Human Health Risks Associated with Harvested Rainwater: Implementation of Biocontrol Strategies

by

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DECLARATION

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

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

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SUMMARY

Rainwater harvesting has been earmarked as an additional fresh water source, which could be utilised to supplement municipal water supplies, especially in water scarce regions. However, various studies have indicated that the microbial quality of this water source is substandard. These microbial contaminants may pose a significant health risk to end-users and it is recommended that treatment systems are implemented to reduce the level of contamination in rainwater. Solar disinfection (SODIS) has been identified as an easy-to-use and cost-effective strategy that could be used to disinfect water. A minimum of 6 hours solar exposure is generally required for effective disinfection of water and photocatalytic nanomaterials such as titanium dioxide (TiO_2) have subsequently been employed to improve SODIS efficiency by decreasing the treatment time. Research has however, indicated that while SODIS is effective in significantly reducing the concentration of microbial contaminants in water sources, various pathogens and opportunistic pathogens employ survival strategies and persist after treatment. A combination of physical, chemical and biological treatments, which target these persistent organisms directly, should therefore be investigated.

For the purpose of this dissertation, the use of *Bdellovibrio bacteriovorus* (*B. bacteriovorus*), a Gram-negative predatory bacterium, was investigated. The primary aim of **Chapter 2** (published in Microbiological Research, 2019) was thus to isolate *B. bacteriovorus* from wastewater and investigate the interaction of this predator with Gram-negative and Gram-positive prey using culture-based (spread plating and double-layer agar overlays) and molecular methods [ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR)]. The predation activity of *B. bacteriovorus* on the different prey cells was assessed and compared in a nutrient poor [diluted nutrient broth (DNB)] and nutrient deficient medium (HEPES buffer). A *B. bacteriovorus* isolate (PF13) was subsequently co-cultured with *Pseudomonas fluorescens* (*P. fluorescens*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecium* (*E. faecium*). Results indicated that *P. fluorescens* (maximum log reduction of 4.21) and *K. pneumoniae* (maximum log reduction of 5.13) were sensitive to predation in DNB and HEPES buffer, while *E. faecium* (maximum log reduction of 2.71) was sensitive to predation in DNB and *S. aureus* (maximum log reduction of 1.80) was sensitive to predation in HEPES buffer. Predation of Gram-positive prey by *B. bacteriovorus* was thus dependent on the specific prey cells used and the media employed to assess these interactions. In contrast, for *P. aeruginosa*, while the culture-based analysis indicated that the cell counts were reduced, the EMA-qPCR analysis indicated that the concentration of *P. aeruginosa* was not significantly reduced in DNB or HEPES buffer. The use of EMA-qPCR can thus aid in accurately monitoring and quantifying both predator and prey cells during co-culture experiments in a time-effective manner.

The aim of **Chapter 3** (published in Water Research, 2020) was to subsequently apply *B. bacteriovorus* PF13 as a pre-treatment to SODIS and solar photocatalytic disinfection. The photocatalyst used was immobilised titanium-dioxide reduced graphene oxide (TiO_2-rGO). Synthetic rainwater was seeded with *K. pneumoniae* and *E. faecium*, with results indicating that the use of

B. bacteriovorus pre-treatment in combination with solar photocatalysis resulted in the greatest reduction in *K. pneumoniae* concentrations in the shortest treatment time, with the cell counts reduced by 9.30 logs to below the detection limit (BDL) within 120 min. In contrast, for *E. faecium* the most effective treatment was solar photocatalysis or SODIS without the *B. bacteriovorus* pre-treatment, as the viable counts of *E. faecium* were reduced by 8.00 logs to BDL (within 210 min) and the gene copies were reduced by ~3.39 logs after 240 min. It was thus evident that the application of *B. bacteriovorus* may specifically enhance the disinfection of Gram-negative bacteria. Additionally, the use of the photocatalyst further enhanced the disinfection of the Gram-negative bacteria, while the same trend was not observed for *E. faecium*. Recirculating the water in solar photocatalytic reactors may, however, enhance disinfection of Gram-positive bacteria, by exerting mechano-osmotic stress on the cells and should be investigated in future research.

As conflicting results regarding the interaction between *B. bacteriovorus* and Gram-positive bacteria have been reported, the aim of **Chapter 4** (published in Microbiological Research, 2020) was to monitor and compare the expression of attack phase (AP) and growth phase (GP) genes of *B. bacteriovorus* in co-culture with Gram-positive and Gram-negative prey. *Bdellovibrio bacteriovorus* PF13 was thus co-cultured with *Escherichia coli* (*E. coli*; control), *K. pneumoniae* and *E. faecium*. Relative qPCR analysis indicated that the AP genes *bd0108* (type IVa pili retraction/extrusion) and *merRNA* (massively expressed riboswitch RNA) were highly expressed in the *B. bacteriovorus* AP cells, whereafter expression in co-culture with all the prey strains was reduced. The *fliC1* gene (flagellar filament) was also expressed at a high level in the AP cells, however, after 240 min of co-culture with *E. faecium* the expression of *fliC1* remained low (at 0.759-fold), while in the presence of the Gram-negative prey, *fliC1* expression increased (in comparison to the expression recorded after 30 min) to 4.62 (*E. coli*) and 2.69-fold (*K. pneumoniae*). In addition, *bd0816* (peptidoglycan-modifying enzyme) and *groES1* (chaperone protein) were not induced in the presence of *E. faecium*, however, after exposure to the Gram-negative prey, *bd0816* expression increased during the early GP, while *groES1* expression gradually increased during the early GP and GP. It was thus concluded that *B. bacteriovorus* senses the presence of potential prey when exposed to Gram-positive and Gram-negative prey however, the GP genes were not induced when *B. bacteriovorus* was co-cultured with *E. faecium*. This indicates that *B. bacteriovorus* may not actively grow in the presence of *E. faecium* and the second predatory cue (which induces active growth of *B. bacteriovorus*) may be lacking under the conditions employed in this study. Limited information on the expression of predatory-specific genes of *B. bacteriovorus* in co-culture with Gram-positive prey cells is available. Recent studies have however, indicated that *B. bacteriovorus* can prey on Gram-positive bacteria and investigating the expression of these predatory-specific genes may elucidate the genetic mechanisms this predator employs to survive in the presence of these atypical prey.

OPSOMMING

Geoeste reënwater is geïdentifiseer as 'n addisionele varswater bron wat gebruik kan word om munisipale water gebruik aan te vul, veral in areas waar water skaars is. Verskeie studies het egter gewys dat die mikrobiese kwaliteit van hierdie water nie op standaard is nie. Hierdie mikrobes kan 'n beduidende gesondheidsrisiko vir verbruikers inhou en daarom moet water behandeling sisteme geïmplementeer word om die vlakke van hierdie mikroörganismes te verlaag. Sonkrag ontsmetting is aangewys as 'n maklike en goedkoop strategie om water te suiwer. Vir effektiewe suiwering, moet die water vir 6 ure aan sonlig blootgestel word en daarom word fotokatalitiese nanomaterial soos titaandioksied (TiO_2) dikwels gebruik om die proses te versnel en sodoende die effektiwiteit van sonkrag ontsmetting te verbeter. Navorsing dui egter daarop dat alhoewel sonkrag ontsmetting mikroörganismes in water verminder, baie patogene en opportunistiese patogene oorlewingsmeganismes gebruik om hierdie tipe behandeling te oorleef. 'n Kombinasie van fisiese, chemiese en biologiese behandelings moet dus ondersoek word om hierdie oorlewende patogene te teiken.

Die gebruik van *Bdellovibrio bacteriovorus* (*B. bacteriovorus*), 'n Gram-negatiwe roofbakterium, is dus vir hierdie dissertasie ondersoek. Die oorhoofse doel van **Hoofstuk 2** (gepubliseer in "Microbiological Research", 2019) was dus om *B. bacteriovorus* uit riool te isoleer en die interaksie tussen hierdie roofbakterium en Gram-negatiewe en Gram-positiewe prooi te ondersoek deur kultuur- (spreiplate en dubbellaag-oorlegsels) en molekulêre metodes [ethidium monoasied kwantitatiewe polimerase ketting reaksie (EMA-kPKR)] te gebruik. Hierdie interaksies is ook in 'n voedingstof-arm medium [verdunde voedingstof boeljon (VVB)] en 'n medium sonder voedingstowwe (HEPES buffer) waargeneem en vergelyk. 'n *B. bacteriovorus* isolaat (PF13) is dus saam met *Pseudomonas fluorescens* (*P. fluorescens*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Staphylococcus aureus* (*S. aureus*) en *Enterococcus faecium* (*E. faecium*) geïnokuleer en toegelaat om te groei. Die resultate het aangedui dat *P. fluorescens* (maksimum log vermindering van 4.21) en *K. pneumoniae* (maksimum log vermindering van 5.13) sensitief was vir predasie in VVB en HEPES buffer, terwyl *E. faecium* (maksimum log vermindering van 2.71) sensitief was vir predasie in VVB en *S. aureus* (maksimum log vermindering van 1.80) sensitief was vir predasie in HEPES buffer. Predasie op die Gram-positiewe bakterieë was dus afhanklik van die spesifieke prooi selle en die medium wat gebruik is om die interaksies te ondersoek. In teenstelling, vir *P. aeruginosa* het die resultate gewys dat die seltellings beduidende verminder is in VVB en HEPES buffer, maar die EMA-kPKR analises het gewys dat die konsentrasie van hierdie organisme nie beduidend afgeneem het nie. Daarom is die gebruik van EMA-kPKR voordelig omdat dit die konsentrasie en lewensvatbaarheid van beide die prooi en roofbakterium kan monitor in tweeledige kulture, op 'n relatiewe vinnige manier.

In **Hoofstuk 3** (gepubliseer in "Water Research", 2020) was die doel om *B. bacteriovorus* PF13 dan te gebruik as 'n voorbehandeling vir sonkrag ontsmetting en fotokatalitiese ontsmetting. Titaandioksied gereduseerde grafeen oksied (TiO_2-rGO) is as die fotokatalis gebruik. Sintetiese

reënwater is met *K. pneumoniae* en *E. faecium* geïnokuleer. Die resultate het aangedui dat die beste kombinasie vir *K. pneumoniae* ontsmetting die fotokatalise met *B. bacteriovorus* voorbehandeling was, aangesien die plaattellings met 9.30 log verminder is tot onder die opsporingslimiet binne 120 min. In teenstelling was die beste behandeling vir *E. faecium* sonkrag ontsmetting of fotokatalitiese ontsmetting sonder die *B. bacteriovorus* voorbehandeling, aangesien die plaattellings verminder is met 8.00 log tot onder die opsporingslimiet (binne 210 min) en die geen kopieë met ~3.39 log verminder is binne 240 min van behandeling. Dit was dus duidelik dat die gebruik van *B. bacteriovorus* die ontsmetting van Gram-negatiewe bakterieë kan verbeter. Die gebruik van die fotokatalis het ook die ontsmetting van die Gram-negatiewe bakterieë verbeter, terwyl dieselfde nie waargeneem is vir *E. faecium* nie. Om die water in die fotokatalitiese behandeling sisteem te sirkuleer mag die ontsmetting van Gram-positiewe bakterieë verbeter deur megano-osmotiese stres op die selle te plaas. Hierdie aspek moet in toekomstige studies ondersoek word.

Teenstrydige resultate aangaande die interaksie tussen *B. bacteriovorus* en Gram-positiewe bakterieë is in die verlede weergegee en daarom was die doel van **Hoofstuk 4** (gepubliseer in “Microbiological Research”, 2020) om die gene wat in die aanvallingsfase (AF) en die groeifase (GF) van *B. bacteriovorus* uitgedruk word, te monitor en te vergelyk terwyl *B. bacteriovorus* aan Gram-positiewe prooi en Gram-negatiewe prooi blootgestel word. *Bdellovibrio bacteriovorus* PF13 is daaropvolgens saam met *Escherichia coli* (*E. coli*; kontrole), *K. pneumoniae* en *E. faecium* in tweeledige kulture geïnokuleer. Relatiewe kPKR analise het daarna aangedui dat die AF gene *bd0108* (tipe IVa pili retraksie) en *merRNA* (massief uitgedrukte riboskakelaar RNA) teen hoë vlakke uitgedruk word in die AF selle, en dat die uitdrukking van hierdie gene daarna afneem in die teenwoordigheid van al die prooi selle. Die *fliC1* geen (filament van die flagella) is ook teen hoë vlakke in die AF selle uitgedruk, maar na 240 min se groei saam met *E. faecium* was die vlak van *fliC1* uitdrukking laag (0.759-voud), terwyl die *fliC1* uitdrukking saam met die Gram-negatiewe bakterieë gestyg het (in vergelykking met die vlakke by 30 min) na 4.62- (*E. coli*) en 2.69-voud (*K. pneumoniae*). Verder is die *bd0816* (peptidoglikaan modifiserende ensiem) en *groES1* (chaperone proteïen) gene nie geïnduseer terwyl *B. bacteriovorus* aan *E. faecium* blootgestel is nie, maar na die roofbakterium aan die Gram-negatiewe bakterieë blootgestel is, het die vlakke van *bd0816* en *groES1* beduidend toegeneem in onderskeidelik die vroeë GF en GF. Hierdie resultate het dus aangedui dat *B. bacteriovorus* waarneem dat moontlike prooi selle naby is wanneer dit blootgestel word aan beide Gram-negatiewe en Gram-positiewe prooi, maar dat die GF gene nie geïnduseer word in die teenwoordigheid van *E. faecium* nie. Dit kan daarop dui dat *B. bacteriovorus* nie kan groei met *E. faecium* as prooi nie en dat die tweede sein (wat aktiewe groei van *B. bacteriovorus* bewerkstellig) afwesig is in hierdie toestande. Beperkte inligting is beskikbaar vir die interaksies tussen *B. bacteriovorus* en Gram-positiewe bakterieë en daarom moet hierdie interaksies op ’n genetiese vlak bestudeer word om vas te stel hoe hierdie roofbakterium oorleef in die teenwoordigheid van hierdie atipiese prooi.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADWG	Australian Drinking Water Guidelines
AOP	Advanced Oxidative Processes
AP	Attack Phase
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BALOs	*Bdellovibrio*-and-Like-Organisms
BDL	Below Detection Limit
CB	Conduction Band
cDNA	Complementary Deoxyribonucleic Acids
CFU	Colony Forming Units
CPC	Compound Parabolic Collector
CSIR	Council for Scientific and Industrial Research
DNA	Deoxyribonucleic Acids
DNB	Diluted Nutrient Broth
DO	Dissolved Oxygen
DWAF	Department of Water Affairs and Forestry
E	Efficiency
EC	Electrical Conductivity
e^-/h^+	Electron/Hole Pairs
EMA	Ethidium Monoazide Bromide
FESEM	Field-Emission Scanning Electron Microscopy
GC	Gene Copies
GP	Growth Phase
HEPES	4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid
LB	Luria Bertani
LLOD	Lower Limit of Detection
LLOQ	Lower Limit of Quantification
NHMRC	National Health and Medical Research Council
NRMMC	Natural Resource Management Ministerial Council
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PET	Polyethylene-Terephthalate
PFU	Plaque Forming Units
PMA	Propidium Monoazide
POU	Point-of-Use
PVC	Poly Vinyl Chloride
qPCR	Quantitative or Real-Time Polymerase Chain Reaction
r^2	Correlation Coefficient
RNA	Ribonucleic Acids
ROS	Reactive Oxygen Species
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SABS	South African Bureau of Standards
SANS	South African National Standards
SDG	Sustainable Development Goals
SODIS	Solar Disinfection
SOPAS	Solar Pasteurisation
STEM	Scanning Transmission Electron Microscopy
TDS	Total Dissolved Solids
TiO_2	Titanium Dioxide
TiO_2-rGO	Titanium Dioxide – reduced Graphene Oxide
UK	United Kingdom
UN	United Nations
UNICEF	United Nations International Children's Emergency Fund
VB	Valence Band
UV	Ultraviolet
VBNC	Viable But Non-Culturable
WHO	World Health Organisation

Chapter 1: Literature Review

(UK spelling is employed)

1.1 Introduction

Globally, 2.2 billion people lack access to a safely managed potable water source, 4.2 billion require safely managed sanitation services, while 3 billion people lack access to basic handwashing facilities [United Nations Children's Fund (UNICEF) and World Health Organisation (WHO), 2019]. More specifically, 319 million people living in sub-Saharan Africa lack access to clean and safe potable water and 102 million people rely on compromised surface water sources to supply their daily hygiene, cooking and cleaning water needs (WHO, 2019). In South Africa, 70.9% of households have access to a basic water supply, which is defined as 25 L per person per day or 6000 L per household per month (Department of Water and Sanitation, 2018). However, in February 2018, the Northern Cape, Western Cape and Eastern Cape provinces were declared national disaster areas as South Africa experienced its worst drought in 23 years (Reuters, 2018). This placed severe pressure on a country classified as water stressed and in the Western Cape region strategies to supplement existing water sources were investigated as a priority by governing authorities. These interventions included implementing stringent water restrictions, extracting groundwater from the Table Mountain and Cape Flats aquifers and implementing pilot-scale desalination plants (GreenCape, 2017; City of Cape Town, 2018a; 2018b; GreenCape, 2018; Ndiritu et al., 2018). At the household level, grey water reuse was recommended for household applications such as toilet flushing (City of Cape Town, 2018b), while borehole water and harvested rainwater were promoted as alternative water sources, which could significantly reduce utilisation of municipal water supplies (City of Cape Town, 2018b).

