Predatory bacteria in combination with solar disinfection and solar photocatalysis for the treatment of rainwater

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Abstract

The predatory bacterium, *Bdellovibrio bacteriovorus*, was applied as a biological pre-treatment to solar disinfection and solar photocatalytic disinfection for rainwater treatment. The photocatalyst used was immobilised titanium-dioxide reduced graphene oxide. The pre-treatment followed by solar photocatalysis for 120 min under natural sunlight reduced the viable counts of *Klebsiella pneumoniae* from 2.00×10^9 colony forming units (CFU)/mL to below the detection limit (BDL) (<1 CFU/100 µL). Correspondingly, ethidium monoazide bromide quantitative PCR analysis indicated a high total log reduction in *K. pneumoniae* gene copies (GC)/mL (5.85 logs after solar photocatalysis for 240 min). In contrast, solar disinfection and solar photocatalysis without the biological pre-treatment were more effective for *Enterococcus faecium* disinfection as the viable counts of *E. faecium* were reduced by 8.00 logs (from 1.00×10^8 CFU/mL to BDL) and the gene copies were reduced by ~3.39 logs (from 2.09×10^6 GC/mL to ~9.00×10^2 GC/mL) after 240 min of treatment. Predatory bacteria can be applied as a pre-treatment to solar disinfection and solar photocatalytic treatment to enhance the removal efficiency of Gram-negative bacteria, which is crucial for the development of a targeted water treatment approach.

Keywords: Harvested rainwater; *Bdellovibrio bacteriovorus*; Biological pre-treatment; Solar disinfection; Photocatalysis

1. Introduction

Domestic rainwater harvesting is employed as a supplementary water source, particularly in water scarce regions. However, the quality of harvested rainwater does not always comply with drinking water standards, and some bacteria of public health concern such as *Pseudomonas*, *Klebsiella*, *Campylobacter* and *Staphylococcus* spp., have been detected in rainwater samples (De Kwaadsteniet et al., 2013). While various treatment methods have been investigated and applied to disinfect rainwater (Dobrowsky et al., 2015; Reyneke et al., 2016), the World Health Organisation (WHO) recognises solar disinfection (SODIS) as a cost-effective, household-based technology, which can be employed to decrease the number of viable pathogenic organisms in contaminated water sources and reduce the incidence of diarrheal disease (Byrne et al., 2011). The protocol involves exposing water in UV-visible transparent containers to direct sunlight for a minimum of 6 h (48 h in cloudy conditions). Nalwanga et al. (2018) investigated the use of SODIS with 2 L polyethylene-terephthalate (PET) bottles for the treatment of harvested rainwater in Uganda. While the viable counts of *Escherichia coli* and faecal enterococci exceeded drinking water standards in the majority of the untreated samples analysed, culture-based analysis indicated that after SODIS, the concentrations of these bacteria were significantly reduced (detailed information on counts not presented) (Nalwanga et al., 2018). The major limitations associated with the use of a simple SODIS system are, however, the small volume of treated water generated (1 to 5 L) and the treatment time required for sufficient disinfection of the water. It is also recommended that the treated water should be used within 24 h as regrowth of bacteria may occur (Makwana et al., 2015).

Different approaches have subsequently been investigated to improve the efficiency of solar disinfection. Ubomba-Jaswa et al. (2010) used a 25 L methacrylate batch reactor fitted with a compound parabolic collector (CPC; concentrates diffuse solar irradiation onto a reactor vessel in order to increase the dose of solar irradiation) to disinfect well water. Complete inactivation of *E. coli* was achieved within 5 h on sunny days and a 3-log reduction was achieved within this time period during overcast conditions (cloudy days). However, some organisms are more resistant to solar disinfection than others. For example, Strauss et al. (2018) reported that while a solar-CPC treatment system effectively reduced the *E. coli* and total coliform counts to below the detection limit (BDL) at

temperatures exceeding 39°C and UV-A radiation exceeding 20 W/m^2, ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) analysis indicated that viable *Legionella* and *Pseudomonas* were detected in all the SODIS-CPC treated samples throughout the sampling period. Clements et al. (2019) used EMA-qPCR to screen solar pasteurized (SOPAS) harvested rainwater for potentially viable bacteria and found that *Klebsiella* spp., amongst others, survived at temperatures > 90°C. It is hypothesised that the survival of bacteria in solar disinfection systems could be due to the possession of heat shock proteins, DNA repair mechanisms (such as *recA*) and their ability to form associations with protozoa (Strauss et al., 2018). Additional treatment techniques are thus required to overcome this bacterial resistance to disinfection strategies and effectively eliminate these pathogens and opportunistic pathogens from water sources.

