

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

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11 Short title: Bacteriophage biocontrol of *Pseudomonas aeruginosa*

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

23

Abstract

24
25 Bacteriophages targeting *Pseudomonas* spp. were isolated and characterised for the biocontrol
26 pre-treatment of harvested rainwater. Bacteriophages PAW33 (isolated using *P. aeruginosa*) and
27 PFW25 (isolated using *P. fluorescens*) were characterised as members of the *Podoviridae* and
28 *Myoviridae* families, respectively. Bacteriophage PAW33 displayed a broad host range against
29 *P. aeruginosa* strains, while PFW25 was more effective in infecting *K. pneumoniae* than
30 *P. fluorescens* (original target organism). PAW33 was subsequently applied in small-scale
31 bacteriophage pre-treatment trials (8 h and 24 h), to evaluate its efficacy in restricting the
32 proliferation of an environmental *P. aeruginosa* S1 68 strain. Following the completion of the
33 bacteriophage pre-treatment trial, the respective samples (bacteriophage pre-treated samples and
34 non-pre-treated control samples) were subjected to treatment in small-scale (vol?) solar
35 disinfection compound parabolic collector (SODIS-CPC) systems for 4 h under natural sunlight. For
36 the 8 h trial followed by SODIS-CPC, similar total log reductions in colony forming units (CFU) and
37 gene copies (GC)/mL were obtained for the bacteriophage pre-treated [3.68 log (CFU) and 2.34
38 log (GC)] and non-pre-treated [3.74 log (CFU) and 2.33 log(GC)] samples. In contrast, culture-
39 based analysis of the 24 h trial samples (followed by SODIS-CPC) indicated that a higher overall
40 log reduction was recorded for the pre-treated sample (4.61 log) in comparison to the non-pre-
41 treated sample (3.91 log), while comparable log reductions were obtained using viability-qPCR
42 (2.32 log and 2.26 log, respectively). Gene expression analysis then indicated that PAW33 pre-
43 treatment for 24 h influenced the ability of *P. aeruginosa* S1 68 to initiate stress response
44 mechanisms during the 4 h SODIS-CPC treatment (downregulation of the *recA* and *lexA* genes)
45 and resulted in the downregulation of the *phzM* gene (virulence factor responsible for pyocyanin
46 production). Bacteriophage PFW33 thus displays promise as a biocontrol pre-treatment strategy of
47 roof-harvested rainwater as it restricts the proliferation of *P. aeruginosa* and may increase the
48 treatment efficiency of primary disinfection methods.

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

50 **1. Introduction**

51 *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen and is primarily associated
52 with nosocomial infections where it may cause pneumonia, urinary tract and skin infections
53 (Driscoll et al., 2007). However, the ubiquitous distribution of *Pseudomonas* spp. in the
54 environment significantly increases the health risk associated with this organism and John et al.
55 (2017) recently reported on an outbreak of community-acquired *P. aeruginosa* pneumonia in Cape
56 Town, South Africa. The authors noted that while no cases of community-acquired *P. aeruginosa*
57 were reported at a local government hospital over a 10-year period (2007 to 2016); over a period of
58 three months (March to May 2017), 9 cases were reported. This outbreak coincided with a severe
59 drought in Cape Town (2017 to 2018), where stringent water restrictions were implemented and
60 residents were increasingly using alternative water sources (e.g. rainwater and grey water). It was
61 hypothesised that the use of these alternative water sources may have led to the exposure of the
62 community members to *P. aeruginosa*.

63 Although various treatment methods have been implemented to reduce the levels or remove
64 pathogens and opportunistic pathogens from environmental water reservoirs, many
65 microorganisms employ survival strategies and are capable of persisting. Certain strains of *P.*
66 *aeruginosa* have been shown to survive conventional disinfection strategies including sub-optimal
67 chlorination (Shrivastava et al., 2004), ultra-violet (UV) radiation (Strauss et al., 2016; 2018) and
68 heat treatment (Strauss et al., 2016; Clements et al., 2019). Using viability-qPCR, Strauss et al.
69 (2016) reported on the detection of *Pseudomonas* spp. in rainwater samples treated at solar
70 pasteurization temperatures > 90 °C and in water samples treated by solar disinfection (SODIS;
71 UV radiation and solar mild-heat) for 8 hours. Similarly, using culture-based methods, Clements et
72 al. (2019) isolated *Pseudomonas* spp. from rainwater samples pasteurized at temperatures >
73 70 °C, with certain isolates identified as *P. aeruginosa* using species-specific primers.
74 *Pseudomonas aeruginosa* has also readily been detected in grey water (Winward et al., 2008;
75 Maimon et al., 2014), with Gross et al. (2007) and Gilboa and Friedler (2008) reporting on the
76 detection of viable *P. aeruginosa* in grey water samples treated in a vertical flow bioreactor and a
77 rotating biological contractor followed by UV disinfection, respectively. The survival of

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

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

106 carbon and anthracite biofilters. Results indicated that the bacteriophage treatment was able to
107 reduce *P. aeruginosa* concentration by 55 to 75% in the two biofilter systems, with minimal impact
108 on the beneficial microorganisms, and thereby contribute to an improvement in effluent water
109 quality.

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

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

133 2. Materials and Methods

134 2.1 Bacterial Strains and Growth Conditions

135 *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, *P. fluorescens* ATCC 13525 and
136 *P. protegens* ATCC 17386 reference strains were obtained from Microbiologics® (St Cloud,
137 Minnesota, USA) and were used for the isolation, propagation and characterisation of the
138 bacteriophages. The bacterial strains (target and non-target bacterial species) utilised for the host
139 range determination of the isolated bacteriophages are indicated in Appendix A, Table S1. All
140 strains were cultured at 30 °C in tryptic soy broth (TSB; Biolab, Merck, Wadeville, South Africa) or
141 on tryptic soy agar (TSA; Biolab, Merck), with the exception of *Legionella* spp. (see Appendix A for
142 *Legionella* spp. growth conditions). For the double-layer plaque assays (double-layer overlays), the
143 TSA medium contained 1.2% (w/v) Agar Bacteriological (Biolab, Merck) in the bottom layer and
144 0.6% agar (w/v) in the soft top layer (Sillankorva et al., 2008).

