***Podoviridae* bacteriophage for the biocontrol of *Pseudomonas aeruginosa* in rainwater**

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Short title: Bacteriophage biocontrol of *Pseudomonas aeruginosa*

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Appendix A: Electronic supplementary information available - *Legionella* spp. growth conditions and modified protocol for host range determination. List of target and non-target bacterial species (host range determination). Sequencing results of the Podo-Hypo-F/R (*Podoviridae*) and Myo-Hypo-F/R (*Myoviridae*) primer sets. qPCR performance characteristics. Summary of cell counts, gene copies and log reductions recorded for the pre-treatment/SODIS-CPC trials. Characterisation results for the isolated bacteriophages (i.e. temperature and pH sensitivity) and results obtained for the bacterial challenge tests.

Abstract

Bacteriophages targeting *Pseudomonas* spp. were isolated and characterised for the biocontrol pre-treatment of harvested rainwater. Bacteriophages PAW33 (isolated using *P. aeruginosa*) and PFW25 (isolated using *P. fluorescens*) were characterised as members of the *Podoviridae* and *Myoviridae* families, respectively. Bacteriophage PAW33 displayed a broad host range against *P. aeruginosa* strains, while PFW25 was more effective in infecting *K. pneumoniae* than *P. fluorescens* (original target organism). PAW33 was subsequently applied in small-scale bacteriophage pre-treatment trials (8 h and 24 h), to evaluate its efficacy in restricting the proliferation of an environmental *P. aeruginosa* S1 68 strain. Following the completion of the bacteriophage pre-treatment trial, the respective samples (bacteriophage pre-treated samples and non-pre-treated control samples) were subjected to treatment in small-scale (vol?) solar disinfection compound parabolic collector (SODIS-CPC) systems for 4 h under natural sunlight. For the 8 h trial followed by SODIS-CPC, similar total log reductions in colony forming units (CFU) and gene copies (GC)/mL were obtained for the bacteriophage pre-treated [3.68 log (CFU) and 2.34 log (GC)] and non-pre-treated [3.74 log (CFU) and 2.33 log(GC)] samples. In contrast, culture-based analysis of the 24 h trial samples (followed by SODIS-CPC) indicated that a higher overall log reduction was recorded for the pre-treated sample (4.61 log) in comparison to the non-pre-treated sample (3.91 log), while comparable log reductions were obtained using viability-qPCR (2.32 log and 2.26 log, respectively). Gene expression analysis then indicated that PAW33 pre-treatment for 24 h influenced the ability of *P. aeruginosa* S1 68 to initiate stress response mechanisms during the 4 h SODIS-CPC treatment (downregulation of the *recA* and *lexA* genes) and resulted in the downregulation of the p*hzM* gene (virulence factor responsible for pyocyanin production). Bacteriophage PFW33 thus displays promise as a biocontrol pre-treatment strategy of roof-harvested rainwater as it restricts the proliferation of *P. aeruginosa* and may increase the treatment efficiency of primary disinfection methods.

Key words: *Podoviridae*; *Pseudomonas* spp.; Solar Disinfection; Virulence; Stress Response

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen and is primarily associated with nosocomial infections where it may cause pneumonia, urinary tract and skin infections (Driscoll et al., 2007). However, the ubiquitous distribution of *Pseudomonas* spp. in the environment significantly increases the health risk associated with this organism and John et al. (2017) recently reported on an outbreak of community-acquired *P. aeruginosa* pneumonia in Cape Town, South Africa. The authors noted that while no cases of community-acquired *P. aeruginosa* were reported at a local government hospital over a 10-year period (2007 to 2016); over a period of three months (March to May 2017), 9 cases were reported. This outbreak coincided with a severe drought in Cape Town (2017 to 2018), where stringent water restrictions were implemented and residents were increasingly using alternative water sources (e.g. rainwater and grey water). It was hypothesised that the use of these alternative water sources may have led to the exposure of the community members to *P. aeruginosa*.

Although various treatment methods have been implemented to reduce the levels or remove pathogens and opportunistic pathogens from environmental water reservoirs, many microorganisms employ survival strategies and are capable of persisting. Certain strains of *P. aeruginosa* have been shown to survive conventional disinfection strategies including sub-optimal chlorination (Shrivastava et al., 2004), ultra-violet (UV) radiation (Strauss et al., 2016; 2018) and heat treatment (Strauss et al., 2016; Clements et al., 2019). Using viability-qPCR, Strauss et al. (2016) reported on the detection of *Pseudomonas* spp. in rainwater samples treated at solar pasteurization temperatures > 90 °C and in water samples treated by solar disinfection (SODIS; UV radiation and solar mild-heat) for 8 hours. Similarly, using culture-based methods, Clements et al. (2019) isolated *Pseudomonas* spp. from rainwater samples pasteurized at temperatures > 70 °C, with certain isolates identified as *P. aeruginosa* using species-specific primers. *Pseudomonas aeruginosa* has also readily been detected in grey water (Winward et al., 2008; Maimon et al., 2014), with Gross et al. (2007) and Gilboa and Friedler (2008) reporting on the detection of viable *P. aeruginosa* in grey water samples treated in a vertical flow bioreactor and a rotating biological contractor followed by UV disinfection, respectively. The survival of

Pseudomonas spp. during water treatment has subsequently been attributed to the initiation of stress response mechanisms, including heat-shock proteins and deoxyribonucleic acid (DNA) repair mechanisms, the ability of the organisms to form biofilms and survive intracellularly within protozoa (Strauss et al., 2016; Clements et al., 2019). As bacteria are able to undergo an adaptive response and build-up resistance to conventional disinfection treatments (Wesche et al., 2009), alternative treatment strategies targeting pathogenic species directly are required.

Bacteriophage therapy or bacteriophage biocontrol has gained increased interest in recent years, due to the specificity with which pathogens may be targeted. Bacteriophages are bacterial viruses, which are ubiquitously distributed in the environment and may interact with bacteria by either causing lysis of the host cell (lytic phages) or integrating their phage genome into the host cell (temperate phages) (Wu et al., 2017). Numerous studies have subsequently reported on the potential of bacteriophage biocontrol to target food-borne pathogens (food safety) (Greer, 2005); biofilm formation on medical devices or treat infectious diseases (human and veterinary medicine) (Clark and March, 2006); to reduce economic losses in agriculture (targeting plant pathogens) and aquaculture (targeting fish pathogens) (Vinod et al., 2006; Frampton et al., 2012); or in bioremediation strategies for the selective removal of bacteria from water (Whitey et al., 2005). While investigating the use of bacteriophages for the biocontrol of *Salmonella* spp. in wastewater, Turki et al. (2012) showed that the isolated bacteriophages were able to reduce the proliferation of the target pathogen (reduction in sample optical density) over time in co-culture experiments. Additionally, using DNA fingerprinting analysis [PCR based amplification of enterobacterial repetitive intergenic consensus (ERIC) sequences], the authors reported on the decreased detection of the enterobacterial community and *Salmonella* spp. in the treated wastewater over time (0 h to 8 h). Similarly, Goldman et al. (2009) reported that bacteriophage treatment could reduce membrane fouling caused by the opportunistic pathogens *Acinetobacter johnsonii*, *Bacillus subtilis* and *P. aeruginosa*, by 40 to 60%. An added benefit of using bacteriophages to target persisting pathogens is the relative ease with which this treatment can be combined with existing disinfection methods. For example, Zhang et al. (2013) applied a mixture of *P. aeruginosa* bacteriophages to selectively remove this organism from water passing through granular activated carbon and anthracite biofilters. Results indicated that the bacteriophage treatment was able to reduce *P. aeruginosa* concentration by 55 to 75% in the two biofilter systems, with minimal impact on the beneficial microorganisms, and thereby contribute to an improvement in effluent water quality.