Advanced oxidative processes (AOP), such as heterogeneous photocatalysis with semiconductor materials (Byrne et al., 2011), have also been explored and Helali et al. (2014) investigated the solar inactivation of *E. coli* with different photocatalysts [i.e., TiO_2 P25, TiO_2 PC500, TiO_2 Ruana and Russelite (Bi_2WO_6)]. With only solar irradiation, 3 to 5 h were required for complete inactivation of *E. coli*. In contrast, the treatment time required for the inactivation of *E. coli* was significantly reduced to between 5 to 30 min for TiO_2 P25, which was the most effective photocatalytic material. We have also previously reported enhanced solar disinfection utilising TiO_2-reduced graphene oxide composites (TiO_2-rGO) (Fernández-Ibáñez et al., 2015; Cruz-Ortiz et al., 2017). Adán et al. (2018) then showed that TiO_2 immobilised on borosilicate glass raschig rings effectively reduced *E. coli* concentrations, in co-culture with *Acanthamoeba* trophozoites, by 3 logs in distilled water after 60 min, while a 2-log reduction was recorded after 180 min for synthetic wastewater. It was thus concluded that immobilised photocatalysts might be practical for water treatment as the post-treatment removal of the photocatalytic material is not required.

An interesting approach to the inactivation of resistant strains is the use of predatory bacteria such as *Bdellovibrio*-and-like-organisms (known as BALOs). These have been identified as potential “live antibiotics” as they are able to prey on and reduce the concentration of predominantly Gram-negative bacteria in co-culture experiments (Socket, 2009). This group of predatory bacteria include species such as *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*. Kadouri et al. (2013)

investigated whether *B. bacteriovorus* and *M. aeruginosavorus* could prey on clinically significant multidrug-resistant Gram-negative bacteria and found that *B. bacteriovorus* HD100 was able to prey on all the host organisms (100%), while *B. bacteriovorus* 109J was able to prey on 93% and *M. aeruginosavorus* ARL-13 was only able to prey on 35% of the host bacteria. Limited research has however been conducted on the application of these predatory bacteria as biocontrol agents for potable water treatment, with most studies focussing on their application as probiotics in aquaculture (Chu & Zhu, 2010; Willis et al., 2016) or as bioremediation agents in wastewater treatment plants (Yu et al., 2017; Ökzan et al., 2018).

Based on the survival of pathogenic microorganisms in treated rainwater, a need exists to investigate a combination of technologies that incorporate biocontrol, physical and chemical treatment. This study thus aimed to apply *B. bacteriovorus* in combination with solar-CPC reactors and solar-CPC treatment with photocatalysis to disinfect rainwater. *Klebsiella pneumoniae* S1 43 (isolated from solar pasteurized rainwater at a treatment temperature above 70°C) (Clements et al., 2019) and *Enterococcus faecium* 8D (isolated from untreated harvested rainwater) (Dobrowsky et al., 2014) were included as test organisms.

2. Materials and Methods

2.1 Coating of Raschig Rings

The design and construction of the CPC is outlined in the Supplementary Information. The TiO_2-rGO composite was synthesised using graphene oxide (GO; Nanoinnova, Spain) and TiO_2 P25 (Aeroxide P25, Evonik, Germany) as previously described by Fernández-Ibáñez et al. (2015). The TiO_2-rGO was immobilised on borosilicate glass raschig rings [5 mm (length) × 5 mm (outer diameter) × 1 mm (glass thickness); Sigma-Aldrich, Germany] for application in the designed small-scale solar-CPC systems. The raschig rings were cleaned as described by Cunha et al. (2018). The TiO_2-rGO (1.5 g) was added to 100 mL absolute methanol to obtain a final concentration of 1.5% w/v. The suspension was sonicated for 15 min and the raschig rings were submerged in the suspension. To evaporate the methanol, the rings in the suspension were added to a rotary evaporator (Heidolph Instruments, Schwabach GmbH, Germany) with the water bath temperature set to 65°C and the rotary speed set

to 120 rpm. Once the methanol was evaporated, the coated raschig rings were dried at 80°C for 90 min and annealed at 400°C for 2 h (with a heating rate of 2°C per min) in air (Cunha et al., 2018). The rings were weighed before and after the coating, and it was determined that the loading of TiO_2-rGO was ca. 0.89 mg/cm^2.

2.2 Solar Treatment Experiments

2.2.1 Prey Bacterial Strains

Klebsiella pneumoniae S1 43 and *E. faecium* 8D were obtained from the Water Resource Laboratory Culture Collection at Stellenbosch University (Department of Microbiology). These bacteria were inoculated into 500 mL Luria Bertani (LB) broth (Biolab, Merck, South Africa) and were incubated at 37°C for 24 to 48 h with shaking at 200 rpm. The bacterial cells were harvested by centrifugation at 11 305 x *g* for 15 min. The bacterial biomass was washed and re-suspended in phosphate buffered saline (PBS) and the optical density (OD) of the re-suspended pellets was measured using the T60 UV-Visible Spectrophotometer (PG Instruments Limited, Thermo Fisher Scientific, South Africa) at 600 nm (OD_{600}). The concentration of the bacterial cells was adjusted with PBS to obtain a final OD_{600} of 1.00 (which corresponded to approximately 10^9 cells/mL) (Feng et al., 2016).