145 2.2 Isolation, Purification and Propagation of Bacteriophages

146 Bacteriophages were isolated by screening various environmental water sources including influent
147 wastewater collected from the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: -
148 33.943505, 18.824584), river water from the Plankenburg River (GPS co-ordinates: -33.927761,
149 18.850544) and roof-harvested rainwater from a rainwater harvesting tank connected to the JC
150 Smuts building at Stellenbosch University (GPS co-ordinates: -33.930858, 18.865611). Selection
151 for *Pseudomonas* spp. bacteriophages was performed as previously described by Sillankorva et al.
152 (2008), with minor modifications. Briefly, following the centrifugation step (10 000 × *g*; 10 min;
153 4 °C), the supernatant from each sample was filtered through a sterile GN-6 Metrical® S-Pack
154 Membrane Disc Filter (Pall Life Sciences, Michigan, USA) with a pore size of 0.45 µm, to remove
155 residual host bacteria from the sample (Vinod et al., 2006). The filtered supernatant was tested for
156 the presence of bacteriophages (Sillankorva et al., 2008), whereafter five repeated rounds of
157 plaque purification and re-infection were performed (Stenholm et al., 2008).

158 The bacteriophages were selected for further studies based on their initial lysis profiles during
159 bacteriophage purification (number and consistency of plaque formation, plaque clarity and plaque

160 size) (Sillankorva et al., 2008). Code identifiers were assigned to the isolated bacteriophages
161 based on the bacteria from which they were isolated (i.e. PA – *P. aeruginosa*; PF – *P. fluorescens*
162 and PP – *P. protegens*), the source of the bacteriophage (e.g. W – wastewater; R – river water)
163 and the plaque number. For example, PAW1 indicates that the bacteriophage was isolated using
164 *P. aeruginosa* (PA), from wastewater (W) and was the first plaque isolated (1).

165 The purified bacteriophages were used to prepare concentrated bacteriophage solutions for use in
166 subsequent experiments using the small-scale liquid culture method as described by Sambrook
167 and Russell (2001), with minor modifications. Briefly, following the onset of bacterial cell lysis, the
168 samples were centrifuged (10 000 × *g*; 10 min; 4 °C) and filtered through a 0.2 µm Acrodisc® PF
169 syringe filter (Pall Life Sciences). The filtered supernatants were centrifuged at 25 000 × *g* for
170 60 min using an Avanti J-E Centrifuge with a JA 20 fixed angle rotor (Beckman Coulter, California,
171 USA). Following centrifugation, the supernatant was removed and the obtained pellet was re-
172 suspended in 1 mL SM-buffer [5.8 g/L sodium chloride (NaCl; Saarchem, Durban, South Africa),
173 2 g/L magnesium sulphate heptahydrate (MgSO₄·7H₂O; Saarchem), 50 mL 1 M Tris, pH 7.5]. The
174 plaque forming units (PFU) per mL concentrated sample were determined by serial dilution (10⁻¹ to
175 10⁻⁵) in SM-buffer and double-layer plaque assays. The concentrated bacteriophage samples in
176 SM-buffer were stored at 4 °C until further use.

177 **2.3 Characterisation of the Isolated Bacteriophages**

178 In order to increase bacteriophage retention on the electron microscopy grids and thereby increase
179 bacteriophage visualisation during electron microscopy analysis, the hydrophilicity and “stickiness”
180 of the 200 mesh carbon-coated Formvar grids (Agar Scientific, Essex, United Kingdom) were
181 increased by using an alcian blue (Electron Microscopy Sciences, Pennsylvania, USA) pre-
182 treatment (1% alcian blue in 1% acetic acid in water) as described by Laue and Bannert (2010).
183 Concentrated bacteriophage samples with a titre of > 10⁹ PFU/mL were then mixed with
184 glutaraldehyde (2.5% v/v; Agar Scientific) for 5 min and 25 µL of the glutaraldehyde treated
185 concentrated bacteriophage sample was loaded onto the alcian blue pre-treated grids and were
186 allowed to settle for 10 min. Hereafter, the sample was stained with 1% uranyl acetate for 2 min.
187 Excess stain was removed using filter paper and the grids were allowed to air dry. The grids were

188 visualised with a Zeiss MERLIN Field Emission Scanning Electron Microscope (FE-SEM; Zeiss,
189 Germany) at the Electron Microbeam Unit of the Central Analytical Facility (CAF) at Stellenbosch
190 University. A Zeiss five-diode Scanning Transmission Electron Detector (Zeiss aSTEMA Detector)
191 and Zeiss Smart SEM software was used to generate STEM images. Beam conditions during
192 analysis on the Zeiss MERLIN FE-SEM were 20 kV accelerating voltage, 150 pA probe current,
193 with a working distance of approximately 3.9 to 4.0 mm. Images were acquired in bright fields
194 mode with the S1 diode activated.

195 **2.4 Analysis of Bacteriophage Nucleic Acids - Restriction Enzyme Digestion and Molecular** 196 **Analysis**

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

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

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

215 ClustalX version 2.0.10 (Larkin et al., 2007) and visualised using GeneDoc version 2.7.00
216 (Nicholas and Nicholas, 1997). Primers targeting the specific gene sequences were designed
217 based on the sequence alignments and are presented in Table 1 along with the respective PCR
218 cycling parameters. Each PCR assay was performed in a final volume of 25 µL and consisted of
219 1X Green GoTaq® Flexi buffer (Promega, Madison, WI, USA), 2.0 mM MgCl₂ (Promega), 0.1 mM
220 dNTP mix (Thermo Scientific Fisher, Finland), 0.1 µM of the respective forward and reverse PCR
221 primers (Table 1), 1.5 U GoTaq® Flexi DNA polymerase (Promega) and 5 µL template DNA.
222 Sterile distilled H₂O was used as a negative control.

