µM EMA (1.19×10^2 gene copies/µL) was utilised (**Fig. 3.1B**).

The effect of various PMA concentrations analysed on viable *L. pneumophila* during PMA-qPCR are also depicted in **Fig. 3.1A**. For the autoclaved sample (dead control) analysed at the respective PMA concentrations, the gene copies were reduced to below the LLOD which ranged from 7 to 13 gene copies/µL (results not shown). Results indicated that PMA treatment of the viable *L. pneumophila* sample at 50 µM produced the lowest log reduction (0.78 log decrease) in 23S rRNA gene copies, as 2.54×10^5 gene copies/µL were detected in comparison to the no viability treatment control (1.56×10^6 gene copies/µL) (**Fig. 3.1A**). However, the result

obtained using 25 µM PMA was comparable, as a 0.82 log reduction (2.33×10^5 gene copies/µL) was recorded in comparison to the no viability treatment control. In contrast, following 100 µM PMA treatment of the viable *L. pneumophila* sample, a 1.02 log decrease in 23S rRNA gene copies was observed (1.51×10^5 gene copies/µL). The effect of various PMA concentrations analysed on heat treated *L. pneumophila* are depicted in **Fig. 3.1B**. Similar to the results observed for the viable *L. pneumophila* sample treated with PMA (**Fig. 3.1A**), the lowest log reduction of viable *L. pneumophila* in the heat treated sample was observed for 50 µM PMA treatment, as the 23S rRNA gene copies only decreased from 8.51×10^4 gene copies/µL in the no viability treatment control to 2.00×10^4 gene copies/µL after 50 µM PMA treatment (0.62 log decrease) (**Fig. 3.1B**). The log reduction obtained in the 25 µM PMA heat treated sample (0.78 log decrease) was again comparable to the log decrease obtained in the 50 µM PMA heat treated sample. However, an increase in PMA concentration to 100 µM then resulted in a greater log decrease of the detected 23S rRNA gene copies (1.26 log decrease).

For the detection of the *oprI* gene from intact *P. aeruginosa,* all qPCR assays following viability treatments (EMA, PMA and DNase treatment, respectively) exhibited an average amplification efficiency (E) of 92% (1.84) and a correlation coefficient (R^2) of 0.99. The effect of various EMA concentrations analysed on viable *P. aeruginosa* during EMA-qPCR are depicted in **Fig. 3.1C**. For the autoclaved sample (dead control) analysed at the respective EMA concentrations, the gene copies were reduced to below the LLOD which ranged from 5 to 12 gene copies/µL (results not shown).

Compared to the no viability treatment control of *P. aeruginosa,* where 1.32×10^6 gene copies/µL were obtained, following 6 µM EMA treatment, 1.03×10^6 gene copies/µL were recorded in the viable *P. aeruginosa* sample. The *oprI* gene copies of viable *P. aeruginosa* were thus only reduced by 0.11 log in comparison to the no viability treatment control (**Fig. 3.1C**). However, an increase in EMA concentration to 12.5 µM resulted in the highest log decrease as 2.06×10^5 gene copies/µL were recorded (0.80 log decrease). It should however be noted that the results obtained for the 35 µM (0.12 log decrease) and 50 µM (0.13 log decrease) EMA treated samples were similar to the results obtained for the viable *P. aeruginosa* treated with 6 µM EMA (**Fig. 3.1C**). Results obtained for the EMA-qPCR analysis of the heat treated *P. aeruginosa* are represented in **Fig. 3.1D**. For the heat treated *P. aeruginosa* no viability treatment control sample, 2.54×10^5 gene copies/µL were detected. In contrast to what was observed for the viable sample (**Fig. 3.1C**), the lowest log reduction (0.74 log decrease) in comparison to the no viability treatment control was obtained following EMA treatment at 12.5 µM EMA (4.37×10^4 gene copies/µL). The greatest log decrease in the heat treated sample was then observed following 35 µM EMA treatment (1.83 log decrease).

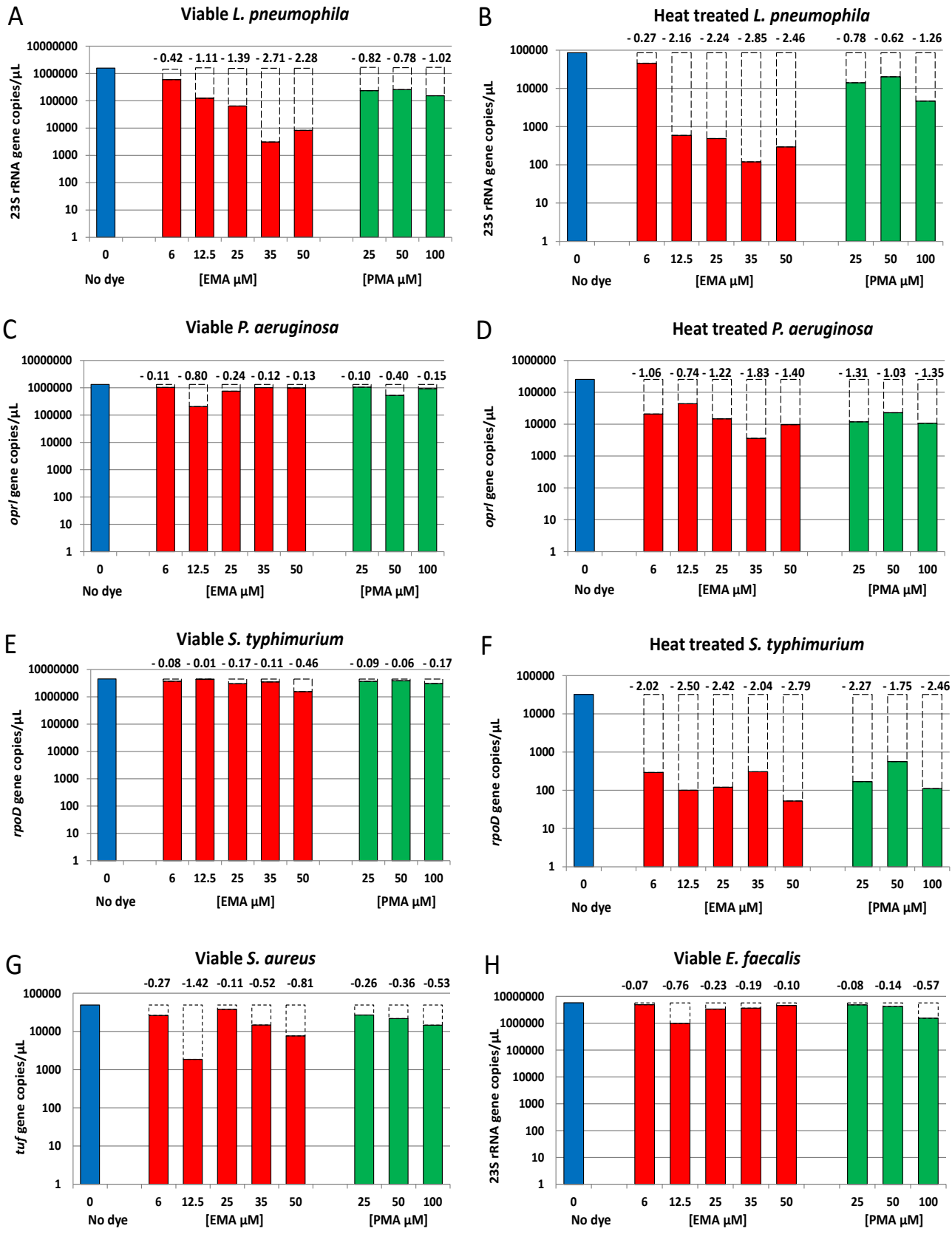

Fig. 3.1. Effect of EMA and PMA concentrations on **(A)** viable and **(B)** heat treated *L. pneumophila*; **(C)** viable and **(D)** heat treated *P. aeruginosa*; **(E)** viable and **(F)** heat treated *S. typhimurium*; **(G)** viable *S. aureus*; **(H)** viable *E. faecalis*. Transparent dashed bars and numerical values represent the log reduction in the sample, as compared to the corresponding no viability treatment control (no dye treatment).

The effect of the various PMA concentrations analysed on viable *P. aeruginosa* are also depicted in **Fig. 3.1C**. For the autoclaved sample (dead control) analysed at the respective PMA concentrations, the gene copies were reduced to below the LLOD which ranged from 5 to 12 gene copies/µL (results not shown). Similar to the trend observed for EMA-qPCR, PMA treatment of the viable *P. aeruginosa* sample at the lowest concentration (25 µM PMA) produced the lowest log reduction (0.10 log decrease) in *oprI* gene copies as 1.05×10^6 gene copies/µL were detected in comparison to the no viability treatment control (1.32×10^6 gene copies/µL) (**Fig. 3.1C**).

The highest log reduction was then observed following 50 µM PMA treatment as 5.25×10^5 gene copies/µL were detected (0.40 log decrease). Results obtained for the PMA-qPCR analysis of the heat treated *P. aeruginosa* are represented in **Fig. 3.1D**. In contrast to what was reported for the viable *P. aeruginosa* sample, 50 µM PMA treatment of the heat treated sample produced the lowest log reduction (1.03 log decrease) in *oprI* gene copies as 2.29×10^4 gene copies/µL were detected in comparison to the no viability treatment control (2.54×10^5 gene copies/µL) (**Fig. 3.1D**). In contrast, the results then indicated that there were 1.19×10^4 gene copies/µL (1.31 log decrease) and 1.07×10^4 gene copies/µL (1.35 log decrease) in the heat treated *P. aeruginosa* sample, following 25 µM PMA and 100 µM PMA treatment, respectively.

All qPCR assays for the detection of the *rpoD* gene from intact *S. typhimurium* following viability treatments (EMA, PMA and DNase treatment, respectively) yielded an average amplification efficiency (E) of 97% (1.94) and a correlation coefficient (R^2) of 0.99. The effect of various EMA concentrations analysed on viable *S. typhimurium* are depicted in **Fig. 3.1E**. For the autoclaved sample (dead control) analysed at the respective EMA concentrations, the gene copies were reduced to below the LLOD which ranged from 4 to 7 gene copies/µL (results not shown). Results indicated that for 12.5 µM EMA treatment, 4.38×10^6 gene copies/µL (0.01 log decrease) were detected, which was comparable to the no viability treatment control, where 4.48×10^6 gene copies/µL were detected. Similarly, 6 µM EMA treatment resulted in a 0.08 log decrease when comparing the detected gene copies to the no viability treatment control. The highest log reduction in *rpoD* gene copies was then observed following 50 µM EMA treatment, as 1.55×10^6 gene copies/µL (0.46 log decrease) were detected (**Fig. 3.1E**). Results obtained for the EMA-qPCR analysis of the heat treated *S. typhimurium* are represented in **Fig. 3.1F**. Analysis of the heat treated *S. typhimurium* no viability treatment control sample indicated that 3.21×10^4 gene copies/µL were present. Results then indicated that following 6 µM EMA treatment, 2.92×10^2 gene copies/µL were detected. The *rpoD* gene copies of *S. typhimurium* were thus reduced by 2.02 log in comparison to the no viability treatment control; however, this was comparable to the 2.04 log decrease observed following 35 µM EMA treatment (3.06×10^2 gene copies/µL). Similar results were then obtained for the remaining EMA concentrations (12.5 µM, 25 µM and 50 µM), as 2.50 (1.00×10^2 gene copies/µL), 2.42

(1.20×10^2 gene copies/µL) and 2.79 (5.26×10^1 gene copies/µL) log reductions were observed, respectively.

The effect of various PMA concentrations analysed on viable *S. typhimurium* during PMA-qPCR are depicted in **Fig. 3.1E**. For the autoclaved sample (dead control) analysed at the respective PMA concentrations, the gene copies were reduced to below the LLOD which ranged from 4 to 7 gene copies/µL (results not shown). Analysis of the viable *S. typhimurium* sample indicated that 4.48×10^6 gene copies/µL were present in the no viability treatment control, which was subsequently reduced to 3.67×10^6 gene copies/µL (25 µM; 0.09 log reduction), 3.89×10^6 gene copies/µL (50 µM; 0.06 log reduction) and 3.06×10^6 gene copies/µL (100 µM; 0.17 log reduction). The results obtained using 25 µM (0.09 log decrease) and 50 µM (0.06 log decrease) PMA treatment were thus comparable to the results obtained using 6 µM EMA treatment (0.08 log decrease). Results for the analysis of the heat treated *S. typhimurium* are represented in **Fig. 3.1F**. Compared to the no viability treatment control of *S. typhimurium,* where 3.21×10^4 gene copies/µL were obtained, results indicated that for the 50 µM PMA concentration, 5.66×10^2 gene copies/µL (1.75 log decrease) were detected. Additionally, following 25 µM and 100 µM PMA treatment, a greater decrease in *rpoD* gene copies was observed, as 1.69×10^2 gene copies/µL (2.27 log decrease) and 1.11×10^2 gene copies/µL (2.46 log decrease) were detected.

For the detection of the *tuf* gene from intact *S. aureus,* all qPCR assays following viability treatments (EMA, PMA and DNase treatment, respectively) yielded an average amplification efficiency (E) of 96% (1.92) and a correlation coefficient (R^2) of 0.99. The effect of various EMA and PMA concentrations analysed on viable *S. aureus* during EMA-qPCR and PMA-qPCR, respectively, are depicted in **Fig. 3.1G**. For the no viability treatment control autoclaved (dead control) and heat treated samples analysed at all the respective EMA and PMA concentrations, the gene copies were reduced to below the LLOD which ranged from 2 to 8 gene copies/µL (results not shown). Compared to the no viability treatment control (4.92×10^4 gene copies/µL), following EMA treatment of the viable *S. aureus* sample at 25 µM, 3.79×10^4 gene copies/µL were detected (0.11 log decrease). In contrast, following 12.5 µM EMA treatment a 1.42 log decrease in *tuf* gene copies was observed as 1.86×10^3 gene copies/µL were detected. For the analysis of viable *S. aureus* using PMA-qPCR (**Fig. 3.1G**), compared to the no viability treatment control where 4.92×10^4 gene copies/µL were obtained, following PMA treatment at 25 µM, 2.69×10^4 gene copies/µL were detected (0.26 log decrease). An increase in PMA concentration to 100 µM then resulted in a 0.53 log reduction, as 1.46×10^4 gene copies/µL were obtained.

All qPCR assays following viability treatments (EMA, PMA and DNase treatment, respectively) for the detection of the 23S rRNA gene from intact *E. faecalis* yielded an average amplification

efficiency (E) of 97% (1.95) and a correlation coefficient (R^2) of 0.99. The effect of various EMA and PMA concentrations analysed on viable *E. faecalis* during EMA-qPCR and PMA-qPCR, respectively, are depicted in **Fig. 3.1H**. For the no viability treatment control autoclaved (dead control) and heat treated samples analysed at all the respective EMA and PMA concentrations, the gene copies were reduced to below the LLOD which ranged from 7 to 16 gene copies/µL (results not shown). Results indicated that following 6 µM EMA treatment, 4.94×10^6 gene copies/µL were obtained, indicating a 0.07 log decrease in comparison to the no viability treatment control, where 5.78×10^6 gene copies/µL were obtained. Following an increase in EMA concentration to 12.5 µM, the detected 23S rRNA gene copies in the viable *E. faecalis* sample decreased by 0.76 log as only 9.95×10^5 gene copies/µL were detected. For the PMA-qPCR analysis, results indicated that at 25 µM and 50 µM, respectively, 4.86×10^6 gene copies/µL and 4.21×10^6 gene copies/µL were detected. This indicates that only a 0.08 and 0.14 log decrease in 23S rRNA gene copies was observed in comparison to the no viability treatment control (5.78×10^6 gene copies/µL). However, after 100 µM PMA treatment, 1.54×10^6 gene copies/µL were detected which indicated the highest observed log decrease (0.57 log decrease).

3.3.2 DNase enzyme assay analysis

The viable, heat treated and dead (autoclaved) *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis* samples were analysed, respectively, using 5 U/mL DNase treatment prior to qPCR analysis. The results obtained for the DNase treatment assay on viable and heat treated samples for all test organisms are represented in **Fig. 3.2**.

Quantitative PCR analysis of the viable *L. pneumophila* sample indicated that 3.14×10^6 gene copies/µL were present in the no viability treatment control (**Fig 3.2**). In comparison, qPCR analysis of the DNase treated sample indicated that 1.54×10^6 gene copies/µL were present in the viable *L. pneumophila* sample. The 23S rRNA gene copies were thus only reduced by 0.30 log. Additionally, in comparison to the no viability treatment control of the heat treated *L. pneumophila* sample where 8.67×10^4 gene copies/µL were detected, a 1.25 log decrease in 23S rRNA gene copies was observed as 4.81×10^3 gene copies/µL were detected in the corresponding DNase treated sample. Moreover, results indicated that in the autoclaved sample (dead control) analysed using DNase treatment, the gene copies were reduced to below the LLOD which ranged from 7 to 13 gene copies/µL (results not shown).

The results obtained for the DNase treatment assay conducted on viable and heat treated *P. aeruginosa* samples are also represented in **Fig. 3.2**. Quantitative PCR analysis of the viable DNase treated sample indicated a 0.12 log decrease in *oprI* gene copies, as 1.01×10^6 gene copies/µL were detected in comparison to 1.32×10^6 gene copies/µL detected in the

corresponding no viability treatment control sample. The *oprI* gene copies in the heat treated *P. aeruginosa* sample then displayed a greater log decrease as 5.01 × 10^4 gene copies/µL were detected in the DNase treated sample, compared to 2.54 × 10^5 gene copies/µL which were detected in the no viability treatment control (0.70 log decrease). Similar to the results reported for *L. pneumophila*, results indicated that the *oprI* gene copies of *P. aeruginosa* in the autoclaved sample (dead control) analysed using DNase treatment, were reduced to below the LLOD which ranged from 5 to 12 gene copies/µL (results not shown).

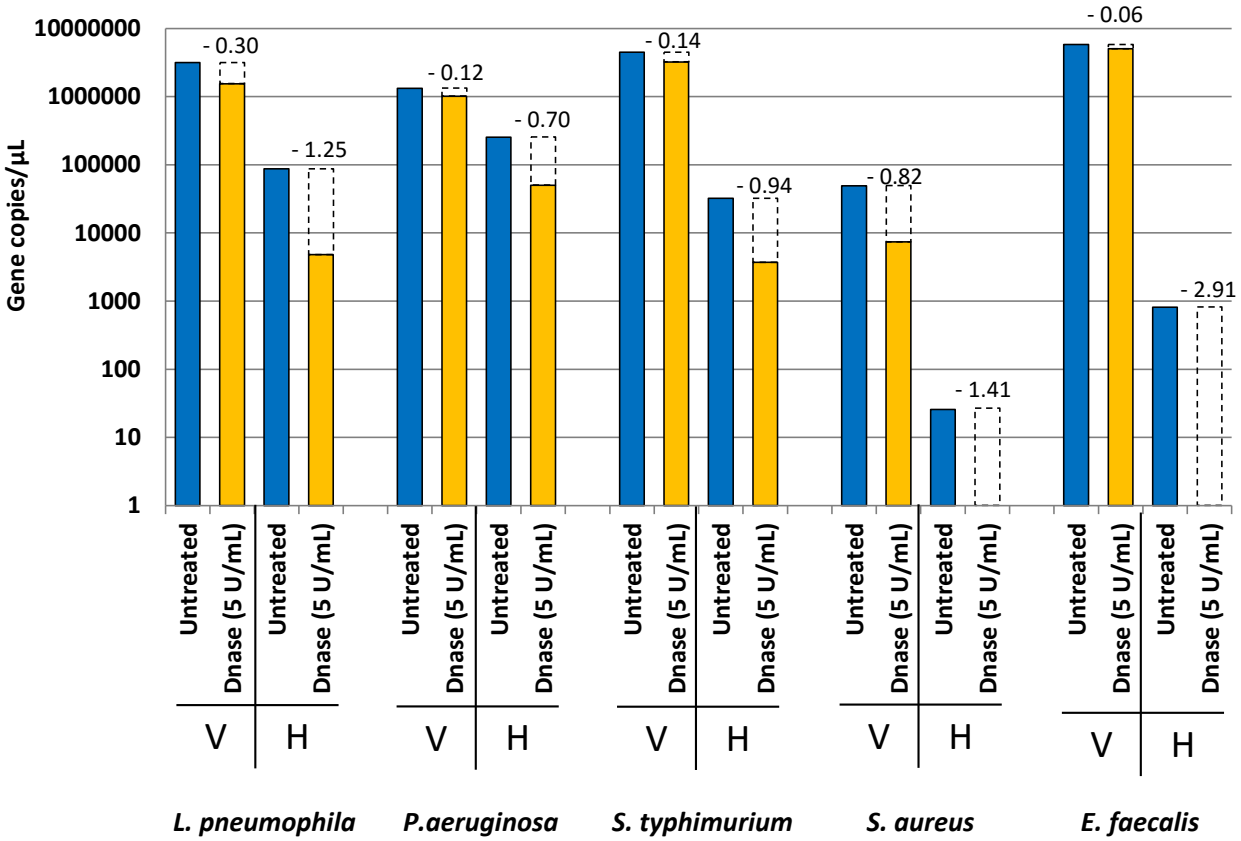

Fig. 3.2. Effect of DNase on viable [V] and heat treated [H] *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*. Transparent dashed bars and numerical values represent the log reduction in the sample, as compared to the corresponding no viability treatment control (no DNase treatment).

The results obtained for the DNase treatment assay on viable and heat treated *S. typhimurium* are represented in **Fig. 3.2**. Similar to the results observed for the viable *P. aeruginosa* sample, a 0.14 log decrease in gene copies was observed in the DNase treated *S. typhimurium* sample, as compared to the corresponding no viability treatment control. For the no viability treatment control, 4.48 × 10^6 gene copies/µL were present in the viable *S. typhimurium* sample, while 3.22 × 10^6 gene copies/µL were detected in the DNase treated sample. Additionally, analysis of the heat treated *S. typhimurium* sample indicated that the *rpoD* gene copies decreased from 3.21 × 10^4 gene copies/µL in the no viability treatment control to 3.70 × 10^3 gene copies/µL in

the DNase treated sample, indicating a 0.94 log decrease. The *rpoD* gene copies were then below the LLOD (4 to 7 gene copies/µL) in the dead (autoclaved) *S. typhimurium* sample (results not shown).