2.2.2 Preparation of the Predatory Bacteria Stock Lysate

Bdellovibrio bacteriovorus PF13 was isolated from wastewater collected from the influent point of the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: 33° 59' 21.13"S 18° 47' 47.75"E) as described by Waso et al. (2019). The predatory bacterium was stored as plaques on double-layer agar plates, with *Pseudomonas fluorescens* ATCC 13525 used as prey cells at 4°C until further experimentation commenced (Dashiff et al., 2011). To apply *B. bacteriovorus* PF13 as a pre-treatment to SODIS, a predator stock lysate (used as the predator inoculum in the pre-treatment experiments) was prepared as described by Dashiff et al. (2011) in the presence of *P. fluorescens* ATCC 13525 as prey cells (Supplementary Information).

2.2.3 Experimental Set Up

Synthetic rainwater was used to ensure that the composition of the medium remained constant throughout the study and was prepared by the method reported by Jones and Edwards (1993). For each test organism (*K. pneumoniae* S1 43 and *E. faecium* 8D), two experimental groups were analysed as follows: for one experimental group (two systems) the test organisms were pre-treated with *B. bacteriovorus*; while for the second experimental group (two systems) no pre-treatment occurred (Fig. 1). Additionally, for each experimental group, one solar-CPC system contained TiO_2-rGO coated raschig rings, while the second system contained uncoated raschig rings (solar disinfection only) (Fig. 1). For the pre-treated samples, 800 mL of synthetic rainwater was seeded with 100 mL of *K. pneumoniae* or *E. faecium* (OD_{600} = 1.00) (section 2.2.1). Subsequently, each sample was inoculated with 100 mL of the *B. bacteriovorus* stock lysate (OD_{600} < 0.2). The co-culture was incubated for 72 h at 30°C with shaking at 200 rpm to allow for the predation of *B. bacteriovorus* on the respective prey cells. For the samples which were not subjected to *B. bacteriovorus* pre-treatment, 900 mL of synthetic rainwater was seeded with 100 mL of *K. pneumoniae* or *E. faecium* cells (OD_{600} = 1.00) (section 2.2.1) (Fig. 1) on the day of solar treatment.

The four solar-CPC reactors were filled with approximately 390 mL of the pre-treated or untreated seeded synthetic rainwater samples and were exposed to natural sunlight for 4 h (Fig. 1). The remaining volume of each sample was kept in the dark and served as dark controls (Fig. 1). Samples (10 mL) were collected from each solar-CPC system at 0, 30, 60, 90, 120, 150, 180, 210, and 240 min. For each of the collected samples, the pH, temperature, total dissolved solids (TDS), and electrical conductivity (EC) were measured with a hand-held Milwaukee Instruments MI806 meter (Spraytech, South Africa), and the dissolved oxygen (DO) was measured using a Milwaukee Instruments M600 meter (Spraytech). The solar irradiance data [maximum UV-A and UV-B radiation and the maximum direct normal irradiance (DNI)] were obtained from the Stellenbosch Weather Services [Stellenbosch University, Faculty of Engineering (http:// weather.sun.ac.za/)], and the ambient temperature data were obtained from the South African Weather Services (Supplementary Information Tables A.1 and A.2). The results for the conductivity, temperature, pH, TDS, and DO collected for the different solar treatment time points (0 to 240 min) for each test organism and

178 experimental design, are summarised in the Supplementary Information Tables A.1 and A.2.
179 Throughout the text the term “solar disinfection” will refer to solar treatment using only the designed
180 solar-CPC system (with uncoated raschig rings), while “solar photocatalytic disinfection” or “solar
181 photocatalysis” will refer to the solar treatment using the designed solar-CPC system in combination
182 with the immobilised TiO_2-rGO. Furthermore, “solar treatment” will be used to refer simultaneously
183 to both disinfection strategies.

184 **2.2.3.1 Culture-based Analysis**

185 To enumerate the *K. pneumoniae* and *E. faecium* cells during the solar treatments [in colony forming
186 units per mL (CFU/mL)], samples (10 mL) were collected as described in section 2.2.3. In addition,
187 for the samples subjected to *B. bacteriovorus* pre-treatment, 10 mL samples were collected before
188 (0 h) and after pre-treatment (72 h). A further 10 mL sample was collected from each of the dark
189 control samples after 240 min (to confirm that the changes in viable organisms occurred as a result
190 of solar or solar photocatalytic disinfection). A 10-fold serial dilution was prepared (ranging from
191 undiluted to 10^{-6}) for each sample (n = 40), and 100 µL of each dilution was spread plated onto LB
192 agar in triplicate. The plates were incubated at 30°C for 12 to 18 h (overnight).

193 In order to verify that the solar treatment effectively removed the predatory bacteria from the pre-
194 treated samples, double-layer agar overlays (as described by Yu et al., 2017) were also prepared
195 using the serial dilutions from the *B. bacteriovorus* pre-treated samples. The plates were incubated
196 at 30°C for up to 7 days and the predatory bacteria were enumerated in plaque forming units per mL
197 (PFU/mL).