223 All samples (digested nucleic acids and PCR products) were analysed by agarose gel
224 electrophoresis in 0.8% agarose (SeaKem® LE Agarose; Lonza, Rockland, ME, USA) containing
225 0.5 µg/mL ethidium bromide, at 50 volts for 180 min (digested nucleic acids) or 80 volts for 80 min
226 (PCR products) with the use of 1X tris/acetic acid/ethylenediaminetetraacetic acid (EDTA) (TAE)
227 buffer followed by visualisation on a Vilber Lourmat gel documentation system (Vilber Lourmat,
228 Collegien, France). The digested samples were compared to undigested bacteriophage nucleic
229 acid and the GeneRuler 1 kb Plus DNA ladder (Thermo Scientific). The genome size of the isolated
230 bacteriophages was estimated by compiling the DNA fragment sizes using the standard ladder (Yu
231 et al., 2013). The obtained PCR products were cleaned and concentrated using the Wizard® SV
232 Gel and PCR Clean-up System (Promega) and were sent for DNA sequencing to the CAF at
233 Stellenbosch University. Sequences were examined using FinchTV version 1.4.0 software and
234 identification completed using the NCBI Basic Local Alignment Search Tool (BLAST)
235 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

236 **2.5 Host Range Determination**

237 The host range of the isolated bacteriophages was determined by spotting 10 µL of the
238 concentrated bacteriophage stock solutions (10⁶ to 10⁷ PFU/mL) on TSA (with the exception of
239 *Legionella* spp.; Appendix A) with 5 mL freshly prepared soft top-agar, which had been inoculated
240 with 50 to 100 µL of the strain to be tested and incubating the plates at 30 °C for 18 h (Sillankorva
241 et al., 2008; Stenholm et al., 2008). The host range for each bacteriophage was determined in
242 triplicate for each bacteriophage-host combination and consisted of screening 20 *Pseudomonas*

243 spp. (target bacterial strains; Appendix A, Table S1) and 57 non-target bacterial strains
244 representative of 14 genera (Appendix A, Table S1). Reference, environmental and clinical isolates
245 of both the target and non-target bacterial strains were included in the host range determination
246 analysis.

247 **2.6 One-step Growth Curve and Bacteriophage Sensitivity to Physical Parameters**

248 Based on the results obtained during the host range determination, *K. pneumoniae* ATCC 10031
249 was used as the host for PFW25 during subsequent experimentation, while *P. aeruginosa* ATCC
250 27853 was used as the host for PAW33. One-step growth curves were performed to determine the
251 latency period and burst size of the isolated bacteriophages, as previously described by
252 Sillankorva et al. (2008), with minor modifications. Briefly, a multiplicity of infection (MOI) of 0.0003
253 was used and samples were collected every 10 min over a period of 2 h. Following overnight
254 incubation at 30 °C, the PFU for each time point was recorded and results were reported as the
255 average number of bacteriophages released per infected host cell. The bacteriophage burst size
256 (number of bacteriophages released per infected host cell) was computed as the ratio between the
257 final bacteriophage count and the initial bacteriophage count recorded during the latency period
258 (Ciacci et al., 2018).

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

268 **2.7 Bacterial Challenge Tests**

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

280 To determine whether bacteriophage resistant mutants had emerged during the bacterial challenge
281 tests, 2 mL aliquots were collected after the 24 h incubation and were centrifuged at 10 000 × *g* for
282 5 min (Spectrafuge™ 24D Digital Microcentrifuge, Labnet International, Edison, USA). The
283 bacterial pellet was re-suspended and was used to inoculate 5 mL freshly prepared soft top-agar,
284 which was poured onto TSA plates. Ten microliters of concentrated bacteriophage stock solutions
285 (10^6 to 10^7 PFU/mL) were spotted onto the surface of the plate and the plates were incubated at
286 30 °C for 18 h. Following incubation, the plates were examined for plaque formation to determine
287 whether the respective host bacterial species were still susceptible to the isolated bacteriophages.
288 The concentration of the respective host bacterial species within the samples, after the 24 h
289 bacterial challenge tests, was determined by preparing serial dilutions (10^{-1} to 10^{-4}) and spread-
290 plating 100 µL of the samples onto TSA, incubating at 30 °C for 18 h and then enumerating the
291 colony forming units (CFU) per mL. Additionally, based on the results obtained for the bacterial
292 challenge test performed on the *P. aeruginosa* ATCC 27853 strain, agglutination tests were
293 performed to screen for lipopolysaccharide (LPS) defective mutants (Le et al., 2014).

294 **2.8 Small-scale Bacteriophage Pre-treatment of Spiked Rainwater and SODIS-CPC Trials**

295 Two small-scale bacteriophage pre-treatment (8 h or 24 h) and SODIS-CPC trials were performed
296 using bacteriophage PAW33 and the environmental isolate *P. aeruginosa* S1 68. Roof-harvested

297 rainwater was collected from a rainwater tank located on a local farm (GPS coordinates:
298 33°56'38.5"S 18°46'26.3"E), where after four 1 L rainwater aliquots were autoclaved three times.
299 An overnight culture of the environmental *P. aeruginosa* S1 68 strain was spiked into each of the
300 four 1 L rainwater aliquots to achieve a final concentration of 1.0×10^7 CFU/mL. Two of the 1 L
301 rainwater aliquots were simultaneously spiked with 500 μ L of a concentrated bacteriophage
302 solution to achieve an MOI of 0.01, with one of the samples incubated for 8 h and the other aliquot
303 incubated for 24 h at 30 °C at 120 rpm on a rotary shaker (New Brunswick Scientific). The
304 remaining two 1 L rainwater aliquots served as the no bacteriophage treatment controls and were
305 spiked with 500 μ L sterile SM-buffer and were also incubated for 8 and 24 h at 30 °C at 120 rpm
306 on a rotary shaker. Ten millilitre aliquots were collected at 0 and 8 h time intervals from the 8 h pre-
307 treatment and corresponding no pre-treatment control samples and at 0, 1, 2, 4, 8, 16 and 24 h
308 time intervals from the 24 h pre-treatment and corresponding no pre-treatment control samples.