The results obtained for the DNase treatment assay on viable and heat treated *S. aureus* are represented in **Fig. 3.2**. Analysis of the no viability treatment control sample indicated that 4.92×10^4 gene copies/µL were present in the viable *S. aureus* sample, while 7.83×10^3 gene copies/µL were detected in the corresponding DNase treated sample (0.82 log decrease). For the no viability treatment control heat treated sample, the *tuf* gene from *S. aureus* was present at 2.58×10^1 gene copies/µL; however, the *tuf* gene copies were reduced to below the LLOD (2 to 8 gene copies/µL) in the corresponding DNase treated sample (1.41 log decrease). Additionally, no *tuf* gene copies were detected in the dead (autoclaved) sample (results not shown).

The results obtained for the DNase treatment assay on viable and heat treated *E. faecalis* are also represented in **Fig. 3.2**. Analysis of the viable sample indicated that 5.79×10^6 gene copies/µL were present in the no viability treatment control sample, while 5.03×10^6 gene copies/µL were detected in the DNase treated sample (0.06 log decrease). Analysis of the no viability treatment control from the heat treated sample then indicated that the 23S rRNA gene from *E. faecalis* was present at 8.16×10^2 gene copies/µL; however, the 23S rRNA copies were reduced to below the LLOD (7 to 16 gene copies/µL) in the corresponding DNase treated sample (2.91 log reduction). Additionally, no 23S rRNA gene copies were detected in the dead (autoclaved) sample (results not shown).

3.3.3 BacTiter-Glo™ Microbial Cell Viability Assay

The BacTiter ATP assay was utilised to determine whether metabolically active (viable) cells were present in the viable, heat treated and dead (autoclaved) samples of *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis,* by measuring ATP in the form of relative light units (RLU) (**Fig. 3.3**).

The BacTiter ATP assay analysis indicated that ATP in the form of RLU was detected at a mean of 5.3×10^5 RLU/100 µL in the viable *L. pneumophila* sample. In comparison, a mean of 2.8×10^4 RLU/100 µL was detected in the heat treated sample (1.27 log reduction). Analysis of the autoclaved (dead) *L. pneumophila* sample then indicated the presence of ATP at a mean concentration of 3.7×10^3 RLU/100 µL (2.14 log reduction). The results thus indicated that a 1.27 log reduction in RLU occurred following heat treatment of the *L. pneumophila* sample and that ATP was still being detected in the autoclaved (dead) sample.

Analysis of the *P. aeruginosa* samples using the BacTiter ATP assay indicated that a mean of 1.3×10^6 RLU/100 µL was present in the viable *P. aeruginosa* sample, which then decreased to a mean of 2.6×10^5 RLU/100 µL in the heat treated sample. Additionally, a mean of 1.9×10^3 RLU/100 µL was recorded in the autoclaved (dead) *P. aeruginosa* sample. Although a 0.72 log reduction in RLU was observed following heat treatment, results indicated that ATP was still present in the autoclaved *P. aeruginosa* sample (2.84 log reduction).

Adenosine triphosphate in the form of RLU was then detected at a mean of 1.7×10^6 RLU/100 µL in the viable *S. typhimurium* sample. Results then indicated that a mean of 2.0×10^5 RLU/100 µL was detected in the heat treated sample (0.94 log reduction), while a mean of 7.8×10^3 RLU/100 µL was recorded for the dead *S. typhimurium* sample (2.34 log reduction). The results thus correspond to the results obtained for the *L. pneumophila* and *P. aeruginosa* samples where a decrease in RLU was observed following heat treatment, however, the results also confirmed the presence of ATP in the autoclaved *S. typhimurium* sample.

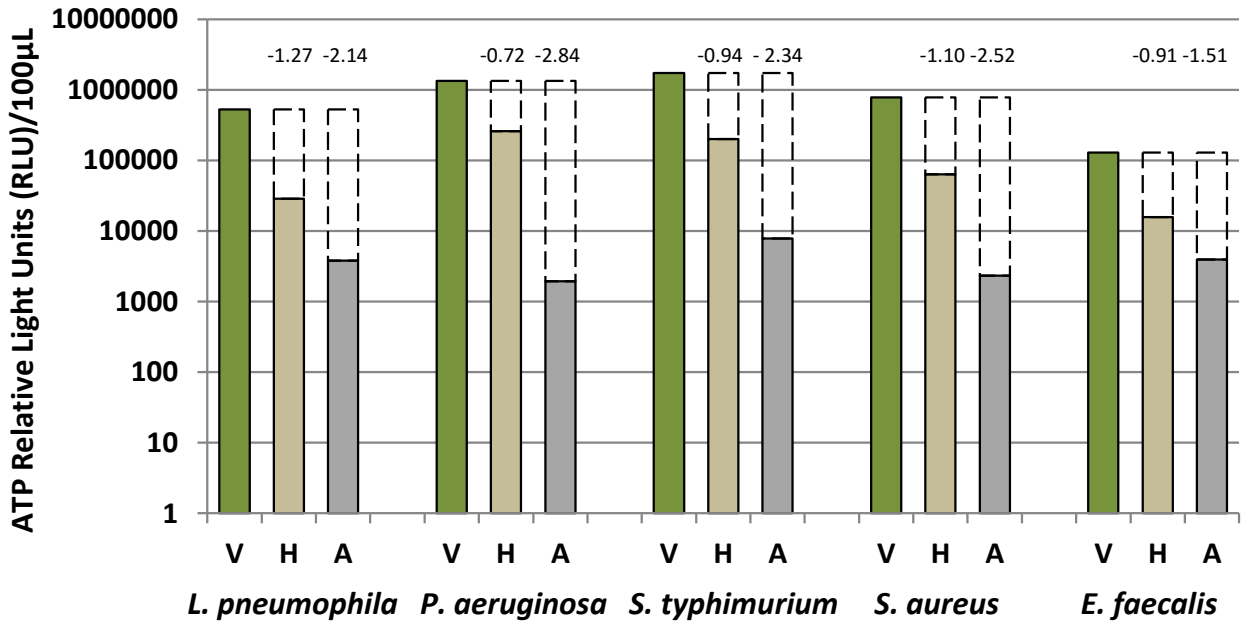

Fig. 3.3. Mean ATP relative light units detected per 100µL spiked water sample [V - viable, H - heat treated and A - autoclaved (dead)] for each of the 5 test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*).

Similarly, for the *S. aureus* sample, a mean ATP RLU concentration of 7.8×10^5 RLU/100 µL and 6.3×10^4 RLU/100 µL were detected in the viable and heat treated *S. aureus* samples, respectively, while a mean concentration of 2.3×10^3 RLU/100 µL was detected in the autoclaved (dead) *S. aureus* sample. A 1.10 log reduction in RLU was thus observed when comparing the viable to the heat treated sample, however, ATP was again still being detected in the autoclaved sample (2.52 log reduction).

Lastly, for the viable *E. faecalis* sample, a mean of 1.3×10^5 RLU/100 µL was detected, while a mean of 1.6×10^4 RLU/100 µL was detected in the heat treated sample (0.91 log reduction). Additionally, 3.9×10^3 RLU/100 µL was recorded in the dead *E. faecalis* sample (1.51 log reduction). The results thus indicated that a 0.91 log reduction in RLU occurred following heat treatment of the *E. faecalis* sample and that ATP was still being detected in the autoclaved sample.

3.3.4 Comparison of the molecular-based viability assays to culture based analysis

Based on the analysis of the overall results obtained for the EMA-qPCR and PMA-qPCR assays on the viable and heat treated *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis* (section 3.3.1), 6 µM EMA and 50 µM PMA were identified as the optimal concentrations, respectively, as the gene copies obtained compared well with the respective no viability treatment controls for all the test organisms. It should be noted that while other optimal concentrations were also identified for the respective organisms, in most cases the results obtained using 6 µM EMA and 50 µM PMA were comparable to these concentrations. Ethidium monoazide bromide-qPCR, PMA-qPCR and the DNase treatment assay in combination with qPCR, were subsequently compared to the results obtained during the culturing analysis. The results obtained using the BacTiter ATP Assay (metabolic responsiveness assay) was not included as metabolically active cells were detected in the autoclaved (dead) samples utilising this assay. This thus indicated that the assay may detect residual ATP in samples following disinfection treatment when metabolically active cells are not present.

Results obtained for the 6 µM EMA and 50 µM PMA concentrations to the DNase treatment assay, applied to the viable *L. pneumophila* sample, revealed that the gene copies detected using the respective assays, resulted in comparable log decreases of 0.39, 0.25 and 0.30 respectively (**Fig. 3.4**). In comparison to the no viability treatment control where 3.14×10^6 gene copies/µL were detected, using 6 µM EMA, 50 µM PMA and the DNase treatment, 1.26×10^6 gene copies/µL, 1.75×10^6 gene copies/µL and 1.54×10^6 gene copies/µL were detected, respectively (**Fig. 3.4**). Culturing analysis then indicated that *L. pneumophila* was present at 4.5×10^6 CFU/mL in the viable sample (**Fig. 3.5**). In contrast, for the heat treated *L. pneumophila* sample, the results obtained for the application of the DNase treatment (4.81×10^3 gene copies/µL; 1.25 log decrease) and 6 µM EMA treatment (5.50×10^3 gene copies/µL; 1.20 log decrease), were comparable. Analysis with 50 µM PMA then resulted in a 2.70 log decrease, in comparison to the no viability treatment control sample (8.67×10^4 gene copies/µL), as only 1.69×10^2 gene copies/µL were detected in the heat treated sample (**Fig. 3.4**). Culturing analysis then confirmed that viable and culturable cells were still present in the heat treated sample as 1.7×10^3 CFU/mL *L. pneumophila* were enumerated (**Fig. 3.5**).

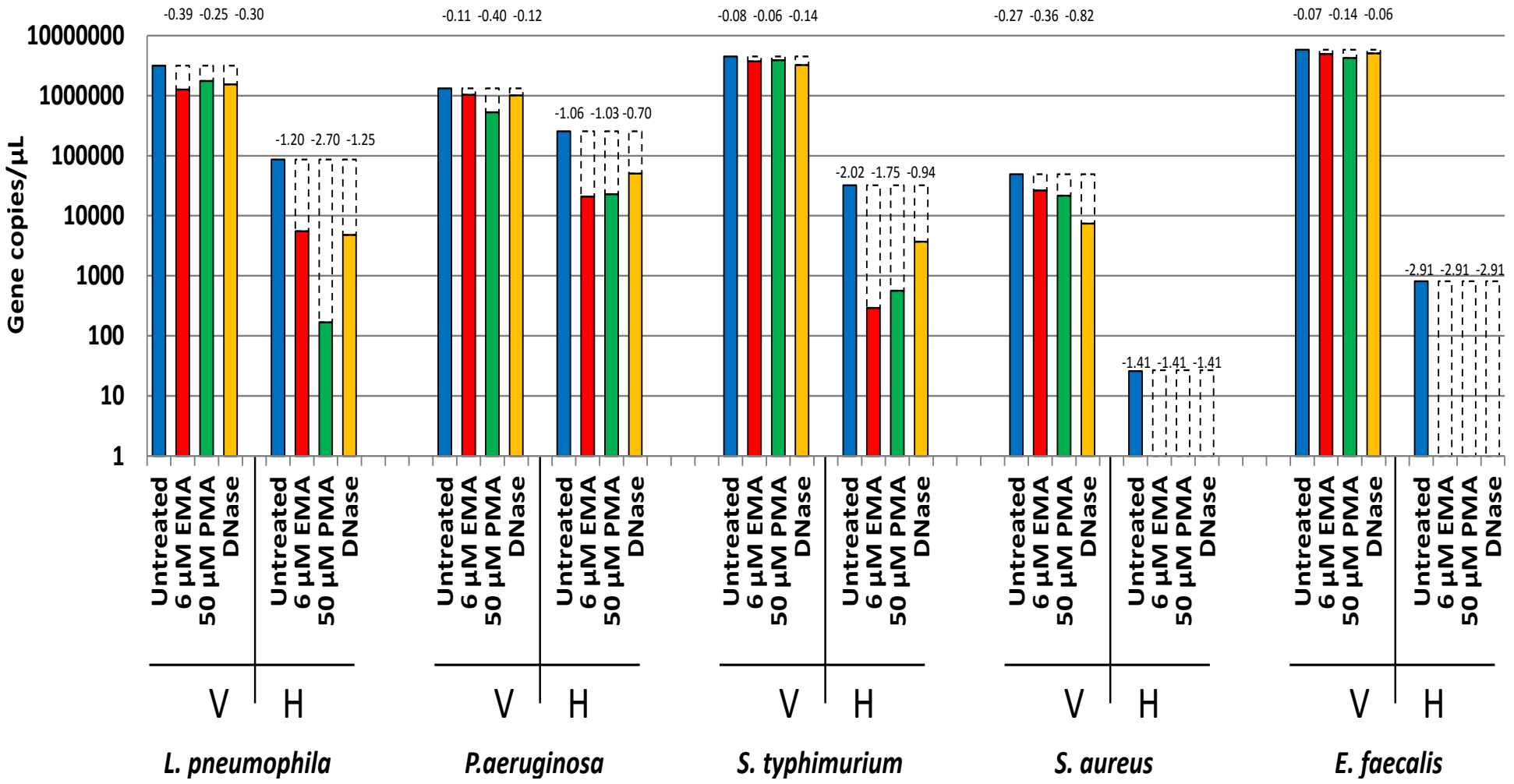

Fig. 3.4. Effect of DNase, EMA and PMA on viable (V) and heat treated (H): *L. pneumophila*; *P. aeruginosa*; *S. typhimurium*; *S. aureus*; *E. faecalis*. Transparent dashed bars and numerical values represent the log reduction in the sample, as compared to the corresponding no viability treatment control (no dye/DNase treatment).

Comparison of the results obtained for the 6 µM EMA, 50 µM PMA (section 3.3.1) and the DNase treatment assay applied to the viable *P. aeruginosa* sample, indicated that the gene copies detected using 6 µM EMA and DNase treatment, were comparable. Compared to the no viability treatment control where 1.32×10^6 gene copies/µL were detected, 1.03×10^6 gene copies/µL (0.11 log decrease) and 1.01×10^6 gene copies/µL (0.12 log decrease) were subsequently detected using 6 µM EMA and DNase treatment, respectively (**Fig. 3.4**).

For the 50 µM, a 0.40 log decrease in gene copies (5.25×10^5 gene copies/µL) was recorded in comparison to the no viability treatment control. Culturing analysis then indicated that *P. aeruginosa* was present at 4.8×10^6 CFU/mL in the viable sample (**Fig. 3.5**). Comparison of the results obtained for the heat treated *P. aeruginosa* sample then indicated that 2.09×10^4 gene copies/µL (1.06 log decrease), 2.29×10^4 gene copies/µL (1.03 log reduction) and 5.01×10^4 gene copies/µL (0.70 log decrease) were detected using 6 µM EMA, 50 µM PMA and DNase treatment, respectively, with 2.54×10^5 gene copies/µL detected in the no viability treatment control (**Fig. 3.4**). The results thus indicated that 6 µM EMA and 50 µM PMA treatment were comparable for the analysis of heat treated *P. aeruginosa* cells however, the lowest log reduction (in comparison to the no viability treatment control) was obtained for the DNase treated sample. Using culture based analysis, viable and culturable cells were still present in the heat treated sample as 4.9×10^4 CFU/mL *P. aeruginosa* were enumerated (**Fig. 3.5**)

Results obtained for the 6 µM EMA and 50 µM PMA (section 3.3.1) concentrations to the DNase treatment assay, applied to the viable *S. typhimurium* sample, revealed that the gene copies detected using EMA, PMA and DNase treatment were comparable. Compared to the no viability treatment control where 4.48×10^6 gene copies/µL were detected, 3.71×10^6 gene copies/µL (0.08 log decrease), 3.89×10^6 gene copies/µL (0.06 log decrease) and 3.22×10^6 gene copies/µL (0.14 log decrease) were detected using 6 µM EMA, 50 µM PMA and DNase treatment, respectively (**Fig. 3.4**). Culturing analysis then indicated that *S. typhimurium* was present at 3.5×10^6 CFU/mL in the viable sample (**Fig. 3.5**). Results for the comparison of the heat treated *S. typhimurium* sample analysed at 6 µM EMA and 50 µM PMA (section 3.3.1) and DNase (section 3.3.2) are represented in **Fig. 3.4**. Compared to the no viability treatment control (3.21×10^4 gene copies/µL), following DNase, 6 µM EMA and 50 µM PMA treatment, respectively, 3.70×10^3 gene copies/µL (0.94 log decrease), 3.06×10^2 gene copies/µL (2.02 log decrease) and 5.66×10^2 gene copies/µL (1.75 log decrease) were detected in the heat treated *S. typhimurium* sample (**Fig. 3.4**). Additionally, culturing analysis confirmed that viable and culturable cells were still present in the heat treated sample as 1.0×10^2 CFU/mL *S. typhimurium* were enumerated (**Fig. 3.5**).

Results obtained using 6 µM EMA and 50 µM PMA (section 3.3.1) to the DNase treatment assay (section 3.3.2) applied to the viable *S. aureus* sample (**Fig 3.4**), indicated that the results

obtained using 6 µM EMA and 50 µM PMA were comparable, while an increased log reduction in *tuf* gene copies was observed in the DNase treated sample. Compared to the no viability treatment control, where 4.92×10^4 gene copies/µL were detected, 2.62×10^4 gene copies/µL (0.27 log decrease), 2.16×10^4 gene copies/µL (0.36 log decrease) and 7.83×10^3 gene copies/µL (0.82 log decrease) were detected using 6 µM EMA, 50 µM PMA and DNase treatment, respectively (**Fig. 3.4**). Culturing analysis then indicated that *S. aureus* was present at 1.2×10^6 CFU/mL in the viable sample (**Fig. 3.5**). It should be noted that while no gene copies were detected following EMA-qPCR and PMA-qPCR analysis of the heat treated *S. aureus* samples, the 6 µM EMA and 50 µM PMA concentrations were utilised for the analysis of the heat treated sample in the current section, as they were the optimal concentrations identified for the viable sample. Analysis of the heat treated *S. aureus* sample indicated that the *tuf* gene from *S. aureus* was present at 2.58×10^1 gene copies/µL in the no viability treatment control sample, however, analysis utilising 6 µM EMA, 50 µM PMA and DNase treatment indicated that the gene copies were reduced to below the LLOD (2 to 8 gene copies/µL). Culturing analysis then supported the results obtained using the molecular-based viability assays as the culturable *S. aureus* in the heat treated sample was also reduced to below the detection limit (< 1 CFU/mL) (**Fig. 3.5**).

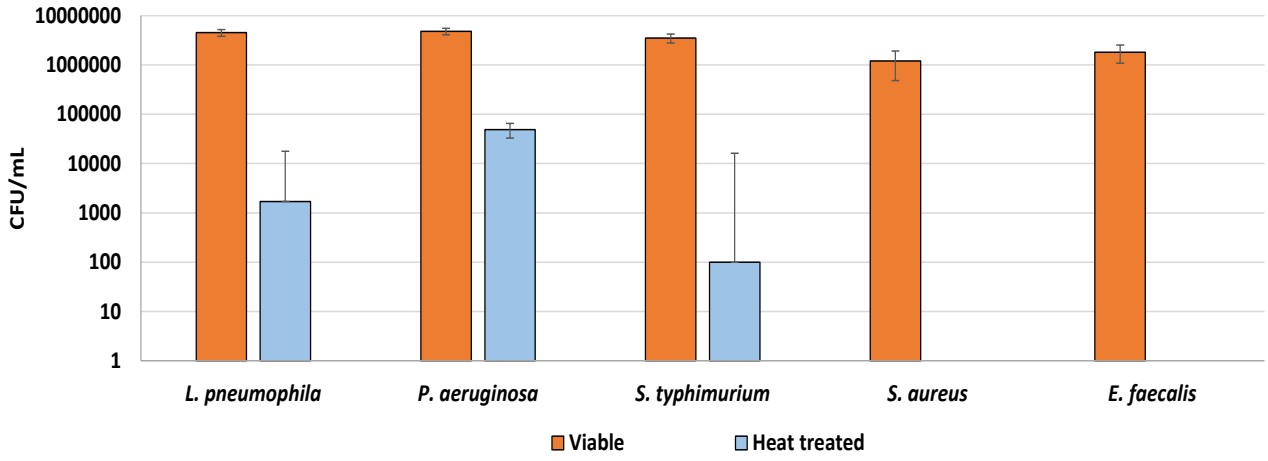

Fig. 3.5. Culturing analysis of the viable and heat treated: *L. pneumophila*; *P. aeruginosa*; *S. typhimurium*; *S. aureus*; *E. faecalis*.

Results obtained using 6 µM EMA and 50 µM PMA (section 3.3.1) to the DNase treatment assay (section 3.3.2) applied to the viable *E. faecalis* sample (**Fig. 3.4**), then revealed that the gene copies detected using the respective assays, were comparable. Compared to the no viability treatment control where 5.79×10^6 gene copies/µL were detected, 5.03×10^6 gene copies/µL (0.06 log decrease), 4.95×10^6 gene copies/µL (0.07 log decrease), and 4.22×10^6 gene copies/µL (0.14 log decrease), were detected using DNase, 6 µM EMA and 50 µM PMA treatment, respectively (**Fig. 3.4**). Culturing analysis then indicated that *E. faecalis* was present at 1.8×10^6 CFU/mL in the viable sample (**Fig. 3.5**). It should be noted that while no gene

copies were detected following EMA-qPCR and PMA-qPCR analysis of the heat treated *E. faecalis* samples, the 6 µM EMA and 50 µM PMA concentrations were utilised for the analysis of the heat treated sample in the current section, as they were the optimal concentrations identified for the viable sample. Analysis of the heat treated *E. faecalis* sample indicated that the 23S rRNA gene from *E. faecalis* was present at 8.16×10^2 gene copies/µL in the no viability treatment control sample, however, analysis utilising 6 µM EMA, 50 µM PMA and DNase treatment indicated that the gene copies were reduced to below the LLOD (7 to 16 gene copies/µL). Culturing analysis then supported the results obtained using the molecular-based viability assays as the culturable *E. faecalis* in the heat treated sample was also reduced to below the detection limit (< 1 CFU/mL) (**Fig. 3.5**).