198 **2.2.3.2 Molecular Analysis**

199 For the molecular analysis of the solar-CPC samples collected at each time point (0 to 240 min) as
200 well as the samples collected before (0 h) and after (72 h) *B. bacteriovorus* pre-treatment, 500 µL of
201 each sample was EMA treated as described by Reyneke et al. (2016). The EMA-treated aliquots
202 were subjected to DNA extractions using the *Quick*-DNA™ Fecal/Soil Microbe Miniprep kit (Zymo
203 Research, Inqaba Biotech, South Africa) as per the manufacturer’s instructions.

Quantitative real-time PCR was subsequently performed to quantify the gene copies (GC) of *B. bacteriovorus*, *K. pneumoniae* and *E. faecium* during the various solar treatments. All qPCR assays were performed using the LightCycler® 96 Instrument (Roche Diagnostics, Mannheim, Germany) and the FastStart Essential DNA Green Master (Roche Diagnostics). All the qPCR primers and cycling parameters are outlined in Table 1, while the qPCR mixture as described by Waso et al. (2018) was utilised. Additionally, the standard curves utilised for GC quantification in the qPCR assays were generated as described by Waso et al. (2019), using conventional PCR and the cycling parameters defined in Table 1.

All the qPCR results were analysed using the Roche LightCycler® 96 Software Version 1.1 and Microsoft Excel 2016. In addition, the lower limit of detection (LLOD) for each qPCR assay was determined as the lowest concentration (GC/µL) consistently detected in the standard curve samples. Furthermore, the lower limit of quantification (LLOQ) for each qPCR sample was determined as the lowest number of GC/µL that could reliably be quantified in the standard curve samples. All GC numbers were converted to GC/mL using the following modified equation (which excludes compensation for sample filtration) (Eq. 1) as described by Rajal et al. (2007):

$$\left(\frac{\text{mL Original Sample}}{\text{mL DNA eluted}}\right) \times (\text{mL used per qPCR assay}) = \text{mL original sample per qPCR assay} \quad \ldots\ldots\ldots\ldots (1)$$

2.3 Data Analysis

All graphs were generated using GraphPad Prism 7.04 (2018). Two-way Analysis of Variance (ANOVA) for Multiple Comparisons with Dunnett's tests (alpha value of 0.05) was utilised to determine whether the concentration of the prey bacteria (*K. pneumoniae* and *E. faecium*) and *B. bacteriovorus* changed significantly during the various solar treatments. Significance was observed at $p < 0.05$.

3. Results

3.1 Impacts of Different Disinfection Strategies on the Survival of *Klebsiella pneumoniae*

For the *B. bacteriovorus* pre-treated samples, the CFU of *K. pneumoniae* were reduced by 1.92 logs during the 72-h pre-treatment, from 2.00×10^9 (before predation) to 2.40×10^7 CFU/mL. The PFU

of *B. bacteriovorus* correspondingly increased by 0.202 logs from 6.53×10^5 PFU/mL (before predation) to 1.04×10^6 PFU/mL. Additionally, EMA-qPCR analysis (characteristics summarised in Table A.3) confirmed that the concentration of *K. pneumoniae* was reduced after predation as the GC of *K. pneumoniae* decreased by 3.51 logs from 2.95×10^8 (before predation) to 9.20×10^4 GC/mL, while the concentration of *B. bacteriovorus* increased by 0.430 logs from 7.96×10^3 (before predation) to 2.14×10^4 GC/mL. Overall, for the dark controls, the plate counts indicated that the concentration of *K. pneumoniae* remained relatively constant with an average of 1.97×10^7 CFU/mL and 7.50×10^8 CFU/mL recorded (after 240 min) for the 72-h *B. bacteriovorus* pre-treated sample and non-pre-treated sample, respectively.

For the *K. pneumoniae* pre-treated sample subsequently exposed to solar photocatalytic treatment, culture-based enumeration indicated that within 120 min the *K. pneumoniae* cell counts were reduced by 7.38 logs from 2.40×10^7 CFU/mL (at 0 min) to BDL (<1 CFU/100 µL) ($p < 0.0001$) (Fig. 2A; Table 2). Thus, considering the reduction in CFU/mL recorded after the *B. bacteriovorus* pre-treatment as well as after the solar photocatalytic treatment, the CFU counts of *K. pneumoniae* were reduced by a total of 9.30 logs ($p < 0.0001$) from the initial concentration of 2.00×10^9 CFU/mL (Table 2). Correspondingly, EMA-qPCR analysis indicated that in total a 5.85 log reduction in the *K. pneumoniae* GC was obtained [from 2.95×10^8 GC/mL (before predation) to 4.19×10^2 GC/mL (after 240 min of solar exposure)] ($p < 0.0001$) (Fig. 2B; Table 2). The culture-based enumeration of the *B. bacteriovorus* cells analysed indicated that in the samples exposed to solar photocatalytic treatment, the PFU of *B. bacteriovorus* was reduced by 6.02 logs from 1.04×10^6 PFU/mL (at 0 min) to BDL ($p < 0.0001$) within 120 min (Fig. A.8A). Similarly, the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus* was reduced by 2.59 logs ($p < 0.0001$) after solar exposure from an initial concentration of 2.14×10^4 GC/mL to 5.49×10^1 GC/mL after 240 min (Fig. A.8B).