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

316

317 **2.8.1 Culture and Viability-qPCR Analysis of Samples**

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

321 However, as *Pseudomonas* spp. may enter a viable but non-culturable state during unfavourable
322 conditions (such as those experienced during disinfection treatments), viability-qPCR assays were
323 included to monitor the gene copy numbers of *P. aeruginosa* S1 68. Briefly, 2 mL of a collected

324 sample was centrifuged at 10 000 × g for 10 min. The obtained pellet was re-suspended in 1 mL
325 saline (0.85% NaCl), whereafter the sample was treated with 6 µM ethidium monoazide bromide
326 (EMA; Biotium, Hayward, CA, USA) as outlined in Reyneke et al. (2017). Deoxyribonucleic acid
327 extraction was completed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research,
328 Inqaba Biotech, South Africa) according to the manufacturers' instructions.

329 Similarly, in order to ensure that only DNA from intact (infective) PAW33 virions were quantified
330 during qPCR analysis, 2 mL of a collected sample was treated with 5 U/mL DNase (Thermo
331 Scientific) as outlined in Reyneke et al. (2017), whereafter the sample was centrifuged at
332 25 000 × g for 60 min. The obtained pellet was re-suspended in the pre-lyse mixture (Buffer T1, B3
333 and Proteinase K) of the NucleoSpin® Tissue kit (Macherey-Nagel) and the DNA extraction was
334 completed as outlined by the manufacturer.

335 **2.8.2 RNA Extraction**

336 In order to determine whether bacteriophage pre-treatment may influence the ability of *P.*
337 *aeruginosa* S1 68 to initiate stress response mechanisms during SODIS-CPC treatment, 5 mL of
338 each collected sample was treated with a 1% phenol/19% ethanol (final v/v) mixture, incubated at 4
339 °C for 45 min and centrifuged at 3 320 × g for 15 min at 4 °C, whereafter the pellet was stored at -
340 80 °C until further analysis (Lambert et al., 2010). Total RNA was extracted from the frozen pellets
341 using TRI Reagent® (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's
342 instructions. Following concentration and purity determination of the extracted RNA, using a
343 NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, Delaware, USA), 0.2 µg of the
344 total RNA was DNase treated (Thermo Fisher) and transcribed into cDNA using the Improm-II™
345 Reverse Transcription System (Promega) and oligo dT primer as described by the manufacturer. A
346 no-template control and a no-reverse transcriptase control were included to confirm complete
347 removal of contaminating genomic DNA from each sample and subsequently included in the
348 respective qPCR assays.

349 **2.8.3 Absolute and Relative qPCR Assays**

350 All absolute and relative qPCR assays were conducted using a LightCycler® 96 (Roche
351 Diagnostics, Risch-Rotkreuz, Switzerland) instrument in combination with the FastStart Essential
352 DNA Green Master Mix (Roche Diagnostics) (Reyneke et al., 2017). For the absolute quantification
353 of PAW33 in the collected samples, the Podo-Hypo-F/R primer pair and cycling parameters
354 outlined in Table 1 were used, while the PS1 5'-ATGAACAACGTTCTGAAATTC-3' and PS2 5'-
355 CTGCGGCTGGCTTTTTCCAG-3' primer pair (Roosa et al., 2014) was used for the absolute
356 quantification of *P. aeruginosa* S1 68 with the following cycling parameters: 95 °C (10 min)
357 followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension
358 at 72 °C for 30 s.

359 For the relative quantification of the *rpoS*, *phzM*, *recA* and *lexA* genes of *P. aeruginosa* S1 68 the
360 primer pairs and cycling parameters as outlined in Table 2 were utilised. The cycle quantification
361 values (C_q) of the reference gene (*rpoS*) were utilised to normalise the calculated C_q values of the
362 target genes (*phzM*, *recA* and *lexA*) amplified from the corresponding samples (ΔC_q), and the fold
363 change ($2^{-\Delta\Delta C_q}$) was compared to the baseline sample (0 h samples) (Dobrowsky et al., 2017). *recA*
364 and *lexA* were selected as target genes as they are involved in the global SOS response initiated
365 by bacteria upon exposure to adverse environmental conditions, particularly those associated with
366 DNA damage (inactivation mechanism of SODIS) (Krebs et al., 2018), while *phzM* is a virulence-
367 associated gene involved in the production of pyocyanin [secondary metabolite (blue-green
368 pigment) produced by *P. aeruginosa*] which has also been hypothesised to protect *P. aeruginosa*
369 from oxidative stress (inactivation mechanism of SODIS) (Hendiani et al., 2019). High-resolution
370 melt curve analysis was included for each qPCR assay in order to verify the specificity of the
371 primer set by ramping the temperature from 65 to 97 °C at a rate of 0.2 °C/s with continuous
372 fluorescent signal acquisition at 15 readings/°C. Standard curves for the quantification of
373 *P. aeruginosa* S1 68 and PAW33 were generated using the methodology outlined in Reyneke et al.
374 (2017).

375 **3. Results**

376 **3.1 Bacteriophage Isolation and Characterisation**

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

381 Based on the STEM micrographs (Fig. 1), both bacteriophages were classified as members of the
382 order *Caudovirales* (tailed bacteriophages). The morphological features of PAW33 (Fig. 1 a)
383 indicated that this bacteriophage belongs to the *Podoviridae* family. Bacteriophages belonging to
384 this family are characterised as having short, “stubby” non-contractile tails (Sepúlveda-Robles et
385 al., 2012). It was observed that the capsid of PAW33 had a hexagonal outline indicating an
386 icosahedral nature, with the capsid diameter recorded as ~63 nm and the tail measuring < 20 nm
387 in length (tapering) (Fig. 1). PFW25 was identified as a *Myoviridae* bacteriophage based on its
388 morphological features (Fig. 1 b). Bacteriophages belonging to this family are characterised as
389 having contractile tails (Sepúlveda-Robles et al., 2012). As was observed for PAW33, the capsid of
390 PFW25 had a hexagonal outline indicating an icosahedral nature; however, the capsid was slightly
391 elongated. The capsid was ~72 nm wide and ~88 nm long, with the contractile tail measuring
392 ~125 nm (Fig. 1).