Rainwater harvesting entails collecting rainwater from a surface such as a rooftop and storing the water in a tank. This water source could diminish some of the pressures placed on existing water supplies, particularly in regions where fresh water is limited. While the benefits of this alternative water source are apparent, the quality of harvested rainwater does not always comply with drinking water standards (Ahmed et al., 2011; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014a; Strauss et al., 2016; Waso et al., 2018) and a variety of opportunistic and pathogenic microorganisms such as *Klebsiella* spp., *Legionella* spp., *Pseudomonas* spp., *Cryptosporidium parvum* (*C. parvum*), *Naegleria fowleri* (*N. fowleri*) and *Campylobacter* spp. have been detected (De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014b; Hamilton et al., 2017; Waso et al., 2017). Various studies have therefore focused on investigating treatment methods to remove microbial contaminants from harvested rainwater in order to reduce or eliminate the potential human health risk. This includes the use of microfiltration and slow-sand filtration systems, chlorine- and ozone-based disinfection, solar disinfection (SODIS) and solar pasteurization (SOPAS) (Amin & Han, 2009; Moreira Neto et al., 2012; Nawaz et al., 2012; Ha et al., 2013; Lopez, 2014; Dobrowsky et al., 2015a; 2015b; Sánchez et al., 2015; Strauss et al., 2016; 2018).

The WHO has however, earmarked SODIS as an easy to implement and cost-effective method to disinfect various water sources at the household level (WHO, 2013). Simple SODIS relies on the combined effect of ultraviolet (UV) radiation and solar mild heat to disinfect contaminated water. The water is usually exposed to direct sunlight in transparent 2 to 5 L polyethylene-terephthalate (PET) bottles for a minimum of 6 hours (Castro-Alférez et al., 2016). Strauss et al. (2018) investigated the use of SODIS (10.6 L borosilicate glass reactor tube) in combination with a compound parabolic collector (CPC) for the treatment of harvested rainwater and found that the SODIS-CPC system effectively reduced the *Escherichia coli* (*E. coli*) and total coliform counts to below the detection limit at temperatures exceeding 39°C and UV-A radiation exceeding 20 W/m^2 (Strauss et al., 2018). However, ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) analysis indicated that viable *Legionella* and *Pseudomonas* spp. were still present in all the SODIS-CPC treated rainwater samples (Strauss et al., 2018). In addition, regrowth of microbial contaminants in solar disinfected water has been observed and it is recommended that the treated water be used within 24 hours (McGuigan et al., 2012).

Various strategies have subsequently been investigated to enhance SODIS efficiency. These include the use of solar mirrors or CPCs in combination with larger reactor tubes and the use of photocatalytic material in suspension or immobilised on support matrices (Byrne et al., 2011; McGuigan et al., 2012). Titanium dioxide (TiO_2) is a semiconductor photocatalytic material and has been applied in combination with SODIS to enhance disinfection efficiency by reducing the treatment time required to degrade microbial contaminants (Byrne et al., 2011; McGuigan et al., 2012). Significant broad-spectrum antimicrobial activity against bacteria (such as *E. coli*), protozoa (such as *Acanthamoeba* spp.) and fungi [such as *Fusarium solani* (*F. solani*)] (Byrne et al., 2011; McGuigan et al., 2012; Fernández-Ibáñez et al., 2015) has also been exhibited. This photocatalytic material mainly exerts its antimicrobial activity by producing reactive oxygen species (ROS) in water upon UV light exposure, whereafter the ROS disrupts the cell membrane, amino acids, fatty acids and nucleic acids, effectively killing microbial cells (Byrne et al., 2011; McGuigan et al., 2012; Ibhadon & Fitzpatrick, 2013).

Biological treatment, such as predatory bacteria or bacteriophages, could also be used to directly target bacteria persisting in treated water sources. The *Bdellovibrio*-and-like-organisms (BALOs) are a well-studied group of predatory bacteria and includes Gram-negative bacteria that predominantly attack and feed on other Gram-negative organisms (Sockett, 2009; Allen et al., 2014). The BALOs include *Bdellovibrio bacteriovorus* (*B. bacteriovorus*), *Bdellovibrio exovorus* (*B. exovorus*), *Micavibrio aeruginosavorus* (*M. aeruginosavorus*), *Bacteriovorax stolpii* (*B. stolpii*) and *Peredibacter starrii* (*P. starrii*). They are characterised by a biphasic life cycle which consists of a non-growing attack phase and an intracellular growth and replicating phase, with the exception of *B. exovorus* and *M. aeruginosavorus*, which do not enter and replicate within their

prey cells, but attach to the outside of the prey cells from where they leach the cell contents in order to replicate (Kadouri et al., 2007; Sockett, 2009; Koval et al., 2013).

Bdellovibrio-and-like-organisms may also be used as “live antibiotics” to combat opportunistic and pathogenic bacteria, especially in the clinical setting, as they can prey on and decrease the concentration of their target cells (Sockett, 2009; Allen et al., 2014; Dharani et al., 2018). In addition, studies have shown that *B. bacteriovorus* can be used as a probiotic in aquaculture to prevent *Shigella* and *Aeromonas hydrophila* (*A. hydrophila*) infections in zebrafish and sturgeon, respectively (Chu & Zhu, 2010; Cao et al., 2012; Willis et al., 2016). Kim et al. (2013) and Özkan et al. (2018) then indicated that the application of *B. bacteriovorus* as a pre-treatment to feed water, can significantly reduce membrane fouling in potable and wastewater treatment plants, effectively improving water treatment. The BALOs may thus be particularly well suited as biocontrol agents for the treatment of harvested rainwater as various Gram-negative opportunistic pathogens, such as *Klebsiella* spp., *Legionella* spp. and *Pseudomonas* spp. amongst others, are frequently detected in this water source and have been found to persist after treatment (Ahmed et al., 2008; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014b; Hamilton et al., 2017; Clements et al., 2019).

Furthermore, while various research groups have indicated that *B. bacteriovorus* mainly preys on Gram-negative organisms, Iebba et al. (2014) and Pantanella et al. (2018) indicated that *B. bacteriovorus* can prey on Gram-positive organisms such as *Staphylococcus aureus* (*S. aureus*). These authors found that this predator can switch from periplasmic predation (in the presence of Gram-negative prey) to epibiotic predation (in the presence of Gram-positive prey), while the predator can also alter its cocktail of secreted hydrolytic enzymes in order to obtain nutrients in the presence of Gram-positive prey (Iebba et al., 2014; Pantanella et al., 2018). Given the contradictory evidence presented for the predation activity of *B. bacteriovorus*, there is a need to monitor the expression of genes associated with the attack and growth phase of *B. bacteriovorus* when exposed to Gram-negative prey cells as compared to when the predator is exposed to Gram-positive prey cells, in order to fully understand how this predator interacts with different prey.

For the purposes of this review rainwater harvesting, the treatment of harvested rainwater, SODIS, SODIS enhancement technologies, BALOs and their interactions with prey cells, will be discussed.

1.2 Rainwater Harvesting

In 2010, it was estimated that approximately 34 000 domestic rainwater harvesting tanks were supplying households with a primary fresh water source in South Africa (Malema et al., 2016). By 2016, this number had increased to almost 70 000 tanks with rainwater harvesting used

extensively particularly in the Eastern Cape and Kwazulu-Natal regions of South Africa (Mwenge Kahinda et al., 2007; Malema et al., 2016). This technology is relatively simple to implement as the rooftop of a house or dwelling can serve as the rainwater catchment area (Lee et al., 2010). The rainwater is then conveyed via a gutter system into a rainwater harvesting tank, where the water is stored prior to use. Common materials used for the construction of rainwater harvesting systems include metal or plastic gutters and downpipes; rooftops constructed from tiles, thatch or galvanised metal sheets; while the harvesting tank may be constructed from concrete or high-density polyethylene (Gould & Nissen-Peterson, 1999; Li et al., 2010; Farreny et al., 2011; Mwenge Kahinda & Taigbenu, 2011).

Although harvested rainwater is generally regarded as a safe water source, studies have indicated that the quality of rainwater is compromised as it generally does not comply with drinking water standards (Abbasi & Abbasi, 2011; Ahmed et al., 2011; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014a; Strauss et al., 2016; Ahmed et al., 2018; Waso et al., 2018). Contaminants can enter rainwater harvesting tanks through bioaerosol deposition and when animal faecal matter, debris and plant material are washed into the tanks from the catchment surface during a rain event (Hamilton et al., 2017). Factors such as the material used to construct the catchment surface, maintenance and cleanliness of the catchment surface and gutters, the design of the harvesting tank (no openings to prevent debris, dust and plant material from entering the tank), and human activity in close proximity to the tanks, may have a significant influence on the quality of harvested rainwater (Mwenge Kahinda et al., 2007).

1.2.1 Harvested Rainwater Quality

There are currently no guidelines stipulated for harvested rainwater quality and therefore drinking water guidelines as stipulated by the South African National Standards (SANS) 241 [South African Bureau of Standards (SABS), 2005], Department of Water Affairs and Forestry[1] (DWAF, 1996), the WHO (WHO, 2011) and the Australian Drinking Water Guidelines (ADWG) [National Health and Medical Research Council (NHMRC) and Natural Resource Management Ministerial Council (NRMMC), 2011], are often used to assess the physico-chemical, chemical and microbial quality of this water source (Dobrowsky et al., 2014a; Hamilton et al., 2016; Strauss et al., 2018). This includes monitoring for various anions (such as chloride, fluoride, sulphate and nitrate), cations (such as iron, lead, aluminium, mercury, potassium and zinc), physico-chemical parameters (such as pH, temperature, total dissolved solids, electrical conductivity and turbidity) as well as indicator organisms (such as *E. coli*, enterococci, faecal coliform, total coliforms and heterotrophic bacteria) (DWAF, 1996; SABS, 2005; WHO, 2011; NHMRC & NRMMC, 2011).

[1] The Department of Water Affairs and Forestry is currently known as the Department of Water and Sanitation

However, numerous studies have indicated that while the chemical quality of harvested rainwater may be affected by several factors such as air pollution (especially in urban areas by industrial activities, vehicle emissions, etc.) and the catchment system (roofing material used, maintenance of the gutter system, etc.), it generally adheres to the levels specified in drinking water standards (De Kwaadsteniet et al., 2013).

In contrast, various indicator organisms and microbial pathogens have been identified in harvested rainwater, which may pose a significant health risk to the end-users. For example, Lee et al. (2010) detected total coliforms and *E. coli* in 91.6% and 72% of harvested rainwater samples, respectively, collected in Gangneung (South Korea), at levels exceeding the WHO drinking water standards (WHO, 1985; Lee et al., 2010). The authors found that the concentration of these indicator organisms increased during summer and fall (autumn) (Lee et al., 2010), which was hypothesised to be due to a lack of catchment maintenance and cleaning during these seasons. Similarly, Ahmed et al. (2012) detected enterococci and *E. coli* in 92% and 63% of harvested rainwater samples, respectively, collected from 24 tanks in Southeast Queensland (Australia), at concentrations exceeding the Australian drinking water guidelines (NHMRC, 2004; Ahmed et al., 2012). In South Africa, Dobrowsky et al. (2014a) found that the levels of *E. coli*, total coliforms, faecal coliforms, enterococci and heterotrophic bacteria detected in harvested rainwater collected from tanks in the peri-urban region of Kleinmond, frequently exceeded the South African (DWAF, 1996; SABS, 2005), Australian (NHMRC & NRMMC, 2011) and WHO drinking water guidelines (WHO, 2011).

Moreover, the various opportunistic and pathogenic microorganisms detected in harvested rainwater includes viruses such as adenovirus, bacteria such as *Salmonella*, *Klebsiella*, *Pseudomonas* and *Legionella* spp. and protozoa such as *Giardia lamblia* (*G. lamblia*), *Cryptosporidium* spp. and *N. fowleri* (Ahmed et al., 2008; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014b; Hamilton et al., 2017; Waso et al., 2017; Ahmed et al., 2018). A few studies have thus focused on elucidating the human health risk posed by these microbial contaminants when this water source is used for domestic purposes and as a source of drinking water (Fewtrell & Kay, 2007; Ahmed et al., 2010; Lim & Jiang, 2013; Hamilton et al., 2017). Subsequently, in 2006, a Legionnaire's disease outbreak in New Zealand [caused by *Legionella pneumophila* (*L. pneumophila*)] was linked to the inhalation of aerosols during showering, in households where harvested rainwater was used as the primary water source (Simmons et al., 2008). Similarly, Hamilton et al. (2017) indicated that inhaling and ingesting harvested rainwater during activities such as showering and garden irrigation, may pose a significant health risk to end-users as a result of the levels of *L. pneumophila* and *Mycobacterium avium* complex (MAC) detected. The authors also noted that the washing of clothes and cars may be the safest applications of harvested rainwater contaminated by these organisms, while toilet flushing and irrigating home grown produce (specifically focusing on lettuce) may be safe applications for end-

users who are not immuno-compromised. Similarly, Ahmed et al. (2010) concluded that ingestion of harvested rainwater posed a significant health risk to the consumer, as a result of the high concentration of *Salmonella* spp. and *G. lamblia* detected.

1.3 Harvested Rainwater Treatment Strategies

The presence of pathogens and opportunistic pathogens in harvested rainwater and the potential human health risks associated with the utilisation of this water source, has resulted in numerous studies investigating and applying various treatment technologies to disinfect harvested rainwater. Treatment strategies may include interventions which prevent contaminants from entering rainwater harvesting tanks (such as first-flush diverters) and post-harvest treatments such as chlorination, filtration, UV treatment, SODIS and SOPAS (Amin & Han, 2009; Moreira Neto et al., 2012; Nawaz et al., 2012; Ha et al., 2013; Lopez, 2014; Dobrowsky et al., 2015a; 2015b; Strauss et al., 2016; 2018).