For the sample pre-treated with *B. bacteriovorus* and subsequently exposed to solar disinfection, the cell counts of *K. pneumoniae* were reduced by 7.38 logs from 2.40×10^7 CFU/mL (at 0 min) to BDL ($p < 0.0001$), after 240 min of solar exposure (Fig. 2A; Table 2). Thus, the cell counts of *K. pneumoniae* were also reduced by a total of 9.30 logs ($p < 0.0001$) from the initial concentration of 2.00×10^9 CFU/mL (Table 2). In addition, the EMA-qPCR analysis indicated that overall the

K. pneumoniae concentration was reduced by 5.41 logs from 2.95×10^8 GC/mL (before predation) to 1.14×10^3 GC/mL (after 240 min of solar exposure) ($p < 0.0001$) (Fig. 2B; Table 2). The *B. bacteriovorus* was reduced by 6.02 logs from 1.04×10^6 PFU/mL (at 0 min) to BDL ($p < 0.0001$) within 150 min in the samples exposed to solar disinfection (Fig. A.8A). Accordingly, the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus* was reduced by 2.28 logs ($p < 0.0001$) after solar exposure, from an initial concentration of 2.14×10^4 GC/mL to 1.12×10^2 GC/mL (Fig. A.8B).

In comparison, the cell counts of *K. pneumoniae* in the non-pre-treated sample exposed to solar photocatalytic treatment were reduced by a total of 6.34 logs after 240 min (from 7.33×10^8 CFU/mL to 3.33×10^2 CFU/mL) ($p < 0.0001$) (Fig. 2A; Table 2), while the molecular analysis indicated that the GC of *K. pneumoniae* in this sample were reduced by a total of 2.67 logs [from 6.41×10^7 GC/mL (initial concentration 0 min) to 1.39×10^5 GC/mL (after 240 min of solar exposure)] ($p < 0.0001$) (Fig. 2B; Table 2). Furthermore, for the non-pre-treated sample exposed to only solar disinfection, the cell counts of *K. pneumoniae* were reduced by 8.87 logs from 7.33×10^8 CFU/mL (at 0 min) to BDL within 210 min ($p < 0.0001$) (Fig. 2A; Table 2). The EMA-qPCR analysis confirmed a reduction in the concentration of the *K. pneumoniae* cells as the GC were reduced by 3.46 logs [from 6.41×10^7 GC/mL (initial concentration 0 min) to 2.24×10^4 GC/mL (after 240 min of solar exposure)] ($p < 0.0001$) in this sample (Table 2).

3.2 Impacts of Different Disinfection Strategies on the Survival of *Enterococcus faecium*

For the *B. bacteriovorus* pre-treated samples, the culture-based enumeration indicated that the *E. faecium* cell counts were reduced by 0.598 logs from 3.57×10^9 (before predation) to 9.00×10^8 CFU/mL (after 72 h of predation). The EMA-qPCR analysis then confirmed that the concentration of *E. faecium* was reduced after 72 h of predation from 8.24×10^5 GC/mL (before predation) to 1.60×10^5 GC/mL with a log reduction of 0.712 recorded. While, *B. bacteriovorus* did not produce any plaques on the double-layer agar overlays when *E. faecium* was utilised as prey, the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus* decreased by 0.167 logs from 1.08×10^4 (before predation) to 7.34×10^3 GC/mL (after 72 h of predation). Overall, for

285 the dark controls, the plate counts indicated that the concentration of *E. faecium* remained relatively
286 constant with an average of 4.17×10^8 CFU/mL and 1.63×10^8 CFU/mL recorded (after 240 min) for
287 the 72-h *B. bacteriovorus* pre-treated sample and non-pre-treated sample, respectively.

288 Subsequently, the cell counts of *E. faecium* recorded for the pre-treated sample exposed to solar
289 photocatalysis were reduced by 3.81 logs from 9.00×10^8 CFU/mL (at 0 min) to 1.40×10^5 CFU/mL
290 (at 240 min) ($p < 0.0001$) (Fig. 3A). Thus, the CFU/mL of *E. faecium* was reduced by a total of
291 4.41 logs from an initial concentration of 3.57×10^9 CFU/mL (Table 2). The EMA-qPCR analysis
292 then indicated that the *E. faecium* concentration was reduced by 1.57 logs from 1.60×10^5 GC/mL
293 (at 0 min) to 4.35×10^3 GC/mL (at 240 min) (Fig. 3B), with an overall reduction of 2.28 logs recorded
294 from an initial concentration of 8.24×10^5 GC/mL (Table 2). As mentioned previously,
295 *B. bacteriovorus* did not produce any plaques on the double-layer agar overlays when *E. faecium*
296 was utilised as prey and the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus*
297 was reduced from an initial concentration of 7.34×10^3 GC/mL (at 0 min) to 8.13×10^2 GC/mL (0.956
298 log reduction; $p < 0.0001$) after 240 min of solar photocatalysis (Fig. A.9).