Additionally, solar radiation can be used to reduce viable pathogenic organisms in water by the exposure of water to natural or concentrated sunlight. When the polluted water is placed in plastic transparent containers to direct sunlight for 6 h, it is known as SODIS. It has been widely investigated as disinfection method that helps reducing the presence of pathogens in water and the incidence of diarrheal diseases. One of the main disadvantages of SODIS is the low efficiency of the treatment for resistant pathogens [McGuigan et al., 2012]. To enhance the efficiency of solar disinfection, the use of compound parabolic collectors (CPC) has been investigated for specific pathogens [Ubomba-Jaswa et al., 2010]. The authors that CPC solar mirrors are a good solution to disinfect 25 L of water containing *E. coli* (6-log and 3-log reduction in 5 h on sunny days, and cloudy conditions, respectively). For resistant microorganisms (*pseudomonas*, *cryptosporidium*, MS2 bacteriophage, etc.) more research must be done to improve their efficient removal. *Legionella*, *Pseudomonas* and *Klebsiella* spp. in solar disinfection systems, including SOPAS, are not completely effective as these bacteria have repair mechanisms and capacity to resist this treatment [Dobrowsky et al., 2016]. Therefore, additional treatment technologies might be explored to effectively eliminate these organisms from water sources.

The primary aim of the current study was thus to isolate and characterise bacteriophages targeting *Pseudomonas* spp. and apply the best performing bacteriophage as a biocontrol pre-treatment (8 and 24 h) of roof-harvested rainwater. Following the completion of the bacteriophage pre-treatment trial, the respective samples (bacteriophage pre-treated samples and non-pre-treated control samples) were subjected to treatment in small-scale SODIS-CPC systems. Culture- and molecular-based (viability-qPCR) analysis were used to quantify *P. aeruginosa* S1 68 and bacteriophage PAW33 during the pre-treatment and SODIS trials, while gene expression assays were used to monitor the expression of *P. aeruginosa* S1 68 stress response and virulence genes.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa (*P. aeruginosa*) ATCC 27853, *P. fluorescens* ATCC 13525 and *P. protegens* ATCC 17386 reference strains were obtained from Microbiologics® (St Cloud, Minnesota, USA) and were used for the isolation, propagation and characterisation of the bacteriophages. The bacterial strains (target and non-target bacterial species) utilised for the host range determination of the isolated bacteriophages are indicated in Appendix A, Table S1. All strains were cultured at 30 °C in tryptic soy broth (TSB; Biolab, Merck, Wadeville, South Africa) or on tryptic soy agar (TSA; Biolab, Merck), with the exception of *Legionella* spp. (see Appendix A for *Legionella* spp. growth conditions). For the double-layer plaque assays (double-layer overlays), the TSA medium contained 1.2% (w/v) Agar Bacteriological (Biolab, Merck) in the bottom layer and 0.6% agar (w/v) in the soft top layer (Sillankorva et al., 2008).

2.2 Isolation, Purification and Propagation of Bacteriophages

Bacteriophages were isolated by screening various environmental water sources including influent wastewater collected from the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: -33.943505, 18.824584), river water from the Plankenburg River (GPS co-ordinates: -33.927761, 18.850544) and roof-harvested rainwater from a rainwater harvesting tank connected to the JC Smuts building at Stellenbosch University (GPS co-ordinates: -33.930858, 18.865611). Selection for *Pseudomonas* spp. bacteriophages was performed as previously described by Sillankorva et al. (2008), with minor modifications. Briefly, following the centrifugation step (10 000 × *g*; 10 min; 4 °C), the supernatant from each sample was filtered through a sterile GN-6 Metricel® S-Pack Membrane Disc Filter (Pall Life Sciences, Michigan, USA) with a pore size of 0.45 μm, to remove residual host bacteria from the sample (Vinod et al., 2006). The filtered supernatant was tested for the presence of bacteriophages (Sillankorva et al., 2008), whereaafter five repeated rounds of plaque purification and re-infection were performed (Stenholm et al., 2008).

The bacteriophages were selected for further studies based on their initial lysis profiles during bacteriophage purification (number and consistency of plaque formation, plaque clarity and plaque size) (Sillankorva et al., 2008). Code identifiers were assigned to the isolated bacteriophages based on the bacteria from which they were isolated (i.e. PA – *P. aeruginosa*; PF – *P. fluorescens* and PP – *P. protegens*), the source of the bacteriophage (e.g. W – wastewater; R – river water) and the plaque number. For example, PAW1 indicates that the bacteriophage was isolated using *P. aeruginosa* (PA), from wastewater (W) and was the first plaque isolated (1).

The purified bacteriophages were used to prepare concentrated bacteriophage solutions for use in subsequent experiments using the small-scale liquid culture method as described by Sambrook and Russell (2001), with minor modifications. Briefly, following the onset of bacterial cell lysis, the samples were centrifuged (10 000 × *g*; 10 min; 4 °C) and filtered through a 0.2 µm Acrodisc® PF syringe filter (Pall Life Sciences). The filtered supernatants were centrifuged at 25 000 × *g* for 60 min using an Avanti J-E Centrifuge with a JA 20 fixed angle rotor (Beckman Coulter, California, USA). Following centrifugation, the supernatant was removed and the obtained pellet was re-suspended in 1 mL SM-buffer [5.8 g/L sodium chloride (NaCl; Saarchem, Durban, South Africa), 2 g/L magnesium sulphate heptahydrate (MgSO4.7H2O; Saarchem), 50 mL 1 M Tris, pH 7.5]. The plaque forming units (PFU) per mL concentrated sample were determined by serial dilution (10^{-1} to 10^{-5}) in SM-buffer and double-layer plaque assays. The concentrated bacteriophage samples in SM-buffer were stored at 4 °C until further use.

2.3 Characterisation of the Isolated Bacteriophages

In order to increase bacteriophage retention on the electron microscopy grids and thereby increase bacteriophage visualisation during electron microscopy analysis, the hydrophilicity and “stickiness” of the 200 mesh carbon-coated Formvar grids (Agar Scientific, Essex, United Kingdom) were increased by using an alcian blue (Electron Microscopy Sciences, Pennsylvania, USA) pre-treatment (1% alcian blue in 1% acetic acid in water) as described by Laue and Bannert (2010). Concentrated bacteriophage samples with a titre of > 10^9 PFU/mL were then mixed with glutaraldehyde (2.5% v/v; Agar Scientific) for 5 min and 25 µL of the glutaraldehyde treated concentrated bacteriophage sample was loaded onto the alcian blue pre-treated grids and were allowed to settle for 10 min. Hereafter, the sample was stained with 1% uranyl acetate for 2 min. Excess stain was removed using filter paper and the grids were allowed to air dry. The grids were visualised with a Zeiss MERLIN Field Emission Scanning Electron Microscope (FE-SEM; Zeiss, Germany) at the Electron Microbeam Unit of the Central Analytical Facility (CAF) at Stellenbosch University. A Zeiss five-diode Scanning Transmission Electron Detector (Zeiss aSTEMA Detector) and Zeiss Smart SEM software was used to generate STEM images. Beam conditions during analysis on the Zeiss MERLIN FE-SEM were 20 kV accelerating voltage, 150 pA probe current, with a working distance of approximately 3.9 to 4.0 mm. Images were acquired in bright fields mode with the S1 diode activated.

2.4 Analysis of Bacteriophage Nucleic Acids - Restriction Enzyme Digestion and Molecular Analysis

To ensure that no potential residual host bacterial DNA was analysed in the subsequent bacteriophage nucleic acid determination, 500 µL of the concentrated bacteriophage samples was treated with DNaseI (Thermo Scientific, Lithuania) as outlined in Reyneke et al. (2017). Following the DNase treatment, bacteriophage nucleic acid was extracted from the concentrated samples using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

The type of nucleic acid was confirmed by treatment with DNaseI (dsDNA; Thermo Scientific), S1 nuclease (ssDNA; Thermo Scientific) and RNase [ribonucleic acid (RNA); Fermentas, Thermo Scientific] (Vinod et al., 2006), while the purified bacteriophage nucleic acids were digested with either *Eco*RI (Roche Diagnostics, Risch-Rotkreuz, Switzerland) or *Cla*I (Fermentas) (Stenholm et al., 2008). All nuclease and restriction enzyme digestions were performed according to the manufacturer's instructions.