3.4 Discussion

Various national and international water quality guidelines recommend the utilisation of culture-based techniques to routinely monitor water quality (Gensberger et al., 2014). However, these methods are laborious and time-consuming and often do not provide a comprehensive analysis of viable cells present in a water sample. The need thus arises for the implementation of rapid and sensitive cultivation independent methods to obtain a complete and accurate viability profile (Van Frankenhuyzen et al., 2011; Barbau-Piednoir et al., 2014; Li et al., 2015).

In order to assess the effectiveness of molecular-based viability assays for the detection of culturable and VBNC cells, five model organisms (three Gram-negative and two Gram-positive) were utilised during the viability treatment trials. The organisms *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis* were selected as they are opportunistic pathogens prevalent in water sources (Ahmed et al., 2008; Dobrowsky et al., 2014; 2015b). Moreover, research has indicated that these organisms may be capable of surviving various disinfection treatments (Hall-Stoodley and Stoodley, 2005; Hauser and Ozer, 2011; Dobrowsky et al., 2016; Reyneke et al., 2016; Strauss et al., 2016). In addition, both Gram-negative and Gram-positive organisms were included in the respective trials as three of the molecular-based viability assays (EMA-qPCR, PMA-qPCR and DNase enzyme assay) are dependent on the selective permeability of compromised cell walls/membranes. A metabolic responsiveness assay targeting the detection of ATP (BacTiter ATP assay) was also utilised as an indication of the presence of viable microbial cells, in the respective viable, heat treated and autoclaved samples.

In addition to the viability assays analysed in the current study, the efficacy of a range of EMA and PMA concentrations (reported in literature) were assessed against viable, heat treated and dead (autoclaved) spiked water samples of the respective organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*). A concentration range for both EMA and PMA was analysed as varying optimal dye concentrations have been reported in literature

for specific organisms and it has also been reported that the sample matrix (e.g. various media or environmental samples) needs to be taken into consideration (Fittipaldi et al., 2012). For the analysis of the viable spiked samples, results indicated that EMA was supressing the qPCR detection of viable cells of the respective test organisms, when compared to the corresponding no viability treatment controls. However, while the degree of suppression (log reduction) varied among the different organisms, a greater overall log reduction was obtained for the viable *L. pneumophila* and *S. aureus* samples treated with EMA (**Fig 3.1**). In contrast, for the analysis of *P. aeruginosa*, *S. typhimurium* and *E. faecalis* in the viable samples, with the exception of the 12.5 µM (0.80 log decrease), 50 µM (0.46 log decrease) and 12.5 µM EMA (0.76 log decrease) concentrations, respectively, the log reductions recorded for the various EMA concentrations ranged from 0.01 log to 0.24 log. It is hypothesised that the log reductions obtained in the viable samples (treated with the varying concentrations of EMA), in comparison to the gene copies detected in the no viability treatment control, may possibly be attributed to the presence of dead bacteria in the viable sample and/or osmotic stress, which may have occurred when the pure cultures were spiked into distilled water (Nogva et al., 2003; Sichel et al., 2007; Delgado-Viscogliosi et al., 2009).

In comparison to the results obtained for the EMA-qPCR analysis of the viable samples, results recorded for the analysis of the heat treated samples then indicated that there was an overall greater log decrease in the gene copies of *L. pneumophila*, *P. aeruginosa*, and *S. typhimurium*, following EMA treatment at all concentrations analysed, in comparison to the corresponding no viability treatment control (**Fig 3.1**). Moreover, results indicated that *S. aureus* and *E. faecalis* gene copies were reduced to below the LLOD in the heat treated samples, for all EMA concentrations analysed. The increased log reductions observed in the heat treated samples in comparison to the log reductions in the viable samples were expected, as the test organisms may be susceptible to the heat treatment (70 °C for 15 min) utilised in the current study and EMA would thus supress the detection of the thermally inactivated (injured or dead) cells. In a study conducted by Delgado-Viscogliosi et al. (2009), EMA concentrations were optimised for the differentiation between viable and dead cells of *L. pneumophila* following various disinfection treatments, including heat disinfection. Similar to the results obtained in the current study, it was reported that EMA treatment at 2.5 µg/mL (6 µM) resulted in a 0.29 log decrease in the detection of viable *L. pneumophila* (in comparison to a no viability treatment control), with the degree of qPCR signal suppression increasing with increasing EMA concentration. Additionally, using 2.5 µg/mL EMA, the authors reported a 3.28 log reduction in the detection of *L. pneumophila* (in comparison to a no viability treatment control), following heat treatment (> 95 °C for 15 min).

Research has however, indicated that the greatest drawback to using EMA as a viability dye is its ability to enter viable cells and its cytotoxic effect on viable bacteria at high concentrations

(Nocker et al., 2006; Fittipaldi et al., 2012). It was therefore suggested by Fittipaldi et al. (2012) that in order to overcome this limitation, lower dye concentrations should be utilised with shorter exposure or incubation times. Additionally, the authors suggested that during the EMA treatment, the sample incubation step should be conducted on ice, as the lower temperature would decrease membrane permeability, making it more difficult for EMA to penetrate viable cells. In the current study, by comparing the log decreases observed for *L. pneumophila* in the viable sample treated at 6 µM and 12.5 µM (0.42 and 1.11 log decreases, respectively) to the 35 µM and 50 µM (2.71 and 2.28 log decreases, respectively) EMA treatments, it was apparent that the ability of EMA to suppress the detection of viable cells, increased with increasing dye concentration. However, the effect of increasing EMA concentrations on viable cells was not observed for all organisms during the current study, as the higher EMA concentrations resulted in lower overall log decreases of the viable *P. aeruginosa*, *S. typhimurium* and *E. faecalis*, with varying results obtained for *S. aureus* (**Fig. 3.1**). Similarly, it has been reported in literature that EMA did not have an effect on the qPCR signal from viable cells of *P. aeruginosa*, *S. typhimurium*, *V. vulnificus* and *Nitrosomonas europaea*, thus allowing for the differentiation between viable and dead cells (Nocker et al., 2006; Wang and Levin, 2006; Flekna et al., 2007). While trying to establish the maximum concentration of EMA, that could be used without affecting viable cells, Wang and Levin (2006) also reported that EMA concentrations of 3 µg/mL (7 µM) had little or no inhibition on the detection of viable *Vibrio vulnificus* (*V. vulnificus*), while concentrations above 5 µg/mL (12 µM) resulted in a significant inhibition.

In the current study, the varying susceptibility to EMA displayed by the test organisms may then be attributed to differences in membrane composition between Gram-negative (complex structure of outer membrane) and Gram-positive (thick peptidoglycan layer) bacteria (Nocker et al., 2006; Fittipaldi et al., 2012). Contradictory results to this theory have however been reported, as Flekna et al. (2007) showed that EMA exhibited a similar effect on viable *Campylobacter jejuni* (Gram-negative) and *Listeria monocytogenes* (*L. monocytogenes*) (Gram-positive). In contrast, Nocker et al. (2006) reported that EMA resulted in a significant loss of DNA from viable *S. aureus* (Gram-positive) compared to *S. typhimurium* (Gram-negative), where the gene copies were comparable to the control. Additionally, inconsistent results for the effect of EMA on viable bacterial species from the same genus have been reported (Nocker et al., 2006; Flekna et al., 2007). The results obtained in the current study however indicate that EMA may have a similar effect on both Gram-negative (*P. aeruginosa* and *S. typhimurium*) and Gram-positive (*E. faecalis*) organisms and that the increased log reductions observed for the viable *L. pneumophila* and *S. aureus* samples was most likely due to additional factors influencing EMA entry into the cells. For example, it has been reported that some bacterial species may be able to actively pump EMA out of the viable cell using metabolically driven multidrug efflux pumps, while the movement of EMA in and out of the cell for other bacteria will be based on diffusion alone (Nogva et al., 2003; Nocker et al., 2006; Flekna et al., 2007). It is

therefore possible that *L. pneumophila* and *S. aureus* were not able to excrete the EMA out of the viable cells when exposed to the high concentrations.

Quantitative PCR was also used to assess the effectiveness of varying PMA concentrations on viable cells of the selected test organisms (*L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*). In contrast to the results obtained for EMA-qPCR, PMA-qPCR analysis indicated that lower log reductions in the viable samples of *L. pneumophila* were observed, for the varying PMA concentrations, in comparison to the corresponding no viability treatment control. Moreover, with the exception of *L. pneumophila* and *S. aureus*, the log reduction in gene copies obtained in the viable samples of *P. aeruginosa*, *S. typhimurium* and *E. faecalis,* at the various concentrations of PMA analysed (in comparison to the no viability treatment control), were comparable to the log reductions obtained for the varying concentrations of EMA analysed (**Fig. 3.1**). However, as the log reductions obtained for the varying concentrations of PMA utilised fluctuated (albeit not significantly) for the respective test organisms, a concentration dependent log reduction was not observed with the use of PMA. Corresponding to literature, results for the current study indicate that viable cells of the selected test organisms (*P. aeruginosa, S. typhimurium* and *E. faecalis*), may be able to tolerate higher concentrations of PMA (Fittipaldi et al., 2012). This can be attributed to the lower cytotoxicity of PMA and the dye's increased selectivity for penetrating only cells with damaged/compromised membranes (Nocker et al., 2006; Fittipaldi et al., 2012). The increased selectivity of PMA to cells with a damaged membrane has been attributed to the higher charge of PMA (two positive charges) as compared to EMA (one positive charge) (Nocker et al., 2006). Propidium monoazide is identical to Propidium iodide (PI), which is commonly used for the discrimination of live and dead bacteria using microscopy or flow cytometry, with the exception that PMA contains an additional azide group, which allows it to bind to DNA upon photoactivation. It has thus been well-documented that PI and PMA enters only permeabilized cells, which are characterised as cells without an intact membrane, are unable to maintain membrane potential (depolarized) and are unable to maintain membrane export (de-energised) (Nebe von Caron et al., 2000; Nocker et al., 2006).

A decrease in the gene copy numbers of the respective test organisms (after heat treatment) was then also recorded when utilising the various PMA concentrations, indicating that PMA-qPCR analysis was able to exclude the detection of DNA from non-viable cells. Similar to the results obtained using EMA-qPCR, PMA-qPCR indicated that intact cells of *L. pneumophila*, *P. aeruginosa* and *S. typhimurium* were present in the heat treated sample, while intact cells of *S. aureus* and *E. faecalis* were not detected using PMA-qPCR. However, studies have shown that PMA does not always adequately penetrate cells with compromised membranes and the number of dead cells in a sample could therefore be underestimated (Fittipaldi et al., 2012). In order to overcome this limitation, it has been suggested that using a higher dye concentration

and increasing incubation temperature (as compared to EMA treatment) will facilitate the entry of PMA into cells more readily (Fittipaldi et al., 2012). As indicated, higher dye concentrations may be used as PMA is more selective for the binding of DNA from cells with compromised membranes and is also less cytotoxic. The results obtained in the current study then corroborated this theory as higher PMA concentrations (25 to 100 µM) could be used in comparison to EMA (6 to 50 µM), with the results for the detection of *P. aeruginosa, S. typhimurium* and *E. faecalis* comparable.

DNase treatment (nuclease treatment) prior to qPCR analysis was also investigated, as a method to exclude the detection of damaged or dead cells of the test organisms (*L. pneumophila, P. aeruginosa, S. typhimurium, S. aureus* and *E. faecalis*) in the viable, heat treated and autoclaved samples. Overall, the results indicated that there was a reduction in the detection of the selected test organisms in the viable samples that had been treated with DNase, in comparison to the no viability treatment controls (**Fig. 3.2**). However, for *P. aeruginosa*, *S. typhimurium* and *E. faecalis* low log reductions of 0.12, 0.14 and 0.06 were recorded, respectively. The results thus correspond to the results reported by Do et al. (2009), where it was concluded that DNase treatment did not have a detectable damaging effect on DNA present in live/intact cells of *S. typhimurium*, *E.coli* and *L. monocytogenes*. However, it has been reported that DNase treatment will have varying effects on different bacterial species as the differences in bacterial cell structure (membrane/cell wall composition) and susceptibility of the cell to the killing mechanism (disinfection treatment), will affect the ability of DNase to enter the cell and degrade the DNA (Shakeri et al., 2014). This may then explain the higher log decreases recorded for the viable *S. aureus* (0.82 log decrease) and *L. pneumophila* (0.30 log decrease) samples following DNase treatment. For *S. aureus* in particular, the increased log reduction observed utilising DNase treatment may be explained by the ability of certain bacteria (including *S. aureus*) to produce extracellular thermostable nucleases (Mann et al., 2009). The incubation of the sample with the 10X DNase reaction buffer at 37 °C may therefore have aided the nucleases already present in the sample to affect the viable cells. It should however be noted that, as was hypothesised for the EMA and PMA treated samples, the log reductions observed in the viable samples of the test organisms in comparison to the no viability treatment controls, may also be attributed to the presence of dead bacteria in the viable sample as well as possible osmotic stress when the pure cultures were spiked into distilled water (Nogva et al., 2003; Sichel et al., 2007; Delgado-Viscogliosi et al., 2009). Analysis of the heat treated samples then revealed that the log reductions observed (compared to the no viability treatment control) for *L. pneumophila* (1.25 log reduction), *P. aeruginosa* (0.70 log reduction) and *S. typhimurium* (0.94 log reduction), were comparable to results reported by Nogva et al. (2003) and Rueckert et al. (2005). In the study conducted by Nogva et al. (2003) a 1 log reduction in *Campylobacter jejuni* was obtained following DNase treatment after heat disinfection (72 °C) of the sample, while Rueckert et al. (2005) reported log reductions ranging from 0.8 to 2.6 using

DNase treatment on samples containing viable and non-viable thermophilic bacilli. Similar to the EMA and PMA results, DNase treatment analysis revealed that the gene copies in the heat treated *S. aureus* and *E. faecalis* samples were also reduced to below the LLOD. It is important to note that limited research on the mechanisms that may influence the effectiveness of the DNase enzyme assay has been conducted, in comparison to the multitude of studies conducted on EMA-qPCR and PMA-qPCR (Do et al., 2009; Shakeri et al., 2014). However, it has been suggested by Shakeri et al. (2014) that the two biggest factors which may influence the success of the DNase enzyme assay is firstly, the high molecular weight of DNase which may inhibit its ability to enter the cell wall of certain dead bacteria. Secondly, it is hypothesised that enzyme inhibitors that irreversibly inhibit the DNase enzyme could be released by cells. Future research should thus focus on elucidating the mechanisms by which DNase treatment may be inhibited.

The BacTiter-Glo™ Microbial Cell Viability Assay (BacTiter ATP assay) was then utilised as a metabolic responsiveness assay, to indicate whether metabolically active *L. pneumophila, P. aeruginosa, S. typhimurium, S. aureus* and *E. faecalis* cells were present in the respective viable, heat treated and autoclaved samples. While a reduction in RLU was observed in the heat treated samples, in comparison to the no viability assay control (**Fig. 3.3**), ATP was still detected in all the samples, including the autoclaved samples of *L. pneumophila, P. aeruginosa, S. typhimurium, S. aureus* and *E. faecalis*. In contrast, using the molecular based viability assays, gene copies were detected in all the viable samples and only the heat treated samples of *L. pneumophila, P. aeruginosa* and *S. typhimurium*. Moreover, no gene copies were detected in any of the autoclaved samples. It is however, important to note that the BacTiter ATP assay monitors for the presence of metabolically active cells by analysing for ATP. Therefore, the "metabolically active cells" detected in the heat treated *S. aureus* and *E. faecalis* samples and the autoclaved samples, may be attributed to the detection of residual ATP. Adenosine triphosphate has been shown to be highly soluble in water and is also extremely stable at a pH range of 6.8 to 7.4. While investigating the stability and behaviour of ATP as an indicator of viable cells, Nescerecka et al. (2016) reported that ATP is stable in deionized water and that the presence of extracellular ATP needs to be considered as it occurs naturally on most surfaces. In the current study, the BacTiter-Glo™ reagent was allowed to hydrolyse (burn-off) any ATP that could have been introduced during the manufacturing process and all analysis were conducted in triplicate, with a sterile milliQ negative control also being analysed to subtract the background luminescence from the samples. The detected ATP in the autoclaved samples may therefore be attributed to the persistence of residual ATP from the dead cells in the samples and not contaminating extracellular ATP. In studies conducted by Berney et al. (2008) and Vital et al. (2012), results then indicated that flow cytometric analysis and the BacTiter ATP assay complemented each other as similar trends were observed in the analysis of samples. However, as the BacTiter ATP assay detected residual ATP in the autoclaved samples (in the current study), it is proposed that the BacTiter ATP assay be used in a complimentary manner to

corroborate data obtained utilising other metabolic responsiveness and molecular-based viability assays.

While the optimal EMA and PMA dye concentrations for the specific test organisms were identified (section 3.3.1), it would be more economically feasible to screen environmental samples with a single dye concentration that would allow for the accurate identification of viable cells from different organisms. Based on the results obtained for the EMA-qPCR and PMA-qPCR analysis, 6 µM EMA and 50 µM PMA were thus identified as the optimal dye concentrations for the detection of all the test organisms (*L. pneumophila, P. aeruginosa, S. typhimurium, S. aureus* and *E. faecalis*) in the viable, heat treated and dead spiked water samples. The 6 µM EMA concentration resulted in the lowest log reduction in the viable samples of *L. pneumophila*, *P. aeruginosa* and *E. faecalis* and was comparable to the lowest log reductions observed for *S. typhimurium* and *S. aureus* at 12.5 µM and 25 µM, respectively. Correspondingly, 6 µM EMA resulted in the lowest log reduction in the heat treated *L. pneumophila* and *S. typhimurium* samples and was comparable to the lowest log reduction observed for *P. aeruginosa* (12.5 µM). While the 25 µM PMA concentration resulted in the lowest log reduction in the viable *P. aeruginosa*, *S. aureus* and *E. faecalis* samples (**Fig. 3.1**), the log reductions for 50 µM PMA were comparable and also resulted in the lowest log reductions for the viable *L. pneumophila* and *S. typhimurium* samples. Additionally, 50 µM PMA yielded the lowest log reductions in the heat treated *L. pneumophila*, *P. aeruginosa* and *S. typhimurium* samples. The recorded log reductions for 6 µM EMA and 50 µM PMA were also comparable in the viable samples of *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*. The identification of 6 µM EMA and 50 µM PMA as the optimal dye concentrations then corresponds to literature where 6 µM EMA (Wang and Levin, 2006; Chang et al., 2009; Chen and Chang, 2009; Delgado-Viscogliosi et al., 2009) and 50 µM PMA (Nocker et al., 2006; Yáñez et al., 2011; Tavernier and Coenye, 2015) have successfully been used to discriminate between viable and dead cells.

As the principle for DNase pre-treatment in combination with qPCR is similar to EMA-qPCR and PMA-qPCR, the results obtained during the DNase assay were compared to the results obtained using 6 µM EMA and 50 µM PMA. In addition culture based analysis was utilised to enumerate the viable and culturable bacteria that were present in the respective samples. For the viable *L. pneumophila, S. typhimurium* and *E. faecalis* samples, the results obtained using EMA-qPCR, PMA-qPCR, and the DNase assay were comparable, as similar log reductions were observed (**Fig 3.4**) with an average of 1.52×10^6 gene copies/µL, 3.60×10^6 gene copies/µL and 4.73×10^6 gene copies/µL detected, respectively. In comparison, culturing analysis indicated that 4.5×10^6 CFU/mL, 3.5×10^6 CFU/mL and 1.8×10^6 CFU/mL were present in the viable samples of *L. pneumophila, S. typhimurium* and *E. faecalis*, respectively. In contrast, for the analysis of the viable *P. aeruginosa* sample using the three molecular based

viability assays, the results recorded for the 6 µM EMA and DNase treatment were comparable as a 0.11 and 0.12 log decrease was recorded for each assay, respectively. In comparison a 0.40 log decrease was observed following 50 µM PMA treatment. For *S. aureus*, it was observed that the gene copies for 6 µM EMA and 50 µM PMA were comparable, as 0.27 and 0.36 log decreases were recorded, respectively, in comparison to the no viability treatment control. Treatment with DNase resulted in an increased log reduction being observed (0.82 log decrease). Culturing analysis then indicated that 4.8×10^6 CFU/mL and 1.2×10^6 CFU/mL were recorded in the viable *P. aeruginosa* and *S. aureus* samples, respectively. In the current study the gene copies/µL obtained during qPCR analysis could not be converted to the number of cells present in the sample, as a varying number of the targeted gene may be present within the organism and the gene copy number may differ between species (Vetrovsky and Baldrian, 2013). Thus, while culturing analysis (CFU/mL) cannot be directly compared to the number of gene copies/µL, numerous studies have conducted correlation analysis using these two variables, with an increase in CFU generally resulting in an increase in gene copies. The results obtained in the current study for the analysis of the viable samples, then indicated that the culturing analysis results were generally within the same log range as the gene copies recorded using molecular based viability analysis. However, an exception to this observation was noted for the viable *S. aureus* sample, where 1.2×10^6 CFU/mL were detected using culturing analysis, while the molecular-based viability assays yielded an average concentration of 1.8×10^4 gene copies/µL. Analysis of the no viability treatment control, however, indicated that 4.9×10^4 gene copies/µL were present in the sample. The ratio of detected gene copies to CFU for *S. aureus* was therefore low, possibly indicating that cells/DNA may have been lost during the sample concentration and/or DNA extraction procedure.