299 The cell counts of *E. faecium* recorded for the pre-treated sample exposed to solar disinfection, were
300 reduced by 6.73 logs from 9.00×10^8 CFU/mL (at 0 min) to 1.67×10^2 CFU/mL (at 240 min)
301 ($p < 0.0001$) (Fig. 3A; Table 2). Therefore, an overall log reduction of 7.33 in *E. faecium* CFU/mL
302 was recorded after the *B. bacteriovorus* pre-treatment and solar disinfection (Table 2). The EMA-
303 qPCR analysis indicated that the concentration of *E. faecium* was reduced by 2.09 logs from
304 1.60×10^5 GC/mL (at 0 min) to 1.29×10^3 GC/mL (at 240 min) ($p < 0.0001$) (Fig. 3B). Overall, the
305 concentration of *E. faecium* was thus reduced by a total of 2.81 logs in the pre-treated sample
306 exposed to solar disinfection, from an initial concentration of 8.24×10^5 GC/mL (Table 2). For the
307 predatory bacteria, the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus* was
308 reduced from an initial concentration of 7.34×10^3 GC/mL (at 0 min) to 8.95×10^2 GC/mL (0.914 log
309 reduction; $p < 0.0001$) after 240 min of solar exposure (Fig. A.9).

310 For the samples which were not pre-treated with *B. bacteriovorus* but exposed to solar disinfection
311 and solar photocatalytic treatment, the culture-based enumeration of *E. faecium* indicated that for

both treatment methods, the cell counts were reduced by 8.00 logs from an initial concentration of 1.00×10^8 CFU/mL to BDL (<1 CFU/100 µL) within 210 min of solar exposure ($p < 0.0001$) (Fig. 3A; Table 2). The EMA-qPCR analysis then indicated that during solar disinfection the concentration of *E. faecium* was reduced by 3.39 logs from 2.09×10^6 GC/mL (at 0 min) to 8.53×10^2 GC/mL (at 240 min) ($p < 0.0001$) (Fig. 3B; Table 2). Similarly, for the sample exposed to solar photocatalytic treatment, the concentration of *E. faecium* was reduced by 3.38 logs from 2.09×10^6 GC/mL (at 0 min) to 8.74×10^2 GC/mL (at 240 min) ($p < 0.0001$) (Fig. 3B; Table 2).

4. Discussion

While disinfection methods are effective in significantly reducing the concentration of microbial contaminants in water sources, various pathogens and opportunistic pathogens employ survival strategies and persist after treatment (Strauss et al., 2018; Clements et al., 2019). It was thus proposed in the current study that a combination of physical, chemical and biological treatments, could prove effective in eliminating disinfection resistant species. *Bdellovibrio bacteriovorus* is known to attach to the cell wall of Gram-negative prey, such as *K. pneumoniae*, through an unknown mechanism or receptor, whereafter the predator rotates to create a pore in the prey cell wall and enters the prey cell's periplasmic space forming a structure called the bdelloplast (Sockett, 2009). Once the predator has invaded the prey cell, it secretes various hydrolytic enzymes to break down the prey cell's constituents and produce progeny (Sockett, 2009). Correspondingly, as *K. pneumoniae* is sensitive to predation, the pre-treatment with *B. bacteriovorus* aided in effectively reducing the concentration of this organism in the seeded water samples. Furthermore, the addition of the photocatalytic material enhanced the disinfection efficiency as the treatment time required to reduce the *K. pneumoniae* CFU to BDL was decreased from 240 min (solar disinfection) to 120 min (solar photocatalysis). Under solar UV-visible exposure, the TiO_2-rGO composite photocatalytic material produces reactive oxygen species (ROS), which significantly disrupts the cell membrane structures and damages DNA and RNA, ultimately leading to cell death (Byrne et al., 2011).

We previously investigated the mechanisms behind the antimicrobial activity of TiO_2-rGO in water using *E. coli* as the model organism (Fernández-Ibáñez et al., 2015; Cruz-Ortiz et al., 2017).

Fernández-Ibáñez et al. (2015) reported that *E. coli* was reduced by 6 logs (within 10 min, less than 2 J/cm^2) under natural sunlight with a photocatalyst loading of 500 mg/L. Probes were used to investigate the primary ROS produced during the disinfection experiments and we found that under UV-visible light, hydrogen peroxide, hydroxyl radicals and singlet oxygen were mainly responsible for the reduction in *E. coli* concentrations. Under visible light irradiation, only singlet oxygen was produced which resulted in the reduction of the *E. coli* concentration (Fernández-Ibáñez et al., 2015; Cruz-Ortiz et al., 2017). Lin et al. (2014) investigated the cytotoxic effects of UV excited TiO_2 on Gram-negative bacteria by also employing *E. coli* as the test organism. With the use of transmission electron microscopy, the authors found that the TiO_2 nanoparticles attached to the outside of the *E. coli* cells, while some microbial cells were also observed to contain internalised nanoparticles. It was concluded that the nanoparticles attached to the cell surface, induced cell distortion, plasmolysis and extensive cell wall and membrane damage. In addition, the authors hypothesised that the attachment of the nanoparticles to the cells resulted in decreased movement of substances into and out of the bacterial cells, ultimately resulting in homeostatic imbalances and cellular metabolic disturbances, which would eventually result in cell death (Lin et al., 2014).