In order to confirm the preliminary classification of the isolated bacteriophages, primers targeting families within the *Caudovirales* order, namely *Podoviridae* and *Myoviridae*, were designed. The PhiSiGns online bacteriophage genes and primers tool as described by Dwivedi et al. (2012) was used to identify signature genes within the respective bacteriophage families. The identified gene sequences were retrieved from the Genbank sequence database of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genbank/) and were aligned using

ClustalX version 2.0.10 (Larkin et al., 2007) and visualised using GeneDoc version 2.7.00 (Nicholas and Nicholas, 1997). Primers targeting the specific gene sequences were designed based on the sequence alignments and are presented in Table 1 along with the respective PCR cycling parameters. Each PCR assay was performed in a final volume of 25 µL and consisted of 1X Green GoTaq® Flexi buffer (Promega, Madison, WI, USA), 2.0 mM $MgCl_2$ (Promega), 0.1 mM dNTP mix (Thermo Scientific Fisher, Finland), 0.1 µM of the respective forward and reverse PCR primers (Table 1), 1.5 U GoTaq® Flexi DNA polymerase (Promega) and 5 µL template DNA. Sterile distilled H_2O was used as a negative control.

All samples (digested nucleic acids and 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, at 50 volts for 180 min (digested nucleic acids) or 80 volts for 80 min (PCR products) with the use of 1X tris/acetic acid/ethylenediaminetetraacetic acid (EDTA) (TAE) buffer followed by visualisation on a Vilber Lourmat gel documentation system (Vilber Lourmat, Collegien, France). The digested samples were compared to undigested bacteriophage nucleic acid and the GeneRuler 1 kb Plus DNA ladder (Thermo Scientific). The genome size of the isolated bacteriophages was estimated by compiling the DNA fragment sizes using the standard ladder (Yu et al., 2013). The obtained PCR products were cleaned and concentrated using the Wizard® SV Gel and PCR Clean-up System (Promega) and were sent for DNA sequencing to the CAF at Stellenbosch University. Sequences were examined using FinchTV version 1.4.0 software and identification completed using the NCBI Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 Host Range Determination

The host range of the isolated bacteriophages was determined by spotting 10 µL of the concentrated bacteriophage stock solutions (10^6 to 10^7 PFU/mL) on TSA (with the exception of *Legionella* spp.; Appendix A) with 5 mL freshly prepared soft top-agar, which had been inoculated with 50 to 100 µL of the strain to be tested and incubating the plates at 30 °C for 18 h (Sillankorva et al., 2008; Stenholm et al., 2008). The host range for each bacteriophage was determined in triplicate for each bacteriophage-host combination and consisted of screening 20 *Pseudomonas* spp. (target bacterial strains; Appendix A, Table S1) and 57 non-target bacterial strains representative of 14 genera (Appendix A, Table S1). Reference, environmental and clinical isolates of both the target and non-target bacterial strains were included in the host range determination analysis.

2.6 One-step Growth Curve and Bacteriophage Sensitivity to Physical Parameters

Based on the results obtained during the host range determination, *K. pneumoniae* ATCC 10031 was used as the host for PFW25 during subsequent experimentation, while *P. aeruginosa* ATCC 27853 was used as the host for PAW33. One-step growth curves were performed to determine the latency period and burst size of the isolated bacteriophages, as previously described by Sillankorva et al. (2008), with minor modifications. Briefly, a multiplicity of infection (MOI) of 0.0003 was used and samples were collected every 10 min over a period of 2 h. Following overnight incubation at 30 °C, the PFU for each time point was recorded and results were reported as the average number of bacteriophages released per infected host cell. The bacteriophage burst size (number of bacteriophages released per infected host cell) was computed as the ratio between the final bacteriophage count and the initial bacteriophage count recorded during the latency period (Ciacci et al., 2018).

The influence of pH on the isolated bacteriophages' stability was evaluated by suspending bacteriophages at ~ 10^6 PFU/mL in 1 mL SM-buffer aliquots, with a pH range of 4.0 to 10.0 (intervals of 1 unit) (Jamal et al., 2015; Ciacci et al., 2018). The bacteriophage solutions were then incubated at room temperature for 1 h, whereafter the bacteriophage titre (PFU/mL) in the solutions were determined using double-layer plaque assays. The effect of temperature on bacteriophage stability was assessed by incubating 2 mL bacteriophage suspensions (~ 10^5 PFU/mL in SM-buffer) at 30 °C (control), 40 °C, 50 °C, 60 °C and 70 °C for 2 h. Samples were collected for bacteriophage titre (PFU/mL) determination at each temperature, using double-layer plaque assays, at 0, 10, 30, 60 and 120 min.

2.7 Bacterial Challenge Tests

To determine the activity of the isolated bacteriophages against the respective host bacterial species (PAW33 – *P. aeruginosa* ATCC 2785; PFW25 – *K. pneumoniae* ATCC 10031), bacterial challenge tests were performed as described by Turki et al. (2012) with minor modifications. Briefly, 50 mL TSB was inoculated with an overnight culture of the respective host bacterial species and corresponding bacteriophage to achieve MOI values of 1, 0.1 and 0.01. A non-infected culture of the respective host bacterial species was included as a negative control. All challenge tests were performed in triplicate. The flasks were incubated at 30 °C on a rotary shaker (New Brunswick Scientific, NY, USA) at 120 rpm for 24 h. Samples were collected every 2 h for the first 8 h and every 4 h thereafter to monitor host bacterial growth within the samples by measuring the optical density at 650 nm with a T60 Visible Spectrophotometer (PG-Instruments Limited, Leicester, UK).

To determine whether bacteriophage resistant mutants had emerged during the bacterial challenge tests, 2 mL aliquots were collected after the 24 h incubation and were centrifuged at 10 000 × *g* for 5 min (Spectrafuge^{TM} 24D Digital Microcentrifuge, Labnet International, Edison, USA). The bacterial pellet was re-suspended and was used to inoculate 5 mL freshly prepared soft top-agar, which was poured onto TSA plates. Ten microliters of concentrated bacteriophage stock solutions (10^6 to 10^7 PFU/mL) were spotted onto the surface of the plate and the plates were incubated at 30 °C for 18 h. Following incubation, the plates were examined for plaque formation to determine whether the respective host bacterial species were still susceptible to the isolated bacteriophages. The concentration of the respective host bacterial species within the samples, after the 24 h bacterial challenge tests, was determined by preparing serial dilutions (10^{-1} to 10^{-4}) and spread-plating 100 µL of the samples onto TSA, incubating at 30 °C for 18 h and then enumerating the colony forming units (CFU) per mL. Additionally, based on the results obtained for the bacterial challenge test performed on the *P. aeruginosa* ATCC 27853 strain, agglutination tests were performed to screen for lipopolysaccharide (LPS) defective mutants (Le et al., 2014).

2.8 Small-scale Bacteriophage Pre-treatment of Spiked Rainwater and SODIS-CPC Trials

Two small-scale bacteriophage pre-treatment (8 h or 24 h) and SODIS-CPC trials were performed using bacteriophage PAW33 and the environmental isolate *P. aeruginosa* S1 68. Roof-harvested rainwater was collected from a rainwater tank located on a local farm (GPS coordinates: 33°56'38.5"S 18°46'26.3"E), where after four 1 L rainwater aliquots were autoclaved three times. An overnight culture of the environmental *P. aeruginosa* S1 68 strain was spiked into each of the four 1 L rainwater aliquots to achieve a final concentration of 1.0 × 10^7 CFU/mL. Two of the 1 L rainwater aliquots were simultaneously spiked with 500 µL of a concentrated bacteriophage solution to achieve an MOI of 0.01, with one of the samples incubated for 8 h and the other aliquot incubated for 24 h at 30 °C at 120 rpm on a rotary shaker (New Brunswick Scientific). The remaining two 1 L rainwater aliquots served as the no bacteriophage treatment controls and were spiked with 500 µL sterile SM-buffer and were also incubated for 8 and 24 h at 30 °C at 120 rpm on a rotary shaker. Ten millilitre aliquots were collected at 0 and 8 h time intervals from the 8 h pre-treatment and corresponding no pre-treatment control samples and at 0, 1, 2, 4, 8, 16 and 24 h time intervals from the 24 h pre-treatment and corresponding no pre-treatment control samples.