Gutter screens may be installed as a pre-treatment strategy to prevent larger debris, such as leaves, from washing into rainwater harvesting tanks (Sánchez et al., 2015). First-flush diverters may also be installed to direct the initial runoff from the rooftop away from the harvesting tank into a flush-pipe (Sánchez et al., 2015). This initial runoff is thought to contain the highest concentration of pollutants, especially during the first rainfall after a prolonged dry weather period (Sánchez et al., 2015). Gikas and Tsihrintzis (2012) then found that while installing a first-flush diverter improved the physico-chemical quality of harvested rainwater, it did not significantly improve the microbial quality as indicator organism concentrations exceeding drinking water standards were still detected in the tank water. Similarly, Mendez et al. (2011) found that the concentration of total and faecal coliforms were reduced, but these organisms were not completely eliminated in harvested rainwater following the diversion of the first-flush. These pre-treatments may thus improve the quality of harvested rainwater, but they are ineffective in eliminating all microbial contaminants and therefore additional post-harvest treatment methods should be employed.

Post-harvest treatment approaches primarily include the use of filtration, thermal and UV disinfection or combinations of these treatment methods. Jordan et al. (2008) investigated the efficiency of a point-of-use (POU) filtration system (20 µm and 5 µm polypropylene progressive-density cartridge filters and a carbon impregnated paper cartridge filter) combined with a high capacity UV steriliser for the disinfection of harvested rainwater in an urban area (Tuscon, Arizona). Results indicated that after filtration and UV treatment, the heterotrophic bacteria were reduced by 98.6%, while total coliforms were reduced to below the detection limit. The authors also reported that the POU system effectively reduced *E. coli* and MS2 bacteriophages by 6 and 5 logs, respectively, in tank water samples spiked with these organisms (Jordan et al., 2008).

Similarly, Dobrowsky et al. (2015a) investigated the use of a polyvinyl (alcohol) (PVA) membrane filter combined with an activated carbon column, for the treatment of harvested rainwater. Based on the results obtained, a >99% reduction in *E. coli*, total coliform and heterotrophic bacteria counts was recorded. Additionally, Moreira Neto et al. (2012) found that the combination of slow-sand filtration and chlorine disinfection resulted in a 3 and 4-log reduction in *E. coli* and total coliform counts, respectively. Based on the traditional culturing analysis of indicator organisms (which is still the golden standard for monitoring water quality), these treatment technologies were effective in reducing microbial contamination in harvested rainwater. However, the major drawback associated with using filtration devices is that filters may become saturated and should be replaced often to ensure adequate treatment of the water, while slow-sand filters require regular maintenance to ensure that the “schmutzdecke” or biofilm layer forms and functions optimally to effectively remove contaminants from the water being treated. Increased costs are also associated with the implementation of UV lights and an electricity source (which may not be available in many rural areas) is generally required.

Thermal disinfection is also considered an effective treatment strategy, with boiling water over a fire the most basic form of implementation. Factors such as the turbidity and pH of the source water, generally do not influence the efficiency of thermal disinfection methods (Burch & Thomas, 1998; Helmreich & Horn, 2009). However, the cost of fuel such as wood, gas and paraffin, is a major drawback associated with this treatment method and may hinder its use, especially in low-income communities (Sobsey, 2002; Islam & Johnston, 2006). Solar pasteurization systems have then become attractive options to treat harvested rainwater as these systems harness free energy from sunlight to heat and subsequently disinfect water. Dobrowsky et al. (2015b) investigated the efficiency of the Apollo™ SOPAS system (closed-coupled system) to treat harvested rainwater. At temperatures exceeding 72°C, the indicator organism counts (i.e. *E. coli*, total coliforms and heterotrophic bacteria) were reduced to below the detection limit. However, using PCR assays, *Pseudomonas* spp. and *Legionella* spp. were detected in treated harvested rainwater at temperatures above 90°C. Using EMA-qPCR, Reyneke et al. (2016) then confirmed that *Legionella* spp. remained viable in rainwater samples pasteurized at a temperature of 95°C. Additionally, it was found that after pasteurization at all temperatures analysed, the concentration of iron, aluminium and lead increased in the treated water. It was then hypothesised by the authors that upon heating, these metals may have leached into the water from the SOPAS system stainless steel holding tank. It was subsequently recommended that alternative materials, such as high-density polyethylene, which is more stable at higher temperatures, be used to prevent the leaching of elements into the treated water (Dobrowsky et al., 2015b; Reyneke et al., 2016).

Strauss et al. (2016) then investigated a SOPAS system with a high-density polyethylene storage tank for the treatment of rainwater. The authors found that at temperatures exceeding 71°C, the *E. coli* and heterotrophic bacterial counts were significantly reduced to below the detection limit.

In addition, the concentration of iron was reduced in all the pasteurized water samples. However, using EMA-qPCR assays, *Legionella* and *Pseudomonas* spp. were still found to persist in the SOPAS treated rainwater at treatment temperatures ranging from 71 to 93°C. It was hypothesised that the persistence of these bacteria in SOPAS systems could be attributed to their association with biofilms, and the potential expression of heat shock proteins (Murga et al., 2001; Fields et al., 2002; Reyneke et al., 2016; Strauss et al., 2016). Additionally, bacteria are able to form associations with protozoa to evade environmental stressors and Dobrowsky et al. (2016) showed that *Legionella* spp. and *Acanthamoeba* spp. were detected in pasteurized harvested rainwater at temperatures exceeding 90°C. It was hypothesised that the *Acanthamoeba* harboured the *Legionella* spp., which may have allowed it to persist during rainwater treatment (Dobrowsky et al., 2016). Thus, while SOPAS systems are effective in producing water that adheres to drinking water standards (based solely on the indicator organism analysis) and offers a cost-effective way of treating harvested rainwater, the WHO specifically identified and promotes SODIS as an easy, practical and cost-effective method to disinfect contaminated water sources, especially in low-income communities (Martín-Domínguez et al., 2005; Byrne et al., 2011; McGuigan et al., 2012; Castro-Alférez et al., 2016; WHO, 2013).

1.3.1 Solar Disinfection

Solar disinfection relies on the combined effect of UV radiation [UV-A (315 to 400 nm) and UV-B (280 to 315 nm)] and solar mild heat to destroy microbial contaminants in water sources (McGuigan et al., 2012). While this method has been used for almost 2000 years to purify water, the bactericidal effect of sunlight was only studied in 1877 by Downing and Blunt (Downing & Blunt, 1877; Castro-Alférez et al., 2016). In 1980, Acra et al. (1980) successfully applied solar energy to purify oral hydration solutions, specifically earmarked for drinking by individuals suffering from severe diarrhoea, whereafter numerous studies started exploring the benefits associated with SODIS and the variables associated with using SODIS to purify water sources (Castro-Alférez et al., 2016).

Simple SODIS involves filling UV-visible transparent plastic (PET) bottles with the water source to be treated and exposing these bottles to natural sunlight for a minimum of 6 hours (Castro-Alférez et al., 2016). The treatment time may be dependent on weather conditions (on overcast days longer treatment times of up to 48 hours may be required) and the sensitivity of the microbial contaminants to UV radiation (Sciacca et at., 2010). Ultraviolet radiation then inactivates microorganisms in the water source using three distinct mechanisms: i) direct inactivation; ii) indirect endogenous inactivation and iii) indirect exogenous inactivation (McGuigan et al., 2012).

During direct inactivation, UV light is absorbed by microbial nucleic acids, which leads to genomic damage (McGuigan et al., 2012). Specifically, the light is absorbed by pyrimidine rings of thymine

and cytosine, which may lead to the formation of bonds between adjacent pyrimidine bases, ultimately forming pyrimidine dimers. The formation of these dimers is problematic as they prevent base-pairing with complementary purines, which changes the shape of the DNA molecule (McGuigan et al., 2012). In turn, this new configuration of the DNA molecule may hinder DNA polymerase from synthesising new DNA molecules. In some cases, the DNA polymerase may skip this newly configured area, which leads to base pair deletions or the DNA polymerase may incorporate a random base pair into this area, which can lead to genetic mutations (McGuigan et al., 2012). To combat this direct inactivation, many bacteria have photolyase enzymes (such as MutS and MutH), recombination repair mechanisms (such as RecA), and an SOS response mechanism, which enables them to repair DNA damage induced by UV irradiation (Willey et al., 2011).

In contrast, during indirect endogenous inactivation UV-A, UV-B and visible light is absorbed by sensitiser molecules within microbial cells (such as porphyrins, flavins, quinones and NADH/NADPH), which leads to the production of intracellular ROS (McGuigan et al., 2012). In addition, during indirect exogenous inactivation, UV-A, UV-B and visible light is absorbed by external sensitisers commonly found in water (such as humic acids and chlorophylls), which subsequently reacts with the oxygen in water to form ROS (McGuigan et al., 2012). These ROS may include free radicals such as superoxide (O_2^-), hydroperoxyl ($HO_2\cdot$), hydroxyl ($HO\cdot$), peroxyl ($ROO\cdot$), alkoxyl ($RO\cdot$) and non-radical species such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), which exert their antimicrobial effect by damaging nucleic acids (single-strand breaks and pyrimidine dimers), oxidising amino acids in proteins and oxidising poly-unsaturated fatty acids in lipids (McGuigan et al., 2012; Castro-Alférez et al., 2016). Furthermore, water molecules may absorb UV irradiation, which ultimately leads to an increase in the water temperature. When the water temperature exceeds 45°C, a synergistic effect between UV irradiation and solar mild heat leads to improved antimicrobial activity, as the elevated water temperature may weaken or damage cell membranes and inhibit DNA repair mechanisms (McGuigan et al., 2012). Numerous studies have thus indicated that SODIS is effective for the inactivation of bacterial, viral and protozoan contaminants in water (McGuigan et al., 1998; Berney et al., 2006; McGuigan et al., 2012; Nalwanga et al., 2014; 2016).

While research has indicated that SODIS is effective in reducing the microbial contaminants in water, limitations associated with this method includes the small volume of treated water that can be produced (between 1 and 5 L per bottle used), the treatment time required to effectively disinfect the water (a minimum of 6 hours is recommended) and the regrowth of microbial contaminants, which may occur if the treated water is stored for prolonged time periods (treated water should be used within 24 hours) (McGuigan et al., 2012; Makwana et al., 2015). Various studies are thus focusing on enhancement technologies to improve SODIS processes. This includes incorporating CPCs and solar mirrors to increase the solar irradiance dose of the water

being treated, the addition of photocatalysts to improve disinfection efficiency and the design of reactors that can treat larger volumes of water (McGuigan et al., 2012).

1.3.2 Solar Disinfection Enhancement Technologies

To enhance the synergistic effect between UV irradiance and solar mild heat and potentially enhance the microbial disinfection efficiency, strategies to increase the temperature within SODIS reactors are being investigated (McGuigan et al., 2012). In addition, during basic SODIS the treatment vessels or reactors are usually only illuminated from the side directly exposed to the sun and the solar radiation may not fully penetrate the water within the SODIS reactor (McGuigan et al., 2012). To mitigate these pitfalls, absorptive materials (such as black polyethylene boxes which absorb solar radiation) may be used to increase the water temperature within a SODIS reactor, while reflective material (such as solar mirrors or CPCs) can be used to reflect the sun's rays directly onto the SODIS reactor, thereby increasing the solar irradiance dose and the subsequent ROS production (Ubomba-Jaswa et al., 2010; McGuigan et al., 2012). Martín-Domínguez et al. (2005) then investigated the use of various solar mirrors (square, rectangular and compound double parabola covered with mirrors, aluminium or aluminised duct tape) in combination with different transparent PET bottles (2 L bottles with no modification, 2 L bottles with a black stripe painted lengthwise or 2 L bottles completely painted black) to treat well water in Mexico. Results showed that the total coliforms were reduced to below detection limit after 8 hours of solar exposure in all the treatment systems using the various solar mirrors as well as in the bottles with no modifications and the bottles with a black stripe painted lengthwise. However, for the bottles which were completely painted black, total coliforms were reduced, but not completely eliminated after the 8 hours of solar exposure (Martín-Domínguez et al., 2005). The authors concluded that while an increased temperature (50 to 65°C) within the SODIS reactors enhanced the disinfection efficiency, the synergistic effect of the UV irradiation and solar mild heat resulted in the complete inactivation of the total coliforms (Martín-Domínguez et al., 2005). Strauss et al. (2016) used a SUNSTOVE 2000™ solar oven (a black polyethylene box with a reflective aluminium surface at the base and a transparent Perspex lid) with 2 L PET bottles to treat harvested rainwater. The temperature of the water reached up to 89°C after 6 hours of solar exposure and at temperatures of 52°C and above, the *E. coli* and heterotrophic bacteria counts were reduced to below the detection limit. Based on the results obtained in these studies, the combination of solar mirrors and absorptive materials enhanced SODIS efficiency by increasing the UV dose and temperature within basic SODIS reactors (plastic bottles).

Research, however, has indicated that CPCs which use non-imaging solar optics to concentrate the sun's rays from different angles, are more effective in reflecting and concentrating diffuse solar irradiance onto a SODIS reactor vessel (McGuigan et al., 2012; Keane et al., 2014). In addition, CPCs have been identified as the most promising intervention to improve SODIS

efficiency for large-scale water purification (> 10 L) (McGuigan et al., 2012; Keane et al., 2014). The main advantages of a CPC includes: i) the use of non-imaging concentration with diffuse focus (able to collect all solar radiation); ii) high efficiency in increasing the solar photon flux (number of electrons generated by solar energy) within the reactor as a result of the homogenous distribution of UV radiation onto the reactor; and, iii) both direct and diffuse solar radiation is used, resulting in high disinfection efficiencies even on overcast days (Keane et al., 2014). Navntoft et al. (2008) compared the SODIS efficiency of a borosilicate glass tube with a CPC to a glass tube without a CPC and a 2 L PET bottle, exposed to natural sunlight. The results indicated that *E. coli* was reduced to below the detection limit within 2 hours in the tube without the CPC, 3 hours were required to reduce the *E. coli* to below the detection limit in the standard PET bottle, while *E. coli* in the system with the CPC was reduced to below the detection limit within 90 minutes (Navntoft et al., 2008). In addition, under cloudy conditions, only the system with the CPC reduced the *E. coli* counts to below the detection limit within the 5-hour treatment time. The authors concluded that the use of the CPC increased the solar irradiance in the reactor, effectively enhancing the SODIS efficiency (Navntoft et al., 2008).

In order to treat larger volumes of water using SODIS, different continuous flow and batch reactors have been designed and employed in combination with solar mirrors or CPCs (Ubomba-Jaswa et al., 2010; McGuigan et al., 2012). Ubomba-Jaswa et al. (2008) compared three SODIS systems: i) a borosilicate glass tube (2.3 L batch reactor); ii) a 14 L recirculating system consisting of two borosilicate glass tubes and a CPC; and iii) a 70 L recirculating system with 20 borosilicate glass tubes fitted with 20 CPCs. The authors also tested the effect of flow rate on the disinfection efficiency in the recirculating systems and employed a flow rate of 2 L/min and 10 L/min. Results indicated that for all three reactors tested, an uninterrupted solar UV dose of > 108 kJ/m^2 was required to completely inactivate 10^6 CFU/mL of *E. coli*. They also found that flow rate had a negative effect on the disinfection rate, which could be due to the fact that the microorganisms are not continuously exposed to UV radiation in flow reactors and could recover from UV stress. Therefore, static batch reactors are often recommended for SODIS.

1.3.2.1 Photocatalysis

In order to improve SODIS efficiency and reduce the treatment time required for the disinfection of a water source, advanced oxidative processes, using photocatalysts, can be employed (Keane et al., 2014). Photocatalysis is defined as the enhancement or acceleration of a photoreaction in the presence of a catalyst (McGuigan et al., 2012). Semiconductor photocatalysis employs semiconductor materials in the presence of light to produce highly oxidative species (Byrne et al., 2011). Semiconductor molecules contain a valence band (VB) occupied by stable energy electrons and empty higher energy conduction bands (CB) (Fisher et al., 2013). When the semiconductor material is irradiated by light, which has a wavelength equal to or greater than the

bandgap of the material, the light energy is absorbed, which results in an electron moving from the VB to the CB (**Fig. 1.1**). This generates electron (e^-)/hole (h^+) pairs (**Fig. 1.1**) (Mills & Le Hunte, 1997; Byrne et al., 2011). These energy carriers (e^- and h^+) migrate to the surface of the photocatalyst, where they can participate in redox reactions, ultimately producing ROS (at the semiconductor/water interface) (McGuigan et al., 2012).