Culturing analysis of the heat treated *L. pneumophila*, *P. aeruginosa* and *S. typhimurium* then revealed that the respective organisms were present at 1.7×10^3 CFU/mL, 4.9×10^4 CFU/mL and 1.0×10^2 CFU/mL, respectively. In comparison, the molecular-based viability assays indicated that 3.49×10^3 gene copies/µL, 3.13×10^4 gene copies/µL and 1.52×10^3 gene copies/µL were detected in the heat treated *L. pneumophila, S. typhimurium* and *E. faecalis* samples, respectively. Moreover, results for the culture-based analysis and molecular viability assays indicated that viable *S. aureus* and *E. faecalis* were not present in the respective heat treated samples. As was observed for the viable samples, the log range of the CFU/mL and gene copies/µL were comparable for the analysis of the heat treated samples. However, the gene copies in the heat treated samples of *L. pneumophila* and *E. faecalis* were slightly (albeit not significantly) higher in samples analysed using the molecular viability assays in comparison to the culturing analysis. Firstly, it has been shown that bacteria, such as *L. pneumophila*, may be present as chain structures (aggregated together or connected) which will essentially be enumerated as 1 CFU during culturing analysis, whereas the individual cells will be quantified during qPCR (Delgado-Viscogliosi et al., 2009). In addition, as the cells had undergone heat

treatment, culturing analysis may be underestimating the concentration of viable cells in the heat treated samples, as the cells may have entered a VBNC state. Therefore, the advantage of using molecular-based techniques will be their ability to detect VBNC organisms in samples following disinfection treatment.

Results thus indicate that the physiological state of the microbial community within a sample, should be considered when deciding which technique to utilise, as results obtained in the current study indicate that both culturing and molecular-based viability assays may be used for the analysis of samples where the microbial community is viable, however, it would be advantageous to use molecular-based viability assays in samples where organisms may be present in a VBNC state. Similarly, while assessing and comparing EMA-qPCR to culture based analysis, Delgado-Viscogliosi et al. (2009), concluded that based on the different physiological states of cells in viable and heat treated samples, culture based analysis could be utilised to analyse samples where no disinfection treatment was utilised, while EMA-qPCR (and by extension PMA-qPCR and DNase treatment in combination with qPCR) could be utilised to analyse cell viability in samples exposed to a disinfection treatment. Molecular-based viability assays are however less labour intensive and results can be generated within 7 hours (Lombard et al., 2016). This may then be advantageous in scenarios where a rapid water quality assessment or organism identification may be required. Additionally, the results generated utilising molecular-based viability assays have been shown to be highly reproducible (Delgado-Viscogliosi et al., 2009; Reyneke et al., 2016; Strauss et al., 2016).

3.5 Conclusions

The current study assessed and compared molecular-based viability assays (EMA-qPCR, PMA-qPCR and DNase treatment in combination with qPCR) and a metabolic responsiveness assay (BacTiter-Glo™ Microbial Cell Viability Assay) to culturing analysis in order to determine which assay would provide a comprehensive indication of viability. The results indicated that EMA, PMA and DNase were able to indicate the presence of viable microbial cells; however, depending on the target organism utilised, treatment with a specific molecular viability assay was more applicable. For example, higher concentrations of EMA, adversely influenced the detection of viable *L. pneumophila*, while DNase treatment may influence the detection of viable *S. aureus* cells, as a greater log decrease in the detection of gene copies was recorded in comparison to EMA and PMA analysis. Additionally, results indicated that overall, 6 µM EMA and 50 µM PMA were the optimal dye concentrations for the detection of the respective test organisms using EMA-qPCR and PMA-qPCR, respectively. However, comparison of the results obtained for the molecular viability assays utilised indicated that the results obtained for 6 µM EMA compared well with the results obtained for 50 µM PMA and DNAse treatment, for the analysis of viable *L. pneumophila*, *P. aeruginosa*, *S. typhimurium, S. aureus* and *E. faecalis*. In

addition, the results for the culturing analysis (CFU) of the viable *S. typhimurium* as well as the viable and heat treated samples of *L. pneumophila* and *P. aeruginosa* were comparable to the gene copies detected using molecular-based viability assays, with no gene copies or CFU detected in the heat treated samples of *S. aureus* and *E. faecalis*. Based on the results obtained it is thus concluded that molecular-based viability assays may be utilised to supplement the "golden standard" culture based analysis. However, molecular-based viability assays may produce a more accurate indication of cell viability when high numbers of VBNC cells are present in a sample. While the results obtained also indicated that DNase pre-treatment in combination with qPCR has the ability to successfully differentiate live from dead cells, future research should identify which organisms, such as *S. aureus*, have the ability to produce thermostable nucleases and subsequently negatively affect analysis of viable cells by DNase treatment. Results obtained in the current study for the BacTiter ATP assay also indicated that while the assay allowed for the rapid and sensitive detection of ATP (indicator of metabolically active cells) in the analysed samples, ATP may persist in samples following disinfection treatment and therefore the presence of residual or extracellular ATP needs to be taken into consideration when analysing samples. Nevertheless, the assay may provide valuable information as to the general viability assessment of samples and would be an excellent secondary analysis tool to utilise in order to substantiate results obtained using other viability techniques.

3.6 References

Ahmed, W., Huygens, F., Goonetilleke, A. & Gardner, T. 2008. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. Appl. Environ. Microb. 74:5490-5496.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Barbau-Piednoir, E., Roosens, N.H., Bertrand, S., Mahillon, J. & Botteldoorn, N. 2013. SYBR® Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels. Appl. Microbiol. Biotechnol. 97:9811-9824.

Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Coucke, W., Roosens, N.H. & Botteldoorn, N. 2014. Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis. J. Microbiol. Meth. 103:131-137.

Bergmark, L., Poulsen, P.H.B., Abu Al-Soud, W., Norman, A., Hansen, L.H. & Sørensen, S.J. 2012. Assessment of the specificity of *Burkholderia* and *Pseudomonas* qPCR assays for detection of these genera in soil using 454 pyrosequencing. FEMS Microbiol. Let. 333:77-84.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H-A., Egli, T. & Hammes, F. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res. 42: 4010-4018.

Bosshard, F., Berney, M., Scheifele, M., Weilenmann, H.U. & Egli, T. 2009. Solar disinfection (SODIS) and subsequent dark storage of *Salmonella typhimurium* and *Shigella flexneri* monitored by flow cytometry. Microbiology+ 155:1310-1317.

Brözel, V.S. & Cloete, T.E. 1991. Resistance of bacteria from cooling waters to bactericides. J. Ind. Microbiol. 8:273-276.

Cenciarini-Borde, C., Courtois, S. & La Scola, B. 2009. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiol. 4:45-64.

Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F. & Watanabe, H. 2009. Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. Appl. Environ. Microbiol. 75:147-153.

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F. & Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. Jpn. J. Infect. Dis. 63:119–123.

Chen, N.T. & Chang, C.W. 2009. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J. Appl. Microbiol. 109:623-634.

Darzynkiewics, Z., Robinson, J.P. & Crissman, H.A. 1994. Methods in cell biology, flow cytometry. Part A, Vol 41. Rev. 2nd ed. California, USA: Academic Press Inc.

Deininger, R.A. & Lee, J. 2006. Modern tools and methods of water treatment for improving living standards. In: Omelchenko, A., Pivovarov, A. A., Swindall, W. J. (Eds.), Proceedings of the NATO Advanced Research Workshop on Modern Tools and Methods of Water Treatment for Improving Living Standards Dnepropetrovsk, Ukraine 19–22 November 2003. Springer, Netherlands, pp. 71–78.

Delgado-Viscogliosi, P., Simonart, T., Parent, V., Dobbelaere, M., Pierlot, E., Menard-Szczebara, F., Gaudard-Ferveur, E., Delabre, K. & Delattre, J.M. 2005. Rapid method for enumeration of viable *Legionella pneumophila* and other *Legionella* spp. in water. Appl. Environ. Microbiol. 71:4086-4096.

Delgado-Viscogliosi, P., Solignac, L. & Delattre, J.M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microb. 75:3502-3512.

Do, H.T.T., Anceno, A.J. & Rakshit, S.K. 2009. DNase I treated DNA-PCR based detection of food pathogens immobilized by metal hydroxides. World J. Microbiol. Biotechnol. 25:1491-1495.

Dobrowsky, P.H., Cloete, T.E. & Khan, W. 2014. Quality assessment of domestic harvested rainwater in the peri-urban region of Kleinmond, Western Cape and the optimisation of point-of-use treatment systems. M.Sc. Thesis. University of Stellenbosch.

Dobrowsky, P.H., Carstens, M., De Villiers, J., Cloete, T.E. & Khan, W. 2015a. Efficiency of a closed-coupled solar pasteurization system in treating roof harvested rainwater. Sci. Total Environ. 536:206-214.

Dobrowsky, P.H., Lombard, M., Cloete, W.J., Saayman, M., Cloete, T.E., Carstens, M., Khan, S. & Khan, W. 2015b. Efficiency of microfiltration systems for the removal of bacterial and viral contaminants from surface and rainwater. Water Air Soil Pollut. 226:1-14.

Dobrowsky, P.H., Khan, S., Cloete, T.E. & Khan, W. 2016. Molecular detection of *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* as vectors for *Legionella* spp. in untreated and solar pasteurized harvested rainwater. Parasit. Vectors 9:539-552.

Dusserre, E., Ginevra, C., Hallier-Soulier, S., Vandenesch, F., Festoc, G., Etienne, J., Jarraud, S. & Molmeret, M. 2008. A PCR-based method for monitoring *Legionella pneumophila* in water samples detects viable but noncultivable legionellae that can recover their cultivability. Appl. Environ. Microbiol. 74:4817–4824.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K. & Morato, J. 2011. Viable real-time PCR in environmental samples: can all data be interpreted directly? Microbial Ecol. 61:7–12.

Fittipaldi, M., Nocker, A. & Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification.J. Microbiol. Meth. 91:276-289.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S. & Hein, I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res. Microbiol. 158:405–412.

Fongaro, G., Nascimento, M.A., Rigotto, C., Ritterbusch, G., Da Silva, A.D.A., Esteves, P.A. & Barardi, C.R.M. 2013. Evaluation and molecular characterization of human adenovirus in drinking water supplies: viral integrity and viability assays. J. Virol. 10:166-174.

Frahm, E. & Obst, U. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Microbiol. Meth. 52:123-131.

Frankfurt, O.S. 1983. Assessment of cell viability by flow cytometric analysis using DNase exclusion. Exp. Cell. Res. 144:478–482.

García, M.T., Jones, S., Pelaz, C., Millar, R.D. & Abu Kwaik, Y. 2007. *Acanthamoeba polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after disinfection. Environ. Microbiol. 9:1267–1277.

Gensberger, E.T., Polt, M., Konrad-Koszler, M., Kinner, P., Sessitsch, A. & Kostic, T. 2014. Evaluation of quantitative PCR combined with PMA treatment for molecular assessment of microbial water quality. Water Res. 67:367-376.

Girones, R., Ferrus, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., De Abreu Correa, A., Hundesa, A., Carratala, A. & Bofil-Mas, S. 2010. Molecular detection of pathogens in water – the pros and cons of molecular techniques. Water Res. 44:4325-4339.

Guy, R.A., Payment, P., Krull, U.J. & Horgen, P.A. 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. 69:5178-5185.

Hall-Stoodley, L. & Stoodley, P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 13:7-10.

Hauser, A. & Ozer, E.A. 2011. *Pseudomonas aeruginosa*. Nature Rev. Microbiol. 9:1-2.

Herpers, B.L., De Jongh, B.M., Van Der Zwaluw, K. & Van Hannen, E.J. 2003. Real-time PCR assay targets the 23S-5S spacer for direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila*. J. Clin. Microbiol. 41:4815-4816.

Hwang, M.G., Katayama, H. & Ohgaki, S. 2006. Effect of intracellular resuscitation of *Legionella pneumophila* in *Acanthamoeba polyphage* cells on the antimicrobial properties of silver and copper. Environ. Sci. Technol. 40:7434–7439.

Keer, J.T. & Birch, L. 2003. Molecular methods for the assessment of bacterial viability. J. Microbiol. Meth. 53:175-183.

Kobayashi, H., Oethinger, M., Tuohy, M.J., Hall, G.S. & Bauer, T.W. 2008. Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide. Lett. Appl. Microbiol. 48:633-638.

Lee, J. & Deininger, R.A. 2001. Rapid quantification of viable bacteria in water using an ATP assay. Am. Lab. 33:24-30.

Lemarchand, K., Berthiaume, F., Maynard, C., Harel, J., Payment, P., Bayardelle, P., Masson, L. & Brousseau, R. 2005. Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. J. Microbiol. Meth. 63:115–26.

Li, H., Xin, H. & Li, S.F.Y. 2015. Multiplex PMA-qPCR assay with internal amplification control for silmultaneous detection of viable *Legionella pneumophila*, *Salmonella typhimurium* and *Staphylococcus aureus* in environmental waters. Environ. Sci. Technol. 49:14249-14256.

Loge, F.J., Thompson, D.E. & Call, D.R. 2002. PCR detection of specific pathogens in water: A risk-based analysis. Environ. Sci. Technol. 36:2754-2759.

Lombard, M., Wolfaardt, G.M., Van Blerk, N. & Khan, W. 2016. Detection, Identification and quantitation of *Cryptosporidium parvum* in water samples and *Ascaris lumbricoides* in sludge samples using real-time polymerase chain reaction coupled with the high-resolution melt curve sssay. M.Sc. Thesis. University of Stellenbosch.

Mann, E.E., Rice, K.C., Boles, B.R., Endres, J.L., Ranjit, D., Chandramohan, L., Tsang, L.H., Smeltzer, M.S., Horswill, A.R. & Bayles, K.W. 2009. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. Plos One 4:e5822.

Masters, C.L., Shallcross, J.A. & Mackey, B.M. 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. J. Appl. Bacteriol. 77:73–79.

Mukhopadhyay, U.K. & Mukhopadhyay, A. 2002. A low-cost, rapid, sensitive and reliable PCR-based alternative method for predicting the presence of possible live microbial contaminants in food. Curr. Sci. India 83:53-56.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. & Donlan, R.M. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology+ 147:3121-3126.

Nebe-von-Caron, G., Stephens, P., Hewitt, C.J., Powell, J.R. & Bradley, R.A. 2000. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J. Microbiol. Meth. 42:97-114.

Nescerecka, A., Juhna, T. & Hammes, F. 2016. Behaviour and stability of adenosine triphosphate (ATP) during chlorine disinfection. Water Res. 101:490-497.

Nocker, A. & Camper, A.K. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl. Environ. Microbiol. 72:1997–2004.

Nocker, A., Cheung, C.Y., Camper, A.K. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Meth. 67:310–320.

Nocker, A. & Camper, A.K. 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiol. Lett. 291:137–142.

Nogva, H.K., Bergh, A., Holck, A. & Rudi, K. 2000. Application of the 5'-Nuclease PCR assay in evaluation and development of methods for quantitative detection of *Campylobacter jejuni*. Appl. Environ. Microbiol. 66:4029-4036.

Nogva, H.K., Dromtorp, S.M., Nissen, H. & Rudi, K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-Nuclease PCR. Biotechniques 34:804-813.

Oliver, J.D. 2000. The public health significance of viable but nonculturable bacteria. In *Nonculturable microorganisms in the environment*. R. Colwell & D. J. Grimes, Eds. Washington, DC, USA: ASM Press. 277-300.

Pyle, B.H., Broadaway, S.C. & McFeters, G.A. 1995. Factors affecting the determination of respiratory activity on the basis of cyanoditolyl tetrazolium chloride reduction with membrane filtration. Appl. Environ. Microbiol. 61:4304-4309.

Reyneke, B., Dobrowsky, P.H., Ndlovu, T., Khan, S. & Khan, W. 2016. EMA-qPCR to monitor the efficiency of a closed-coupled solar pasteurization system in reducing *Legionella* contamination of roof-harvested rainwater. Sci. Total Environ. 553:662-670.

Rueckert, A., Ronimus, R.S. & Morgan, H.W. 2005. Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus.* J. Appl. Microbiol. 99:1246–1255.

Sakai, H., Procop, G.W., Kobayashi, N., Togawa, D., Wilson, D.A., Borden, L., Krebs, V. & Bauer, T.W. 2004. Simultaneous detection of *Staphylococcus aureus* and coagulase-negative

staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. J. Clin. Microbiol. 42:5739-5744.

Shakeri, M., Shahidi, F., Mortazavi, A., Bahrami, A.R. & Nassiri M.R. 2014. Application of PCR technique in combination with DNAse treatment for detection of viable *Lactobacillus acidophilus* bacteria. J. Food Quality 37:291–295.

Shannon, K.E., Lee, D.Y., Trevors, J.T. & Beaudette, L.A. 2007. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci. Total Environ. 382:121-129.

Sheridan, G.E., Masters, C.I., Shallcross, J.A. & MacKey, B.M. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. 64:1313–1318.

Sichel, C., Blanco, J., Malato, S. & Fernandez-Ibanez, P. 2007. Effects of experimental conditions on *E. coli* survival during solar photocatalytic water disinfection. J. Photoch. Photobio. A 189:239-246.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A. & Yoshida, S. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol. Immunol. 51:763–775.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Yoshida, S. 2008. Method to detect only live bacteria during PCR amplification. J. Clin. Microbiol. 46:2305–2313.

Strauss, A., Dobrowsky, P.H., Ndlovu, T., Reyneke, B., Khan, W. 2016. Comparative analysis of solar pasteurization versus solar disinfection for the treatment of harvested rainwater. BMC Microbiol. 16:289-305.

Tavernier, S. & Coenye, T. 2015. Quantification of *Pseudomonas aeruginosa* in multispecies biofilms using PMA-qPCR. PeerJ 3:e787; DOI 10.7717/peerj.787.

Trevors, J.T. 2012. Can dead bacterial cells be defined and are genes expressed after cell death? J. Microbiol. Meth. 90:25-29.

Van Frankenhuyzen, J.K., Trevors, J.T., Lee, H., Flemming, C.A. & Habash, M.B. 2011. Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. J. Microbiol. Meth. 87:263-272.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S. & Vesper, A. 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). J. Microbiol. Meth. 72:180–184.

Vetrovsky, T. & Baldrian, P. 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. Plos One 8:e57923. doi:10.1371/journal.pone.0057923

Viancelli, A., Garcia, L.A.T., Kunz, A., Steinmetz, R., Esteves, P.A. & Barardi, C.R.M. 2012. Detection of circoviruses and porcine adenoviruses in water samples collected from swine manure treatment systems. Res. Vet. Sci. 93:538-543.

Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L. & Hammes, F. 2012. Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. Water Res. 46:4665-4676.

Wang, S. & Levin, R.E. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J. Microbiol. Meth. 64:1–8.

Wang, Y., Claeys, L., Van Der Ha, D., Verstraete, W. & Boon, N. 2010. Effects of chemically and electrochemically dosed chlorine on *Escherichia coli* and *Legionella beliardensis* assessed by flow cytometry. Appl. Microbiol. Biotechnol. 87:331-341.

Wolffs, P., Norling, B. & Radstrom, P. 2005. Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. J. Microbiol. Meth. 60:315-323.

Yáñez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L. & Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Meth. 85:124–130.

Chapter 4:

General Conclusions and Recommendations

(UK spelling is employed)

General Conclusions and Recommendations

Rainwater harvesting has been earmarked by many international governmental authorities as an intervention strategy that allows for the provision of an on-site water supply in areas where water distribution infrastructure is lacking (Li et al. 2010; Mwenge Kahinda et al. 2010). In an effort to provide the inhabitants of rural areas and urban informal settlements with an alternative water source, the South African government has also earmarked rainwater as a sustainable, supplementary water source (Malema et al. 2016). However, numerous studies have demonstrated that microbial and chemical contaminants may be associated with rainwater sources as a result of geographical location, proximity to pollution sources and the system design (Ahmed et al. 2008; 2011; Helmreich & Horn, 2009; Li et al. 2010). It is therefore essential that adequate treatment technologies be implemented, especially in developing countries, in order to ensure that the water is safe to utilise for all daily water requirements.

The Water Research Commission project K5/2124//3 (2014) titled, "*Point of use disinfection systems designed for domestic rainwater harvesting (DRWH) tanks for improved water quality in rural communities*," completed by members of our research group, focused not only on routinely monitoring the chemical and microbial quality of tank water, but also investigated the application of various treatment technologies such as slow sand filtration, activated carbon/nanofiber filtration and solar pasteurization (SOPAS), for the reduction of the level of microbial contaminants in rainwater. The results obtained demonstrated that the closed-coupled SOPAS treatment system utilised effectively reduced the total coliform, *Escherichia coli* (*E. coli*) and heterotrophic bacteria counts to below the detection limit (> 99.9%) in rainwater samples pasteurized above 72 °C (Dobrowsky et al. 2015). Moreover, the system allowed for the treatment of larger volumes of water in comparison to the other tested treatment technologies (slow sand, activated carbon, and nanofiber filtration). However, the SOPAS system adversely affected the chemical quality of the harvested rainwater during pasteurization treatment, as aluminium (Al), lead (Pb), nickel (Ni) and iron (Fe) were detected at levels exceeding various national and international drinking water guidelines [Department of Water Affairs and Forestry (DWAF, 1996); South African National Standards (SANS) 241 (South African Bureau of Standards (SABS), 2005); Australian Drinking Water Guidelines (ADWG) (NHMRC and NRMMC, 2011); World Health Organisation (WHO, 2011)], following SOPAS treatment (Dobrowsky et al. 2015; Reyneke et al. 2016). As it was hypothesised that these metals were leaching from the stainless steel storage tank used in the SOPAS system, a new SOPAS system (Phungamanzi^{TM}) with a storage tank constructed from high grade polyethylene was designed by a South African manufacturer.