Four SODIS-CPC reactors (described in Waso et al. 2019), with a treatment capacity of 500 mL, were filled with the 8 h or 24 h pre-treated samples or the 8 h or 24 h no pre-treatment control samples, respectively, and were exposed to natural sunlight for 4 h. The remaining rainwater from each sample (± 400 mL) was incubated at room temperature in the dark over the same time period to serve as the “dark controls” for the SODIS-CPC trial. Samples (10 mL) were collected from each SODIS-CPC system at 0, 1, 2, 3 and 4 h. The temperature of the collected samples was monitored with a hand-held mercury thermometer (ALLA France®, Chemillé, France).

2.8.1 Culture and Viability-qPCR Analysis of Samples

Culture-based analysis [10-fold serial dilutions and spread plating (*P. aeruginosa* S1 68) or double-layer agar overlays (PAW33) (described in section 2.2 and 2.7)] were used to enumerate the *P. aeruginosa* S1 68 CFU/mL and PAW33 PFU/mL.

However, as *Pseudomonas* spp. may enter a viable but non-culturable state during unfavourable conditions (such as those experienced during disinfection treatments), viability-qPCR assays were included to monitor the gene copy numbers of *P. aeruginosa* S1 68. Briefly, 2 mL of a collected sample was centrifuged at 10 000 × *g* for 10 min. The obtained pellet was re-suspended in 1 mL saline (0.85% NaCl), whereafter the sample was treated with 6 µM ethidium monoazide bromide (EMA; Biotium, Hayward, CA, USA) as outlined in Reyneke et al. (2017). Deoxyribonucleic acid extraction was completed using the Quick-DNA^{TM} Fecal/Soil Microbe Miniprep Kit (Zymo Research, Inqaba Biotech, South Africa) according to the manufacturers' instructions.

Similarly, in order to ensure that only DNA from intact (infective) PAW33 virions were quantified during qPCR analysis, 2 mL of a collected sample was treated with 5 U/mL DNase (Thermo Scientific) as outlined in Reyneke et al. (2017), whereafter the sample was centrifuged at 25 000 × g for 60 min. The obtained pellet was re-suspended in the pre-lyse mixture (Buffer T1, B3 and Proteinase K) of the NucleoSpin® Tissue kit (Macherey-Nagel) and the DNA extraction was completed as outlined by the manufacturer.

2.8.2 RNA Extraction

In order to determine whether bacteriophage pre-treatment may influence the ability of *P. aeruginosa* S1 68 to initiate stress response mechanisms during SODIS-CPC treatment, 5 mL of each collected sample was treated with a 1% phenol/19% ethanol (final v/v) mixture, incubated at 4 °C for 45 min and centrifuged at 3 320 × g for 15 min at 4 °C, whereafter the pellet was stored at -80 °C until further analysis (Lambert et al., 2010). Total RNA was extracted from the frozen pellets using TRI Reagent® (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. Following concentration and purity determination of the extracted RNA, using a NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA), 0.2 µg of the total RNA was DNase treated (Thermo Fisher) and transcribed into cDNA using the Improm-II^{TM} Reverse Transcription System (Promega) and oligo dT primer as described by the manufacturer. A no-template control and a no-reverse transcriptase control were included to confirm complete removal of contaminating genomic DNA from each sample and subsequently included in the respective qPCR assays.

2.8.3 Absolute and Relative qPCR Assays

All absolute and relative qPCR assays were conducted using a LightCycler® 96 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) instrument in combination with the FastStart Essential DNA Green Master Mix (Roche Diagnostics) (Reyneke et al., 2017). For the absolute quantification of PAW33 in the collected samples, the Podo-Hypo-F/R primer pair and cycling parameters outlined in Table 1 were used, while the PS1 5'-ATGAACAACGTTCTGAAATTC-3' and PS2 5'-CTGCGGCTGGCTTTTTCCAG-3' primer pair (Roosa et al., 2014) was used for the absolute quantification of *P. aeruginosa* S1 68 with the following cycling parameters: 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 relative quantification of the *rpoS*, *phzM*, *recA* and *lexA* genes of *P. aeruginosa* S1 68 the primer pairs and cycling parameters as outlined in Table 2 were utilised. The cycle quantification values (C_q) of the reference gene (*rpoS*) were utilised to normalise the calculated C_q values of the target genes (*phzM*, *recA* and *lexA*) amplified from the corresponding samples (ΔC_q), and the fold change ($2^{-\Delta\Delta Cq}$) was compared to the baseline sample (0 h samples) (Dobrowsky et al., 2017). *recA* and *lexA* were selected as target genes as they are involved in the global SOS response initiated by bacteria upon exposure to adverse environmental conditions, particularly those associated with DNA damage (inactivation mechanism of SODIS) (Krebs et al., 2018), while *phzM* is a virulence-associated gene involved in the production of pyocyanin [secondary metabolite (blue-green pigment) produced by *P. aeruginosa*] which has also been hypothesised to protect *P. aeruginosa* from oxidative stress (inactivation mechanism of SODIS) (Hendiani et al., 2019). High-resolution melt curve analysis was included for each qPCR assay in order to verify the specificity of the primer set by ramping the temperature form 65 to 97 °C at a rate of 0.2 °C/s with continuous fluorescent signal acquisition at 15 readings/°C. Standard curves for the quantification of *P. aeruginosa* S1 68 and PAW33 were generated using the methodology outlined in Reyneke et al. (2017).

3. Results

3.1 Bacteriophage Isolation and Characterisation

Bacteriophages were selected for further analysis based on the number and consistency of plaque formation, plaque clarity (clear plaques selected over turbid plaques) and plaque size. Consequently, bacteriophages PAW33 (isolated using *P. aeruginosa* ATCC 27853) and PFW25 (isolated using *P. fluorescens* ATCC 13525) were selected for further analysis.

Based on the STEM micrographs (Fig. 1), both bacteriophages were classified as members of the order *Caudovirales* (tailed bacteriophages). The morphological features of PAW33 (Fig. 1 a) indicated that this bacteriophage belongs to the *Podoviridae* family. Bacteriophages belonging to this family are characterised as having short, “stubby” non-contractile tails (Sepúlveda-Robles et al., 2012). It was observed that the capsid of PAW33 had a hexagonal outline indicating an icosahedral nature, with the capsid diameter recorded as ~63 nm and the tail measuring < 20 nm in length (tapering) (Fig. 1). PFW25 was identified as a *Myoviridae* bacteriophage based on its morphological features (Fig. 1 b). Bacteriophages belonging to this family are characterised as having contractile tails (Sepúlveda-Robles et al., 2012). As was observed for PAW33, the capsid of PFW25 had a hexagonal outline indicating an icosahedral nature; however, the capsid was slightly elongated. The capsid was ~72 nm wide and ~88 nm long, with the contractile tail measuring ~125 nm (Fig. 1).

3.2 Nucleic Acid Analysis and PCR-based Identification of PAW33 and PFW25

Results obtained following restriction endonuclease digestion with DNaseI (dsDNA), S1 nuclease (ssDNA) and RNase (RNA) confirmed that the bacteriophages were dsDNA viruses, which corresponds to their classification in the *Caudovirales* order. The DNA fragments obtained after digestion with *Eco*RI or *Cla*I indicated that PAW33 had an estimated molecular weight of 73kb, while the molecular weight of PFW25 could not be estimated following digestion.