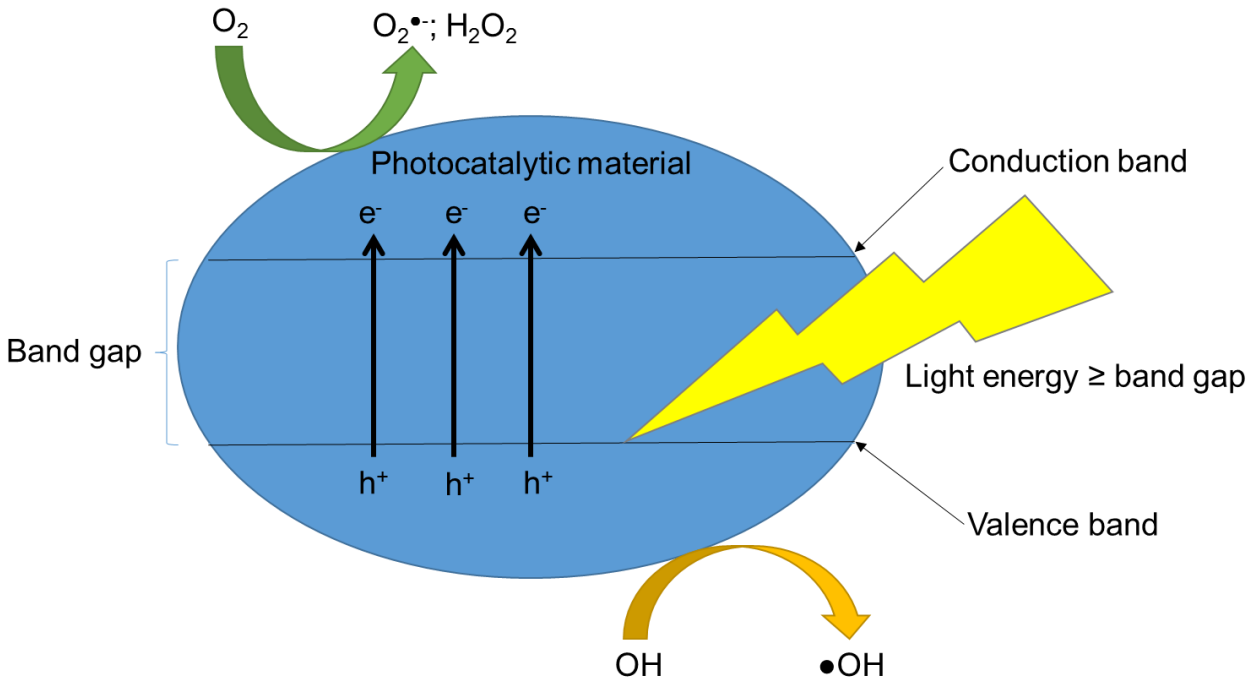

Fig. 1.1: Illustration of the mechanism of photocatalysis [Adapted from Byrne et al. (2010)]

The ROS are highly active and indiscriminate (especially hydroxyl radicals) and can degrade various microorganisms and chemical pollutants in water (**Fig. 1.1**) (Byrne et al., 2011; McGuigan et al., 2012). Various semiconductor materials have been investigated for their photocatalytic properties. These include metal oxides such as zinc oxide (ZnO), Iron (III) oxide (Fe_2O_3), TiO_2, zirconium dioxide (ZrO_2), vanadium pentoxide (V_2O_5), tin oxide (SnO_2) as well as metal sulphides such as cadmium sulphide (CdS) and zinc sulphide (ZnS). Of these, the most common photocatalyst investigated for water treatment is TiO_2 (Byrne et al., 2011; McGuigan et al., 2012).

1.3.2.1.1 Titanium Dioxide

Titanium dioxide is a large bandgap semiconductor [a bandgap of 3.0 electronvolt (eV) in rutile phase (prismatic crystal form; red material) and 3.2 eV in anatase phase (tetragonal crystal form; yellow to blue material)], which requires UV illumination for photocatalytic activity (Ibhadon & Fitzpatrick, 2013). This compound is considered one of the most fascinating materials for homogeneous [photocatalyst and reactants exist in the same phase (solid or liquid)] and heterogeneous [photocatalysts and reactants exist in different phases (solid, liquid or gas)]

photocatalysis, as it can be used to oxidise organic and inorganic substrates in air and water through redox reactions (Ibhadon & Fitzpatrick, 2013). In addition, this metal oxide can be effectively applied for water purification as it is cost-effective, chemically and photochemically stable, is easy to obtain and to date, no known human toxicity has been reported (Byrne et al., 2011; Tang et al., 2018).

While many benefits are associated with the application of TiO_2, two factors hinder its widespread application for water treatment (Byrne et al., 2011). The first pitfall is associated with the light required to induce photocatalytic activity. As mentioned, TiO_2 is a wide bandgap semiconductor and high energy light is required to induce photocatalytic activity (Byrne et al., 2011). Specifically, the bandgap of TiO_2 is in the range of 3.0 to 3.2 eV (depending on the phase of the material – anatase or rutile) (Byrne et al., 2011). This implies that lower wavelength light (≤ 400 nm) with higher energy is required and therefore, the photocatalytic activity of TiO_2 is only induced when the material is irradiated with UV light (Byrne et al., 2011). This limits the application of TiO_2 in larger industrial processes as it requires UV lamps to be installed in order to harness the photocatalytic activity of the material (Byrne et al., 2011). Various research groups have thus investigated modifying TiO_2 to reduce the bandgap of the material, and in so doing induce visible light activity (Byrne et al., 2011; Ibhadon & Fitzpatrick, 2013; Tang et al., 2018). Research has subsequently indicated that internal doping with metal and non-metal ions introduces impurities to the bandgap of TiO_2, effectively reducing the bandgap and conferring visible light activity (Tang et al., 2018). However, these impurities can act as recombination centres for photo-generated e^-/h^+ pairs, potentially limiting the photocatalytic activity of the semiconductor (Tang et al., 2018). Surface sensitisation is thus the preferred method for enhancing TiO_2 photocatalytic activity. An appropriate sensitiser should have a narrower bandgap than TiO_2 and the CB of the material should be more positive, or the VB should be more negative than that of TiO_2 (Tang et al., 2018). In addition, the loading of the sensitiser should be controllable and the combination of the sensitiser with TiO_2 should be achieved through conventional methods (Tang et al., 2018). Various TiO_2 composite photocatalysts have thus been investigated for water purification, which includes the combination of TiO_2 with copper, platinum, eosin Y dye and various carbon materials such as carbon nanotubes and graphene (Tang et al., 2018).

Graphene was isolated from graphite in 2004 by Geim and Novoselov (Novoselov et al., 2004; Tang et al., 2018). This two-dimensional material has a high electron mobility, a high specific surface area, good thermal conductivity and exhibits remarkable mechanical strength (Tang et al., 2018). In addition, graphene has a bandgap of zero (0 eV) and has been identified as the perfect sensitiser to be used for photocatalytic activity (Tang et al., 2018). The zero bandgap of graphene, in combination with TiO_2, thus allows for visible light activity of the composite material, while its high electron mobility allows for the rapid transport of photo-induced electrons between the graphene and TiO_2, which suppresses the recombination of the photo-generated e^-/h^+ pairs

in the metal oxide (Tang et al., 2018). Furthermore, graphene's high specific surface area serves as a favourable scaffold for the attachment of TiO_2 particles, which in turn allows for the enhanced absorption of various pollutants to the surface of the TiO_2-graphene composite material (Tang et al., 2018). Titanium dioxide-graphene composite materials have subsequently been used for water treatment, air purification, water splitting and CO_2 reduction, amongst other applications (Tang et al., 2018).

Fernández-Ibáñez et al. (2015) compared the disinfection efficiency of TiO_2-reduced graphene oxide (TiO_2-rGO) to TiO_2 in suspension under simulated solar irradiance using *E. coli* and *F. solani* spores. *Escherichia coli* counts were reduced to below the detection limit within 10 minutes of solar irradiance in the presence of 500 mg/L TiO_2-rGO, while 16 minutes was required for 500 mg/L TiO_2 to achieve a similar reduction. In contrast, the *F. solani* spores were reduced to below the detection limit within 35 minutes using 10 mg/L TiO_2-rGO, while 35 mg/L TiO_2 was required to reduce the concentration of the *F. solani* spores to below the detection limit in the same time period. The authors also compared the photocatalytic activity of TiO_2-rGO at 500 mg/L under visible light, using a UV cut-off filter (thus only using light wavelengths greater than 380 nm), to simulated solar irradiance (without the UV cut-off filter). Results indicated that for TiO_2-rGO, the *E. coli* disinfection rate was similar under visible light and simulated solar irradiance. In comparison, when using simulated solar irradiance, the *E. coli* disinfection rate for TiO_2 was similar to the disinfection rates observed for TiO_2-rGO, however using the UV cut-off filter (thus only using visible light irradiation) the disinfection efficiency of TiO_2 was reduced and the disinfection time increased from approximately 10 to 30 minutes. It was thus concluded that the reduced graphene oxide conferred visible light activity on the composite photocatalyst and increased the material's disinfection efficiency (Fernández-Ibáñez et al., 2015).

Cruz-Ortiz et al. (2017) then elucidated the primary mechanisms behind the visible light disinfection efficiency of TiO_2-rGO by comparing the activity of TiO_2-rGO to the activity of TiO_2 in suspension. A UV cut-off filter was used to simulate visible light irradiation, while solar irradiance without the UV filter (thus UV and visible light irradiance) was also employed. A 6-log reduction in *E. coli* counts was achieved within 120 minutes using TiO_2 with simulated solar irradiance, while the use of TiO_2-rGO resulted in a 6-log reduction within 90 minutes. In contrast, using only visible light, the use of TiO_2-rGO resulted in a 5.7 log reduction in *E. coli* counts, while the use of TiO_2 only resulted in a log reduction of 1.7 (which was comparable to the light control experiment where no photocatalyst was used). The authors also identified the primary ROS produced during the disinfection experiments and found that under UV-visible light (simulated solar irradiance), H_2O_2, $HO\cdot$ and $^1O_2$ were produced, while under visible light (UV cut-off filter) only $^1O_2$ was produced. These results thus confirmed that the combination of reduced graphene oxide with TiO_2 produces a visible light active photocatalytic material.

The second pitfall hindering the widespread application of photocatalysts in water treatment is the fact that the nanomaterial (i.e. TiO_2 nanoparticles) needs to be removed from the treated water prior to use or consumption. Olabarrieta et al. (2018) investigated the use of microfiltration and ultrafiltration to remove TiO_2 nanoparticles from water, with comparable removal efficiencies obtained for these two filtration methods. However, regardless of the pore size of the filters used, TiO_2 particles passed through all the filtration systems, which stopped as soon as a cake layer of the nanoparticles formed on the surface of the membrane (Olabarrieta et al., 2018). The authors recommended that ultrafiltration systems be employed to remove TiO_2 from water, as backwashes were required more often for the microfiltration system to sustain optimal operation as compared to the ultrafiltration system, which implies that less maintenance would be required for an ultrafiltration device (Olabarrieta et al., 2018).

To mitigate the need to develop effective, yet costly, filtration systems for the removal of the photocatalytic material from water after disinfection, various research groups have recommended that the nanomaterials be immobilised onto support matrices (Fernández et al., 1995; Comparelli et al., 2004; Borges et al., 2015; Adán et al., 2018; Paredes et al., 2019). Oblak et al. (2018) immobilised different TiO_2 photocatalytic powders (P25, P90 and PC500) onto stainless steel mesh and fibre glass strips and investigated the efficiency of these immobilised nanomaterials to degrade phenol and the commercial textile dye Reactive Blue 19 in a flow-through photoreactor. All the immobilised photocatalysts degraded phenol by 38 to 94% and Reactive Blue 19 by 72 to 99% after 240 minutes of irradiation. Cunha et al. (2018) immobilised TiO_2 onto borosilicate glass spheres, which were used in a CPC reactor and analysed under simulated solar irradiance, to degrade methylene blue. The authors found that methylene blue was degraded by 96% within 90 minutes of solar exposure. Similarly, Aguas et al. (2018) immobilised TiO_2 onto borosilicate glass tubes and low-density polyethylene pellets and used these coated supports in a CPC reactor to reduce the concentration of *E. coli* and *Enterobacter cloacae* (*E. cloacae*). Results indicated that, for the reactor where the TiO_2 coated pellets were employed, the *E. coli* and *E. cloacae* counts were reduced by 4.9 logs within 180 minutes of treatment and 11.2 kJ/L of radiation. In comparison, the bacterial counts were reduced by 2 logs in the CPC reactor where the photocatalyst was coated onto the glass tubes. It was hypothesised that the low-density polyethylene pellets had a larger surface area for coating with the photocatalyst and subsequently resulted in a higher disinfection efficiency (Aguas et al., 2018). Immobilised photocatalysts can thus effectively be used in water treatment systems to degrade various compounds and microorganisms found in water sources, without the need to include a filtration system after treatment.

While the use of chemical and physical enhancement strategies to improve SODIS efficiency has been well established, these studies have mostly determined disinfection efficiency using culture-based methods. In contrast, recent studies employing molecular-based methods (EMA-qPCR and

EMA coupled with Illumina next-generation sequencing) to monitor the efficiency of SODIS for the treatment of harvested rainwater, confirmed that microorganisms persist after disinfection (Strauss et al., 2018; 2019). Investigating the use of biological agents, which could be employed to overcome bacterial resistance to disinfection by targeting persistent bacteria directly, is thus warranted. In this regard, predatory bacteria may be particularly useful and could be combined with physical and chemical disinfection methods to purify contaminated water sources.

1.4 Predatory Bacteria

Bdellovibrio-and-like organisms are a group of Gram-negative bacteria that are miniscule (0.2 to 0.5 µm in width and 0.5 to 2.5 µm in length), highly motile predators of predominantly other Gram-negative bacteria (Sockett & Lambert, 2004; Rotem et al., 2014). *Bdellovibrio* was first isolated by Stolp and Petzold (1962), while investigating bacteriophages and until recently *B. bacteriovorus* was the only recognised species belonging to the BALOs (Rotem et al., 2014). Advances in molecular techniques has however, shown that the BALOs form diverse monophyletic groups, which include the delta-proteobacteria families Bdellovibrionaceae, Bacteriovoraceae and Peredibacteraceae and the alpha-proteobacteria genus *Micavibrio* (Davidov & Jurkevitch, 2004; Jurkevitch & Davidov, 2006; Rotem et al., 2014). They inhabit a wide variety of aquatic and terrestrial habitats such as the rhizosphere of plants, bulk soil, rivers, brackish water, fish ponds, water and wastewater (Jurkevitch et al., 2000; Iebba et al., 2014; Avidan et al., 2017; Kongrueng et al., 2017). Since the discovery of these predatory bacteria, various research groups have elucidated the physiology, ecology, taxonomy and the mechanisms involved in predator-prey interactions as well as the mechanisms involved in their biphasic cell cycle (Rotem et al., 2014). Most of the research has however, been conducted on *B. bacteriovorus*, which is the best characterised predatory bacterium belonging to the BALOs (Strauch et al., 2006).

1.4.1 The Life Cycle of the *Bdellovibrio*-and-Like-Organisms

The BALOs are characterised by a unique biphasic life cycle, which may be divided into two distinct phases: i) the attack phase and ii) the growth phase. The growth phase can be further subdivided based on the predation strategy employed by the predator i.e. either an epibiotic predation strategy (the predator attaches to the outer membrane of the prey cell from where the predator leaches the cellular contents in order to replicate) or a periplasmic predation strategy (the predator invades the periplasmic space of the prey bacterium and consumes the prey cell's contents in order to replicate). Epibiotic predators grow by binary fission, while periplasmic predators grow filamentously and split into progeny cells upon maturation, whereafter the progeny are released to scavenge for new prey cells (Rotem et al., 2014).