However, while Lin et al. (2014) evaluated the photocatalyst in suspension, in the current study, the photocatalyst was immobilised onto glass raschig rings and exposed to real solar irradiation. Sordo et al. (2010) compared the use of TiO_2 in suspension to TiO_2 immobilised onto a glass tube (used as the reactor vessel) and raschig rings for the disinfection of *E. coli* in a recirculating solar treatment system. The authors found that the disinfection of *E. coli* in the reactor with the TiO_2 coated raschig rings, was comparable to the disinfection obtained in the reactor with TiO_2 in suspension, while disinfection efficiency was not enhanced in the glass tube reactor vessel coated with the photocatalyst. It was hypothesised that the high disinfection efficiency obtained with the raschig ring immobilised photocatalyst was due to the greater contact area generated, which increased exposure of the *E. coli* cells to hydroxyl radicals produced during the photocatalytic process. However, the authors also noted that the flow rate generated in the recirculating system containing the raschig rings, greatly enhanced the disinfection efficiency of the reactor as strong mechanical stress was exerted on the bacterial cells (Sordo et al., 2010). The use of raschig rings as support materials for

367 the immobilisation of photocatalysts is thus advantageous as post-treatment removal of the material
368 is not required. In addition, immobilising the photocatalyst creates a greater contact area which may
369 increase the exposure of the cells to the photocatalytic material. Furthermore, if a flow rate is applied,
370 mechanical stress is exerted on the cells.

371 Apart from using photocatalytic material in two of the solar-CPC reactors in the current study, all the
372 water samples (pre-treated with *B. bacteriovorus* and non-pre-treated) were exposed to solar
373 treatment under CPC concentrated solar UV-A radiation. The CPC mirrors were used for the solar
374 treatment reactors as it significantly enhances any kind of solar water treatment by improving the
375 solar UV energy income by a concentration factor of 1 (Keane et al., 2014). Navntoft et al. (2008)
376 demonstrated that the use of a CPC accelerated the reduction of 6-log *E. coli* K12 under solar
377 disinfection by 90 minutes as compared to a PET plastic bottle. Based on the solar UV-A dose
378 calculated for the *K. pneumoniae* trials (Supplementary Information), a similar UV-A dose was
379 obtained within 120 min of solar exposure (25.83 J cm^{-2}), to the dose reported in literature (27 J/cm^2)
380 to achieve a 5-log reduction in *E. coli* K12 by solar disinfection in a 2 L-PET bottle filled with clear
381 transparent water (Castro-Alférez et al., 2018). Additionally, the dose obtained in the current study
382 was 10 times higher than the 1.8 J/cm^2 (10 min at 30 W/m^2 of solar UV-A) required to achieve a 6-
383 log reduction of *E. coli* K 12 using the same catalyst (TiO_2-rGO) suspended as a slurry at a
384 concentration of 500 mg/L (Fernández-Ibáñez et al., 2015). Similar solar dose values were obtained
385 for the *E. faecium* trial. Thus, sufficient solar irradiation was obtained to reduce the concentration of
386 *K. pneumoniae* and *E. faecium* during the current study.

387 Correspondingly, the most efficient treatment strategy for the reduction of *E. faecium* was the use of
388 solar disinfection or solar photocatalytic disinfection without *B. bacteriovorus* pre-treatment. While it
389 is generally theorised that *B. bacteriovorus* does not prey on Gram-positive bacteria, studies have
390 indicated that this predator can prey on *Staphylococcus aureus* (Iebba et al., 2014; Pantanella et al.,
391 2018). The lytic enzymes produced by *B. bacteriovorus* have also been shown to disrupt biofilms
392 produced by Gram-positive bacteria, while proteases produced by *B. bacteriovorus* can decrease
393 the efficiency of *S. aureus* invasion into human epithelial cells (Monnappa et al., 2014). Furthermore,
394 using culture-based methods and EMA-qPCR, we have recently reported that *B. bacteriovorus* PF13

can reduce the concentration of *S. aureus* and *E. faecium* in co-culture experiments (Waso et al., 2019). Thus, while it is warranted to investigate the effect of *B. bacteriovorus* pre-treatment on the disinfection of Gram-positive bacteria, in this study pre-treatment with *B. bacteriovorus* PF13 did not significantly reduce the concentration of *E. faecium*.