Following the design and optimisation of numerous primer sets, the Podo-Hypo-F/R (targeting *Podoviridae*) and Myo-Hypo-F/R primer sets (targeting *Myoviridae*) (Table 1), were selected to confirm the preliminary classification of the isolated bacteriophages and enable the quantification of the bacteriophages during the pre-treatment and SODIS-CPC trials. Conventional PCR analysis of DNA obtained from PAW33 using the Podo-Hypo-F/R primer set resulted in the amplification of a 225 bp product. Sequencing analysis of the amplicon indicated that PAW33 shared sequence similarity with *Pseudomonas* bacteriophages LP14 (GenBank accession no: MH356729.1), YH30 (GenBank accession no: KP994390.1), phi176 (GenBank accession no: KM411960.1) and Pa2 (GenBank accession no: NC_027345.1), respectively (Appendix A, Table S2). These bacteriophages are listed as belonging to the *Podoviridae* family, which further corroborates the preliminary classification of PAW33. The Podo-Hypo-F/R primer set did not amplify DNA from PFW25.

Conventional PCR analysis of DNA obtained from PFW25 using the Myo-Hypo-F/R primer set resulted in the amplification of a 254 bp product. Sequencing analysis of the amplicon indicated that PFW25 shared sequence similarity with *Klebsiella* phage vB_Kpn_F48 (GenBank accession no: MG746602.1) (Appendix A, Table S2). Although the sequence similarity corresponded to a *Klebsiella* bacteriophage, vB_Kpn_F48 was classified as a *Myoviridae* bacteriophage by Ciacci et al. (2018). This result thus corroborates the preliminary classification of PFW25 as a *Myoviridae* bacteriophage. Additionally, the Myo-Hypo-F/R primer set did not amplify DNA from PAW33.

3.3 Host range determination for PAW33 and PFW25

The host range of the isolated bacteriophages was assessed against various target (*Pseudomonas* spp.) and non-target bacterial species (Appendix A, Table S1), with activity recorded as the presence of clear zones or plaques (++), turbid zones or plaques (+) or no growth inhibition (-). While no activity was observed for PAW33 against the 57 non-target bacterial species analysed, lytic activity was observed against 92% (*n* = 11) of the *P. aeruginosa* strains and 25% (*n* = 2) of the other *Pseudomonas* spp. tested (Fig. 2). In contrast, PFW25 displayed activity against the non-target bacterial species, *K. pneumoniae* ATCC 10031 and ATCC 333305 (results not shown), but none of the other non-target bacterial species analysed. PFW25 also displayed lytic activity against 75% (*n* = 3) of the *P. fluorescens* strains analysed and against 25% (*n* = 3) of the other *Pseudomonas* spp. tested (Fig. 2).

3.4 Bacteriophage Growth Characteristics and Sensitivity to Physical Parameters

Under the conditions studied (ambient temperature of 20 to 22 °C and aerobic conditions), PAW33 displayed a latency period of ~80 min, a rise period of ~50 min and a burst size of ~136 PFU per infected cell, when co-cultured with *P. aeruginosa* ATCC 27853 (results not shown). As indicated, *K. pneumoniae* ATCC 10031 was used to elucidate the life cycle of bacteriophage PFW25 during the one-step growth experiments and subsequent experiments. Under the conditions studied, PFW25 displayed a latency and rise period of ~30 min each, while the burst size of PFW25 was ~47 PFU per infected cell (results not shown).

Results from the temperature stability tests indicated that the infectivity of PAW33 remained stable between 30 °C and 50 °C; however, a significant decrease (~5 log) in infectivity was observed after 10 min at 70 °C and 60 min at 60 °C (Appendix A, Fig. S1 a). A similar temperature sensitivity profile was observed for PFW25 as its infectivity remained stable between 30 °C and 50 °C. PFW25 infectivity gradually decreased by ~1 log after 60 min at 60 °C and then remained relatively constant for the remaining 60 min. In comparison, a significant decrease (~ 4 log) in PFW25 infectivity was recorded after 30 min at 70°C (Appendix A, Fig. S1 b). Results for the pH stability tests indicated that PAW33 retained infectivity after incubation at pH values ranging from 6.0 to 9.0, while a 0.19 log, 0.41 log and 0.51 log decrease in infectivity was observed following incubation at pH 4, 5 and 10 (as compared to the mean PFU recorded for pH 6.0 to 9.0) (Appendix A, Fig. S1 c). In comparison, the infectivity of PFW25 remained relatively constant after incubation at pH values ranging from 5.0 to 8.0; however, at pH 4.0, 9.0 and 10.0, a 0.43 log, 0.25 log and 0.55 log decrease, in PFW25 infectivity was observed (as compared to the mean PFU recorded for pH 5.0 to 8.0), respectively (Appendix A, Fig. S1 d).

3.5 Efficiency of PAW33 and PFW25 to Control Target Host Growth

Results for the bacterial challenge tests indicated that the untreated *P. aeruginosa* control increased significantly ($p = 0.00004$) during h 2 to 6 as an increase in sample turbidity was observed, whereafter bacterial growth started to plateau, remaining relatively constant over the next 18 h (Appendix A, Fig. S2 a). In comparison, at all three MOI's analysed, PAW33 was effectively able to inhibit the proliferation of *P. aeruginosa* ATCC 27853 during the first 12 h of co-culture, whereafter steady increases in *P. aeruginosa* growth was observed (Appendix A,

Fig. S2 a). Although, PAW33 was not able to completely eliminate the *P. aeruginosa* population, culture-based analysis following the 24 h co-culture indicated that the *P. aeruginosa* CFU were 1.30 log (p = 0.0038), 1.08 log (p = 0.0048) and 1.06 log (p = 0.0046) lower in the samples treated at an MOI of 1, 0.1 and 0.01, respectively, in comparison to the untreated bacterial control (results not shown). In order to determine whether the increase in *P. aeruginosa* growth in the PAW33 treated samples was due to the emergence of resistance to the bacteriophage, bacterial cells were harvested and susceptibility to PAW33 was assessed using the spot-test method. Results indicated that the *P. aeruginosa* population were still susceptible to PAW33; however, bacteriophage resistant mutants had emerged. These colonies were characterised by the production of a red pigment, which resulted in a red mutant phenotype observed on the TSA plates (results not shown). Visualisation of these colonies using microscopy and comparison to the untreated control samples (not treated with PAW33 during co-culture) revealed that these bacteriophage resistant mutants clumped together following the agglutination test, indicating that their bacterial cell surface was LPS defective.

Results for the bacterial challenge tests indicated that limited growth was observed in the untreated *K. pneumoniae* ATCC 10031 control during the first 4 h, whereafter bacterial growth increased significantly (p = 0.00003) during the next 6 h and then started to plateau, remaining constant over the next 12 h (Appendix A, Fig. S2 b). In comparison, PFW25 was effectively able to inhibit the proliferation of *K. pneumoniae* during the first 16 h of co-culture for all three MOI ratio's tested; however significant increases in *K. pneumoniae* growth was observed between 16 and 24 h (Appendix A, Fig. S2 b). Culture-based analysis following the 24 h co-culture indicated that the *K. pneumoniae* CFU were 0.94 log (p = 0.0122), 1.05 log (p = 0.0129) and 0.85 log (p = 0.0187) lower in the samples treated at an MOI of 1, 0.1 and 0.01, respectively, as compared to the untreated bacterial control (results not shown). Spot test analysis of the culture following completion of the co-culture experiments indicated that the *K. pneumoniae* population was still susceptible to PFW25; however, bacteriophage resistant mutants had also emerged as turbid plaques (in comparison to clear plaques observed when the untreated *K. pneumoniae* controls were subjected to PFW25 during the spot test analysis) were visible.