During the attack phase, the BALOs are small, vibroid to rod-shaped cells that move at high speeds, facilitated by a single sheathed flagellum (Sockett & Lambert, 2004, Davidov et al., 2006; Strauch et al., 2006). Research has in fact indicated that *B. bacteriovorus* can swim up to 100 times its cell length per second, which is ten times faster than the speed recorded for *E. coli* (Rittenberg, 1983; Lambert et al., 2006; Strauch et al., 2006). Studies conducted on the attack phase have indicated that the predators exhibit weak chemotaxis towards respirable substrates, specific amino acids and other organic compounds (Lambert et al., 2003; Strauch et al., 2006). Thus, while the exact mechanism behind the predator locating and recognising prey cells is not fully elucidated, it has been hypothesised that the BALOs can use this weak chemotactic attraction to specific compounds to locate areas in environmental niches where a higher density of prey cells may be present (Strauch et al., 2006). To corroborate the hypothesis that chemotaxis may play a role in prey cell localisation and detection, Lambert et al. (2003) found that the *B. bacteriovorus* 109J strain possessed a methyl-accepting chemotaxis protein, while Strauch et al. (2006) found 20 of these proteins as well as chemotactic machinery, which can signal environmental changes to the flagellar motor, in *B. bacteriovorus* HD100. Earlier research has however, indicated that the location of prey by BALOs predominantly occurs through random collisions between prey and predator cells, which implies that predation is directly dependent on the cell density of both the predator and prey (Varon & Shilo, 1980; Strauch et al., 2006). Thus, while the exact mechanism of how the predators locate their prey is largely unknown, once they collide with and attach to the prey cells, they either invade the periplasmic space of the prey cell or remain attached to the outside where they proliferate.

Bdellovibrio bacteriovorus was isolated in Chapter 2 of this dissertation and was utilised for all subsequent experiments conducted for Chapters 3 and 4. Therefore, for the purposes of this review the periplasmic predation strategy (**Fig. 1.2**) employed by *B. bacteriovorus* will be discussed and specific genes (which have been annotated and their function elucidated) that are upregulated and hypothesised to be vital for this predation strategy will be described.

1.4.1.1 Periplasmic Growth of Bdellovibrio bacteriovorus

The life cycle of *B. bacteriovorus* starts with free-swimming attack phase predators scavenging for potential prey cells (**Fig. 1.2a** and **Fig. 1.2b**). Upon collision with a prey cell, *B. bacteriovorus* reversibly attaches to the cell (**Fig. 1.2b** and **Fig. 1.2c**). If the predator is able to utilise the prey cell as a host, this attachment becomes irreversible (**Fig. 1.2c**), while if a collision between the predator and a non-suitable prey cell occurs, the predator will detach from the cell and continue to search for suitable prey (Rendulic et al., 2004; Strauch et al., 2006). It is not known what triggers the switch from reversible to irreversible attachment (Karunker et al., 2013), however it has been hypothesised that BALOs recognise cell wall components in order to identify potential prey, which could be used as hosts for replication (Strauch et al., 2006).

Research into the cell wall components of *B. bacteriovorus* indicated that these bacteria have unique structural features including Lipid A moieties, which lack negatively charged groups and outer membrane protein F-like (OmpF-like) structures. The latter form pores and allow for the passive diffusion of small molecules across the cell membrane and serves as a recognition site for bacteriophages in *E. coli* (Schwudke et al., 2003). Additionally, it was discovered that *B. stolpii* has sphingolipids in its cell wall, which is unusual as sphingolipids are generally associated with eukaryotic cells (Watanabe et al., 2001). Furthermore, host-independent mutants of *B. stolpii* lacked these sphingolipids and it was therefore assumed that these lipids are linked to the predation activity of this predator, although this has not been shown for *B. bacteriovorus*. In terms of the targeted prey cells, it has been hypothesised that the receptor sites involved in the attachment of the predator to the prey cell resides in the lipopolysaccharide (LPS) core, however, to date specific receptor sites have not been identified (Strauch et al., 2006).

In contrast, Gray and Ruby (1991) suggested that bdellovibrios possess a general but adaptable and efficient ability to recognise prey cells, which does not require a receptor or prey cell wall component. Thus, while no specific predator cell wall structure or prey receptor has been identified, research has indicated that once *B. bacteriovorus* irreversibly attaches to a prey cell, it aggressively rotates and bores a hole into the prey cell envelope (**Fig. 1.2c**) (Rittenberg, 1983; Rendulic et al., 2004; Strauch et al., 2006). The predator then enters the periplasmic space of the prey cell via this pore and subsequently secretes peptidoglycan-modifying enzymes such as glycanases, lipases and peptidases to seal this pore and modify the prey cell wall in order to create a niche within which it can proliferate (generally referred to as the bdelloplast) (**Fig. 1.2d**) (Rittenberg, 1983; Strauch et al., 2006; Lerner et al., 2012; Rotem et al., 2015). Additionally, the predator loses its flagella once it enters a prey cell (**Fig. 1.2d**) and produces various hydrolytic enzymes to break down the cellular constituents in order to utilise the prey cell's DNA, RNA and fatty acids, etc., to produce progeny (Rittenberg, 1983; Rendulic et al., 2004; Lambert et al., 2006; Strauch et al., 2006; Lambert et al., 2010). The predatory bacteria then proliferate by elongating and growing filamentously and once the filament has reached a size several times that of the free-living predatory cells, septation into progeny cells commence (**Fig. 1.2e** and **Fig. 1.2f**) (Strauch et al., 2006). The number of progeny produced is largely dependent on the prey cell utilised as host and can range from 3 to 4 progeny in small prey cells, to more than 90 in multinucleate *E. coli* (Kessel & Shilo, 1976; Diedrich, 1988). The new progeny are also equipped with flagella (Lambert et al., 2006). Subsequently, the prey cell is lysed and the released progeny are able to hunt for new prey (**Fig. 1.2f** and **Fig. 1.2a**).

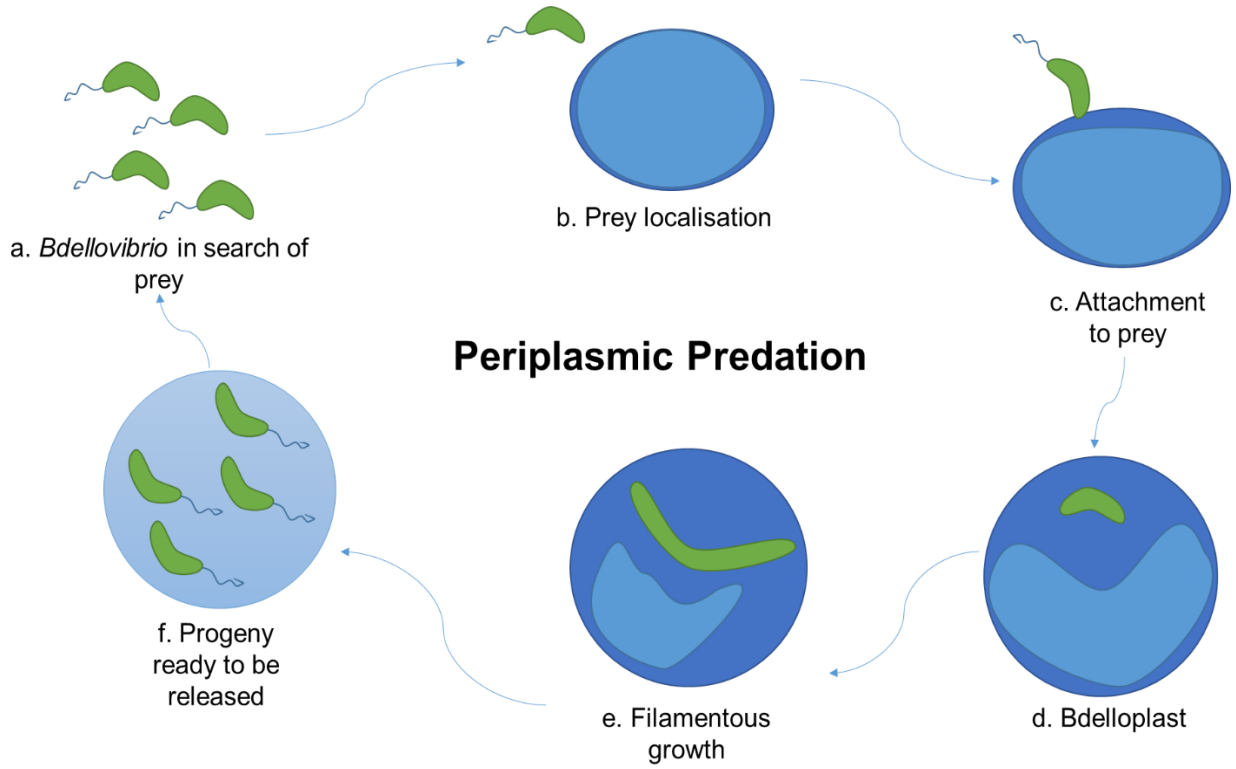

Fig. 1.2 Schematic representation of the periplasmic growth strategy employed by *B. bacteriovorus* [Adapted from Rotem et al. (2016)]

1.4.1.2 Genes Involved in *Bdellovibrio bacteriovorus* Predation

Using gene expression, oligonucleotide microarrays and reverse transcription qPCR, Lambert et al. (2010) attempted to identify the genes expressed during the attack phase and during the predation or growth stage (the "predatosome") of the life cycle of *B. bacteriovorus*. Overall, approximately 40% of the genome encoded for genes potentially involved in the predatory life cycle of the bacterium. Specifically, the authors found that during the *B. bacteriovorus* attack phase, 99 hypothetical proteins (with unknown functions), various transcriptional regulators, pili and flagella genes involved in motility, cell surface proteins, protective genes and GGDEF related proteins (regulatory protein domain important for phosphorylation or oxygen sensing), are upregulated. In contrast, 240 genes were significantly upregulated during the growth/predation stage. Of these genes, 174 encoded for hypothetical proteins presumed to be essential to the predatosome of this predator (Lambert et al., 2010).

Karunker et al. (2013) subsequently used Illumina high-throughput sequencing to further characterise the genes expressed during the attack and growth phase of *B. bacteriovorus* in co-culture with *E. coli*. The authors confirmed that 67% of the genes expressed by *B. bacteriovorus* were active during the growth phase (1557 genes), while 15% were active during the attack phase (353 genes) and 18% of the genes were simultaneously expressed during the growth and attack phase (Karunker et al., 2013). For the genes expressed during the attack phase, 59% encoded

for uncharacterised hypothetical proteins and the remaining 41% were found to be involved in chemotaxis, motility and cell surface composition. The growth phase genes were then found to mostly encode for ribosome biogenesis, cell division, DNA polymerase, chromosome partitioning proteins and energy metabolism (Karunker et al., 2013).

Thus, while various genes have been identified and were found to be upregulated during the growth or attack phase of *B. bacteriovorus*, various research groups are still conducting research to determine the functions of the various hypothetical proteins expressed during the life cycle of *B. bacteriovorus* in order to fully elucidate the mechanisms involved in their predation strategy (Karunker et al., 2013). Rotem et al. (2015) specifically identified genes upregulated during the attack, early-growth or attachment and growth phases of *B. bacteriovorus* and published a model on the potential regulatory pathways involved in the predation activity of *B. bacteriovorus* (**Fig. 1.3**). Therefore, for the purpose of this review, the specific genes identified and annotated by Rotem et al. (2015) will be discussed as a few of these genes were investigated in gene expression assays in Chapter 4 of this study.

It is important to note that *B. bacteriovorus* has been found to predominantly prey on Gram-negative organisms (Dashiff et al., 2011; Kadouri et al., 2013), and as such the life cycle and genes involved in the predation activity of *B. bacteriovorus* have mainly been studied in the presence of the model Gram-negative bacterium, *E. coli*. However, Iebba et al. (2014) and Pantanella et al. (2018) showed that *B. bacteriovorus* is able to prey on *S. aureus*, while the predator was also able to reduce biofilms formed by this Gram-positive organism. Specifically, Iebba et al. (2014) investigated the ability of *B. bacteriovorus* to prey on *Pseudomonas aeruginosa* (*P. aeruginosa*) and *S. aureus* strains isolated from cystic fibrosis patients. It was observed that *B. bacteriovorus* was able to effectively prey on planktonic *P. aeruginosa* and *S. aureus* cell cultures. Additionally, the predator was able to significantly reduce the *P. aeruginosa* and *S. aureus* biofilm biomass.

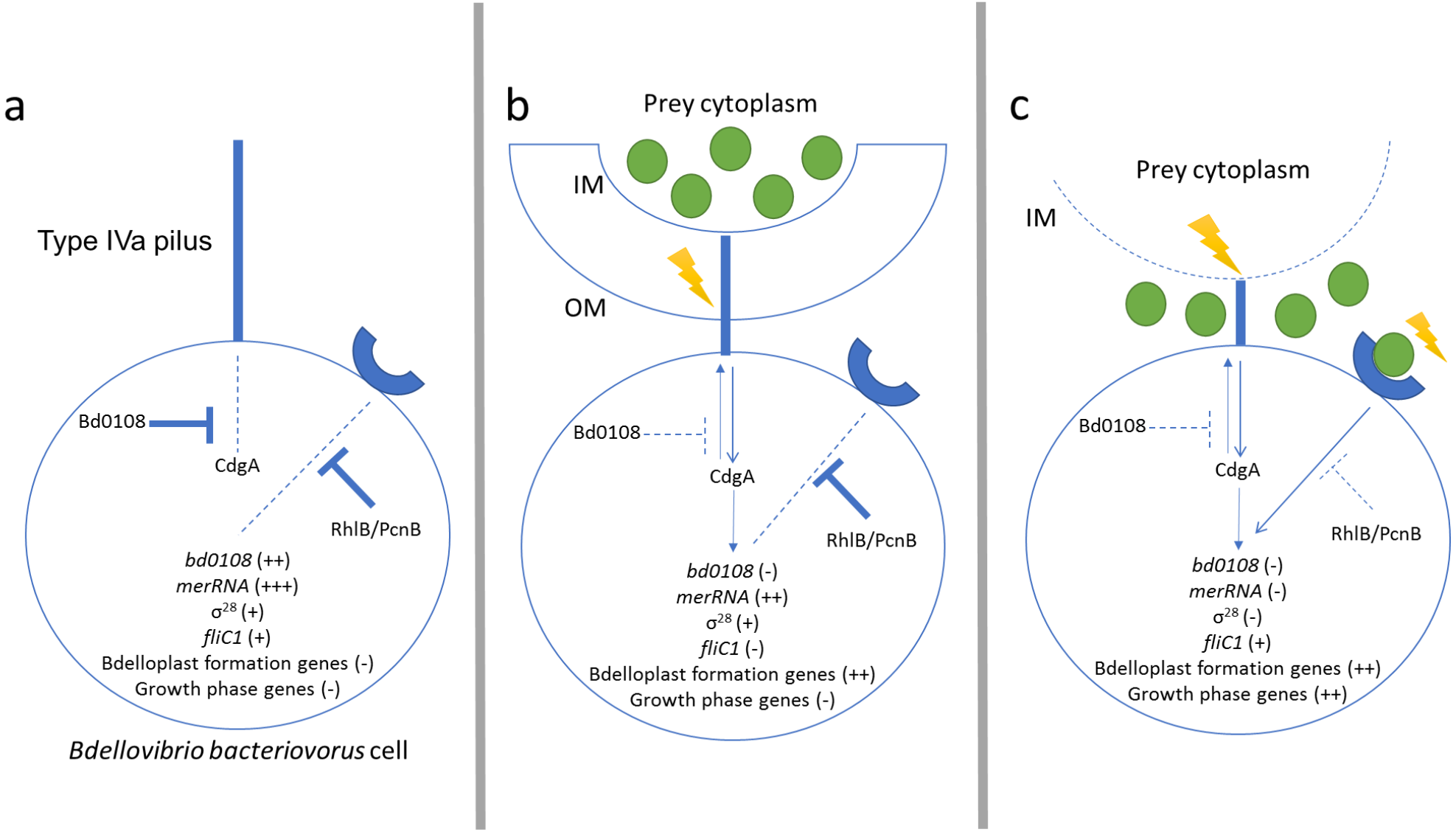

Fig. 1.3 Model of the potential regulatory pathways associated with the predation activity of *B. bacteriovorus* during the attack phase (a), attachment/penetration phase (b) and intracellular growth phase (c) [Adapted from Rotem et al., (2015)]

Using the hanging drop method and bright field microscopy, it was further observed that *B. bacteriovorus* displayed periplasmic predation in the presence of *P. aeruginosa* cells and switched to epibiotic predation when exposed to *S. aureus* cells. It was thus hypothesised that *B. bacteriovorus* was able to lyse the *S. aureus* cells by secreting specific lytic enzymes, which facilitated the epibiotic predation observed (Iebba et al., 2014). This was corroborated by Pantanella et al. (2018) who postulated that in the absence of natural or ideal prey (i.e. Gram-negative bacteria) or in nutrient deficient environments, *B. bacteriovorus* may alter its production of lytic enzymes and its predation strategy in order to utilise Gram-positive organisms as a potential survival strategy. However, further research is required to fully elucidate the interaction of the predator with Gram-negative and Gram-positive prey and monitor the gene expression during the attack and growth phase.