393 **3.2 Nucleic Acid Analysis and PCR-based Identification of PAW33 and PFW25**

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

399 Following the design and optimisation of numerous primer sets, the Podo-Hypo-F/R (targeting
400 *Podoviridae*) and Myo-Hypo-F/R primer sets (targeting *Myoviridae*) (Table 1), were selected to
401 confirm the preliminary classification of the isolated bacteriophages and enable the quantification
402 of the bacteriophages during the pre-treatment and SODIS-CPC trials. Conventional PCR analysis
403 of DNA obtained from PAW33 using the Podo-Hypo-F/R primer set resulted in the amplification of

404 a 225 bp product. Sequencing analysis of the amplicon indicated that PAW33 shared sequence
405 similarity with *Pseudomonas* bacteriophages LP14 (GenBank accession no: MH356729.1), YH30
406 (GenBank accession no: KP994390.1), phi176 (GenBank accession no: KM411960.1) and Pa2
407 (GenBank accession no: NC_027345.1), respectively (Appendix A, Table S2). These
408 bacteriophages are listed as belonging to the *Podoviridae* family, which further corroborates the
409 preliminary classification of PAW33. The Podo-Hypo-F/R primer set did not amplify DNA from
410 PFW25.

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

418 **3.3 Host range determination for PAW33 and PFW25**

419 The host range of the isolated bacteriophages was assessed against various target (*Pseudomonas*
420 spp.) and non-target bacterial species (Appendix A, Table S1), with activity recorded as the
421 presence of clear zones or plaques (++) , turbid zones or plaques (+) or no growth inhibition (-).
422 While no activity was observed for PAW33 against the 57 non-target bacterial species analysed,
423 lytic activity was observed against 92% ($n = 11$) of the *P. aeruginosa* strains and 25% ($n = 2$) of the
424 other *Pseudomonas* spp. tested (Fig. 2). In contrast, PFW25 displayed activity against the non-
425 target bacterial species, *K. pneumoniae* ATCC 10031 and ATCC 333305 (results not shown), but
426 none of the other non-target bacterial species analysed. PFW25 also displayed lytic activity against
427 75% ($n = 3$) of the *P. fluorescens* strains analysed and against 25% ($n = 3$) of the other
428 *Pseudomonas* spp. tested (Fig. 2).

429 **3.4 Bacteriophage Growth Characteristics and Sensitivity to Physical Parameters**

430 Under the conditions studied (ambient temperature of 20 to 22 °C and aerobic conditions), PAW33
431 displayed a latency period of ~80 min, a rise period of ~50 min and a burst size of ~136 PFU per
432 infected cell, when co-cultured with *P. aeruginosa* ATCC 27853 (results not shown). As indicated,
433 *K. pneumoniae* ATCC 10031 was used to elucidate the life cycle of bacteriophage PFW25 during
434 the one-step growth experiments and subsequent experiments. Under the conditions studied,
435 PFW25 displayed a latency and rise period of ~30 min each, while the burst size of PFW25 was
436 ~47 PFU per infected cell (results not shown).

437 Results from the temperature stability tests indicated that the infectivity of PAW33 remained stable
438 between 30 °C and 50 °C; however, a significant decrease (~5 log) in infectivity was observed after
439 10 min at 70 °C and 60 min at 60 °C (Appendix A, Fig. S1 a). A similar temperature sensitivity
440 profile was observed for PFW25 as its infectivity remained stable between 30 °C and 50 °C.
441 PFW25 infectivity gradually decreased by ~1 log after 60 min at 60 °C and then remained relatively
442 constant for the remaining 60 min. In comparison, a significant decrease (~ 4 log) in PFW25
443 infectivity was recorded after 30 min at 70°C (Appendix A, Fig. S1 b). Results for the pH stability
444 tests indicated that PAW33 retained infectivity after incubation at pH values ranging from 6.0 to
445 9.0, while a 0.19 log, 0.41 log and 0.51 log decrease in infectivity was observed following
446 incubation at pH 4, 5 and 10 (as compared to the mean PFU recorded for pH 6.0 to 9.0) (Appendix
447 A, Fig. S1 c). In comparison, the infectivity of PFW25 remained relatively constant after incubation
448 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
449 0.55 log decrease, in PFW25 infectivity was observed (as compared to the mean PFU recorded for
450 pH 5.0 to 8.0), respectively (Appendix A, Fig. S1 d).

451 **3.5 Efficiency of PAW33 and PFW25 to Control Target Host Growth**

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

458 Fig. S2 a). Although, PAW33 was not able to completely eliminate the *P. aeruginosa* population,
459 culture-based analysis following the 24 h co-culture indicated that the *P. aeruginosa* CFU were
460 1.30 log ($p = 0.0038$), 1.08 log ($p = 0.0048$) and 1.06 log ($p = 0.0046$) lower in the samples treated
461 at an MOI of 1, 0.1 and 0.01, respectively, in comparison to the untreated bacterial control (results
462 not shown). In order to determine whether the increase in *P. aeruginosa* growth in the PAW33
463 treated samples was due to the emergence of resistance to the bacteriophage, bacterial cells were
464 harvested and susceptibility to PAW33 was assessed using the spot-test method. Results
465 indicated that the *P. aeruginosa* population were still susceptible to PAW33; however,
466 bacteriophage resistant mutants had emerged. These colonies were characterised by the
467 production of a red pigment, which resulted in a red mutant phenotype observed on the TSA plates
468 (results not shown). Visualisation of these colonies using microscopy and comparison to the
469 untreated control samples (not treated with PAW33 during co-culture) revealed that these
470 bacteriophage resistant mutants clumped together following the agglutination test, indicating that
471 their bacterial cell surface was LPS defective.