3.6 Small-scale Bacteriophage Pre-treatment of Spiked Rainwater Followed by SODIS-CPC

3.6.1 Culture-based and Viability-qPCR Quantification of *P. aeruginosa* S1 68 and PAW33

The potential of bacteriophages to serve as a biocontrol pre-treatment (8 h and 24 h) of roof-harvested rainwater was investigated using PAW33 and the environmental *P. aeruginosa* S1 68 strain (Fig. 3). The performance characteristics of the viability-qPCR analysis of *P. aeruginosa* S1 68 and PAW33 are provided in Appendix A Table S3, while Appendix A Table S4 summarises the concentration and overall log reduction data for the 8 h and 24 h trials, followed by SODIS-CPC.

For the 8 h trial (Fig. 3 a), culture-based analysis of the non-pre-treated control sample indicated that the *P. aeruginosa* S1 68 CFU counts increased by 0.36 log, from 1.38×10^7 CFU/mL to 3.19×10^7 CFU/mL, over the 8 h incubation period. Subsequent exposure of the non-pre-treated sample to a 4 h SODIS-CPC treatment resulted in a total log reduction of 3.74 ($p = 0.0109$) in *P. aeruginosa* S1 68 CFU counts (2.50×10^3 CFU/mL recorded after SODIS-CPC), from the initial concentration of 1.38×10^7 CFU/mL (Appendix A, Table S4). Correspondingly, viability-qPCR analysis indicated that the *P. aeruginosa* S1 68 GC increased by 0.49 log over the 8 h incubation period, from 6.13×10^5 GC/mL to 1.88×10^6 GC/mL, with a reduction to 2.80×10^3 GC/mL recorded following SODIS-CPC treatment [2.33 total log reduction ($p = 0.0087$)] (Fig. 3 a; Appendix A, Table S4). Culture-based and viability-qPCR analysis of the corresponding dark control sample (collected after the 4 h SODIS-CPC treatment), indicated that the concentration of *P. aeruginosa* S1 68 remained relatively constant with 2.13×10^7 CFU/mL and 1.81×10^6 GC/mL recorded, respectively (results not shown).

Culture-based analysis of the corresponding 8 h bacteriophage pre-treated sample indicated that the *P. aeruginosa* S1 68 CFU/mL increased by 0.26 log from 1.24×10^7 CFU/mL to 2.28×10^7 CFU/mL, where after the SODIS-CPC treatment reduced the cell counts to 2.58×10^3 CFU/mL [3.68 total log reduction ($p = 0.0299$) from the initial CFU of 1.24×10^7] (Fig. 3 a; Appendix A, Table S4). Similarly, viability-qPCR analysis indicated that the *P. aeruginosa* S1 68 gene copies (GC) only increased by 0.19 log during the 8 h pre-treatment, from 6.98×10^5 GC/mL to 1.09×10^6 GC/mL, whereafter the gene copies were reduced to 3.15×10^3

GC/mL during the SODIS-CPC treatment [2.34 total log reduction ($p = 0.0033$)] (Appendix A, Table S4). Monitoring of PAW33 in the 8 h pre-treated sample indicated that the PFU/mL decreased by 0.28 log from 6.00×10^4 PFU/mL to 3.16×10^4 PFU/mL, while 1.20×10^2 PFU/mL were detected following the SODIS-CPC treatment [2.70 total log reduction ($p = 0.0023$)] (Appendix A, Fig. S3 a). In contrast, the PAW33 GC/mL increased by 0.48 log (1.80×10^4 GC/mL to 5.37×10^4 GC/mL) during the 8 h pre-treatment, whereafter the gene copies remained relatively constant, as 1.42×10^4 GC/mL were recorded following the SODIS-CPC treatment [0.12 total log reduction ($p = 0.1909$)] (Appendix A, Table S4 and Fig. S3 a). Culture-based and viability-qPCR analysis of the corresponding dark control sample (collected after the 4 h SODIS-CPC treatment), indicated that the concentration of *P. aeruginosa* S1 68 remained relatively constant with 8.63×10^6 CFU/mL and 1.23×10^6 GC/mL recorded, respectively, while PAW33 also remained constant as 3.16×10^4 PFU/mL and 1.92×10^4 GC/mL were recorded (results not shown).

For the 24 h trial, culture-based analysis of the non-pre-treated control sample, indicated that *P. aeruginosa* S1 68 increased by 0.67 log, from 2.08×10^7 CFU/mL to 9.42×10^7 CFU/mL over the 24 h incubation period (Fig. 3 b). The *P. aeruginosa* S1 68 cell counts were subsequently reduced to 2.5×10^3 CFU/mL (from an initial CFU of 2.08×10^7) following the SODIS-CPC treatment [3.91 total log reduction ($p = 0.0101$)] (Appendix A, Table S4). Similarly, viability-qPCR analysis of the non-pre-treated control sample indicated that the *P. aeruginosa* S1 68 GC/mL marginally increased from 2.71×10^6 GC/mL to 3.28×10^6 GC/mL after 24 h (0.08 log increase) (Fig. 3 b). An overall total reduction of 2.26 log ($p = 0.0239$) in GC was then observed following the SODIS-CPC treatment (GC reduced to 1.47×10^4 GC/mL) (Appendix A, Table S4). Culture-based and viability-qPCR analysis of the corresponding dark control sample (collected after the 4 h SODIS-CPC treatment), indicated that the concentration of *P. aeruginosa* S1 68 remained relatively constant with 9.63×10^7 CFU/mL and 7.98×10^6 PFU/mL recorded, respectively (results not shown).

Culture-based analysis of the corresponding bacteriophage pre-treated sample from the 24 h trial indicated that PAW33 was able to restrict the proliferation of *P. aeruginosa* S1 68 in the pre-treated sample, as the *P. aeruginosa* S1 68 CFU counts only increased by 0.14 log, from 2.03×10^7

CFU/mL to 2.79×10^7 CFU/mL (Fig. 3 b). Subsequent SODIS-CPC treatment of the pre-treated sample reduced the *P. aeruginosa* S1 68 CFU counts to 5.0×10^2 CFU/mL [4.61 log reduction overall ($p = 0.0079$)], from the initial count of 2.03×10^7 (Appendix A, Table S4). Similarly, viability-qPCR analysis of the pre-treated sample indicated a 0.30 log increase (5.31×10^5 GC/mL to 1.06×10^6 GC/mL) in *P. aeruginosa* S1 68 GC/mL during the 24 h pre-treatment, whereafter the GC were reduced to 1.07×10^4 GC/mL due to the SODIS-CPC treatment [2.32 log reduction overall ($p = 0.0128$)] (Fig. 3 b; Appendix A, Table S4). Enumeration of the PAW33 plaque counts in the pre-treated sample indicated that the PFU/mL increased from 8.0×10^4 PFU/mL to 4.0×10^5 PFU/mL (0.70 log increase) during the 24 h pre-treatment, whereafter a decrease to 1.3×10^2 PFU/mL was recorded following the SODIS-CPC treatment [2.79 log reduction overall ($p = 0.0115$)] (Appendix A, Fig. S3 b). In comparison, the PAW33 GC/mL remained relatively constant in the pre-treated sample during the 24 h trial, as 2.50×10^4 GC/mL were detected at both 0 and 24 h, while 4.8×10^3 GC/mL were detected following SODIS-CPC treatment [0.72 log reduction overall ($p = 0.0270$)] (Appendix A, Table S4). Culture and viability-qPCR analysis of the corresponding dark control sample (collected after the 4 h SODIS-CPC treatment), indicated that the concentration of *P. aeruginosa* S1 68 remained relatively constant with 4.62×10^7 CFU/mL and 6.45×10^6 GC/mL recorded, respectively, while PAW33 also remained relatively constant as 1.93×10^5 PFU/mL and 2.10×10^4 GC/mL were recorded (results not shown).