1.4.1.2.1 Attack Phase Genes

During the attack phase of the *B. bacteriovorus* cell cycle, non-replicating cells scavenge the environment for suitable prey. One of the genes (*bd0108*) found to be upregulated during the attack phase (**Fig. 1.3a**), is hypothesised to control the extrusion/retraction of a type IVa pilus (Rotem et al., 2015). Type IVa pili (encoded for by genes *bd1290* or *pilA*) are essential for the predation activity exhibited by *B. bacteriovorus* (Evans et al., 2007; Mahmoud & Koval, 2010). These pili are associated with many functions in other bacterial genera (such as *Pseudomonas*, *Nesseria* and *Myxococcus*) such as twitching motility, host cell adherence and invasion and fruiting body formation, and they are known for the considerable contractile force they exhibit. It has therefore been hypothesised that in *B. bacteriovorus*, type IVa pili are important for prey cell adherence (attachment) and invasion (Capeness et al., 2013). Rotem et al. (2015) found that these pili are specifically used to sense whether a prey cell has been encountered and for prey cell entry. These authors then hypothesised that during the attack phase, *B. bacteriovorus* expresses a default transcriptional profile, during which prey sensing is downregulated. Thus, the predatory bacterium does not receive a signal that a prey cell has been encountered and as such *bd0108* is expressed (**Fig. 1.3a**). Rotem et al. (2015) further theorised that the first predatory cue is related to the predator sensing an intact bacterial prey envelope via the type IVa pili. Once the pili comes into contact with a prey cell, a signal is received within the predator cell, which in turn represses the expression of *bd0108* (**Fig. 1.3b**). The *bd0108* gene is thus also essential in the regulatory pathway involved in the sensing of a prey cell and specifically represses signal transduction through CdgA (**Fig. 1.3a**), which is a sensor histidine kinase which forms part of the cyclic di-GMP network (secondary messengers) in *B. bacteriovorus*. The cyclic di-GMP network of *B. bacteriovorus* is known to consist of five diguanylate cyclases (DGCs) and CdgA is an inactive DGC known to be essential for rapid prey invasion. Additionally, a second predatory cue (a second signal that a prey cell has been encountered), which is transduced by a late signalling pathway through an unknown receptor, has been found to be repressed by RhlB or PcnB (RNA

degradosome subunits) (**Fig. 1.3a**). In *B. bacteriovorus*, the genes *bd0108*, *rhlB* and *pcnB*, are thus essential for obligatory predatory growth, while host-independent variants of *B. bacteriovorus* have been found to have mutations in these genes. The repression of the signal transduction of the first and second predatory cues then result in the upregulation of the *merRNA* gene (which encodes for a massively transcribed cyclic di-GMP riboswitch) and sigma factor 28 (σ^{28}) (**Fig. 1.3a**).

Bdellovibrio bacteriovorus attack phase cells are also known for their high-speed motility, which is conferred by a single sheathed flagella. It is therefore not surprising that during the attack phase, flagella encoding genes are expressed (Lambert et al., 2006). However, as mentioned previously, once *B. bacteriovorus* enters a prey cell, the predator loses its flagella (Lambert et al., 2006). Therefore, expression of flagella genes can be associated with the attack phase of *Bdellovibrio*. Karunker et al. (2013) found that while there are six *fliC* genes in the *B. bacteriovorus* genome, the *fliC1* gene was upregulated 1636-fold during the attack phase (**Fig. 1.3a**), emphasising the importance of flagella in the hunt and location of prey cells and the survival of *B. bacteriovorus*. The *fliC1* gene specifically encodes for the middle section of the flagellar filament (Iida et al., 2009).

1.4.1.2.2 Invasion and Growth Phase Genes

Once the attack phase predator irreversibly binds to a prey cell, the gene expression profile of *B. bacteriovorus* shifts from an attack phase programme to a recognition programme. The first predation cue (first signal transduction) is sensed by *B. bacteriovorus* and the signal is transduced by the second messenger CdgA (**Fig. 1.3b**) (Rotem et al., 2015). This alters the extrusion/retraction state of the type IVa pilus as CdgA interacts with the pilus regulatory protein complex. This effect is thought to be essential for prey cell penetration (Rotem et al., 2015).

Bdellovibrio bacteriovorus then creates a pore in the cell wall and enters the periplasmic space of the prey cell (attachment or early growth phase). The predator subsequently secretes peptidoglycan-modifying enzymes to seal the pore and forms the bdelloplast (Lambert et al., 2010; Lerner et al., 2012; Rotem et al., 2015). The genes coding for the peptidoglycan-modifying enzymes include *bd0816*, *bd3459*, *bd3575*, *bd1358* and *bd3279*, which encodes for D-ala-D-ala carboxypeptidases, lytic murein transglycosylase, putative peptidoglycan binding protein and a polysaccharide de-acetylase (Lambert et al., 2010; Lerner et al., 2012). These enzymes allow the predator to modify the peptidoglycan of the prey cell during the formation of the bdelloplast in order to create a niche for growth, whereafter the peptidoglycan of the prey cell is broken down to provide the predator with building blocks to produce progeny and to lyse the prey cell once the progeny have matured (Lambert et al., 2010; Lerner et al., 2012). In particular, two genes, *bd0816* and *bd3459*, which encode for peptidoglycan DD-endopeptidases, modify the invaded cell wall

by hydrolysing the structural 3-4 peptide crosslinks (Lambert et al., 2010; Lerner et al., 2012; Lambert et al., 2015). This effectively allows for the modification of the prey cell shape to a rounded invaded form, which in turn prevents the entry of another predatory bacterium, by indicating occupation by the predator and ultimately enhancing predation activity (Lambert et al., 2015).

Furthermore, CdgA partially represses *merRNA* expression, while σ^{28} remains active (**Fig. 1.3b**) (Rotem et al., 2015). The second late signal transduction (second predatory cue), which has been speculated to be essential for predatory bacteria to actively grow within an invaded prey cell, is then silenced during this phase (Rotem et al., 2015). Therefore, the predatory cells are now in an intermediary or transitionary state (early growth phase), which can last up to 1 hour (Rotem et al., 2015). This intermediary phase can thus be characterised by the expression of peptidoglycan-modifying genes such as *bd0816* and *bd3459*.

Once the second predatory cue is received and the late signalling pathway is activated, the predatory bacteria enter the growth phase, during which they actively proliferate within an invaded prey cell (**Fig. 1.3c**) (Rotem et al., 2015). It has been hypothesised that the second predatory cue is linked to the cytosol of the prey cell, and this cue is used by the predator to determine if the prey cell can provide sufficient nutrients for a growing predatory cell (Rotem et al., 2015). During this phase, the σ^{28} regulon, *merRNA* and bdelloplast-formation genes are silenced, while the core growth phase genes are upregulated (**Fig. 1.3c**). Lambert et al. (2010) found that several sensor-regulator genes [transcriptional regulator genes (*bd0136*, *bd1634*, *bd3063*), two-component sensor-kinase genes (*bd3359*, *bd3360*) and a CarD-like transcriptional regulator gene (*bd2320*)] were upregulated during the growth phase of *B. bacteriovorus*. These genes are presumably involved in sensing the conditions in the bdelloplast, the secretion of hydrolytic enzymes, *Bdellovibrio* septation and prey cell lysis. Thus, during predation/growth, genes encoding for 173 hypothetical proteins, 6 transcriptional regulators, nucleic acid synthesis, chaperones, peptidoglycan metabolising enzymes, hydrolytic enzymes (protease, esterases, helicases and endonucleases), TonB-like proteins, ABC transporters, ATP synthase and Fe-S clusters, are upregulated.

In summary, research has indicated that the attack phase of *B. bacteriovorus* can be characterised by the expression of *bd0108*, the upregulation of *merRNA* and the expression of genes encoding for flagella such as *fliC*, while the bdelloplast-formation and growth phase genes are not expressed during this phase (**Fig. 1.3a**) (Lambert et al., 2006; Karunker et al., 2013; Rotem et al., 2015). Therefore, during Chapter 4 of this dissertation, the expression of *bd0108*, *merRNA* and *fliC1* were utilised to monitor the attack phase of *B. bacteriovorus*. Additionally, the recognition phase (attachment or early growth phase) of *B. bacteriovorus* can be characterised by the expression of genes encoding for peptidoglycan-modifying enzymes (such as *bd0816*) and

the growth phase can be characterised by genes important for the intracellular growth of the predator such as genes essential for DNA replication and those encoding for chaperone proteins (which aid in folding functional proteins after translation of mRNA and is therefore essential for the production of progeny cells). Therefore, the *bd0816* gene was utilised to monitor the recognition phase (attachment or early growth phase) and *groES1* (encoding for a chaperone protein) was selected to monitor the growth phase of *B. bacteriovorus* in Chapter 4 of this study.

1.4.2 Potential Applications of Predatory Bacteria

Although various aspects of the BALO predatory behaviour remains to be elucidated, *Bdellovibrio* spp. are able to effectively reduce prey cell populations of pathogens and opportunistic pathogens and these predators and their lytic enzymes may thus serve as promising alternatives to current therapeutic or biocontrol agents (Sockett & Lambert, 2004; Markelova, 2010). Most studies have focused on investigating the application of BALOs as “live antibiotics” in a clinical setting to combat multi-drug resistant infections (Dashiff et al., 2011; Kadouri et al., 2013; Shanks et al., 2013; Iebba et al., 2014; Sun et al., 2017). For example, Dharani et al. (2018) investigated the susceptibility of colistin-resistant *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Acinetobacter baumannii* (*A. baumannii*) to predation by *B. bacteriovorus* and *M. aeruginosavorus* and compared it to the susceptibility of their colistin-sensitive counterparts. While the colistin-resistant and –sensitive strains were found to be sensitive to predation by *B. bacteriovorus* and *M. aeruginosavorus*, *B. bacteriovorus* was able to significantly reduce biofilms formed by colistin-resistant *E. coli* and *A. baumannii*. Similarly, Dashiff et al. (2011) found that both *B. bacteriovorus* and *M. aeruginosavorus* were able to prey on and reduce the concentration of multidrug-resistant pathogens, which included *Acinetobacter spp., Burkholderia cepacia, K. pneumoniae* and *P. aeruginosa,* in co-culture experiments. Investigation into their application as live antimicrobial agents is also warranted as the BALOs have been shown to not elicit an immune response by human macrophages and they have been detected in the gut of healthy individuals (Iebba et al., 2013; Shanks et al., 2013; Monnappa et al., 2014).

Researchers have also suggested that BALOs may be used as biocontrol agents in the food, aquaculture and water treatment industries. Cao et al. (2012) investigated *Bdellovibrio* strain F16 as a potential probiotic for sturgeon aquaculture and the effect of this predator on the fish pathogen *A. hydrophila* was also analysed. It was observed that *Bdellovibrio* strain F16 effectively reduced *A. hydrophila* concentrations in co-culture, while no haemolytic activity on eukaryotic cell lines by the predator was detected (Cao et al., 2012). Similarly, Kongrueng et al. (2017) isolated *Bacteriovorax* spp. and investigated the ability of different strains to reduce the concentration of *Vibrio parahaemolyticus* (*V. parahaemolyticus*) in co-culture, as this organism causes acute hepatopancreatic necrosis disease in shrimp, leading to great financial losses in shrimp aquaculture. The authors found that all the *Bacteriovorax* strains could prey on the disease-

causing *V. parahaemolyticus* strains. In addition, *Bacteriovorax* could be used to prevent infection of the shrimp by *V. parahaemolyticus,* as the mortality rate of post-larval shrimp inoculated with 10^7 CFU/mL of *V. parahaemolyticus* and various concentrations of *Bacteriovorax* strain BV-A [10^2 to 10^6 plaque forming units (PFU)/mL] was reduced by an average of 27.5 to 52.5% (Kongrueng et al., 2017).

Yu et al. (2017) investigated the ability of *Bdellovibrio* spp. to act as a biolysis agent, which could be applied to improve the dewaterability of excess wastewater sludge. It was hypothesised that the application of the predator could improve sludge disposal efficiency and could possibly serve as an environmentally friendly alternative to chemical sludge pre-treatment. The authors found that without any additional pH adjustments or chemical additives, the application of *Bdellovibrio* spp. significantly disrupted the sludge, subsequently releasing the intracellular water, which significantly improved sludge dewaterability and improved the sludge disposal efficiency. Kim et al. (2013) and Özkan et al. (2018) then investigated the effect of applying *B. bacteriovorus* as a pre-treatment to filtration during potable and wastewater treatment, respectively. Results from both studies indicated that the application of *B. bacteriovorus* as a pre-treatment to the feed water could significantly reduce membrane fouling in water treatment plants as the initial microbial load in the water was reduced. Yilmaz et al. (2014) investigated the use of *B. bacteriovorus* to remove preformed biofilms from membrane filters used in wastewater treatment and found that the predatory bacterium effectively improved the flux across the membrane after treatment as compared to the control membrane. This implies that using *B. bacteriovorus* as a pre-treatment or applying the predator to reduce preformed biofilms on membrane filters, can effectively improve filtration efficiency and extend the lifespan of membrane filters applied in industry.

Bdellovibrio-and-like-organisms thus have a distinct advantage as biocontrol agents as many current agents target a specific organism, while BALOs could potentially target multiple Gram-negative opportunistic and pathogenic organisms because of their wide prey range (Olanya & Lakshman, 2015). Additionally, no acquired (genetic) resistance to the BALOs have been documented, as it is unlikely that the BALOs target specific cell surface receptors on prey bacteria (Shemesh & Jurkevitch, 2004; Dwidar et al., 2012; Olanya & Lakshman, 2015). Moreover, the BALOs are able to target actively growing prey cells, prey cells in stationary phase and metabolically inactive cells (Olanya & Lakshman, 2015). Despite these benefits it is important to note that research has indicated that the predation activity of particularly *B. bacteriovorus* is predominantly limited to Gram-negative organisms, while other predatory bacteria [such as *Micavibrio* and *Cupriavidus* (Pantanella et al., 2018)] have been found to prey on Gram-positive organisms. Research elucidating the full potential prey range of the BALOs, particularly *B. bacteriovorus*, is thus required. The BALOs are also not able to attack Gram-negative bacteria which have a protective S-layer (Sinha et al., 2014). Additionally, predatory bacteria do not completely eradicate prey cell populations, even at high predator to prey ratios (Sinha et al., 2014;

Olanya & Lakshman, 2015). This is because prey cell populations exhibit an inherent plastic phenotypic resistance towards predation (Shemesh & Jurkevitch, 2004). This resistance mechanism is not associated with a genetic mutation in the prey cells or the acquisition of genetic material (Shemesh & Jurkevitch, 2004). It is a general phenotypic adaptation observed in residual prey populations after exposure to a predator and has been hypothesised to be related to low prey cell density. Research has thus indicated that the prey population should have a cell density of 10^5 CFU/mL for effective predation by the predator (Shemesh & Jurkevitch, 2004).