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

486 3.6 Small-scale Bacteriophage Pre-treatment of Spiked Rainwater Followed by SODIS-CPC

487 3.6.1 Culture-based and Viability-qPCR Quantification of *P. aeruginosa* S1 68 and PAW33

488 The potential of bacteriophages to serve as a biocontrol pre-treatment (8 h and 24 h) of roof-
489 harvested rainwater was investigated using PAW33 and the environmental *P. aeruginosa* S1 68
490 strain (Fig. 3). The performance characteristics of the viability-qPCR analysis of *P. aeruginosa* S1
491 68 and PAW33 are provided in Appendix A Table S3, while Appendix A Table S4 summarises the
492 concentration and overall log reduction data for the 8 h and 24 h trials, followed by SODIS-CPC.

493 For the 8 h trial (Fig. 3 a), culture-based analysis of the non-pre-treated control sample indicated
494 that the *P. aeruginosa* S1 68 CFU counts increased by 0.36 log, from 1.38×10^7 CFU/mL to
495 3.19×10^7 CFU/mL, over the 8 h incubation period. Subsequent exposure of the non-pre-treated
496 sample to a 4 h SODIS-CPC treatment resulted in a total log reduction of 3.74 ($p = 0.0109$) in
497 *P. aeruginosa* S1 68 CFU counts (2.50×10^3 CFU/mL recorded after SODIS-CPC), from the initial
498 concentration of 1.38×10^7 CFU/mL (Appendix A, Table S4). Correspondingly, viability-qPCR
499 analysis indicated that the *P. aeruginosa* S1 68 GC increased by 0.49 log over the 8 h incubation
500 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
501 recorded following SODIS-CPC treatment [2.33 total log reduction ($p = 0.0087$)] (Fig. 3 a; Appendix
502 A, Table S4). Culture-based and viability-qPCR analysis of the corresponding dark control sample
503 (collected after the 4 h SODIS-CPC treatment), indicated that the concentration of *P. aeruginosa*
504 S1 68 remained relatively constant with 2.13×10^7 CFU/mL and 1.81×10^6 GC/mL recorded,
505 respectively (results not shown).

506 Culture-based analysis of the corresponding 8 h bacteriophage pre-treated sample indicated that
507 the *P. aeruginosa* S1 68 CFU/mL increased by 0.26 log from 1.24×10^7 CFU/mL to
508 2.28×10^7 CFU/mL, where after the SODIS-CPC treatment reduced the cell counts to
509 2.58×10^3 CFU/mL [3.68 total log reduction ($p = 0.0299$) from the initial CFU of 1.24×10^7] (Fig.
510 3 a; Appendix A, Table S4). Similarly, viability-qPCR analysis indicated that the *P. aeruginosa* S1
511 68 gene copies (GC) only increased by 0.19 log during the 8 h pre-treatment, from
512 6.98×10^5 GC/mL to 1.09×10^6 GC/mL, whereafter the gene copies were reduced to 3.15×10^3

513 GC/mL during the SODIS-CPC treatment [2.34 total log reduction ($p = 0.0033$)] (Appendix A, Table
514 S4). Monitoring of PAW33 in the 8 h pre-treated sample indicated that the PFU/mL decreased by
515 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
516 following the SODIS-CPC treatment [2.70 total log reduction ($p = 0.0023$)] (Appendix A, Fig. S3 a).
517 In contrast, the PAW33 GC/mL increased by 0.48 log (1.80×10^4 GC/mL to 5.37×10^4 GC/mL)
518 during the 8 h pre-treatment, whereafter the gene copies remained relatively constant, as
519 1.42×10^4 GC/mL were recorded following the SODIS-CPC treatment [0.12 total log reduction
520 ($p = 0.1909$)] (Appendix A, Table S4 and Fig. S3 a). Culture-based and viability-qPCR analysis of
521 the corresponding dark control sample (collected after the 4 h SODIS-CPC treatment), indicated
522 that the concentration of *P. aeruginosa* S1 68 remained relatively constant with 8.63×10^6 CFU/mL
523 and 1.23×10^6 GC/mL recorded, respectively, while PAW33 also remained constant as 3.16×10^4
524 PFU/mL and 1.92×10^4 GC/mL were recorded (results not shown).

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

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

541 CFU/mL to 2.79×10^7 CFU/mL (Fig. 3 b). Subsequent SODIS-CPC treatment of the pre-treated
542 sample reduced the *P. aeruginosa* S1 68 CFU counts to 5.0×10^2 CFU/mL [4.61 log reduction
543 overall ($p = 0.0079$)], from the initial count of 2.03×10^7 (Appendix A, Table S4). Similarly, viability-
544 qPCR analysis of the pre-treated sample indicated a 0.30 log increase (5.31×10^5 GC/mL to
545 1.06×10^6 GC/mL) in *P. aeruginosa* S1 68 GC/mL during the 24 h pre-treatment, whereafter the
546 GC were reduced to 1.07×10^4 GC/mL due to the SODIS-CPC treatment [2.32 log reduction
547 overall ($p = 0.0128$)] (Fig. 3 b; Appendix A, Table S4). Enumeration of the PAW33 plaque counts in
548 the pre-treated sample indicated that the PFU/mL increased from 8.0×10^4 PFU/mL to 4.0×10^5
549 PFU/mL (0.70 log increase) during the 24 h pre-treatment, whereafter a decrease to 1.3×10^2
550 PFU/mL was recorded following the SODIS-CPC treatment [2.79 log reduction overall
551 ($p = 0.0115$)] (Appendix A, Fig. S3 b). In comparison, the PAW33 GC/mL remained relatively
552 constant in the pre-treated sample during the 24 h trial, as 2.50×10^4 GC/mL were detected at both
553 0 and 24 h, while 4.8×10^3 GC/mL were detected following SODIS-CPC treatment [0.72 log
554 reduction overall ($p = 0.0270$)] (Appendix A, Table S4). Culture and viability-qPCR analysis of the
555 corresponding dark control sample (collected after the 4 h SODIS-CPC treatment), indicated that
556 the concentration of *P. aeruginosa* S1 68 remained relatively constant with 4.62×10^7 CFU/mL and
557 6.45×10^6 GC/mL recorded, respectively, while PAW33 also remained relatively constant as $1.93 \times$
558 10^5 PFU/mL and 2.10×10^4 GC/mL were recorded (results not shown).