3.6.2 Expression of SOS Response- and Virulence-associated Genes of *P. aeruginosa* S1 68

The performance characteristics of the relative qPCR assays are provided in Appendix A, Table S3. For the 8 h trial, a similar increase in *phzM* gene expression was observed for both the pre-treated and non-pre-treated control samples during the 8 h incubation period (Fig 4). While a decrease in *phzM* gene expression was observed for both samples following the SODIS-CPC treatment, the expression level was still up-regulated. In comparison, while an upregulation in *recA* gene expression was observed in the non-pre-treated control and pre-treated samples during the 8 h incubation period as well as in the non-pre-treated control sample following the SODIS-CPC treatment, downregulation of *recA* was observed for the pre-treated sample following the SODIS-CPC treatment (Fig. 4). *lexA* was then up-regulated in both the non-pre-treated control and pre-treated samples during the 8 h incubation period, whereafter downregulation was observed for both samples during the SODIS-CPC treatment (Fig. 4). Although changes in gene expression were observed for the collected samples, the fold change in expression was not significant (< 5-fold change) for any of the analysed target genes.

For the 24 h trial, *phzM*, *recA* and *lexA* were up-regulated in the non-pre-treated control sample during the 24 h incubation period, whereafter downregulation of the *phzM* and *lexA* genes were observed following SODIS treatment (Fig. 4). In comparison, although *recA* expression in the non-pre-treated control decreased during the SODIS-CPC treatment, the overall level was still up-regulated. Results then indicated that for the PAW33 pre-treated samples, *phzM*, *recA* and *lexA* were down-regulated during the 24 h trial, with continued downregulation of the genes observed following the SODIS treatment (Fig. 4). However, similar to the results obtained for the 8 h incubation trial, the observed fold changes in gene expression were not significant (< 5-fold change).

4. Discussion

Bacteria are able to undergo an adaptive response and build-up resistance to stressful environments, such as those experienced during conventional water treatment methods. As bacteriophages may allow for the selective removal of problematic pathogens within water samples (Goldman et al., 2009; Turki et al., 2012; Zhang et al., 2013), bacteriophage biocontrol was investigated and combined with SODIS-CPC in order to reduce the concentration and limit the proliferation of *P. aeruginosa* in rainwater.

Lytic bacteriophages displaying activity against *Pseudomonas* spp. were subsequently isolated from numerous environmental sources, with PAW33 (isolated using *P. aeruginosa*) and PFW25 (isolated using *P. fluorescens*) selected for further characterisation. Electron microscopy and nucleic acid analysis indicated that both PAW33 and PFW25 belong to the order *Caudovirales* and more specifically the *Podoviridae* and *Myoviridae* families, respectively. Subsequently, the pH and temperature sensitivity of PAW33 and PFW25 was assessed as various chemical and physical parameters (such as those associated with rainwater harvesting systems) may influence the viability/infectivity of bacteriophages by damaging their structural elements (e.g. head and tail structures) (Jończyk et al., 2011). Results however, indicated that both bacteriophages were stable and retained their infectivity upon exposure to the physico-chemical parameters commonly associated with untreated harvested rainwater (pH 6.2 to 8.4; 19 to 26 °C) and temperatures experienced within large-scale SODIS systems (39 to 59 °C) (Reyneke et al., 2018; Strauss et al., 2018).

Caudovirales bacteriophages are associated with more than 140 prokaryotic genera with varying degrees of host specificity reported (9th International Committee on Taxonomy of Viruses Report, 2011; Kęsik-Szeloch et al., 2013). The host range determination then indicated that PAW33 was able to infect reference, environmental and clinical isolates of *P. aeruginosa*, with notable activity displayed against the multidrug-resistant *P. aeruginosa* T1 clinical isolate (Havenga et al., 2019) and numerous environmental strains previously isolated from a solar pasteurization system connected to a rainwater harvesting tank (Appendix A, Table S1). Additionally, as PAW33 was able to infect two environmental *P. fluorescens* strains, it was classified as having a broad host range against *P. aeruginosa* strains, with limited activity against other *Pseudomonas* spp. and no activity against the non-target bacteria. In comparison, PFW25 was able to infect three *P. fluorescens* strains, two environmental and one clinical isolate of *P. aeruginosa* (Fig. 2) and two *K. pneumoniae* ATCC strains (results not shown). The activity displayed against *K. pneumoniae* and the efficiency of plating when PFW25 was cultured with *K. pneumoniae* ATCC 10031, coupled with the sequence similarity (hypothetical protein targeted by the Myo-Hypo-F/R primer set) displayed to bacteriophage vB_Kpn_F48, indicated that PFW25 may be better suited to target *K. pneumoniae* strains. Similarly, Wu et al. (2007) reported on the isolation of a *Myoviridae* bacteriophage (Kpp95) using *K. pneumoniae*, which was subsequently classified as having a broad host range, as the bacteriophage displayed lytic activity against *K. pneumoniae*, *K. oxytoca*, *Enterobacter agglomerans* and *Serratia marcescens*.

For the bacterial challenge tests, while the bacteriophages PAW33 and PFW25 were able to inhibit the growth of their respective target hosts (*P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 10031), bacteriophage resistant *P. aeruginosa* and *K. pneumoniae* mutants had emerged.

Specifically, for *P. aeruginosa* ATCC 27853, the bacteriophage resistant mutants were characterised by the production of a red pigment and were classified as being LPS defective (based on an agglutination test). A similar observation was made by Le et al. (2014), where it was demonstrated that a chromosomal DNA deletion (gene fragment containing the *hmgA* and *galU* genes) conferred bacteriophage resistance to *P. aeruginosa*, with the deletion of *hmgA* resulting in the accumulation of a red compound (homogentisic acid) and the deletion of *galU* resulting in the loss of the O-antigen (which is required for bacteriophage adsorption). Moreover, as LPS is an important virulence factor within Gram-negative bacterial pathogens, the authors reported that, in a mouse infection model, the bacteriophage resistant *P. aeruginosa* were significantly attenuated (Le et al., 2014). Thus, while the emergence of bacteriophage resistant bacteria is a major concern when employing bacteriophage biocontrol, these bacteria (such as the *P. aeruginosa* obtained in the current study following exposure to PAW33) may be less virulent (Le et al., 2014) and thereby pose a lower health risk to the end-user. Bacteriophages do however, have the ability to develop counter strategies to by-pass bacterial resistance mechanisms and thereby ensure the survival of the bacteriophage population and in so doing continue to restrict the proliferation of the target bacterial population (Samson et al., 2013). These strategies include, amongst others, the modification of the bacteriophage receptor binding proteins, which recognise new receptors/adsorption sites on bacteria, the production of enzymes to degrade bacterial capsules or exopolysaccharides, and the modification of the bacteriophage genome to circumvent restriction-modification systems (restriction enzyme digestion) in bacteria (Samson et al., 2013).

The efficiency of bacteriophage biocontrol as a rainwater pre-treatment strategy was ultimately assessed using PAW33 as the biocontrol agent and *P. aeruginosa* S1 68 (environmental isolate obtained from rainwater pasteurized at 70 °C) as the target organism. Based on observations from the bacterial challenge tests (Appendix A, Fig. S2 a) and a supplementary bacterial challenge test conducted in sterile rainwater on *P. aeruginosa* ATCC 27853 and *P. aeruginosa* S1 68 (results not shown), two pre-treatment times, namely 8 h and 24 h, were assessed. It was hypothesised that the bacteriophage pre-treatment would firstly restrict the proliferation of the target host pathogen during the pre-treatment period and secondly sensitise the overall bacterial population to the primary treatment strategy (i.e. SODIS-CPC).