Thus, while the predatory bacteria can potentially be applied as a safe and environmentally friendly biological control agent, based on the fact that they do not completely eradicate prey cell populations, they would ideally be applied in pre-treatment strategies. Employing these predators in pre-treatment systems, in combination with physical or chemical treatment methods, may result in increased treatment efficiencies as bacterial populations are directly targeted by the predatory bacteria, whereafter physical treatment methods could remove any residual bacterial populations.

1.5 Project Aims

The use of predatory bacteria (such as *B. bacteriovorus*) as biocontrol agents has distinct advantages over other biocontrol strategies, as genetic or acquired resistance to predation has not been documented, predatory bacteria are able to prey on encapsulated bacteria (which are resistant to phage attack) and they can be used to target a range of Gram-negative bacteria (Olanya & Lakshman, 2015). The primary aim of this study was thus to investigate the efficiency of *B. bacteriovorus* to reduce the concentration of microorganisms frequently detected in untreated harvested rainwater and persisting in treated rainwater samples, by applying *B. bacteriovorus* as a pre-treatment to SODIS and solar photocatalytic disinfection. A secondary aim was to investigate the interaction between *B. bacteriovorus* and Gram-positive prey (considered non-ideal prey) and compare this interaction to their interaction with Gram-negative prey by employing gene expression assays.

These aims were achieved as follows:

Chapter 2: Assessment of predatory bacteria and prey interactions using culture-based methods and EMA-qPCR (published in Microbiological Research)

- *Bdellovibrio* spp. were isolated and purified from wastewater using standard culture-based methods (double-layer agar overlays) and all isolates were identified using conventional polymerase chain reaction (PCR) and sequencing analysis.
- A *Bdellovibrio* isolate (*B. bacteriovorus* PF13) was subsequently co-cultured with three Gram-negative organisms [*P. aeruginosa, Pseudomonas fluorescens* (*P. fluorescens*) and *Klebsiella pneumoniae* (*K. pneumoniae*)] and two Gram-positive organisms

[*S. aureus* and *Enterococcus faecium* (*E. faecium*)] in diluted nutrient broth (DNB) and HEPES buffer to investigate whether the predator can prey on these target organisms. *Pseudomonas*, *Klebsiella*, *Enterococcus* and *Staphylococcus* spp. are frequently detected in harvested rainwater, while *Pseudomonas* and *Klebsiella* spp. have been shown to persist after the implementation of various disinfection strategies (Ahmed et al., 2008; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014b; Hamilton et al., 2017; Clements et al., 2019). Furthermore, DNB was employed as a nutrient poor medium and HEPES buffer was employed as a nutrient deficient medium, which allowed for the investigation and comparison of the predator's interaction with the different prey cells under nutrient limiting and nutrient deficient conditions.

- The co-cultures were monitored using spread plating, double-layer agar overlays and EMA-qPCR in order to identify an optimum technique to monitor the effect of *B. bacteriovorus* on the various prey bacteria.

Chapter 3: Predatory bacteria in combination with solar disinfection and solar photocatalysis for the treatment of rainwater (published in Water Research)

- The research for this chapter was conducted in collaboration with Ulster University (Northern Ireland), where the PhD candidate was based for ten weeks. The TiO_2-rGO composite photocatalyst was synthesised and immobilised onto glass raschig rings by the PhD candidate (during her stay at Ulster University).
- A small-scale SODIS-CPC reactor was also designed by the collaborators at Ulster University and four systems were manufactured in South Africa using locally available materials.
- To test the disinfection efficiency of the SODIS-CPC systems, synthetic rainwater was seeded with *K. pneumoniae* (isolated from solar pasteurized rainwater at a treatment temperature above 70°C) (Clements et al., 2019) and *E. faecium* (isolated from untreated harvested rainwater) (Dobrowsky et al., 2014b). *Klebsiella pneumoniae* was selected as the Gram-negative target bacteria as Clements et al. (2019) indicated that this organism survived heat treatment of rainwater above 70°C and thus persists after rainwater treatment. *Enterococcus faecium* was selected as the Gram-positive prey as this bacterium is commonly utilised to monitor the quality of water sources. In addition, results from Chapter 2 indicated that these two organisms are sensitive to predation by *B. bacteriovorus* PF13 in co-culture experiments.
- For each test organism (*K. pneumoniae* and *E. faecium*), two experimental groups were analysed as follows: for one experimental group (two systems) the synthetic rainwater samples seeded with the test organisms were pre-treated with *B. bacteriovorus* for 72 hours; while for the second experimental group (two systems) no pre-treatment occurred. Additionally, for each experimental group, one solar-CPC system contained

TiO_2-rGO coated raschig rings (solar photocatalysis), while the second system contained uncoated raschig rings (SODIS only). The four solar-CPC reactors were filled with the pre-treated or untreated seeded synthetic rainwater samples and were exposed to natural sunlight for 4 hours.

- Samples were collected before and after *B. bacteriovorus* pre-treatment and every 30 minutes during the solar and solar photocatalytic treatments from time point 0 to 240 minutes (4 hours).
- The viability of *B. bacteriovorus*, *K. pneumoniae* and *E. faecium* was monitored using culture-based methods (spread plates and double-layer agar overlays) and EMA-qPCR, to determine if the treatments effectively eradicated the bacterial cells.

Chapter 4: Expression of attack and growth phase genes of *Bdellovibrio bacteriovorus* in the presence of Gram-negative and Gram-positive prey (published in Microbiological Research)

- The molecular response (gene expression) of *B. bacteriovorus* when exposed to Gram-negative prey cells was compared to the predator's response when exposed to Gram-positive prey cells. Subsequently *B. bacteriovorus* PF13 was co-cultured with *E. coli* (Gram-negative control to compare results obtained to results reported in literature), *K. pneumoniae* (Gram-negative prey) and *E. faecium* (Gram-positive prey), respectively, for 4 hours (time required for *B. bacteriovorus* to complete one life cycle).
- Total RNA was extracted from the co-cultures at specific time points during the attack phase (0 minutes), attachment phase (15 to 30 minutes) and growth phase (240 minutes) of the predatory life cycle.
- The expression of genes associated with flagella production (*fliC1*), the host-independent locus (*bd0108*) and massively expressed riboswitch RNA (*merRNA*), which are specific to the attack phase of *B. bacteriovorus*, a peptidoglycan-modifying enzyme (*bd0816*), which is specifically expressed during the attachment phase (early growth phase) and a chaperone protein (*groES1*), which is expressed during the growth phase, were monitored using reverse transcription relative qPCR.
- The expression of the genes when *B. bacteriovorus* was co-cultured with the Gram-negative organisms (*E. coli* and *K. pneumoniae*) was subsequently compared to the gene expression in co-culture with *E. faecium* to elucidate how *B. bacteriovorus* adapts when exposed to different prey cells.

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Assessment of predatory bacteria and prey interactions using culture-based methods and EMA-qPCR

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Abstract

Traditional culture-based enumeration methods were compared with the ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) technique to assess *Bdellovibrio*-and-like-organisms (BALOs) predator-prey interactions. Gram-negative [*Pseudomonas* spp. and *Klebsiella pneumoniae* (*K. pneumoniae*)] and Gram-positive [*Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecium* (*E. faecium*)] organisms were employed as prey cells, while a *Bdellovibrio bacteriovorus* strain (PF13) was used as the predator. The co-culture experiments were also compared in diluted nutrient broth (DNB) and HEPES buffer. In both media, *K. pneumoniae* (maximum log reduction of 5.13) and *Pseudomonas fluorescens* (*P. fluorescens*) (maximum log reduction of 4.21) were sensitive to predation by *B. bacteriovorus* PF13 as their cell counts and gene copies were reduced during all the co-culture experiments, while the concentration of *B. bacteriovorus* PF13 increased. The concentration of *B. bacteriovorus* PF13 also increased in the presence of *S. aureus* (HEPES buffer) and *E. faecium* (DNB), indicating that the predator interacted with these Gram-positive prey in order to survive. Moreover, as no predator plaques were produced in the co-culture experiments with *P. aeruginosa* (DNB and HEPES buffer), *S. aureus* (DNB and HEPES buffer) and *E. faecium* (HEPES buffer), EMA-qPCR proved to be beneficial in monitoring the concentration of *B. bacteriovorus*. In conclusion, the cell counts and/or EMA-qPCR analysis for the HEPES buffer and DNB assays were successfully employed to monitor the predation of *P. fluorescens* and *K. pneumoniae* by *B. bacteriovorus,* while *E. faecium* was sensitive to predation in DNB and *S. aureus* was sensitive to predation in HEPES buffer.

Keywords: *Bdellovibrio bacteriovorus*; predation; EMA-qPCR; Gram-positive prey; diluted nutrient broth; HEPES buffer

2.1 Introduction

Predatory bacteria such as *Bdellovibrio, Micavibrio* and *Bacteriovorax* spp. play important roles as “ecological balancer species” in the natural environment (Jurkevitch et al., 2000; Allen et al., 2014; Iebba et al., 2014). These Gram-negative bacteria are collectively referred to as *Bdellovibrio*-and-like-organisms (BALOs) and flourish in a variety of aquatic and terrestrial habitats such as bulk soil (Jurkevitch et al., 2000), the rhizosphere of plants (Jurkevitch et al., 2000), rivers (Sar et al., 2015), estuaries (brackish water) (Schoefield & Williams, 1989), fish ponds (Chu & Zhu, 2010), water and wastewater treatment plants (Feng et al., 2016; Yu et al., 2017) and they have been isolated from the human gut (Iebba et al., 2013). They thrive by preying on other Gram-negative bacteria either in an epibiotic or periplasmic manner (Kadouri et al., 2013; Avidan et al., 2017).

It is however, challenging to investigate the interaction of the predator with the prey cells in their natural habitat and BALO predator-prey interactions are predominantly studied using culture-based methods. Diluted nutrient broth (DNB) is the most widely used medium to assess bacterial predator-prey interactions and while it contains lower concentrations of nutrients in comparison to full strength nutrient broth (usually 1/10 strength of nutrient broth), HEPES buffer suspension does not contain any nutrients and only provides the cations (magnesium and calcium) required by predatory bacteria for effective predation (Koval, 2006; Rotem et al., 2014). Numerous pitfalls have however, been associated with the accuracy of the methods employed to investigate predator-prey interactions. Firstly, BALOs vary significantly in their prey range and the prey cells employed in isolation experiments may not be specific for the predatory bacteria being investigated (Koval, 2006; Williams & Piñeiro, 2006; Zheng et al., 2008). Additionally, the environmental strains of prey cells may not be amenable to cultivation in the laboratory, which further hinders the discovery and investigation of new or unique BALOs and their prey interactions (Williams & Piñeiro, 2006; Zheng et al., 2008; Rotem et al., 2014). Moreover, while the enumeration of the predator cells using plaque forming units (PFU) on double-layer agar overlays is cost-effective, some BALOs have difficulty preying on bacteria in soft agar, which may subsequently hinder plaque formation and influences the accuracy of the plaque counts obtained during co-culture experiments (Koval, 2006). The enumeration of the predatory bacteria is also not a time-effective process, as the plaques may only become visible after 2-7 days of incubation (Koval, 2006; Jurkevitch, 2006).

Accurate methods of investigating and studying the BALO-prey cell interactions are thus required and the use of molecular-based techniques, specifically viability qPCR which incorporates Ethidium Monoazide Bromide (EMA) or Propidium Monoazide (PMA), may be advantageous as the growth and degradation of the predator and prey cells can be monitored directly in co-culture experiments. Viability dyes such as EMA or PMA, effectively reduce the amplification of DNA from

cells with compromised membranes (presumed non-viable) or extracellular DNA and therefore can be utilised to monitor the gene copies (GC) from predominantly viable cells (Cenciarini-Borde et al., 2009; Seinige et al., 2014). The use of viability dyes could thus account for the viable but non-culturable (VBNC) community as these cells have intact membranes and their DNA will subsequently be quantified with qPCR after viability dye treatment. Moreover, as some BALOs may have difficulty forming plaques on double-layer agar overlays, viability qPCR could be employed to monitor their growth in co-culture experiments. This technique could thus potentially aid in accurately monitoring and quantifying both predator and prey cells during co-culture experiments in a time-effective manner as results can be generated within 24 hours (Cenciarini-Borde et al., 2009; Seinige et al., 2014).

The aim of the current study was thus to compare the standard culture-based methods, employed to monitor the interaction of *Bdellovibrio* spp. with different prey cells, with the molecular-based EMA-qPCR method. A secondary aim was to investigate and compare the interactions of the isolated *Bdellovibrio* spp. with opportunistic pathogenic bacteria in DNB and HEPES buffer. To achieve these aims *Bdellovibrio* spp. were isolated from wastewater as literature has indicated that these bacteria are abundant in wastewater treatment plants (Yu et al., 2017). The potential of the isolated predatory bacteria to prey on Gram-negative organisms was assessed using American Type Culture Collection (ATCC) isolates of *Pseudomonas aeruginosa* (*P. aeruginosa*), *Pseudomonas fluorescens* (*P. fluorescens*) and *Klebsiella pneumoniae* (*K. pneumoniae*) as prey in co-culture experiments. As contradictory evidence on the ability of BALOs to prey on Gram-positive bacteria has been presented (Dashiff et al., 2011; Iebba et al., 2014; Monnappa et al., 2011), the Gram-positive prey, *Staphylococcus aureus* (*S. aureus*) ATCC 25925 and a clinical *Enterococcus faecium* (*E. faecium*) isolate were included in this study.

2.2 Materials and Methods

2.2.1 Wastewater Sample Collection and Processing

A wastewater sample (1 L) was collected from the influent point of the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: 33°59'21.13"S 18°47'47.75"E) in a sterile 1 L Schott bottle. Twenty millilitres (20 mL) of the sample was incubated at 30°C for 1 h with shaking at 200 rpm. The incubated aliquot was subsequently centrifuged at 500 rpm for 10 min, whereafter the supernatant was filtered through a 1.2 µm cellulose nitrate filter (47 mm; Sartorius Biolab Products, Kimix, South Africa) (Feng et al., 2016). The filtrate was utilised for the subsequent *Bdellovibrio* spp. isolation experiments.

2.2.2 *Bdellovibrio*-and-like-organisms Isolation

Pseudomonas spp. [*P. aeruginosa* ATCC 27853, *P. fluorescens* ATCC 13525 and *Pseudomonas protegens* (*P. protegens*) ATCC 17386] were selected as prey bacteria for the isolation of *Bdellovibrio* spp. in the current study as research has indicated that *Pseudomonas* spp. are sensitive to predation (Dashiff et al., 2011; Feng et al., 2016). The method as outlined by Feng et al. (2016) was used for the isolation of the *Bdellovibrio* spp.

2.2.3 *Bdellovibrio*-and-like-organisms Purification

To purify the putative BALOs, plaques with varying diameters were removed from the agar using a sterile blade. The plaques were inserted into 70 mL DNB (pH 7.2) [0.1 g/L of Lab Lemco Powder (Oxoid, Hampshire, England), 0.2 g/L of yeast extract (Biolab, Midrand, South Africa), 0.5 g/L peptone bacteriological (Biolab), 0.5 g/L sodium chloride (NaCl; Kimix, Cape Town, South Africa), 3 mM magnesium chloride ($MgCl_2$; Oxoid) and 2 mM calcium chloride ($CaCl_2$; Biolab)] which contained 1 mL of the respective *Pseudomonas* spp. prey cell cultures (10^9 cells/mL) (Feng et al., 2016; Yu et al., 2017). The inocula were incubated at 30°C at 200 rpm and were monitored for a reduction in OD every 24 h (Yu et al., 2017). When the incubation medium was cleared (OD < 0.20) (Im et al., 2014), serial dilutions (10^{-2} to 10^{-8}) were prepared, whereafter 500 µL of each dilution was mixed with 500 µL fresh prey cells in 5 mL molten soft-top agar which was then poured onto DNB agar plates (Yu et al., 2017). The plates were incubated at 30°C. This process was repeated five times to obtain pure BALO strains (Yu et al., 2017).