The primary aim of **Chapter 2** was thus to monitor the operational sustainability and efficacy of two small-scale Phungamanzi^{TM} (Sites 1 and 2) SOPAS systems and one large-scale (Site 3)

SOPAS system installed in Enkanini informal settlement (Stellenbosch) for the treatment of rainwater. In order to achieve this aim, unpasteurized and pasteurized tank water samples were collected from the small-scale SOPAS systems located at Sites 1 [52 °C, 60 °C, 67 °C, 72 °C, 73 °C, 75 °C (2 samples) and 85 °C] and 2 (53 °C, 58 °C and 66 °C), while unpasteurized, pasteurized (55 °C, 61 °C, 66 °C, 71 °C, 72 °C and 79 °C) and stored pasteurized tank water samples were collected from the large-scale SOPAS system at Site 3. The microbial quality (culture-based analysis for indicator organisms) and chemical quality (cation and anion concentration determination) of the harvested rainwater before and after SOPAS was monitored with the results compared to various national and international drinking water guidelines [DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011)]. However, as research has shown that there is a poor correlation between the presence of indicator organisms and pathogenic microorganisms (Ahmed et al. 2008), conventional polymerase chain reaction (PCR) assays were utilised to screen for pathogens (including *Legionella* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Klebsiella* spp., amongst others) generally associated with harvested rainwater. The most readily detected pathogens were then quantified using quantitative PCR (qPCR) assays. Additionally, a metabolic responsiveness assay (BacTiter-Glo™ Microbial Cell Viability Assay) was utilised to monitor the efficiency of the SOPAS systems in reducing the level of metabolically active cells (ATP) in the pasteurized rainwater. The operational sustainability of the systems was also monitored for a period of one year (September 2015 to September 2016) in order to determine whether the systems were beneficial to the households using them, whether the system components were durable, how much water could be produced and how much pasteurized rainwater was being utilised by the households.

Results for the anion and cation analysis indicated that all the collected water samples from Sites 1 and 2 (unpasteurized and pasteurized) and Site 3 (unpasteurized and stored pasteurized), were within the respective drinking water guidelines' limits [DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011)]. However, results for the cation analysis indicated that zinc (Zn) was detected at levels exceeding the recommended drinking water guidelines in both the unpasteurized and pasteurized tank water samples collected from Sites 1 and 2, with no significant difference (Site 1: $p = 0.21$; Site 2: $p = 0.76$) in concentration being observed following SOPAS. The increased Zn concentrations were then attributed to the galvanised Zn roofing material utilised at Sites 1 and 2, as research has demonstrated that rainwater collected from roofing systems constructed from galvanised Zn sheets may contain Zn concentrations ranging from 0.1 to 10 mg/L, depending on the type of Zn sheeting used (Heijerick et al. 2002). Cation analysis also indicated that arsenic (As) was detected at levels exceeding the recommended drinking water guidelines in the both the unpasteurized and stored pasteurized tank water samples collected from Site 3, with no

significant difference (p = 0.18) in concentration being observed following SOPAS. It was then hypothesised that the increased As concentrations may be attributed to the paint utilised to coat the galvanized zinc roofing material (catchment area) at Site 3, as research has indicated that certain paints may contain As (Kopylov et al. 2007; Anomaly, 2009). Numerous coating materials are commercially available that are used to extend the life span of a roofing material, by providing protection against ultra-violet (UV) light exposure as well as water leaks during a rainfall event (Berdahl et al. 2008). These coatings range from metal-based paints to rubber and elastomeric materials. It is thus important that future research should focus on the chemical quality of rainwater harvested from surfaces coated with these commercially available products, in order to determine which types of coatings may be used on rainwater harvesting catchment systems. Based on the results obtained in the current study and results reported in literature, chemical contaminants of rainwater should be routinely investigated. Moreover, as it has recently been reported that emerging chemical contaminants, such as endocrine-disruptors, are ubiquitous within water sources (Altenburger et al. 2015), it is recommended that the scope of chemical contaminants regularly analysed for, is increased to include these chemical compounds. Future studies should thus focus on identifying whether these emerging chemical contaminants are prevalent in rainwater sources and then possibly investigate treatment technologies that would allow for their successful removal from the water source.

Corresponding to other reports (Ahmed et al. 2008; 2010; 2011; Radaidah et al. 2009; Dobrowsky et al. 2014), results recorded for the culturing analysis revealed that the unpasteurized tank water samples collected from Sites 1, 2 and 3 were not within the respective drinking water guidelines' limits, as total coliforms, *E. coli* and heterotrophic plate counts (HPC) were recorded at levels exceeding the recommended guideline limits, while faecal coliforms were sporadically detected at levels which also contravened the recommended guidelines [DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011)]. Analysis of the pasteurized tank water samples collected from Sites 1 and 2 indicated that a minimum pasteurization temperature of 53 °C was required to reduce the levels of total coliforms and *E. coli* to below the detection limit (BDL; < 1 CFU/100 mL), while a minimum temperature of 66 °C was required to reduce the HPC to within drinking water standards. Similarly, results obtained for the large-scale system indicated that a minimum pasteurization temperature of 55 °C was required to reduce the total coliforms and *E. coli* to within drinking water standards, as all counts were BDL (< 1 CFU/100 mL) in the pasteurized (manifold system) and stored pasteurized tank water (1500 L storage tank) samples. Enumeration of the HPC revealed that a minimum pasteurization temperature of 71 °C was required to reduce the HPC to within recommended drinking water limits for the large-scale system (Site 3). However, HPC recorded in all the stored pasteurized water samples analysed exceeded the recommended drinking water guidelines. The results obtained for the samples following SOPAS treatment

therefore correspond to results reported in literature where researchers studying the thermal inactivation kinetics of indicator organisms (Spinks et al. 2003; 2006; Despins et al. 2009) and solar pasteurization of rainwater (Coombes et al. 2000; Dobrowsky et al. 2015; Strauss et al. 2016), indicated that temperatures ranging from 55 °C to 72 °C have the ability to reduce microbial contamination in rainwater to within drinking water standards [DWAF (1996), SANS 241 (SABS, 2005), ADWG (NHMRC and NRMMC, 2011) and WHO (2011)]. However, the increased HPC observed in the stored pasteurized tank water samples collected from the large-scale system (Site 3), indicated that microbial regrowth was occurring in the stored pasteurized tank water with 16S rRNA PCR analysis identifying *Flectobacillus* spp., *Acidovorax* spp., *Pseudomonas* spp., *Dechlorosoma* spp., *Bordetella* spp., *Novosphingobium* spp. and *Sphingomonas* spp., as the primary bacterial contaminants.

The BacTiter-Glo™ Microbial Cell Viability Assay was then able to corroborate the results obtained during the culturing analysis, but also provided additional information, as the assay detected the presence of ATP (which serves as an indicator of metabolically active cells) in tank water samples collected after SOPAS treatment, where no culturable bacteria were detected using HPC analysis. This result then indicated that metabolically active cells may have been present in the collected pasteurized tank water samples, but that the cells had possibly entered a viable but non-culturable (VBNC) state. Additionally, tank water samples where HPC were reduced to BDL (< 1 CFU/100 mL) were analysed for a period of two weeks in order to determine the storage time period of the treated rainwater (i.e. before microbial regrowth occurred). Analysis of the two samples collected at 67 °C (Site 1) and 66 °C (Site 2) where HPC were reduced to BDL (< 1 CFU/100 mL), but the BacTiter-Glo™ Microbial Cell Viability Assay indicated the presence of metabolically active cells, then exhibited microbial regrowth after 6 days using culture based analysis, while samples pasteurized above 71 °C (Site 3) could be stored for up to two weeks. The BacTiter-Glo™ Microbial Cell Viability Assay has been used in numerous studies to monitor water quality, including water quality following disinfection treatment (Berney et al. 2008; Bosshard et al. 2009; Vital et al. 2012; Nescerecka et al. 2016; Reyneke et al. 2016). Corresponding to literature, the assay can be used to supplement results during HPC analysis and analyse for microbial regrowth in water samples following disinfection treatment (Berney et al. 2008; Bosshard et al. 2009; Vital et al. 2012; Nescerecka et al. 2016; Reyneke et al. 2016). In studies conducted by Bosshard et al. (2009) and Reyneke et al. (2016) results indicated that ATP (as an indicator of metabolically active cells) decreased in water samples by > 90% following solar disinfection and SOPAS treatment, respectively. The results thus correspond to the results reported in the current study, where an overall ATP reduction of 93%, 91% and 86% (solar manifold system), was recorded following SOPAS at Sites 1, 2 and 3, respectively.

Results obtained from the conventional PCR analysis identified *Legionella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp., as the most readily detected opportunistic pathogens associated with rainwater. Quantitative PCR revealed that while the gene copies of *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp., were reduced during SOPAS (small- and large-scale systems) at all temperatures analysed, gene copies from the respective organisms were still being detected at the highest pasteurization temperatures analysed for each site (Site 1 – 85 °C; Site 2 – 66 °C; Site 3 – 79 °C). In addition, while *Staphylococcus* spp. were of the most readily detected pathogens in the unpasteurized and pasteurized (stored pasteurized for Site 3) tank water samples, during qPCR analysis for *Staphylococcus* spp. multiple melting peaks were obtained per sample during the melt curve analysis, which invalidated the quantification of this organism. Future research should thus focus on designing genus-specific primers/probes for the detection of *Staphylococcus* spp. using qPCR assays. It should be noted that an increase in the detection of gene copies of *Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp., was observed during qPCR analysis of the stored pasteurized tank water samples collected from Site 3, which correspond to the results obtained for the HPC culturing and the BacTiter-Glo™ Microbial Cell Viability Assay where increases in the measured variables were observed in the stored pasteurized tank water samples (Site 3). The detection of *Legionella* spp. and *Pseudomonas* spp. in pasteurized tank water samples following solar pasteurization treatment correspond to results reported in literature, where researchers indicated that intact cells of *Legionella* spp. and *Pseudomonas* spp., were present in tank water following SOPAS above 90 °C (Dobrowsky et al. 2016; Reyneke et al. 2016; Strauss et al. 2016). Moreover, while SOPAS was able to reduce the level of microbial contamination in the pasteurized tank water samples collected from Sites 1, 2 and 3 (excluding the stored pasteurized tank water – Site 3), the detection of opportunistic pathogens in the water remains a cause for concern.

As conventional PCR and qPCR cannot provide an indication of viability, it is recommended that future studies should utilise molecular-based viability assays in combination with quantitative microbial risk assessment (QMRA) studies as this will enable the accurate assessment of the human health risks associated with using the water for various domestic purposes. For example, *Legionella pneumophila* (*L. pneumophila*) pose a health risk when aerosolised particles are inhaled by susceptible individuals, while *Campylobacter jejuni* (*C. jejuni*) needs to be ingested. By taking the infection mechanisms, concentration of *L. pneumophila* and *C. jejuni* in rainwater and the exposure volumes due to the inhalation or ingestion of rainwater at splash parks into consideration, De Man et al. (2014) was then able to establish that a mean exposure time of at least 3.5 min may lead to a risk of human infection by these two organisms. Based on the results obtained in the current study, rainwater treatment technologies that are able to effectively remove both microbial and chemical contaminants are thus required. This may

possibly be achieved by combining various treatment technologies. For example, SOPAS can be combined with filtration technologies, by simply attaching a filtration mechanism to the outlet tap of the SOPAS system. The SOPAS system will therefore remove the majority of microbial contaminants, while the filtrations system may remove residual microbial and chemical contaminants.

After the small-scale (Sites 1 and 2) and large-scale (Site 3) SOPAS systems had been installed in Enkanini, a workshop (WRC Report Project No. K5/2368//3, 2016) was conducted with members from each household that were participating in the study. The primary aim of the workshop was to explain the principle of the rainwater harvesting SOPAS treatment systems as well as to outline the maintenance of the systems and the primary water uses of the treated water. Furthermore, a rainwater harvesting poster and pamphlet (containing the core user information), was provided to each of the ten households involved in the pilot research phase of the project. Additionally, the operational sustainability of the systems was monitored for a period of one year (September 2015 to September 2016). Based on this analysis, the systems were effectively able to provide the inhabitants of the informal settlement with an alternative water source, with the water being used for various domestic purposes, including washing and laundry. For the small-scale SOPAS systems located at Sites 1 and 2, three households (Site 1 - House 1, 2 and 3; Site 2 - Church 1, 2 and 3) had access to the system, respectively. Monitoring of the pasteurized tank water usage indicated that at Site 1, 93 L/week, 78 L/week and 96 L/week, pasteurized tank water was being used by House 1, 2 and 3, respectively, during the weeks when pasteurized tank water was available. In comparison, at Site 2, 97 L/week, 112 L/week and 127 L/week, of pasteurized tank water was being used by Church 1, 2 and 3, respectively. Moreover, results indicated that the households' municipal tap water usage at both Sites 1 and 2, decreased during the weeks when pasteurized tank water was available. Four households (ERC 1, 2, 3 and 4) had access to the large-scale SOPAS system located at Site 3. Monitoring of the pasteurized tank water usage indicated that 146 L/week, 123 L/week, 130 L/week and 85 L/week, of pasteurized tank water was being used by ERC 1, 2, 3 and 4, respectively, during the weeks when pasteurized tank water was available. However, during May 2016 to August 2016 when less pasteurized tank water was being used, the households' weekly municipal tap water usage increased to 476 L/week, 295 L/week and 115 L/week, for ERC 1, 2 and 4, respectively. During the course of the study, the maintenance of the respective SOPAS systems, by the participating households, was mostly limited to cleaning the borosilicate glass tubes once a week and ensuring that nothing obstructed the SOPAS systems (for example hanging laundry over the system). One borosilicate glass tube had to be replaced for each of the systems; however, the replacement of the tubes did not require specialised equipment and can occur within 10 min. It was also reported by the households utilising the systems at Sites 1 and 2 that less paraffin/gas was being used by the

respective households to heat water for use in domestic activities as hot water was being produced by the small-scale systems. In addition, the households using the system at Site 3, then indicated that they would also preferably utilise a system where hot water was provided (water does not stay heated after it exits the SOPAS system into the 1 500 L storage tank). Future research should thus focus on designing polymeric materials that are able to retain heat, which could then be used for the rainwater storage tank. Additionally, consideration should be given to the design of rainwater harvesting tanks, as dust particles entered the 1500 L storage tank via the overflow opening and the rainwater harvesting tank lid. Research has also been conducted on the use of first-flush mechanisms that can be connected to the conveyance system (gutters). Essentially the initial inflow of rainwater (thought to contain the majority of contaminants) will be redirected away from the tank at the start of a rain event (Gikas & Tsihrintzis, 2012). However, based on the limited space available between the gutter system and the rainwater tank at an informal dwelling, it would be beneficial if the first-flush diverters are designed and incorporated directly into the rainwater harvesting tank.

In South Africa specifically, approximately 70 000 households use rainwater as their primary water source (Malema et al. 2016) and it is envisioned that rainwater harvesting may be the solution to providing individuals residing in rural areas and urban informal settlements with an alternative water source; however, an in-depth understanding of the dynamics in specifically informal settlements is required for the successful implementation of these technologies. Overall, while the use of Enkanini as a study site had numerous advantages, a major disadvantage in installing the rainwater harvesting SOPAS treatment systems in an informal settlement is space availability. The dwellings are built very close to one another and the Enkanini informal settlement is established on the steep slope of the Onder Papegaaiberg, which limits ideal system implementation. It is therefore recommended, that before planning and designing water treatment systems, such as the small-scale and large-scale SOPAS systems utilised in the current study, sufficient land be secured. In addition, as each dwelling within Enkanini differs in shape and size, it is thus also recommended that each rainwater harvesting and SOPAS system be designed and planned based on the configuration and location of the specific household. A “one size fits all” approach for the implementation of alternative technologies cannot be implemented for all informal settlements or all households within a specific settlement. When identifying possible locations for rainwater harvesting and SOPAS systems, it is also important to consider the systems effect on neighbouring properties, whether it is possible flooding or perhaps blocking a walkway or interfering with power cables etc. Any decisions made should thus also take the possible effect on non-participating individuals into consideration so as to not impede on their everyday life. Moreover, factors that should be considered when designing these passive systems should include: the ground preparation, the slope of the roof, the direction in which the roofing sheets have been placed on the catchment

area and the material and treatment used for the roofing material. During the current project individuals who have worked on various Sustainability Institute projects in Enkanini, were also recruited as co-researchers on the project. Their insight and relationship with the community assisted with any troubleshooting incidents and provided credibility to the “new researchers”.

While cultivation dependent methods may contribute knowledge on the detection of viable and culturable microorganisms present in a water source, a huge proportion of the microbial population, which enter a viable but not culturable state, are excluded from this analysis. A need therefore exists for the implementation of rapid and sensitive cultivation independent methods that would allow for the accurate detection of all the microbial contaminants present in a water source (Van Frankenhuyzen et al. 2011; Barbau-Piednoir et al. 2014; Prest et al. 2014; Li et al. 2015). The primary aim of **Chapter 3** was thus to compare molecular-based viability assays to a metabolic responsiveness assay for the accurate determination of microbial cell viability. In order to achieve this aim, the molecular-based viability assays ethidium monoazide bromide (EMA)-qPCR, propidium monoazide (PMA)-qPCR and DNase treatment in combination with qPCR and the metabolic responsiveness BacTiter-Glo™ Microbial Cell Viability Assay, were compared to culture based analysis, for their ability to accurately determine cell viability in water samples inoculated with a bacterial monoculture. Three Gram-negative (*Legionella* spp., *Pseudomonas* spp. and *Salmonella* spp.) and two Gram-positive (*Staphylococcus* spp. and *Enterococcus* spp.) bacteria commonly associated with water sources were selected as test organisms. Various EMA and PMA dye concentrations were also analysed, based on a range of concentrations that have been reported in literature, in order to determine which dye is optimal and at what concentration (Nocker et al. 2006; Soejima et al. 2007; Kobayashi et al. 2008; Chen and Chang, 2009; Delgado-Viscogliosi et al. 2009; Yáñez et al. 2011; Tavernier & Coenye, 2015).

Results from the EMA-qPCR and PMA-qPCR analysis of the viable samples for all test organisms, indicated that variable log reductions were recorded after the application of both EMA and PMA treatments, as log reductions ranging from 0.01 to 2.71 (EMA) and 0.06 to 1.02 (PMA) were observed in comparison to the no viability treatment control. The log reductions observed in the viable samples following EMA and PMA treatment (in comparison to the no viability treatment control), were then attributed to the presence of dead bacteria in the viable samples as well as possible osmotic stress, which may have occurred when the pure cultures were spiked into distilled water (Nogva et al. 2003; Sichel et al. 2007; Delgado-Viscogliosi et al. 2009). Moreover, results indicated that greater log reductions were obtained for the heat treated samples in comparison to the corresponding no viability treatment control [EMA – 0.27 to 2.85; PMA – 0.62 to 2.46]. This was attributed to the fact that dead or membrane-compromised organisms were amplified by qPCR analysis in the no viability treatment control, thereby overestimating the presence of microorganisms (Delgado-Viscogliosi et al. 2009; Fittipaldi et al.

2012). It is however crucial that the viability dyes utilised, not significantly influence the detection of viable cells, as this would lead to an underestimation of the microbial population when analysing environmental water samples (Fittipaldi et al. 2012; Li et al. 2015). Various factors may also influence the effectiveness of EMA-qPCR and PMA-qPCR to indicate microbial cell viability. For example, it has been reported that some bacterial species may be able to actively pump EMA out of the viable cell using metabolically driven multidrug efflux pumps, while the entry of EMA into other bacteria will be based on diffusion alone (Nogva et al. 2003; Nocker et al. 2006; Flekna et al. 2007). In addition, research has indicated that gene copy numbers, such as ribosomal genes, can differ substantially amongst different bacterial species (Klappenbach et al. 2001). Moreover, it has been demonstrated that the target amplicon length of the gene may influence the effectiveness of viability treatments (EMA-qPCR and PMA-qPCR) as the detection of longer DNA sequences correlates more closely with viability, as compared to the detection of shorter target amplicons (Soejima et al. 2007; Chang et al. 2010; Contreras et al. 2011; Loozen et al. 2011). This is mainly due to longer DNA sequences being more readily excluded by nucleic acid binding dyes. While assessing the viability of heat-killed *L. pneumophila* using both EMA and PMA, Chang et al. (2010) reported that a difference in the number of dead cells was obtained depending on the whether the 16S rRNA (454 bp) or 5S rRNA (108 bp) genes were amplified during qPCR. Similarly, it was reported by Loozen et al. (2011) that a greater log reduction in the detection of *Aggregatibacter actinomycetemcomitans* during qPCR was obtained when targeting a 200 bp DNA fragment as compared to an 82 bp DNA fragmant. Thus, in the current study it is hypothesised that the significant suppression of qPCR signals from the viable samples of *L. pneumophila* (259 bp) and *S. aureus* (475 bp) may be attributed to the longer target amplicon length for each organism during the qPCR analysis, in comparison to *S. typhimurium* (75 bp) and *E. faecalis* (75 bp), where shorter amplicons were targeted.

DNase treatment in combination with qPCR was an additional viability assay that was assessed during the current study. Results indicated that log reductions ranging from 0.06 to 0.82 were obtained for the viable samples following DNase treatment. In comparison, log reductions ranging from 0.70 to 2.91 were recorded for the heat treated samples. As was reported for EMA-qPCR and PMA-qPCR analysis, the observed decrease in the detection of viable cells in the DNase treated samples, in comparison to the untreated control, was attributed to the presence of dead bacteria in the viable sample as well as possible osmotic stress, when spiking the test organisms into distilled water (Nogva et al. 2003; Sichel et al. 2007; Delgado-Viscogliosi et al. 2009). Additionally, the log decreases observed in the heat treated samples following DNase treatment was attributed to the exclusion of DNA from dead or membrane compromised cells. In comparison to EMA-qPCR and PMA-qPCR, limited information on the entry of DNase into cells is known. It was however, reported by Shakeri et al. (2014) that organisms which produce extracellular thermostable nucleases (including *S. aureus*), may be more susceptible to

DNase treatment. The BacTiter-Glo™ Microbial Cell Viability Assay was also used to monitor the presence of metabolically active cells in the viable, heat treated and autoclaved (dead) samples. While for most of the test organisms analysed, the results obtained substantiated the results recorded utilising molecular-based viability assays, a major disadvantage of using the BacTiter-Glo™ Microbial Cell Viability Assay was the detection of residual ATP in the autoclaved samples. However, overall the results indicated that the assay was a valuable indicator of the presence/absence of viability in the viable and heat treated samples and could therefore be included when monitoring water disinfection treatments, if the presence of residual extracellular ATP is considered (Vital et al. 2012; Nescerecka et al. 2016).