559 **3.6.2 Expression of SOS Response- and Virulence-associated Genes of *P. aeruginosa* S1 68**

560 The performance characteristics of the relative qPCR assays are provided in Appendix A, Table
561 S3. For the 8 h trial, a similar increase in *phzM* gene expression was observed for both the pre-
562 treated and non-pre-treated control samples during the 8 h incubation period (Fig 4). While a
563 decrease in *phzM* gene expression was observed for both samples following the SODIS-CPC
564 treatment, the expression level was still up-regulated. In comparison, while an upregulation in *recA*
565 gene expression was observed in the non-pre-treated control and pre-treated samples during the
566 8 h incubation period as well as in the non-pre-treated control sample following the SODIS-CPC
567 treatment, downregulation of *recA* was observed for the pre-treated sample following the SODIS-
568 CPC treatment (Fig. 4). *lexA* was then up-regulated in both the non-pre-treated control and pre-

569 treated samples during the 8 h incubation period, whereafter downregulation was observed for
570 both samples during the SODIS-CPC treatment (Fig. 4). Although changes in gene expression
571 were observed for the collected samples, the fold change in expression was not significant (< 5-
572 fold change) for any of the analysed target genes.

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

582 **4. Discussion**

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

589 Lytic bacteriophages displaying activity against *Pseudomonas* spp. were subsequently isolated
590 from numerous environmental sources, with PAW33 (isolated using *P. aeruginosa*) and PFW25
591 (isolated using *P. fluorescens*) selected for further characterisation. Electron microscopy and
592 nucleic acid analysis indicated that both PAW33 and PFW25 belong to the order *Caudovirales* and
593 more specifically the *Podoviridae* and *Myoviridae* families, respectively. Subsequently, the pH and
594 temperature sensitivity of PAW33 and PFW25 was assessed as various chemical and physical
595 parameters (such as those associated with rainwater harvesting systems) may influence the

596 viability/infectivity of bacteriophages by damaging their structural elements (e.g. head and tail
597 structures) (Jończyk et al., 2011). Results however, indicated that both bacteriophages were stable
598 and retained their infectivity upon exposure to the physico-chemical parameters commonly
599 associated with untreated harvested rainwater (pH 6.2 to 8.4; 19 to 26 °C) and temperatures
600 experienced within large-scale SODIS systems (39 to 59 °C) (Reyneke et al., 2018; Strauss et al.,
601 2018).

602 *Caudovirales* bacteriophages are associated with more than 140 prokaryotic genera with varying
603 degrees of host specificity reported (9th International Committee on Taxonomy of Viruses Report,
604 2011; Kęsik-Szeloch et al., 2013). The host range determination then indicated that PAW33 was
605 able to infect reference, environmental and clinical isolates of *P. aeruginosa*, with notable activity
606 displayed against the multidrug-resistant *P. aeruginosa* T1 clinical isolate (Havenga et al., 2019)
607 and numerous environmental strains previously isolated from a solar pasteurization system
608 connected to a rainwater harvesting tank (Appendix A, Table S1). Additionally, as PAW33 was able
609 to infect two environmental *P. fluorescens* strains, it was classified as having a broad host range
610 against *P. aeruginosa* strains, with limited activity against other *Pseudomonas* spp. and no activity
611 against the non-target bacteria. In comparison, PFW25 was able to infect three *P. fluorescens*
612 strains, two environmental and one clinical isolate of *P. aeruginosa* (Fig. 2) and two *K. pneumoniae*
613 ATCC strains (results not shown). The activity displayed against *K. pneumoniae* and the efficiency
614 of plating when PFW25 was cultured with *K. pneumoniae* ATCC 10031, coupled with the sequence
615 similarity (hypothetical protein targeted by the Myo-Hypo-F/R primer set) displayed to
616 bacteriophage vB_Kpn_F48, indicated that PFW25 may be better suited to target *K. pneumoniae*
617 strains. Similarly, Wu et al. (2007) reported on the isolation of a *Myoviridae* bacteriophage (Kpp95)
618 using *K. pneumoniae*, which was subsequently classified as having a broad host range, as the
619 bacteriophage displayed lytic activity against *K. pneumoniae*, *K. oxytoca*, *Enterobacter*
620 *agglomerans* and *Serratia marcescens*.

621 For the bacterial challenge tests, while the bacteriophages PAW33 and PFW25 were able to inhibit
622 the growth of their respective target hosts (*P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC
623 10031), bacteriophage resistant *P. aeruginosa* and *K. pneumoniae* mutants had emerged.

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

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

651 during the pre-treatment period and secondly sensitise the overall bacterial population to the
652 primary treatment strategy (i.e. SODIS-CPC).

653 Culture-based and viability-qPCR analysis indicated that PAW33 was able to restrict the
654 proliferation of the *P. aeruginosa* S1 68 in the rainwater during both the 8 h and 24 h pre-treatment
655 trials. However, while similar total CFU and GC log reductions were obtained for the pre-treated
656 [3.68 log (CFU) and 2.34 log (GC)] and non-pre-treated control samples [3.74 log (CFU) and 2.33
657 log (GC)] for the 8 h trial (followed by SODIS-CPC); culture-based analysis indicated that a higher
658 overall log reduction was recorded for the 24 h bacteriophage pre-treated sample followed by
659 SODIS-CPC (4.61 log) in comparison to the non-pre-treated sample (3.91 log). Additionally,
660 culture-based analysis indicated that after the 24 h bacteriophage pre-treatment trial, faster
661 inactivation of *P. aeruginosa* S1 68 occurred during the first hour (1.73 log reduction) of the
662 SODIS-CPC treatment. A similar observation was recently reported by Al-Jassim et al. (2018)
663 where the ability of bacteriophages to sensitise a pathogenic New Delhi metallo β -lactamase-
664 positive *E. coli* to SODIS was investigated. Results from the study indicated that exposure to
665 bacteriophages increased the susceptibility of *E. coli* to SODIS, with faster inactivation of the *E.*
666 *coli* observed (treatment time reduced from 4 h to 2 h). Additionally, using gene expression
667 analysis, the authors reported that the exposure of *E. coli* to the bacteriophage resulted in a
668 downregulation of cell wall functions, the ability to scavenge reactive oxygen species and DNA
669 repair mechanisms, effectively rendering the *E. coli* more susceptible to SODIS treatment. It is
670 however important to note that the Al-Jassim et al. (2018) study utilised a combination of
671 bacteriophages at a high treatment concentration (MOI = 1), the bacteriophage and SODIS
672 treatment occurred simultaneously and an artificial light source was used to simulate SODIS. In
673 contrast, in the current study a lower treatment concentration (MOI = 0.01) of a single
674 bacteriophage was used as a pre-treatment strategy to SODIS-CPC under natural sunlight. Thus,
675 while the bacteriophage pre-treatment for 24 h, followed by SODIS-CPC, resulted in the highest
676 total log reduction (4.61 log) of *P. aeruginosa* S1 68 CFU/mL, the target host could not be
677 completely eradicated using this combination treatment strategy, as 5.0×10^2 CFU/mL was still
678 recorded following SODIS-CPC treatment. Additionally, while viability-qPCR analysis indicated that