Culture-based and viability-qPCR analysis indicated that PAW33 was able to restrict the proliferation of the *P. aeruginosa* S1 68 in the rainwater during both the 8 h and 24 h pre-treatment trials. However, while similar total CFU and GC log reductions were obtained for the pre-treated [3.68 log (CFU) and 2.34 log (GC)] and non-pre-treated control samples [3.74 log (CFU) and 2.33 log (GC)] for the 8 h trial (followed by SODIS-CPC); culture-based analysis indicated that a higher overall log reduction was recorded for the 24 h bacteriophage pre-treated sample followed by SODIS-CPC (4.61 log) in comparison to the non-pre-treated sample (3.91 log). Additionally, culture-based analysis indicated that after the 24 h bacteriophage pre-treatment trial, faster inactivation of *P. aeruginosa* S1 68 occurred during the first hour (1.73 log reduction) of the SODIS-CPC treatment. A similar observation was recently reported by Al-Jassim et al. (2018) where the ability of bacteriophages to sensitise a pathogenic New Delhi metallo β-lactamase-positive *E. coli* to SODIS was investigated. Results from the study indicated that exposure to bacteriophages increased the susceptibility of *E. coli* to SODIS, with faster inactivation of the *E. coli* observed (treatment time reduced from 4 h to 2 h). Additionally, using gene expression analysis, the authors reported that the exposure of *E. coli* to the bacteriophage resulted in a downregulation of cell wall functions, the ability to scavenge reactive oxygen species and DNA repair mechanisms, effectively rendering the *E. coli* more susceptible to SODIS treatment. It is however important to note that the Al-Jassim et al. (2018) study utilised a combination of bacteriophages at a high treatment concentration (MOI = 1), the bacteriophage and SODIS treatment occurred simultaneously and an artificial light source was used to simulate SODIS. In contrast, in the current study a lower treatment concentration (MOI = 0.01) of a single bacteriophage was used as a pre-treatment strategy to SODIS-CPC under natural sunlight. Thus, while the bacteriophage pre-treatment for 24 h, followed by SODIS-CPC, resulted in the highest total log reduction (4.61 log) of *P. aeruginosa* S1 68 CFU/mL, the target host could not be completely eradicated using this combination treatment strategy, as 5.0×10^2 CFU/mL was still recorded following SODIS-CPC treatment. Additionally, while viability-qPCR analysis indicated that comparable total log reductions [2.32 log (pre-treated) and 2.26 log (non-pre-treated)] in *P. aeruginosa* S1 68 concentrations were obtained for the 24 h trial samples, gene copies were still detected after SODIS-CPC, indicating that viable but non-culturable cells may be present within the samples. The survival of the *P. aeruginosa* S1 68 following the combination treatment is however, not surprising as *Pseudomonas* spp. may initiate a range of stress responses during both the planktonic or biofilm life cycles, including the production of heat shock proteins and the initiation of DNA repair mechanisms, amongst others, and thereby switch to a more tolerant phenotype to facilitate its survival under adverse conditions (Fux et al., 2005; Breidenstein et al., 2011). However, as highlighted by Al-Jassim et al. (2018), the ability of bacteria to initiate these stress response mechanisms may be severely impaired following/during exposure to bacteriophages.

Gene expression analysis was subsequently included to monitor the SOS response-associated *recA* and *lexA* genes, while *phzM* (gene associated with pyocyanin production) was monitored as the bacterial challenge tests indicated that decreased pyocyanin was produced by bacteriophage resistant *P. aeruginosa* ATCC 27853. Results for the 8 h trial indicated that while *recA* and *lexA* expression levels were decreased in the PAW33 treated sample during the 8 h incubation period (as compared to the non-pre-treated sample), the overall expression level was still up-regulated, with downregulation only observed following SODIS-CPC treatment. In comparison, *phzM* gene expression was up-regulated in the no treatment control and PAW33 treated samples during the 8 h incubation trial and the subsequent SODIS-CPC treatment. Results for the 24 h trial then indicated that *phzM*, *recA* and *lexA* were down-regulated in the PAW33 pre-treated sample during the 24 h incubation period, with continued downregulation observed following the SODIS-CPC treatment. *recA* and *lexA* are known to be up-regulated in bacteria in response to adverse conditions as part of the SOS response mechanism and are primarily involved in DNA repair mechanisms (Krebs et al., 2018). The downregulation of *recA* and *lexA* in the PAW33 pre-treated *P. aeruginosa* S1 68, particularly during the 4 h SODIS-CPC treatment, indicates that the bacteriophage pre-treatment for both 8 and 24 h may have influenced the ability of the target host bacterium to initiate stress response mechanisms during the primary treatment strategy (i.e. SODIS-CPC). However, based on the results obtained, a prolonged bacteriophage pre-treatment period may initiate the change in gene expression as *recA* and *lexA* were down-regulated during the 24 h incubation period. Additionally, while assessing the influence of sub-lethal photodynamic inactivation [sPDI; photo-oxidative stress caused by the generation of reactive oxygen species (ROS) after a photosensitiser molecule was excited by visible light], Hendiani et al. (2019) reported that pyocyanin production (*phzM* expression) in *P. aeruginosa* ATCC 27853 as well as strains P2 and P3, increased during sPDI, with the authors hypothesising that the over-expression of pyocyanin played a possible protective role against sPDI-induced oxidative stress. As *phzM* was down-regulated in both the 24 h bacteriophage pre-treatment and subsequent SODIS-CPC samples, in comparison to the observed up-regulation in the 8 h trail samples, it is hypothesised that the decreased *phzM* expression may be due to the presence of bacteriophage resistant *P. aeruginosa* S1 68 cells within the sample (as was observed for the bacterial challenge tests). The bacteriophage pre-treatment for 24 h may thus have influenced the ability of the bacteriophage resistant *P. aeruginosa* S1 68 cells to initiate pyocyanin production as a stress response mechanism, rendering the bacterial cells more susceptible to primary disinfection strategies (such as SODIS-CPC). Additionally, as pyocyanin is considered a virulence factor of *P. aeruginosa* (Hendiani et al., 2019), its downregulation in the 24 h PAW33 pre-treated samples indicates that bacteriophage pre-treatment may decrease pathogen virulence. The overall results thus indicate that a longer bacteriophage pre-treatment may be required for the bacteriophages to adequately influence target host stress response mechanisms.

5. Conclusions

Results from the study indicate that PAW33 has the potential to be used in biocontrol strategies for the selective removal of *P. aeruginosa* from roof-harvested rainwater as this *Podoviridae* bacteriophage was able to effectively restrict the proliferation of *P. aeruginosa* S1 68 for up to 24 h. Additionally, an increase in the susceptibility of *P. aeruginosa* S1 68 to the SODIC-CPC disinfection treatment was observed after the 24 h bacteriophage pre-treatment trial, as a total log reduction of 4.61 was recorded. However, while gene copies and CFU were still detected after SODIS-CPC for both the 8 h and 24 h trials, it important to note that the efficiency of the bacteriophage pre-treatment may be improved by using a combination of bacteriophages (Gu et al., 2016), while the SODIS-CPC treatment efficiency may be further improved by increasing the SODIS treatment time (6 to 8 h SODIS exposures recommended in literature) (Strauss et al., 2016).

Additionally, although the fold changes observed during gene expression analysis were not significant, results from the 8 and 24 h bacteriophage pre-treatment trial indicated that the *P. aeruginosa* S1 68 exhibited a reduced ability to initiate conventional stress response mechanisms (*recA and lexA*), while the expression of pyocyanin (*phzM*; virulence factor) was also down-regulated during the 24 h bacteriophage pre-treatment trial. The ability of bacteriophage biocontrol to influence pathogen stress response mechanisms and virulence during treatment should thus be further investigated. Moreover, as biofilm formation is a key survival strategy employed by *P. aeruginosa*, the biofilm disruption and anti-adhesive abilities of PAW33 should be investigated in future studies.

Author Contributions Conceived and designed the experiments: BR and WK. Performed the experiments: BR. Designed the primers: SK. Designed the small-scale SODIS-CPC systems: PFI. Analysed the data: BR and WK. Contributed reagents/materials/analysis tools: WK and SK. Compiled the manuscript: BR and WK. Edited the manuscript: SK and PFI.

Compliance with Ethical Standards

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Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of Interest The authors declare that they have no conflict of interest.

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