Following the completion of the EMA-qPCR and PMA-qPCR analysis, 6 µM EMA and 50 µM PMA were then identified as the optimal dye concentrations for the detection of viable *L. pneumophila*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *E. faecalis*, as low log reductions were recorded (viable and heat treated samples) in comparison to the no viability treatment control. Comparison of the 6 µM EMA and 50 µM PMA results to the DNase treatment assay then indicated that for most of the test organisms, the results obtained using the 6 µM EMA concentration was comparable to either 50 µM PMA or the DNase treatment. In addition, the gene copies obtained (using the molecular-based viability assays) in the viable samples, for most of the respective test organisms, were comparable to the CFU/mL enumerated during culturing analysis. However, analysis of the heat treated *S. typhimurium* sample revealed that the gene copies (1.52×10^3 gene copies/µL) detected using the molecular based viability assays were higher than the 1.0×10^2 CFU/mL detected using culturing analysis. It was hypothesised that VBNC cells may have been present in the sample as a result of the heat treatment and these cells were not enumerated using culture based analysis. However, based on the results obtained overall, both culturing analysis and the molecular-based viability assays can be utilised to indicate the presence or absence (viable *E. faecalis* and *S. aureus* not detected using culturing analysis or the molecular-based viability assays following heat treatment) of viable microbial cells. It is therefore recommended that molecular-based viability assays may be used as a valuable supplementary analysis tool when investigating water sources. However, it is important to note that the application of molecular-based viability assays will have certain advantages over culture based analysis. These advantages include decreased analysis time, as results may be obtained within 7 hours when using molecular-based viability assays (Lombard et al. 2016), in comparison to the 24 to 96 hrs required to generate results using culture based analysis. Moreover, researchers have also combined the use of EMA and PMA with microscopy and flow cytometry, demonstrating that nucleic acid binding dyes may be combined with other platforms/ technologies to detect cell viability (Fittipaldi et al. 2012). Additionally, the results generated utilising molecular-based viability assays have been shown to be highly reproducible (Delgado-Viscogliosi et al. 2009; Reyneke et al. 2016, Strauss et al. 2016).

4.1 References

Ahmed, W., Huygens, F., Goonetilleke, A. & Gardner, T. 2008. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. *Applied and Environmental Microbiology*. 74(17):5490-5496.

Ahmed, W., Vieritz, A., Goonetilleke, A. & Gardner, T. 2010. Health risk from the use of roof-harvested rainwater in Southeast Queensland, Australia, as potable or nonpotable water, determined using quantitative microbial risk assessment. *Applied and Environmental Microbiology*. 76(22):7382-7391.

Ahmed, W., Gardner, T. & Toze, S. 2011. Microbiological quality of roof-harvested rainwater and health risks: A review. *Journal of Environmental Quality*. 40(1):1-9.

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barceló, D., Seiler, T.B., Brion, F., Busch, W., Chipman, K., López De Alda, M., De Aragão Umbuzeiro, G., Escher, B.I., Falciani, F., Faust, M., Focks, A., Hilscherova, K., Hollender, J., Hollert, H., Jäger, F., Jahnke, A., Kortenkamp, A., Krauss, M., Lemkine, G.F., Munthe, J., Neumann, S., Schymanski, E.L., Scrimshaw, M., Segner, H., Slobodnik, J., Smedes, F., Kughathas, S., Teodorovic, I., Tindall, A.J., Tollefsen, K.E., Walz, K.H., Williams, T.D., Van Den Brink, P.J., Van Gils, J., Vrana, B., Zhang, X. & Brack, W. 2015. Future water quality monitoring – adapting tools to deal with mixtures of pollutants in water resource management. *Science of the Total Environment*. 512-513:540-551.

Anomaly, J. 2009. Harm to others: the social cost of antibiotics in agriculture. *Journal of Agricultural and Environmental Ethics*. 22:423-435.

Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Coucke, W., Roosens, N.H. & Botteldoorn, N. 2014. Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis. *Journal of Microbiological Methods*. 103:131-137.

Berdahl, P., Akbari, H., Levinson, R. & Miller, W.A. 2008. Weathering of roofing materials – an overview. *Construction and Building Materials*. 22:423-433.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H-A., Egli, T. & Hammes, F. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Research*. 42(14):4010-4018.

Bosshard, F., Berney, M., Scheifele, M., Weilenmann, H.U. & Egli, T. 2009. Solar disinfection (SODIS) and subsequent dark storage of *Salmonella typhimurium* and *Shigella flexneri* monitored by flow cytometry. *Microbiology*. 155:1310-1317.

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F. & Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. *Japanese Journal of Infectious Diseases*. 63:119–123.

Chen, N.T. & Chang, C.W. 2009. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. *Journal of Applied Microbiology*. 109:623-634.

Contreras, P.J., Urrutia, H., Sossa, K. & Nocker, A. 2011. Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment. *Journal of Microbiological Methods*. 87:89–95.

Coombes, P.J., Argue, J.R. & Kuczera, G. 2000 Figtree Place: a case study in water sensitive urban development (WSUD). *Urban Water*. 1:335-343.

Delgado-Viscogliosi, P., Solignac, L. & Delattre, J-M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. *Applied and Environmental Microbiology*. 75(11):3502-3512.

De Man, H., Bouwknegt, M., Van Heijensbergen, E., Leenen, E.J.T.M., Van Knapen, F., De Roda Husman, A.M. 2014. Health risk assessment for splash parks that use rainwater as source water. *Water Research*. 54:254-261.

Department of Water Affairs and Forestry (DWAF). 1996. *South African Water Quality Guidelines* 2nd ed. Volume 1: Domestic Water Use. Pretoria, South Africa: CSIR Environmental Services. ISBN 0-7988-5338-7.

Despins, C., Farahbakhsh, K. & Leidl, C. 2009. Assessment of rainwater quality from rainwater harvesting systems in Ontario, Canada. *Journal of Water Supply: Research and Technology – AQUA*. 58(2):117–134.

Dobrowsky, P.H., Cloete, T.E. & Khan, W. 2014. Quality assessment of domestic harvested rainwater in the peri-urban region of Kleinmond, Western Cape and the optimisation of point-of-use treatment systems. M.Sc. Thesis. University of Stellenbosch.

Dobrowsky, P.H., Carstens, M., De Villiers, J., Cloete, T.E. & Khan, W. 2015. Efficiency of a closed-coupled solar pasteurization system in treating roof harvested rainwater. *Science of the Total Environment*. 536:206-214.

Dobrowsky, P.H., Khan, S., Cloete, T.E. & Khan, W. 2016. Molecular detection of *Acanthamoeba* spp., *Naegleria fowleri* and *Vermamoeba* (*Hartmannella*) *vermiformis* as vectors

for *Legionella* spp. in untreated and solar pasteurized harvested rainwater. *Parasites and Vectors*. 9:539-552.

Fittipaldi, M., Nocker, A. & Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods*. 91(2):276-289.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S. & Hein, I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Research in Microbiology*. 158(5):405–412.

Gikas, G.D. & Tsihrintzis, V.A. 2012. Assessment of water quality of first-flush roof runoff and harvested rainwater. *Journal of Hydrology*. 466-467:115-126.

Heijerick, D.G., Janssen, C.R., Karlen, C., Wallinder, I.O. & Leygraf, C. 2002. Bioavailability of zinc in runoff water from roofing materials. *Chemosphere*. 47:1073-1080.

Helmreich, B. & Horn, H. 2009. Opportunities in rainwater harvesting. *Desalination*. 248(1):118-124.

Klappenbach, J.A., Saxman, P.R., Cole, J.R. & Schmidt, T.M. 2001. rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Research*. 29:181–184.

Kobayashi, H., Oethinger, M., Tuohy, M.J., Hall, G.S. & Bauer, T.W. 2008. Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide. *Letters in Applied Microbiology*. 48:633-638.

Kopylov, N.I., Kaplin, Y.M., Litinov, V.P. & Kaminskii, Y.D. 2007. Large-scale use of arsenic in the production of antifouling coatings. *Theoretical Foundations of Chemical Engineering*. 41:780-785.

Lombard, M., Wolfaardt, G.M., Van Blerk, N. & Khan, W. 2016. Detection, Identification and quantitation of *Cryptosporidium parvum* in water samples and *Ascaris lumbricoides* in sludge samples using real-time polymerase chain reaction coupled with the high-resolution melt curve sssay. M.Sc. Thesis. University of Stellenbosch.

Loozen, G., Boon, N., Pauwels, M., Quirynen, M. & Teughels, W. 2011. Live/dead realtime polymerase chain reaction to assess new therapies against dental plaque-related pathologies. *Molecular Oral Microbiology*. 26:253–261.

Li, Z., Boyle, F. & Reynolds, A. 2010. Rainwater harvesting and greywater treatment systems for domestic application in Ireland. *Desalination*. 260(1):1-8.

Li, H., Xin, H. & Li, S.F.Y. 2015. Multiplex PMA-qPCR assay with internal amplification control for simultaneous detection of viable *Legionella pneumophila*, *Salmonella typhimurium* and *Staphylococcus aureus* in environmental waters. Environmental Science and Technology 49:14249-14256.

Malema, S., Abia, L.K.A., Mwenge Kahinda, J. & Ubomba-Jaswa, E. 2016. Gaining a better understanding of the factors that influence the quality of harvested rainwater in South Africa – a review. *WISA 2016 Biennial conference.* 16 - 19 May 2016. Durban, South Africa.

Mwenge Kahinda, J., Taigbenu, A.E. & Boroto, R.J. 2010. Domestic rainwater harvesting as an adaptation measure to climate change in South Africa. *Physics and Chemistry of the Earth*. 35(13-14):742-751.

Nescerecka, A., Juhna, T. & Hammes, F. 2016. Behaviour and stability of adenosine triphosphate (ATP) during chlorine disinfection. *Water Research*. 101:490-497.

NHMRC and NRMMC (ADWG). 2011*. Australian drinking water guidelines Paper 6 National Water Quality Management Strategy*. National Health and Medical Research Council, National Resource Management Ministerial Council Commonwealth of Australia, Canberra.

Nocker, A., Cheung, C.Y. & Camper, A.K. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*. 67(2):310–320.

Nogva, H.K., Dromtorp, S.M., Nissen, H. & Rudi, K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-Nuclease PCR. *Biotechniques.* 34:804-813.

Prest, E.I., El-Chakhtoura, J., Hammes, F., Saikaly, P.E., Van Loosdrecht, M.C.M. & Vrouwenvelder, J.S. 2014. Combining flow cytometry and 16S rRNA gene pyrosequencing: A promising approach for drinking water monitoring and characterization. *Water Research*. 63:179-189.

Radaidah, J., Al-Zboon, K., Al-Harahsheh, A. & Al-Adamat, R. 2009. Quality assessment of harvested rainwater for domestic uses. *Jordan Journal of Earth and Environmental Sciences*. 2:26–31.

Reyneke, B., Dobrowsky, P.H., Ndlovu, T., Khan, S. & Khan, W. 2016. EMA-qPCR to monitor the efficiency of a closed-coupled solar pasteurization system in reducing *Legionella* contamination of roof-harvested rainwater. *Science of the Total Environment*. 553:662-670.

Shakeri, M., Shahidi, F., Mortazavi, A., Bahrami, A.R. & Nassiri, M.R. 2014. Application of PCR technique in combination with DNAse treatment for detection of viable *Lactobacillus acidophilus* bacteria. *Journal of Food Quality*. 37:291–295.

Sichel, C., Blanco, J., Malato, S. & Fernandez-Ibanez, P. 2007. Effects of experimental conditions on *E. coli* survival during solar photocatalytic water disinfection. *Journal of Photochemistry and Photobiology A*. 189:239-246.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A. & Yoshida, S. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. *Microbiology and Immunology*. 51(8):763–775.

South African Bureau of Standards (SABS). 2005. South African National Standards (SANS) 241: In *Drinking water quality management guide for water services authorities*. 6th ed. Annexure 1. ISBN 0-626-17752-9.

Spinks, A.T., Coombes, P., Dunstan, R.H. & Kuczera, G. 2003. Water quality treatment processes in domestic rainwater harvesting systems. *Proceedings of the 28th International Hydrology and Water Resources Symposium*. 10-13 November 2003. Wollongong, Australia.

Spinks, A.T., Dunstan, R.H., Harrison, T., Coombes, P. & Kuczera, G. 2006. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. *Water Research*. 40(6):1326–1332.

Strauss, A., Dobrowsky, P.H., Ndlovu, T., Reyneke, B., & Khan W. 2016. Comparative analysis of solar pasteurization versus solar disinfection for the treatment of harvested rainwater. *BMC Microbiology*. 16:289-305.

Tavernier, S. & Coenye, T. 2015. Quantification of *Pseudomonas aeruginosa* in multispecies biofilms using PMA-qPCR. *PeerJ* 3:e787; DOI 10.7717/peerj.787.

Van Frankenhuyzen, J.K., Trevors, J.T., Lee, H., Flemming, C.A. & Habash, M.B. 2011. Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. *Journal of Microbiological Methods*. 87:263-272.

Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L. & Hammes, F. 2012. Flow cytometry and adenosine tri-phosphate analysis: Alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. *Water Research*. 46:4665-4675.

Water Research Commission (WRC), Project No. K5/2124//3. 2014. Quality of harvested rainwater and application of point-of-use treatment systems. Available:

http://www.wrc.org.za/Pages/DisplayItem.aspx?ItemID=10973&FromURL=%2FPages%2FKH_DocumentsList.aspx%3Fdt%3D1%26ms%3D4%3B7%3B%26d%3DQuality+of+harvested+rainwater+and+application+of+point+of+use%26start%3D1. (Accessed 20 May 2016).

Water Research Commission (WRC), Project No. K5/2368//3. 2016. *Design, Construction and Monitoring of Sustainable Domestic Rainwater Harvesting Treatment Systems in Enkanini Informal Settlement, Stellenbosch*. Report to the Water Research Commission, Project No. K5/2368 by Department of Microbiology, Stellenbosch University. Stellenbosch, South Africa.

World Health Organisation (WHO). 2011. *Guidelines for drinking-water quality.* Rev. 4th ed. World Health Organisation. Geneva, Switzerland: WHO Press. ISBN: 978-92-4- 154815-1.

Yáñez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L. & Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. *Journal of Microbiological Methods*. 85:124–130.

Appendix A:

Construction and installation of the domestic rainwater harvesting solar pasteurization treatment systems in Enkanini informal settlement (Stellenbosch) and water usage by the participating households

(UK spelling is employed)

1. Site selection and description

Based on the quality of the roofing material and space availability, an Enkanini resident's house, that was part of the Stellenbosch Sustainability Institute iShack project, was selected as Site 1 (**Fig. A1**). A local Church was selected as Site 2, as the Pastor and his family lived on the Church grounds, while the Enkanini Research Centre (ERC) was selected as Site 3, with the research centre also serving as the home of a co-researcher (Mr Yondela Tyawa) on the project (**Fig. A1**). All participating individuals granted consent for the systems to be implemented next to their respective properties. The systems were designed to be used by the specific household where the system was installed. However, after the systems had been installed, the research group was informed that neighbouring households also wanted to use the systems. These households were therefore included in the study and representatives from each household attended the workshop [Water Research Commission (WRC) Report Project No. K5/2368//3, 2016] that was held in Enkanini after the systems had been installed. Each of the small-scale rainwater harvesting solar pasteurization (SOPAS) systems located at Sites 1 and 2 (**Fig. A1**), were used by three households, respectively. Moreover, four households had access to the large-scale rainwater harvesting solar pasteurization treatment system located at Site 3.

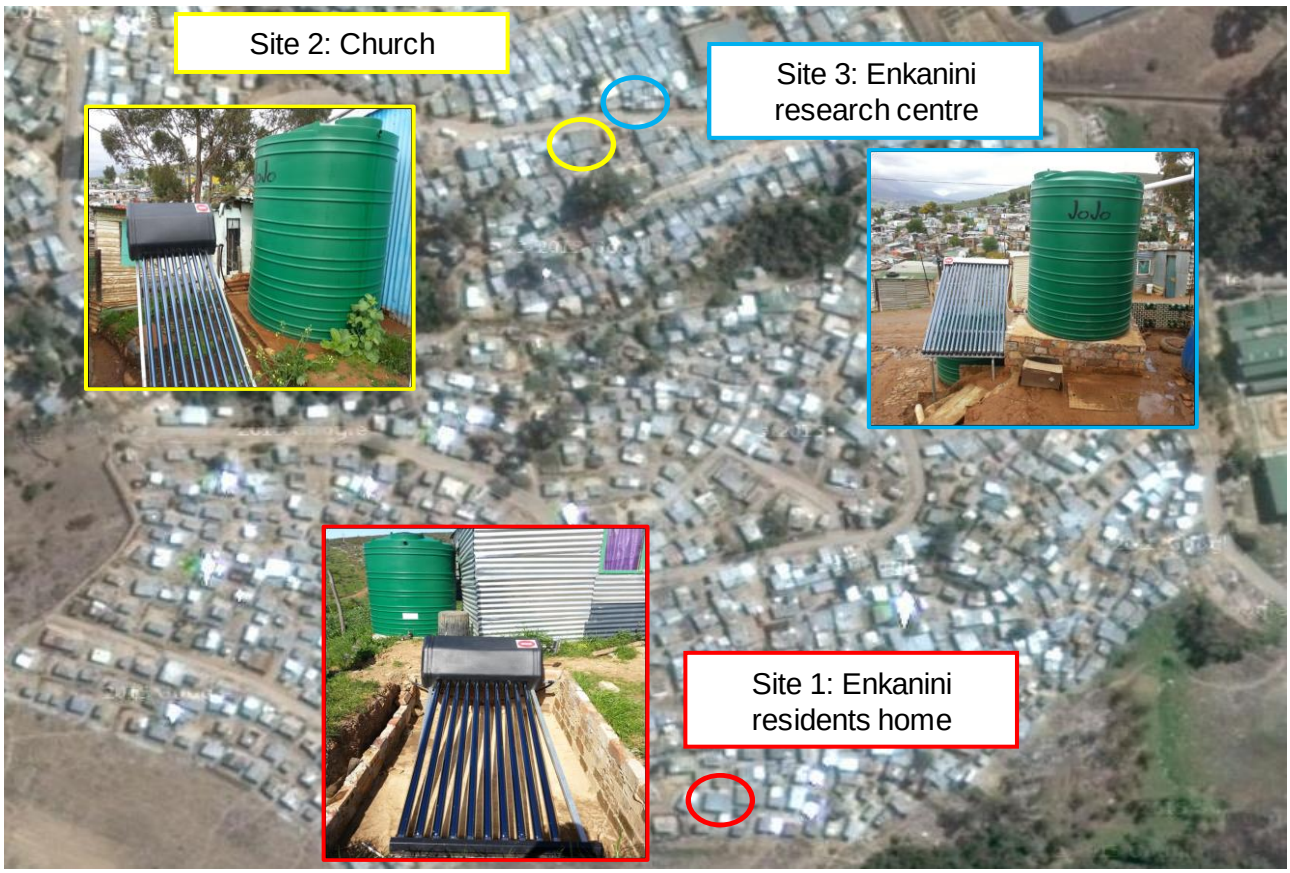

Fig. A1. Two small-scale rainwater harvesting solar pasteurization systems and a large-scale rainwater harvesting solar pasteurization treatment system were installed in Enkanini informal settlement. Site 1: Enkanini resident's home. Site 2: Church. Site 3: Enkanini Research Centre.

2. Design and construction of the rainwater harvesting treatment systems

The information on the number of people using the water and the average water demand of each person per day was obtained through communication with the owner of the home for Site 1, the Pastor of the Church for Site 2 and the custodian of the ERC for Site 3. By taking the roof size and roofing material into consideration for each site, the SamSamWater Rainwater Harvesting Tool (2015) was then utilised to determine the average water availability and water demand per month over a calendar year, which could then be used to determine the optimal size of the rainwater harvesting tanks that were required at Sites 1, 2 and 3, respectively. It is important to note that each system was initially designed and installed for one household to use. The information for the specific household was therefore used and is reported below. However, multiple households had access to the systems after they had been installed.

2.1 Site 1: Small-scale system located at an Enkanini resident's home

Roof size:	15.27 m^2
Roof type:	Zinc sheets (iron/metal sheets)
Number of people using the water:	3
Average water demand:	20 L per person per day
Total average water demand:	60 L per day

Although the owner of the home reported that the average water demand of each person per day was 20 L, the United Nations (2010) recommends a guideline value of 25 L/person per day for persons living in South Africa. The total average water demand for the household was therefore recalculated as 75 L per day.

A metal roof such as the zinc metal sheeting used at Site 1, has a runoff coefficient of 0.9, which means that 90% of the rain that comes into contact with the roof can be harvested (SamSamWater Rainwater Harvesting Tool, 2015). Based on this runoff coefficient and a roof area of 15.27 m^2, the total average annual amount of water that can be collected from the roof is 8 100 L. The total water demand of the household is 75 L per day, which equals to an approximate amount of 2 250 L per month. The total water demand would then be approximately 27 400 L per year. This implies that the households total daily water demand of 75 L will be met for 108 days of the year. However, if the households were to limit their treated tank water usage to 22 L per day, thus using it as a supplementary water source, the rainwater harvesting system may provide water on site for the entire year. Based on the calculations and space availability, a 2 500 L vertical JoJo domestic rainwater harvesting (DRWH) tank was installed at Site 1 (**Fig. A2**). The diameter of the 2 500 L tank is 1.42 m and the height of the tank is 1.8 m (JoJo Tanks, 2016).