679 comparable total log reductions [2.32 log (pre-treated) and 2.26 log (non-pre-treated)] in
680 *P. aeruginosa* S1 68 concentrations were obtained for the 24 h trial samples, gene copies were still
681 detected after SODIS-CPC, indicating that viable but non-culturable cells may be present within the
682 samples. The survival of the *P. aeruginosa* S1 68 following the combination treatment is however,
683 not surprising as *Pseudomonas* spp. may initiate a range of stress responses during both the
684 planktonic or biofilm life cycles, including the production of heat shock proteins and the initiation of
685 DNA repair mechanisms, amongst others, and thereby switch to a more tolerant phenotype to
686 facilitate its survival under adverse conditions (Fux et al., 2005; Breidenstein et al., 2011).
687 However, as highlighted by Al-Jassim et al. (2018), the ability of bacteria to initiate these stress
688 response mechanisms may be severely impaired following/during exposure to bacteriophages.

689 Gene expression analysis was subsequently included to monitor the SOS response-associated
690 *recA* and *lexA* genes, while *phzM* (gene associated with pyocyanin production) was monitored as
691 the bacterial challenge tests indicated that decreased pyocyanin was produced by bacteriophage
692 resistant *P. aeruginosa* ATCC 27853. Results for the 8 h trial indicated that while *recA* and *lexA*
693 expression levels were decreased in the PAW33 treated sample during the 8 h incubation period
694 (as compared to the non-pre-treated sample), the overall expression level was still up-regulated,
695 with downregulation only observed following SODIS-CPC treatment. In comparison, *phzM* gene
696 expression was up-regulated in the no treatment control and PAW33 treated samples during the
697 8 h incubation trial and the subsequent SODIS-CPC treatment. Results for the 24 h trial then
698 indicated that *phzM*, *recA* and *lexA* were down-regulated in the PAW33 pre-treated sample during
699 the 24 h incubation period, with continued downregulation observed following the SODIS-CPC
700 treatment. *recA* and *lexA* are known to be up-regulated in bacteria in response to adverse
701 conditions as part of the SOS response mechanism and are primarily involved in DNA repair
702 mechanisms (Krebs et al., 2018). The downregulation of *recA* and *lexA* in the PAW33 pre-treated
703 *P. aeruginosa* S1 68, particularly during the 4 h SODIS-CPC treatment, indicates that the
704 bacteriophage pre-treatment for both 8 and 24 h may have influenced the ability of the target host
705 bacterium to initiate stress response mechanisms during the primary treatment strategy (i.e.
706 SODIS-CPC). However, based on the results obtained, a prolonged bacteriophage pre-treatment

707 period may initiate the change in gene expression as *recA* and *lexA* were down-regulated during
708 the 24 h incubation period. Additionally, while assessing the influence of sub-lethal photodynamic
709 inactivation [sPDI; photo-oxidative stress caused by the generation of reactive oxygen species
710 (ROS) after a photosensitiser molecule was excited by visible light], Hendiani et al. (2019) reported
711 that pyocyanin production (*phzM* expression) in *P. aeruginosa* ATCC 27853 as well as strains P2
712 and P3, increased during sPDI, with the authors hypothesising that the over-expression of
713 pyocyanin played a possible protective role against sPDI-induced oxidative stress. As *phzM* was
714 down-regulated in both the 24 h bacteriophage pre-treatment and subsequent SODIS-CPC
715 samples, in comparison to the observed up-regulation in the 8 h trial samples, it is hypothesised
716 that the decreased *phzM* expression may be due to the presence of bacteriophage resistant
717 *P. aeruginosa* S1 68 cells within the sample (as was observed for the bacterial challenge tests).
718 The bacteriophage pre-treatment for 24 h may thus have influenced the ability of the bacteriophage
719 resistant *P. aeruginosa* S1 68 cells to initiate pyocyanin production as a stress response
720 mechanism, rendering the bacterial cells more susceptible to primary disinfection strategies (such
721 as SODIS-CPC). Additionally, as pyocyanin is considered a virulence factor of *P. aeruginosa*
722 (Hendiani et al., 2019), its downregulation in the 24 h PAW33 pre-treated samples indicates that
723 bacteriophage pre-treatment may decrease pathogen virulence. The overall results thus indicate
724 that a longer bacteriophage pre-treatment may be required for the bacteriophages to adequately
725 influence target host stress response mechanisms.

726 **5. Conclusions**

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

735 al., 2016), while the SODIS-CPC treatment efficiency may be further improved by increasing the
736 SODIS treatment time (6 to 8 h SODIS exposures recommended in literature) (Strauss et al.,
737 2016).

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

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748 experiments: BR. Designed the primers: SK. Designed the small-scale SODIS-CPC systems: PFI.
749 Analysed the data: BR and WK. Contributed reagents/materials/analysis tools: WK and SK.
750 Compiled the manuscript: BR and WK. Edited the manuscript: SK and PFI.

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761

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