Based on the results obtained for the *E. faecium* trials, the addition of the photocatalyst also did not significantly enhance the disinfection efficiency. Gutiérrez-Alfaro et al. (2015) compared three systems to disinfect potable water inoculated with wastewater containing *E. coli*, *Enterococcus* spp. and *Clostridium perfringens*: a 2 L PET bottle; a 2 L PET bottle with an internal cylinder coated with TiO_2 doped with zinc; and a glass reactor (9 L) with a TiO_2 coated inner cylinder. In all the systems analysed, *E. coli* was readily reduced to BDL, while *Enterococcus* spp. and *C. perfringens* were more resistant to disinfection. In addition, the immobilised photocatalyst used in the 2 L PET bottles only enhanced the disinfection efficiency of the SODIS bottles by 0.43 logs for *E. coli*, 0.45 logs for *Enterococcus* spp. and 0.28 logs for *C. perfringens* under natural sunlight (Gutiérrez-Alfaro et al., 2015). The authors ultimately concluded that Gram-positive bacteria, which have more complex cell walls, are more resistant to disinfection in comparison to Gram-negative bacteria. However, they found that recirculating the water in the solar photocatalytic systems, increased turbulence and contact between the catalyst and the bacteria, significantly enhancing the disinfection efficiency, especially for Gram-positive bacteria (Gutiérrez-Alfaro et al., 2015). Veneiri et al. (2014) also investigated the disinfection of *Enterococcus faecalis* using TiO_2 P25 (200 mg/L to 1500 mg/L) and SODIS under simulated sunlight, using culture-based methods and qPCR. The culturing results indicated that at the highest TiO_2 concentration (1500 mg/L), *E. faecalis* was reduced by 7 logs to BDL after approximately 40 min of treatment. Similarly, while qPCR analysis indicated that a 7-log reduction in the GC of *E. faecalis* was obtained after 120 min of treatment, the GC were not reduced to BDL in any of the treated samples. The authors concluded that viable but non-culturable (VBNC) *E. faecalis* cells were still present in the treated samples and that the SODIS treatment time should be extended in order to eradicate *E. faecalis* (Veneiri et al., 2014).

Similarly, in the current study, for all the treatment combinations analysed, EMA-qPCR results indicated that the GC of *K. pneumoniae* and *E. faecium* were not reduced to BDL, signifying that

VBNC cells may still have persisted. While numerous research groups have detected *Klebsiella* spp. in untreated harvested rainwater (De Kwaadsteniet et al., 2013), the *K. pneumoniae* strain (S1 43) employed in the current study was isolated from SOPAS rainwater at a treatment temperature above 70°C (Clements et al., 2019). The thermal tolerance of *Klebsiella* spp. has been associated with the expression of heat shock proteins or can be acquired through plasmids encoding for ClpK ATPase (Bojer et al., 2011). Moreover, *K. pneumoniae* have prominent capsules which have been hypothesised to protect this organism from bactericidal stressors such as UV irradiation and antibiotic agents (Veneiri et al., 2017; Dorman et al., 2018). In contrast, the *E. faecium* strain (8D) employed was isolated from untreated harvested rainwater (Dobrowsky et al., 2014). *Enterococcus* spp. are known to tolerate a wide range of environmental conditions and they have been found to exhibit increased resistance to UV disinfection (McKinney & Pruden, 2012; Maraccini et al., 2012). Some strains of enterococci have been found to possess intracellular carotenoids which may act as quenchers of intracellularly produced ROS upon exposure to sunlight, ultimately protecting the cell from increasing oxidative stress and providing *Enterococcus* spp. with a competitive advantage against sunlight-induced inactivation (Maraccini et al., 2012). Gram-negative and Gram-positive bacteria also possess DNA repair mechanisms, which can repair damage induced by UV irradiation, and allow bacterial cells to persist and survive after UV disinfection (McGuigan et al., 2012). Thus, while the molecular analysis results obtained in the current study indicated that significant reductions ($p < 0.0001$) in GC were recorded (Fig. 2B and 3B), further work may include extending the solar disinfection and solar photocatalytic treatment time.

5. Conclusions

Based on the results obtained, *B. bacteriovorus* may be applied to decrease the concentration of Gram-negative bacteria, such as *K. pneumoniae*, prior to solar disinfection. This is crucial as many pathogenic Gram-negative bacteria have been found to persist after the implementation of various disinfection strategies. Solar disinfection or solar photocatalytic treatment successfully reduced the concentration of *E. faecium* and it is likely that forced convection in a solar photocatalytic system may further enhance the effect of the photocatalytic material on the disinfection of Gram-positive bacteria.

Furthermore, as hydroxyl radicals produced during photocatalysis significantly disrupts the cell membrane of bacteria (Polo-López et al., 2017), the use of EMA-qPCR is recommended to supplement culture-based analysis and should therefore be included in future studies monitoring such water treatment systems. As natural water sources will contain mixed bacterial communities, future research should investigate the effect of predatory bacteria pre-treatment on mixed bacterial communities in natural water sources, to assess the overall effect of *B. bacteriovorus* pre-treatment.

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476 **Declaration of Interest**

477 The authors declare no conflict of interest.

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