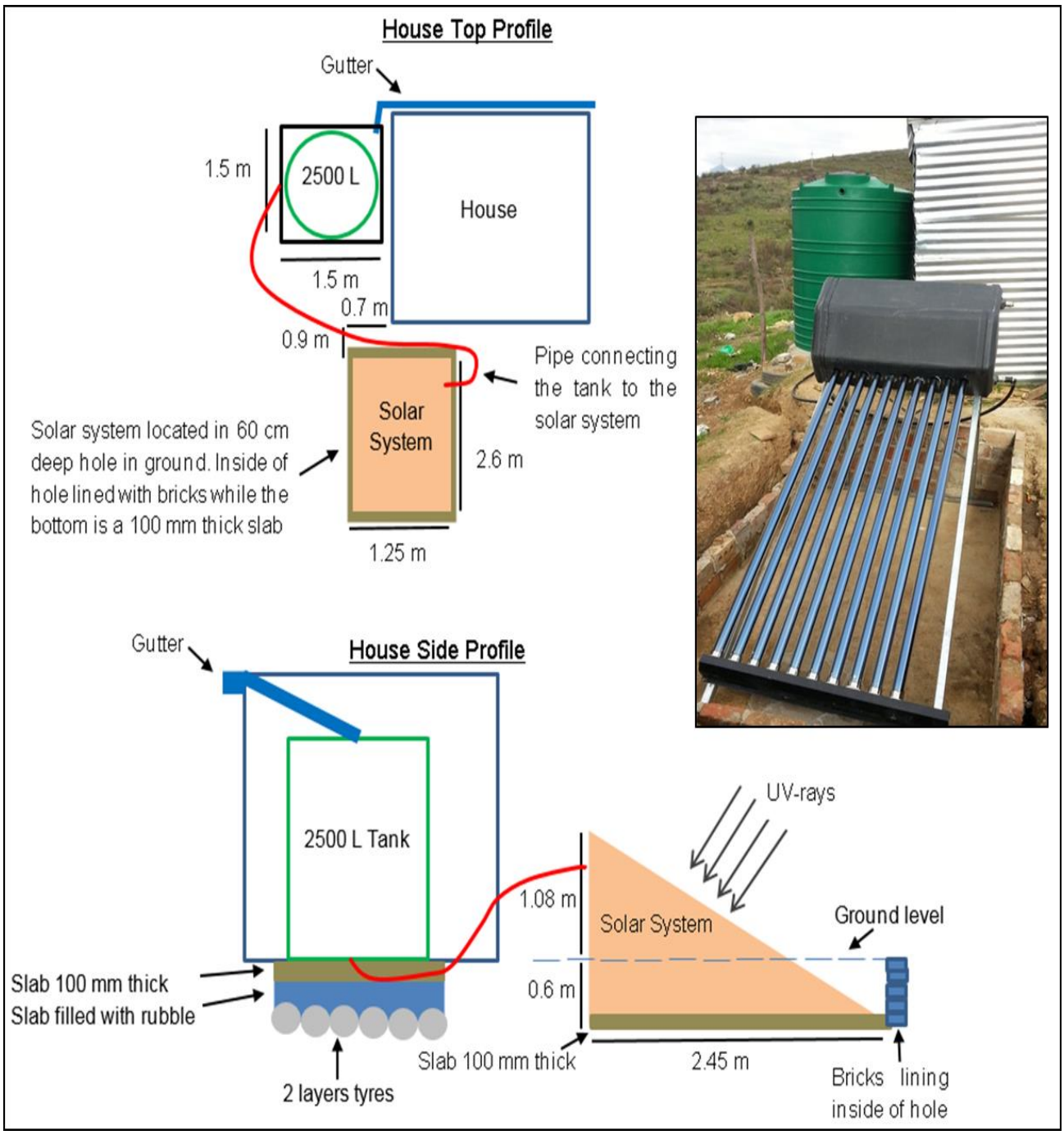

Fig. A2. A schematic diagram of the top and side profile of the small-scale rainwater harvesting solar pasteurization treatment system constructed at Site 1 with a picture of the system after installation.

As indicated in **Fig. A2**, a 2 500 L rainwater harvesting tank was connected to a pasteurization system containing a 125 L storage tank and 10 × 2 m borosilicate tubes. A total cost analysis for the installation of the small-scale solar pasteurization rainwater harvesting treatment system at Site 1 is outlined in **Table A1**. It should however be noted that while the costs associated with constructing the system at Site 1 (and at Site 2) are high, costs would decrease upon large-scale production and implementation of the solar pasteurization systems and by using day-labour workers in the community to help with the installation of the systems. The life expectancy of the

rainwater harvesting solar pasteurization system also needs to be taken into consideration. For example, a JoJo rainwater harvesting tank has a guarantee of 8 years, however, if properly installed they have been known to exceed a life span of 20 years (JoJo Tanks, 2016). Commercially available solar pasteurization systems have also been reported to have warranties ranging from 5 to 10 years and life expectancies ranging from 10 to 20 years (International Technology Sourcing Solar, 2016). It is also important to note that the water produced by these systems will be hot upon collection and that the households could save money by using less paraffin and gas for heating water to use for domestic purposes.

Table A1: Cost analysis for the installation of the small-scale treatment system at Site 1.

ITEM	PRICE
Site clearance and levelling	R 160
Labour	R 1 767
Operational Costs*	R 2 100
Construct new concrete plinth on compacted base	R 1 975
2 500 L vertical DRWH tank	R 2 246
Tank adaptors for connection to solar geyser	R 500
Gutter complete with facia and support brackets	R 700
Install downpipe connection and associated fittings	R 200
Solar system	R 4 000
System stand	R 500
Commissioning the system	R 2 500
Total (excluding VAT)	**R 16 648**
VAT	**R 2 331**
Total (including VAT)	**R 18 979**

* Sand/gravel sourcing and delivery, transport/fuel, tool hire

2.2 Site 2: Small-scale system located at the Church grounds

Roof size:	55.9 m^2
Roof type:	Zinc sheets (iron/metal sheets)
Number of people using the water:	5
Average water demand:	25 L per person per day
Total average water demand:	125 L per day

The zinc metal sheeting used at Site 2 has a runoff coefficient of 0.9, which means that 90% of the rain that comes into contact with the roof can be harvested (SamSamWater Rainwater Harvesting Tool, 2015). Based on this runoff coefficient and a roof area of 55.9 m^2, the total average yearly amount of water that can be collected from the roof is 29 800 L. The total water demand of the household is 125 L per day, which equals to approximately 3 750 L per month. The total water demand would then be 45 600 L per year. Based on the information obtained, the total amount of water that can be collected from this roof is not enough to fulfil the total water demand. However, a rainwater harvesting system with a storage reservoir of 7 400 L could provide 82 L of water per day, which is 65% of the total demand. Due to space availability, a 5 000 L vertical JoJo DRWH

tank was however installed at Site 2 (**Fig. A3**), which will account for ~44% of the households' total water demand. As the amount of harvested rainwater does not meet the full demand of the household, the rainwater source may act as a supplementary rather than a sole domestic water supply.

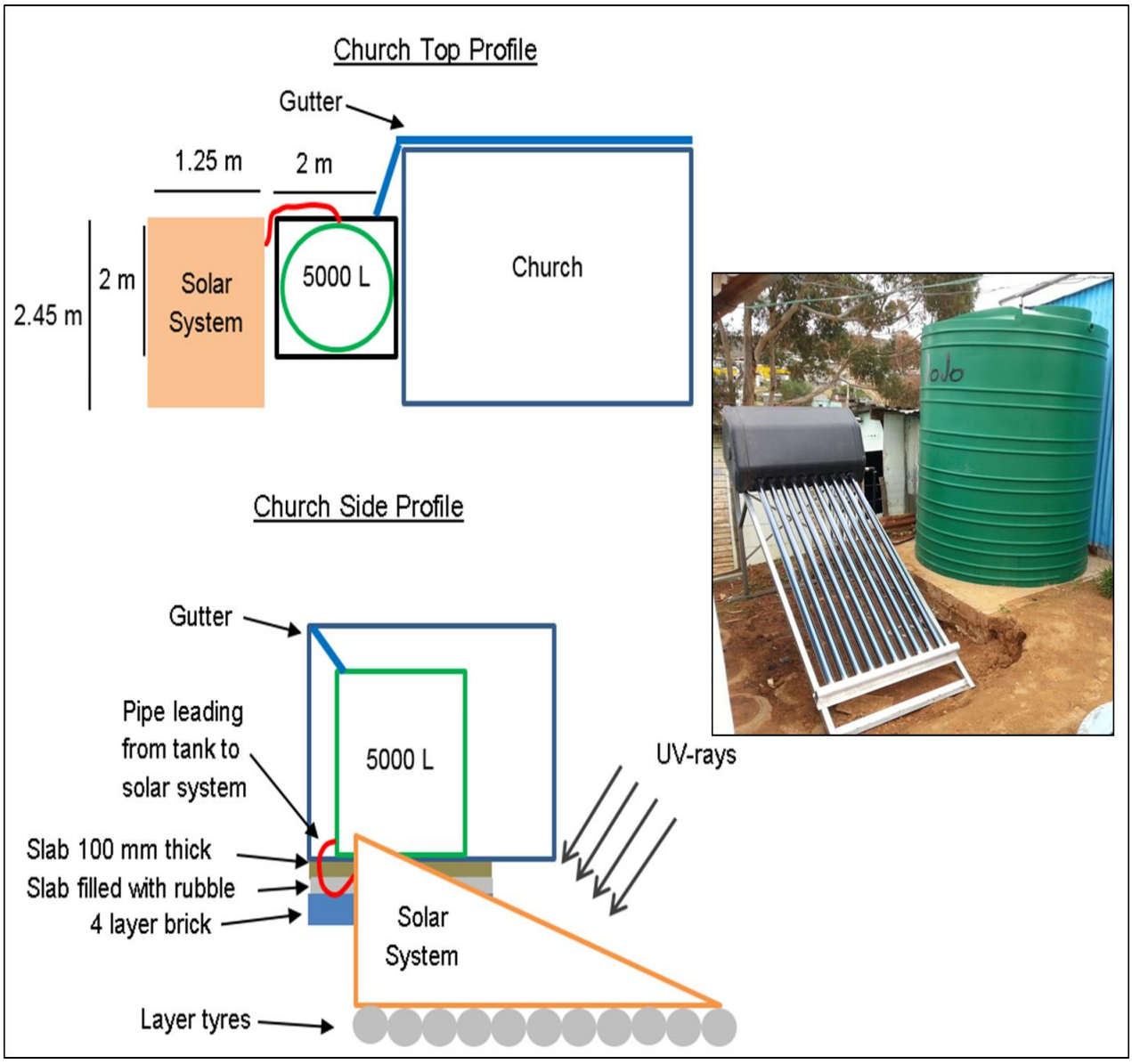

Fig. A3. A schematic diagram of the top and side profile of the small-scale rainwater harvesting solar pasteurization treatment system constructed at Site 2 with a picture of the system after installation.

As indicated in **Fig. A3**, a 5 000 L rainwater harvesting tank was connected to a pasteurization system containing a 125 L storage tank and 10 × 2 m borosilicate tubes. A total cost analysis for the installation of the small-scale solar pasteurization rainwater harvesting treatment system at Site 2 is outlined in **Table A2**.

Table A2: Cost analysis for the installation of the small-scale treatment system at Site 2.

ITEM	PRICE
Site clearance and levelling	R 160
Labour	R 1 467
Operational Costs*	R 2 100
Construct new concrete plinth on compacted base	R 1 450
5 000 L vertical DRWH tank	R 3 428
Tank adaptors for connection to solar geyser	R 500
Gutter complete with facia and support brackets	R 893
Install downpipe connection and associated fittings	R 200
Solar system	R 4 000
System stand	R 500
Commissioning the system	R 2 500
Total (excluding VAT)	**R 17 198**
VAT	**R 2 408**
Total (including VAT)	**R 19 606**

* Sand/gravel sourcing and delivery, transport/fuel, tool hire

2.3 Site 3: Large-scale system located at the Enkanini Research Centre

Roof size:	88.5 m^2
Roof type:	Zinc sheets (iron/metal sheets)
Number of people using the water:	3
Average water demand:	30 L per person per day
Total average water demand:	90 L per day

Similar to Sites 1 and 2, the zinc metal sheeting used at Site 3 has a runoff coefficient of 0.9, which means that 90% of the rain that comes into contact with the roof can be harvested (SamSamWater Rainwater Harvesting Tool, 2015). Based on this runoff coefficient and a roof area of 88.5 m^2, the total average yearly amount of water that can be collected from the roof is 47 200 L. The total water demand of the household is 90 L per day, which equals to about 2 700 L per month. The total water demand would then be 32 900 L per year. Based on the information obtained, the total amount of water that can be collected from this roof is sufficient to fulfil the total water demand of the household. During 7 months of the year (April, May, June, July, August, September and October) the amount of water that can be collected from the roof is larger than the water demand. This excess water can thus be stored to be used in the months where the water availability is less than the demand. The optimum size for a storage reservoir for this rainwater harvesting system is 4 900 L. However, a 5 000 L vertical JoJo DRWH tank was installed at the ERC (**Fig. A4**).

For the large-scale solar pasteurization rainwater harvesting treatment system, Crest Africa designed a CREST EVT Collector, i.e. a manifold system containing 18 evacuated borosilicate tubes. A 1500 L storage tank was then installed alongside the system in order to capture the treated water (> 75 °C) from the manifold system (thermal release valve opens at 75 °C) (**Fig. A4**).

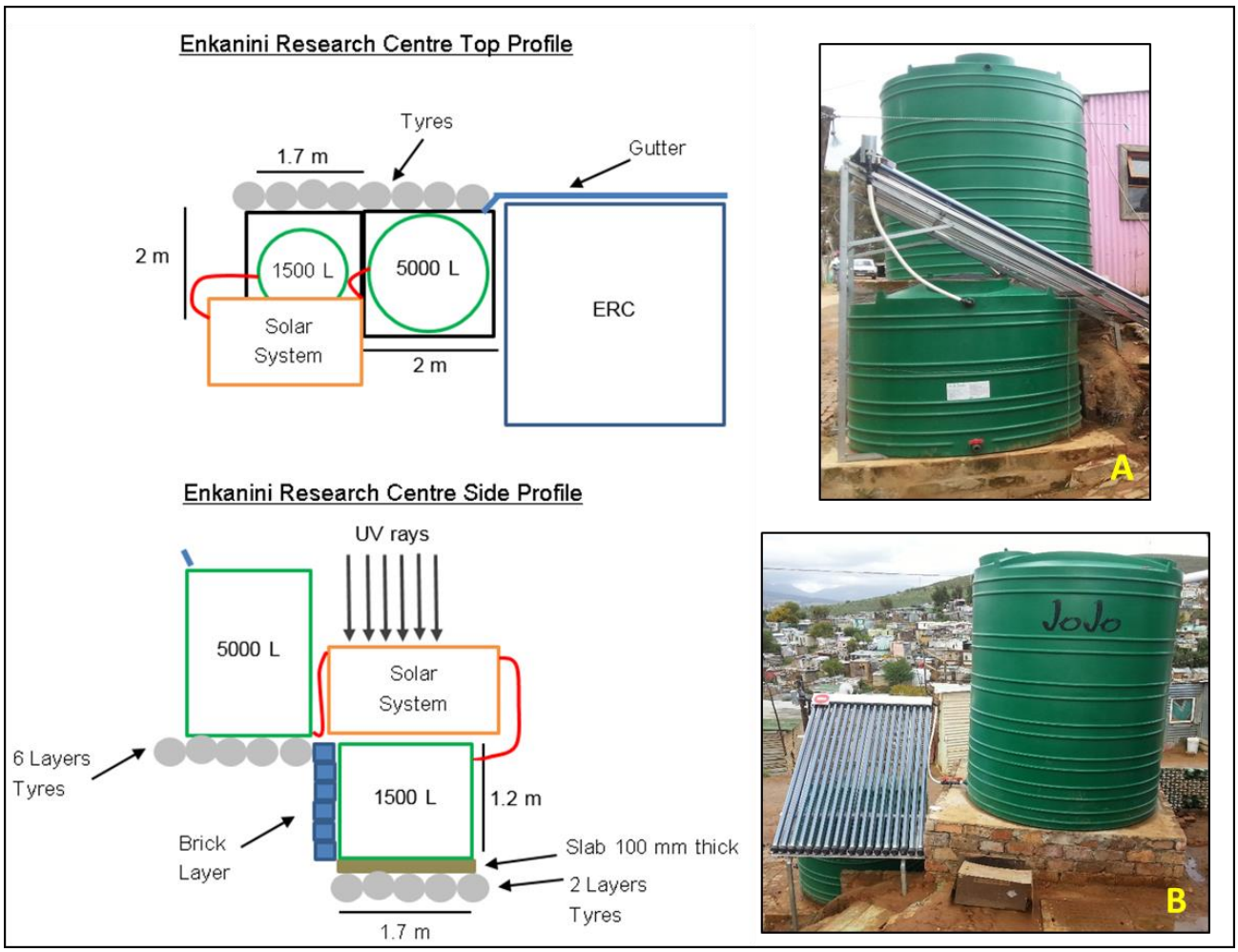

Fig. A4. A schematic diagram of the top and side profile of the large-scale rainwater harvesting solar pasteurization treatment system constructed at Site 3 with pictures of the system after installation.

A total cost analysis for the installation of the large-scale solar pasteurization rainwater harvesting treatment system at Site 3 is outlined in **Table A3**.

Table A3: Cost analysis for the installation of the large-scale treatment system at Site 3.

ITEM	PRICE
Site clearance and levelling	R 200
Labour	R 2 067
Operational Costs*	R 2 100
Construct new concrete plinth on compacted base	R 1 664
5 000 L vertical DRWH tank	R 3 428
1 500 L vertical DRWH tank	R 1 463
Tank adaptors for connection to solar geyser	R 1 000
Collector – 18 Evacuated tubes including manifold and stand	R 7 950
Thermostatic solar release valve	R 1 000
Commissioning the system	R 2 500
Total (excluding VAT)	**R 23 372**
VAT	**R 3 272**
Total (including VAT)	**R 26 644**

* Sand/gravel sourcing and delivery, transport/fuel, tool hire

3. Monitoring the rainwater and municipal water usage by the households utilising the solar pasteurization treatment systems

In order to monitor the water usage for each of the ten participating households, a co-researcher on the project located in Enkanini was provided with a log book for each site. The co-researcher visited the participating households every Friday of the trial period (September 2015 to September 2016) and posed the following two questions (answers recorded in the log books):

1. How much water was collected from the solar systems per day (how many times per day using the 20 L water container provided)?

2. How much water did you collect from the standpipe/municipal tap systems per day (how many times using another container)?

The answers were recorded directly into the log books and a summary of the results are represented in **Tables A4** (Site 1), **A5** (Site 2) and **A6** (Site 3). It is important to note that the volumes presented in each table per site, represent an estimation based on verbal responses provided by participants in each household. A workshop was conducted in September 2015, in Enkanini informal settlement and representatives from each of the ten participating households attended. The aim of the workshop was to inform the end users about the concept of rainwater harvesting, the general quality of rainwater and how the solar pasteurization systems function and should be maintained (WRC Report Project No. K5/2368//3, 2016).

3.1 Small-scale solar pasteurization systems (Sites 1 and 2)

In total three households had access to the small-scale solar pasteurization rainwater treatment system installed at Site 1. For ease of analysis, the households were designated and identified using the codes House 1 (5 residents), House 2 (3 residents) and House 3 (3 residents). During the first week, after the workshop (September 2015) had been presented in Enkanini, the three households using the small-scale solar pasteurization system located at Site 1 (House 1, 2 and 3) used 100, 90 and 80 L of pasteurized tank water, respectively (**Table A4**). This volume then increased to 190, 180 and 170 L during the following week, with a mean volume of 165, 145 and 135 L of pasteurized tank water utilised by the three households per week, respectively, during the first month of implementation (September 2015).

However, at Site 1 during October 2015, the research team noted that pressure was building up in the solar pasteurization system and that tank water was not effectively flowing from the rainwater harvesting tank into the pasteurization system (**Table A4**). After the problem had been addressed, monitoring of the water usage continued from the end of November 2015 until 11 January 2016, whereafter it was reported that the rainwater harvesting tank was empty [summer period in the Western Cape with limited rainfall (33.6 mm total rainfall) recorded during this period].

Table A4: Weekly rainwater (pasteurized tank water) and municipal tap water usage by each of the three households utilising the small-scale system located at Site 1 during September 2015 to September 2016.

Date	Weekly Rainwater Usage (L)			Weekly Tap Water Usage (L)		
	House 1	House 2	House 3	House 1	House 2	House 3
07-Sep-15	100	90	80	180	180	120
14-Sep-15	190	180	170	240	220	140
21-Sep-15	200	180	160	200	220	140
28-Sep-15	170	130	130	220	220	200
05-Oct-15	System not working efficiently, pressure release pipe inserted and system lowered			160	280	140
12-Oct-15				240	380	320
19-Oct-15				200	300	280
26-Oct-15				220	350	300
02 to 23-Nov-2015	Co-researcher unable to monitor the water usage during this time period as he was involved in a car accident					
30-Nov-15	140	120	140	200	300	275
07-Dec-15	65	85	140	250	380	200
14-Dec-15	55	50	120	225	180	275
21-Dec-15	80	50	120	250	180	250
28-Dec-15	15	50	60	200	180	250
04-Jan-16	55	35	55	250	200	250
11-Jan-16	Borosilicate glass tube broken			300	280	325
18-Jan to 28-Mar-2016	Rainwater harvesting tank empty (January) Stellenbosch rainfall season only starts end of March					

Date	Weekly Rainwater Usage(L)			Weekly Tap Water Usage (L)		
	House 1	House 2	House 3	House 1	House 2	House 3
04-Apr-16	30	15	20	75	180	175
11-Apr-16	75	35	90	175	200	350
18-Apr-16	50	30	55	225	240	375
25-Apr-16	25	20	20	350	280	350
02-May-16	Insufficient volume of water produced by solar pasteurization system			275	300	225
09-May-16				375	300	325
16-May-16				275	280	400
23-May-16	Solar pasteurization system closed to enable research team to monitor pasteurized tank water quality			350	400	350
30-May-16				350	300	350
06-Jun-16				350	320	400
13-Jun-16				450	300	450
20-Jun-16				350	320	525
27-Jun-16				575	340	525
04-Jul-16				450	300	425
11-Jul-16				450	300	425
18-Jul-16				400	360	425
25-Jul-16				400	575	700
01-Aug-16				380	550	550
08-Aug-16				380	625	425
15-Aug-16				540	700	550
22-Aug-16				340	575	375
29-Aug-16				380	550	425
05-Sep-16				240	400	325

As expected, the solar pasteurized tank water usage decreased during the “dry months” of November 2015 to January 2016. From the end of November 2015 until the end of December 2015, a mean volume of 71, 71 and 116 L of pasteurized tank water was subsequently utilised per week by the households located at House 1, 2 and 3, respectively. Moreover, during the week before the primary rainwater tank was completely empty (first week of January 2016), the weekly pasteurized tank water usage for House 1, 2 and 3 decreased even further to 55, 35 and 55 L, respectively (**Table A4**).

The pasteurized rainwater usage by each of the three households was then monitored from April 2016, as the rainfall period started during March 2016 (30.4 mm total rainfall recorded during March 2016). However, as Site 1 has the smallest rainwater harvesting system with regard to the rainwater harvesting tank size (2 500 L) and the size of the catchment area (15.27 m^2), the volume of harvested rainwater could not sufficiently meet the demands of the households, as only 49.2 mm rainfall was recorded for April 2016. During April 2016, House 1, 2 and 3 used a mean of 45, 25 and 46 L of pasteurized tank water, respectively on a weekly basis.

The overall pasteurized tank water usage decreased even further during the month of May 2016, as low rainfall (21.3 mm total rainfall) was experienced during this time period and the households reported that an insufficient volume of water was being produced by the system for all their domestic needs. Moreover, as soon as pasteurized rainwater was available in the solar system, the households utilised the water and subsequently emptied the 125 L storage tank. Subsequently, an insufficient volume of water was being produced by the solar pasteurization system for the research team to finish their analysis on the quality of the pasteurized tank water.

When comparing the pasteurized tank water usage to the tap water usage at Site 1, an increase in tap water usage was observed during the months when less pasteurized rainwater was available [October 2015 (pressure release pipe needed to be inserted into the system), December 2015 to January 2016 (rainwater harvesting tank started to empty and borosilicate glass tube broke), June 2016 to September 2016 (solar pasteurization system closed)]. From September 2015 to October 2015, the households located at House 1, 2 and 3 then reported using a mean of 208, 270 and 205 L tap water per week, respectively. The mean volume of tap water utilised per week from the last week of November 2015 to January 2016 was recorded as 240, 243 and 261 L, respectively. When the monitoring of the water usage continued during April 2016 (after the rainfall season had started), the households’ mean tap water usage per week for April to May 2016 was recorded as 250, 254 and 314 L, respectively. As indicated, during May 2016 the solar pasteurization system was locked as the households reported that the rainwater harvesting tank and subsequently the 125 L solar pasteurization storage tank were being drained rapidly after a low rainfall event. Thus an increase in the households’ tap water usage was observed from May 2016 to September 2016

and the households' mean tap water usage increased to 385, 410 and 430 L, respectively (**Table A4**).

As warm pasteurized tank water is collected from the solar pasteurization system, the residents in the participating households communicated with the research team that the water was primarily used for bathing, laundry and general house cleaning activities. Since the water was already hot, less paraffin/gas was being used by the households as they would normally first heat municipal tap water to perform some of these tasks (personal communication). Analysis of the results also indicated that proximity to the solar pasteurization system influenced the usage of pasteurized tank water. For example, the household of House 3 was located closest to the system and subsequently used the greatest volume of pasteurized water, when compared to households of House 1 and 2, which were located approximately 15 metres from Site 1.

In total three households had access to the small-scale solar pasteurization rainwater treatment system installed at the local church (Site 2). For ease of analysis, each household was designated and identified by the codes Church 1 (1 resident), Church 2 (4 residents) and Church 3 (4 residents). During the first week of September 2015, after the workshop had been presented, 90, 60 and 65 L of pasteurized tank water was utilised by the residents of Church 1, 2 and 3, respectively. As noted for Site 1, this volume increased during the second week, with 160, 130 and 120 L of pasteurized tank water being used, respectively (**Table A5**).

On average during the first two months (September to October 2015) 108, 114 and 123 L of pasteurized tank water was being used by each household per week (Church 1, 2 and 3), respectively. As the rainwater harvesting tank (5 000 L) and catchment system (55.9 m^2) utilised at Site 2 are larger than the tank and roof surface area at Site 1 (2 500 L and 15.27 m^2), an increased volume of pasteurized tank water was available during the dry months (November 2015 to January 2016 - 33.6 mm total rainfall).

From the last week of November 2015 (co-researcher was in a car accident and no data was collected for the first three weeks of November 2015) to December 2015, the mean pasteurized rainwater utilised per week by each of the three households at Site 2 was recorded as 87, 110 and 130 L (Church 1, 2 and 3), respectively. However, on the 21st of December 2015 it was reported by one of the participating households (Site 2) that the tap connected to the solar pasteurization system was not functioning optimally. The tap was subsequently replaced.

When the water usage monitoring commenced in April 2016, the mean pasteurized rainwater usage for each of the three households (Church 1, 2 and 3) was reported as 43, 36 and 46 L per week, respectively (April 2016 to June 2016 – 153 mm total rainfall).

Table A5: Weekly rainwater (pasteurized tank water) and municipal tap water usage by each of the three households utilising the small-scale system located at Site 2 during September 2015 to September 2016.

Date	Weekly Rainwater Usage (L)			Weekly Tap Water Usage (L)		
	Church 1	Church 2	Church 3	Church 1	Church 2	Church 3
07-Sep-15	90	60	65	0	220	220
14-Sep-15	160	130	120	0	160	440
21-Sep-15	140	160	140	0	140	200
28-Sep-15	100	160	140	60	240	260
05-Oct-15	100	120	140	0	220	220
12-Oct-15	60	60	80	200	240	280
19-Oct-15	80	130	160	0	280	140
26-Oct-15	130	90	140	0	140	140
02 to 23-Nov-2015	Co-researcher unable to monitor the water usage during this time period as he was involved in a car accident					
30-Nov-15	60	90	180	0	200	180
07-Dec-15	120	150	80	0	220	300
14-Dec-15	80	90	Holiday	0	140	220
21-Dec-15	Outlet tap of solar pasteurization system faulty. Water level in rainwater tank very low			0	140	220
28-Dec-15				0	130	260
04-Jan-16				0	220	220
11-Jan-16				0	80	100
18-Jan to 28-Mar-2016	Rainwater harvesting tank empty (January) Stellenbosch rainfall season only starts end of March					

Date	Weekly Rainwater Usage (L)			Weekly Tap Water Usage (L)		
	Church 1	Church 2	Church 3	Church 1	Church 2	Church 3
04-Apr-16	15	20	20	0	180	125
11-Apr-16	100	45	80	0	240	325
18-Apr-16	60	50	30	0	280	300
25-Apr-16	75	75	80	0	380	325
02-May-16	50	60	70	0	300	400
09-May-16	35	40	55	0	280	375
16-May-16	60	15	35	0	400	425
23-May-16	25	40	40	0	340	500
30-May-16	30	10	40	0	400	350
06-Jun-16	20	25	30	0	320	400
13-Jun-16	5	15	25	0	300	500
20-Jun-16	Borosilicate glass tube broken			0	360	555
27-Jun-16	0	0	0	0	360	400
04-Jul-16	30	40	30	0	380	350
11-Jul-16	75	60	40	0	340	450
18-Jul-16	50	45	65	0	360	475
25-Jul-16	70	40	70	0	220	575
01-Aug-16	70	70	65	0	200	625
08-Aug-16	65	30	70	0	200	600
15-Aug-16	70	50	60	65	260	525
22-Aug-16	65	35	60	80	220	575
29-Aug-16	50	30	55	80	260	575
05-Sep-16	40	25	25	50	100	425

During June 2016, a borosilicate glass tube of the solar pasteurization system was damaged. As the borosilicate glass tubes enter directly into the storage tank, the pasteurization system could not be used during this time period as the water would flow directly out of the broken tube. The system was thus closed until the tube was replaced later that week. Following the replacement of the borosilicate glass tube, the mean volume of pasteurized tank water utilised per household (Church 1, 2 and 3) per week until September 2016 was 59, 43 and 54 L, respectively (**Table A5**), with 159 mm of total rainfall being recorded for July to September 2016.

The corresponding mean tap water usage per week by each of the three households located at Site 2, from September 2015 to October 2015 was then recorded as 33, 205 and 238 L (Church 1, 2 and 3), respectively. From November 2015 to January 2016 (low to no rainfall – 33.6 mm total rainfall) the mean tap water usage recorded per week for the Church 1, 2 and 3 households was 0, 161 and 214 L, respectively. When the water usage monitoring commenced during April 2016, the respective households' mean tap water usage per week for April 2016 to May 2016 (medium rainfall – 70.5 mm rainfall) was recorded as 0, 311 and 347 L, respectively. During the high rainfall period (June 2016 to September 2016 – 240 mm total rainfall), the mean tap water usage recorded per week per household (Church 1, 2 and 3) was 20, 277 and 502 L, respectively.

Interestingly, the household designated as "Church 1" predominantly used pasteurized tank water during the monitoring period (September 2015 to September 2016). One occupant currently resides in this participating household and as indicated he only used municipal tap water from September 2015 to October 2015 and then again during August 2016 (**Table A5**). Upon further investigation, the co-researcher informed the research team that during this time period the resident at Church 1 was occasionally using approximately 10 to 15 L of municipal tap water per week from the water supply being collected by the Church 3 household. However, as with Site 1, proximity to the solar pasteurization system influenced the usage of the pasteurized tank water, as Church 1 and Church 3 were located closest to the system and subsequently used the greatest volume of pasteurized water, in comparison to the household at Church 2, which was located approximately 30 m from the system located at Site 2.

3.2 Large-scale solar pasteurization system (Site 3)

The tank water and tap water usage for each of the four households using the large-scale solar pasteurization system located at Site 3, were also monitored. In total four households have access to the large-scale solar pasteurization rainwater treatment system installed at the ERC (Site 3). For ease of analysis, each household was designated and identified by the codes ERC 1 (4 residents), ERC 2 (4 residents), ERC 3 (3 residents) and ERC 4 (3 residents). During the first week of September 2015, after the workshop had been presented, 80, 100, 100 and 80 L of stored pasteurized tank water was utilised by the residents of ERC 1, 2, 3 and 4, respectively (**Table A6**).

As was noted for Sites 1 and 2, this volume increased during the second week, with 150, 180, 140 and 120 L of stored pasteurized tank water being used by the respective households (ERC 1, 2, 3 and 4). Overall a decrease in pasteurized tank water usage was observed during October 2015 in comparison to September 2015, as the mean weekly pasteurized tank water usage for each of the four households decreased from 143 to 128 L (ERC 1), 135 to 105 L (ERC 2), 125 to 118 L (ERC 3) and 120 to 68 L (ERC 4), respectively. From the last week of November 2015 to January 2016, ERC 1 and ERC 2 used a mean volume of 137 and 129 L of stored pasteurized tank water per week, while limited water usage was recorded for the ERC 3 and 4 households, since the residents were not in Enkanini during that time period (**Table A6**). As the rainwater harvesting tank (5 000 L) and catchment system (88.5 m^2) utilised at Site 3 is the largest of the three systems, pasteurized tank water was available during the "dry months", which are characterised by low to no rainfall (November 2015 to February 2016 – 62.7 mm total rainfall recorded during this period). Subsequently the rainwater harvesting tank only ran dry during the beginning of March 2016, when it was reported that an insufficient volume of water was available in the 1 500 L storage tank.

Monitoring of tank water usage then continued from April 2016, after the rainfall season had commenced during the latter part of March 2016. During April 2016, the four households reported using a mean volume of 165, 120, 118 and 93 L stored pasteurized tank water per week (49.2 mm total rainfall recorded). At the start of May 2016 the ERC 3 household permanently relocated from Enkanini and thus no water usage information is available for this household from the 2nd of May 2016 until the end of the monitoring period (September 2016). During May 2016 (21.3 mm total rainfall), the mean pasteurized tank water utilised per week by each of the three remaining households (ERC 1, 2 and 4) decreased to 54, 72 and 24 L, respectively.

During June 2016, sporadic use of the stored pasteurized tank water was being reported. The research team then liaised with the participating households at Site 3, who articulated that during the winter months, the stored pasteurized tank water was too cold (3 to 4 °C) to use for daily domestic activities, including bathing. Additionally, as a result of poor weather conditions (cloudy overcast conditions), less pasteurized water was being produced by the solar manifold system in comparison to that which was normally being produced during the summer months. The households that utilise the large-scale solar pasteurization system also indicated that they would utilise the system more frequently if hot water was supplied to the households (similar to that which was being supplied by the small-scale solar pasteurization systems), as hot water could be used for domestic purposes. These factors then contributed to the decrease in rainwater usage by the households during the winter months. Future studies should therefore focus on designing a storage tank that is insulated and is able to keep the pasteurized rainwater warm throughout the day.

Table A6: Weekly rainwater (pasteurized tank water) and municipal tap water usage by each of the four households utilising the large-scale system located at Site 3 during September 2015 to September 2016.

Date	Weekly Rainwater Usage (L)				Weekly Tap Water Usage (L)			
	ERC 1	ERC 2	ERC 3	ERC 4	ERC 1	ERC 2	ERC 3	ERC 4
07-Sep-15	80	100	100	80	200	160	160	90
14-Sep-15	150	180	140	120	275	200	280	120
21-Sep-15	140	120	120	140	250	200	200	90
28-Sep-15	200	140	140	140	270	220	200	170
05-Oct-15	180	90	80	40	165	140	220	140
12-Oct-15	80	110	110	80	175	320	200	310
19-Oct-15	110	60	140	70	245	160	280	100
26-Oct-15	140	160	140	80	250	140	140	70
02 to 23-Nov-2015	Co-researcher unable to monitor the water usage during this time period as he was involved in a car accident							
30-Nov-15	200	160	220	80	200	160	200	70
07-Dec-15	140	140	80	70	425	200	140	125
14-Dec-15	140	140	Holiday	50	200	140	Holiday	70
21-Dec-15	140	140		Holiday	250	160		Holiday
28-Dec-15	140	110			250	140		
04-Jan-16	140	150			350	140		
11-Jan-16	60	60			100	60		
18-Jan to 28-Mar-2016	Rainwater harvesting tank empty (March) Stellenbosch rainfall season only starts end of March							

Date	Weekly Rainwater Usage (L)				Weekly Tap Water Usage (L)			
	ERC 1	ERC 2	ERC 3	ERC 4	ERC 1	ERC 2	ERC 3	ERC 4
04-Apr-16	140	60	130	130	300	175	175	70
11-Apr-16	160	80	90	30	325	300	300	90
18-Apr-16	120	110	40	0	350	250	250	150
25-Apr-16	240	230	210	210	350	175	175	80
02-May-16	90	150	Permanently relocated from Enkanini	70	350	160	Permanently relocated from Enkanini	80
09-May-16	60	70		50	450	180		110
16-May-16	0	0		0	450	300		80
23-May-16	0	0		0	480	340		140
30-May-16	120	140		0	400	280		150
06-Jun-16	Sporadic use of stored pasteurized tank water reported			Sporadic use of stored pasteurized tank water reported	400	320		100
13-Jun-16					550	320		110
20-Jun-16					425	280		120
27-Jun-16					525	340		100
04-Jul-16					550	300		140
11-Jul-16					550	280		80
18-Jul-16					525	280		110
25-Jul-16	10	80		20	625	480		80
01-Aug-16	20	100		30	575	380		110
08-Aug-16	40	110		0	650	340		130
15-Aug-16	Sporadic use of stored pasteurized tank water reported			Sporadic use of stored pasteurized tank water reported	675	400		140
22-Aug-16					675	320		100
29-Aug-16					675	420		170
05-Sep-16					450	260		160

The mean tap water usage per week recorded for September 2015 to October 2015 for each of the four households (ERC 1, 2, 3 and 4) was 229, 193, 210 and 136 L, respectively. From the end of November 2015 to January 2016, ERC 1 and ERC 2 used a mean of 253 and 143 L tap water per week, respectively. The ERC 3 and 4 households each used a mean of 170 and 88 L of tap water respectively, in the weeks before leaving on holiday. Monitoring of the tap water usage then continued from April 2016. During April 2016, the 4 households reported using a mean of 331, 225, 225 and 98 L tap water per week, respectively. As previously indicated, the ERC 3 household permanently relocated away from Enkanini and thus no tap water usage data from April 2016 is available for this household. For May 2016, the mean tap water usage for each of the three remaining households (ERC 1, 2 and 4) was recorded as 426, 252 and 112 L, respectively. From June 2016 to September 2016, the mean weekly tap water usage by each of the three remaining households increased to 561, 337 and 118 L, respectively.

4. References

International Technology Sourcing Solar. 2016. *How do evacuated tubes work?* Available: http://www.itssolar.co.za/its-downloads [2016 May, 20].

JoJo Tanks. 2016. *About JoJo tanks*. Available: http://www.jojotanks.co.za/faqs [2016, October 20].

SamSamWater Rainwater Harvesting Tool. 2015. Available: http://www.samsamwater.com/rain/ [2015, June 20].

United Nations. 2010. *The human right to water and sanitation*. Media brief. Available: http://www.un.org/waterforlifedecade/pdf/human_right_to_water_and_sanitation_media_brief.pdf [2014, October 28].

Water Research Commission (WRC), Report Project No. K5/2368//3. (2016). *Design, Construction and Monitoring of Sustainable Domestic Rainwater Harvesting Treatment Systems in Enkanini Informal Settlement, Stellenbosch*. Report to the Water Research Commission, Project No. K5/2368//3 by Department of Microbiology, Stellenbosch University. Stellenbosch, South Africa.

Appendix B:

Conventional PCR Results

Table B1: Results obtained during the conventional PCR analysis of the unpasteurized (n = 8) and pasteurized (n = 8) tank water samples collected from Site 1 for the detection of indigenous rainwater pathogens.

Organism	Unpasteurized 52°C	Pasteurized 52°C	Unpasteurized 60°C	Pasteurized 60°C	Unpasteurized 67°C	Pasteurized 67°C	Unpasteurized 72°C	Pasteurized 72°C	Unpasteurized 73°C	Pasteurized 73°C	Unpasteurized 75°C	Pasteurized 75°C	Unpasteurized 75°C	Pasteurized 75°C	Unpasteurized 85°C	Pasteurized 85°C
Aeromonas spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillus spp.	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
Klebsiella spp.	0	0	1	0	1	0	1	1	1	1	1	0	1	0	0	0
Legionella spp.	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Pseudomonas spp.	0	0	1	1	1	0	1	1	1	0	1	1	1	0	1	0
Salmonella spp.	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0
Serratia spp.	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0
Shigella spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus spp.	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	0
Streptomyces spp.	0	0	1	1	0	0	1	1	1	0	1	0	1	0	1	0
Yersinia spp.	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Adenovirus	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0

***** 0 = absent/ 1 = present

Table B2: Results obtained during the conventional PCR analysis of the unpasteurized (n = 3) and pasteurized (n = 3) tank water samples collected from Site 2 for the detection of indigenous rainwater pathogens.

Organism	Unpasteurized 53°C	Pasteurized 53°C	Unpasteurized 58°C	Pasteurized 58°C	Unpasteurized 66°C	Pasteurized 66°C
Aeromonas spp.	0	0	0	0	0	0
Bacillus spp.	0	0	0	0	0	0
Klebsiella spp.	0	0	0	0	1	0
Legionella spp.	1	1	1	1	1	1
Pseudomonas spp.	1	1	1	1	1	1
Salmonella spp.	1	1	1	1	1	1
Serratia spp.	0	0	0	0	0	0
Shigella spp.	0	0	0	0	0	0
Staphylococcus spp.	1	1	1	0	1	1
Streptomyces spp.	1	1	1	1	1	0
Yersinia spp.	0	0	0	0	0	0
Adenovirus	0	0	1	1	0	0

***** 0 = absent/ 1 = present

Table B3: Results obtained during the conventional PCR analysis of the unpasteurized (n = 6) and stored pasteurized (n = 6) tank water samples collected from Site 3 for the detection of indigenous rainwater pathogens.

Organism	Unpasteurized (55°C)	Stored Pasteurized (55°C)	Unpasteurized (61°C)	Stored Pasteurized (61°C)	Unpasteurized (66°C)	Stored Pasteurized (66°C)	Unpasteurized (71°C)	Stored Pasteurized (71°C)	Unpasteurized (72°C)	Stored Pasteurized (72°C)	Unpasteurized (79°C)	Stored Pasteurized (79°C)
Aeromonas spp.	0	0	0	0	0	0	0	0	0	0	0	0
Bacillus spp.	1	0	0	1	0	1	1	0	1	0	0	1
Klebsiella spp.	1	1	0	0	1	1	1	0	1	0	1	1
Legionella spp.	1	1	0	1	1	1	1	1	1	1	1	1
Pseudomonas spp.	0	0	1	0	1	1	1	0	0	0	1	1
Salmonella spp.	1	0	0	0	0	0	1	0	0	0	0	1
Serratia spp.	1	1	0	1	1	1	0	0	1	0	1	1
Shigella spp.	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus spp.	1	1	0	1	1	1	1	1	1	1	1	1
Streptomyces spp.	0	0	0	0	0	1	0	0	0	0	1	1
Yersinia spp.	0	0	0	0	0	0	0	0	0	0	0	0
Adenovirus	0	1	0	0	0	1	0	0	1	0	0	1

***** 0 = absent/ 1 = present