2.2.4 DNA Extractions and Conventional PCR for BALOs Identification

To confirm the isolation of BALOs, the isolates and *Pseudomonas* spp. co-cultures were subjected to DNA extractions using the boiling method as previously described by Ndlovu et al. (2015). The DNA extracts were then subjected to conventional PCR for the identification of *Bdellovibrio* spp., using the primers Bd347F – GGAGGCAGCAGTAGGGAATA and Bd549R – GCTAGGATCCCTCGTCTTACC (Van Essche et al., 2009). Each PCR reaction consisted of 1X Green GoTaq® Reaction Buffer (Promega Corp, Madison, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.9 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega Corp, Madison, USA) and 5 µL of DNA in a final volume of 25 µL. The cycling parameters consisted of a denaturation step at 94°C for 10 min, followed by 40 cycles of 94°C for 30 s and 60°C for 30 s and a final elongation step at 72°C for 10 min. Each PCR assay included a sterile milliQ negative control.

The PCR products were electrophoresed on an agarose gel (1.5%) stained with ethidium bromide (0.5 µg/mL) in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer, for 1 h at 80 V. The products were visualised using the Vilber Lourmat gel documentation system (Vilber Lourmat,

Collégien, France) to confirm the presence of the desired amplicon (202 bp). Representative PCR products (*n* = 9) were cleaned, sequenced and the sequence data analysed as outlined in Waso et al. (2016). The DNA sequences of representative isolates that showed > 97% similarity (< 3% diversity) to *Bdellovibrio* spp. were recorded and the DNA obtained from the isolates identified as *Bdellovibrio* spp. were subsequently utilised as positive control DNA in all the molecular-based assays.

2.2.5 Comparison of Culture-Based and Molecular Methods to Assess Predation

2.2.5.1 Predator Stock Lysate

Isolate PF13 (isolated by co-culturing with *P. fluorescens* ATCC 13525 and designated as isolate number 13) was identified as a *Bdellovibrio bacteriovorus* (*B. bacteriovorus*) strain by sequencing analysis (as described above) and was selected for all predation assays as this isolate consistently produced plaques on *Pseudomonas* prey. For the predation assays, a predator stock lysate of *B. bacteriovorus* PF13 was prepared as described by Dashiff et al. (2011). The stock lysate was subsequently used as the predator inoculum in all the predation assays. In addition, 10 mL of the predator stock lysate was filtered through a 0.22 µm Nylon filter (47 mm; Starlab Scientific, Kimix, South Africa) three times, to remove all the *B. bacteriovorus* cells from the suspension. The absence of the predator from the resulting filtrate was confirmed by double-layer agar overlays as described in the preceding sections. This filtrate then served as the inoculum for the predation negative control in all predation assays as described by Kadouri et al. (2013).

2.2.5.2 Predation Assays

In order to determine whether *B. bacteriovorus* PF13 was able to prey on opportunistic pathogens, *B. bacteriovorus* PF13 was co-cultured with *P. fluorescens* ATCC 13525, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 333305, *S. aureus* ATCC 25925 and a clinical isolate of *E. faecium* (designated as *E. faecium* Clinical). These isolates were obtained from the Water Resource Laboratory Culture Collection at the Department of Microbiology (Stellenbosch University).

To compare the efficiency of *B. bacteriovorus* PF13 to reduce the concentration of the above-mentioned prey cells in co-culture, the experiments were performed in both DNB and HEPES buffer [25mM, pH 7.2; supplemented with 3 mM $MgCl_2$ (Oxoid) and 2 mM $CaCl_2$ (Biolab)] (Shemesh & Jurkevitch, 2004). All the prey strains were grown in 100 mL LB at 37°C for 24 h. The prey cell cultures were then centrifuged at 8 000 rpm for 15 min, whereafter the pellet was washed and resuspended in either DNB or supplemented HEPES buffer (OD_{600} = 1.00). For each co-culture experiment, 98 mL of DNB or HEPES buffer was subsequently inoculated as described by Yu et al. (2017). For each set of co-culture flasks, a predation negative control was included, which consisted of 98 mL of DNB/HEPES buffer inoculated with 1 mL of the respective prey cell

cultures and 1 mL of filtered predator stock lysate (which lacked predator cells). The co-cultures and predation negative controls were incubated at 30°C for 120 h with shaking at 200 rpm. These experiments were repeated three times.

2.2.5.2.1 Culture-Based Analysis of the Co-Culture Experiments

Three millilitre (3 mL) aliquots were collected from each co-culture flask and the predation negative control at 0, 48, 96 and 120 h. Serial dilutions (10^{-2} to 10^{-9}) were prepared from each aliquot and 100 µL of each dilution was spread plated onto LB agar to determine the colony forming units (CFU)/mL of the respective prey cell cultures, in duplicate. In addition, soft-top agar overlays were prepared as described above, to enumerate the PFU of *B. bacteriovorus* PF13 in co-culture with the various prey cells.

2.2.5.2.2 Ethidium Monoazide Treatment and DNA Extractions of the Co-Culture Experiments

For the molecular analysis of the co-culture experiments, 500 µL of each 3 mL time series aliquot (0, 48, 96 and 120 h) was EMA treated as described by Reyneke et al. (2017), whereafter the EMA-treated aliquots were subjected to DNA extractions using the Zymo Research Soil Microbe DNA Miniprep™ kit as per the manufacturer's instructions.

2.2.5.2.3 Quantitative Real-Time PCR

Quantitative real-time PCR was performed to quantify the GC of *B. bacteriovorus* PF13 in co-culture with *P. fluorescens* ATCC 13525*, P. aeruginosa* ATCC 27853*, K. pneumoniae* ATCC 333305*, S. aureus* ATCC 25925 and *E. faecium* Clinical and to quantify the GC of each of the respective prey cells in co-culture for each time point (0, 48, 96 and 120 h) in both the DNB and HEPES buffer. All qPCR assays were performed using the LightCycler® 96 Instrument (Roche Diagnostics, Mannheim, Germany) and the FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) as outlined in Waso et al. (2018). All the qPCR primers and cycling parameters are outlined in **Table 2.1**.

Table 2.1 The primers and cycling parameters utilised for the quantification of *B. bacteriovorus* PF13, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E. faecium* with the EMA-qPCR assays

Organisms	Primer	Primer Sequences (5' – 3')	qPCR Cycling Parameters	Conventional PCR Cycling Parameters	Gene (product size in bp)	Melting Peak (°C)	Reference
***Bdellovibrio* spp.**	Bd347F	GGAGGCAGCAGT AGGGAATA	2 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; final elongation of 10 min at 72°C	16S rRNA (202)	84.30± 1.00	Van Essche et al., 2009
	Bd549R	GCTAGGATCCCTC GTCTTACC					
***Enterococcus* spp.**	ECST784 F	AGAAATTCCAAAC GAACTTG	10 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	5 min at 95°C; 30 cycles of 30 s at 94°C, 60 s at 59°C and 60 s at 72°C; final elongation of 10 min at 72°C	23S rRNA (75)	79.15 ± 1.00	Frahm and Obst, 2003
	ENC854R	CAGTGCTCTACCT CCATCATT					
***Klebsiella* spp.**	gyrA-A	CGCGTACTATACG CCATGAACGTA	10 min at 95°C; 50 cycles of 60 s at 94°C, 30 s at 50°C and 30 s at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	3 min at 95°C; 35 cycles of 60 s at 94°C, 30 s at 50°C and 30 s at 72°C; final elongation of 10 min at 72°C	*Gyrase A* (383)	87.70 ± 1.00	Brisse and Verhoef, 2001
	gyrA-C	ACCGTTGATCACT TCGGTCAGG					
***Pseudomonas* spp.**	PS1	ATGAACAACGTTC TGAAATTC	10 min at 95°C; 50 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	10 min at 95°C; 50 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; final elongation of 10 min at 72°C	*oprI* (249)	88.70 ± 1.00	Bergmark et al., 2012
	PS2	CTGCGGCTGGCTT TTTCCAG					
***Staphylococcus* spp.**	PanStaph F	CAATGCCACAAAC TCG	10 min at 95°C; 45 cycles of 30 s at 95°C, 30 s at 61°C and 30 s at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	10 min at 95°C; 45 cycles of 30 s at 95°C, 30 s at 61°C and 30 s at 72°C; final elongation of 10 min at 72°C	*tuf* (462)	81.50 ± 1.00	Sakai et al., 2004
	PanStaph R	GCTTCAGCGTAGT CTA					

For GC quantification, a standard curve was included (in duplicate) for each of the respective qPCR assays. Each standard curve was generated by performing conventional PCR (with the primers and cycling parameters as outlined in **Table 2.1**) on positive control DNA extracted (using the Zymo Research Soil Microbe MiniPrep™ kit as per manufacturer's instructions) from *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 333305, *E. faecium* Clinical isolate, *S. aureas* ATCC 25295 and *B. bacteriovorus* PF13. The conventional PCR mixture for *Bdellovibrio* spp. consisted of 1X Green GoTaq® Reaction Buffer (Promega Corp, Madison, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.9 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega Corp, Madison, USA) and 5 µL of DNA in a final volume of 25 µL. For *E. faecium* the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.2 µM of each primer, 1.5 U GoTaq® Flexi DNA Polymerase and 2 µL of DNA were added. For *K. pneumoniae* the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.3 µM of each primer and 1.5 U GoTaq® Flexi DNA Polymerase were added. For *Pseudomonas* spp. and *S. aureus* the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.1 µM (*Pseudomonas* spp.) and 1.0 µM (*S. aureus*) of each primer and 1.5 U GoTaq® Flexi DNA Polymerase were added. The standard curve for each target organism was then generated as outlined by Waso et al. (2018).

Lastly, high resolution melting curve analysis was included for all of the SYBR Green qPCR assays in order to verify the specificity of the assays. The temperature was thus increased from 40 to 97 °C at a rate of 0.2 °C/s with continuous fluorescent signal acquisition of 5 readings/°C to generate the melting curves.

2.2.6 Data and Statistical Analyses

All the qPCR performance characteristics were analysed using the Roche LightCycler® 96 Software Version 1.1 and Microsoft Excel 2016. In addition, the lower limit of detection (LLOD) was determined as the actual GC/µL values consistently and accurately detected per qPCR assay for the standard with the lowest GC. All GC numbers were converted to GC/mL using the following modified equation (excluding the compensation for sample filtration) (Eq. 1) as described by Rajal et al. (2007):

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

All graphs were generated and data analyses were performed using GraphPad Prism 7.04 (2018). Two-way Analysis of Variance (ANOVA) and unpaired t-tests using the Holm-Šídák method were utilised to determine whether there were significant differences between the concentrations of the prey cells detected (using culture-based enumeration and EMA-qPCR quantification) in the co-

culture experiments versus the initial inoculum and predator-free controls. Significance was observed as $p < 0.05$.

2.3 Results

2.3.1 *Bdellovibrio* spp. Isolation

In total 55 putative BALO strains were isolated from the wastewater sample collected from the Stellenbosch Wastewater Treatment Plant. Representative PCR products ($n = 9$) were sequenced to confirm the detection of *Bdellovibrio* spp. Seven of the representative PCR products were identified as *B. bacteriovorus* (**Table 2.2**), while *B. bacteriovorus* isolate PF13 was selected for all predation assays as this isolate produced numerous and large plaques when cultured on a lawn of *P. fluorescens* ATCC 13525 prey.

Table 2.2 Sequencing results obtained for the representative PCR products sequenced for presumptive BALO strains identification

BALO Isolate	BLAST result	Sequence Similarity (%)	Accession Number
PP1[a]	*B. bacteriovorus* W, complete genome	99	CP002190.1
PP9[a]	*B. bacteriovorus* strain SOIR-1 16S Ribosomal RNA gene	100	MG230309.1
PP17[a]		100	
PF13[b]		100	
PF17[b]		100	
PF20[b]		100	
PA8[c]	*B. bacteriovorus* W, complete genome	97	CP002190.1

[a] *P. protegens* 17386 used as prey, [b] *P. fluorescens* ATCC 13525 used as prey, [c] *P. aeruginosa* ATCC 27853 used as prey

2.3.2 Predation by *B. bacteriovorus* PF13 on *Pseudomonas* spp.

The ability of the predator *B. bacteriovorus* PF13 to attack *P. fluorescens* and *P. aeruginosa* was assessed in DNB and HEPES buffer using culture-based methods [spread plates (CFU/mL) and double-layer agar overlays (PFU/mL)] and EMA-qPCR (GC/mL) (qPCR performance characteristics summarised in **Table 2.3**). For the prey bacteria, the cell counts (CFU/mL) and gene copies (GC/mL) obtained after predation were compared to the cell counts and GC obtained in the initial inoculum and the predator-free control.

Table 2.3 Performance characteristics for the EMA-qPCR assays utilised to quantify *B. bacteriovorus*, *P. fluorescens*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E. faecium*

qPCR Performance Characteristics	*B. bacteriovorus*	*P. fluorescens*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*	*E. faecium*
Efficiency (*E*)	103.3 ± 2.66	94.50 ± 4.00	93.75 ± 3.25	95.50 ± 5.00	96.50 ± 1.00	101 ± 0.03
y-intercept	32.99 ± 1.39	36.72 ± 2.49	37.09 ± 1.97	35.13 ± 3.38	34.41 ± 0.22	34.00 ± 0.19
Correlation Coefficient (r^2)	0.99 ± 0.008	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.005	1.00 ± 0.00	0.99 ± 0.01
Lower Limit of Detection (LLOD) (GC/µL)	2.31 ± 2.06	105.34 ± 38.5	71.86 ± 3.85	2.15 ± 1.76	15.23 ± 2.44	3.23 ± 1.88
Slope	-3.17 ± 0.13	-3.64 ± 0.24	-3.67 ± 0.20	-3.59 ± 0.30	-3.50 ± 0.07	-3.28 ± 0.12

Similarly, for the predatory bacteria, the PFU/mL and GC/mL were compared to the plaque counts and GC obtained in the initial inoculum to determine if the concentrations of these bacteria changed significantly in co-culture.

As indicated in **Fig. 2.1a** and **Table 2.4**, in comparison to the predator-free control, the *P. fluorescens* ATCC 13525 cell counts were reduced significantly by 3.24 logs ($p = 0.00057$) after 120 h of predation in DNB. Correspondingly, the plaque counts of *B. bacteriovorus* PF13 increased by 5.44 logs at the expense of *P. fluorescens* ATCC 13525 and the maximum cell density was reached after 96 h of predation (**Table 2.5**). In contrast to the culture-based enumeration of *P. fluorescens* ATCC 13525 in co-culture with *B. bacteriovorus* PF13 in DNB, the EMA-qPCR results indicated that the GC of *P. fluorescens* ATCC 13525 fluctuated. However, overall, a log reduction of 1.24 was observed for the GC recorded for the predator-free control versus the GC recorded at the end of predation at 120 h (**Fig. 2.1b**; **Table 2.4**). In accordance with this decrease, the GC of *B. bacteriovorus* PF13 increased by 4.33 logs and reached the maximum GC concentration after 48 h of predation (**Table 2.5**).

When *P. fluorescens* ATCC 13525 was exposed to the predator in HEPES buffer, the cell counts were also significantly reduced (4.21 log reduction; $p = 0.0128$) after 120 h of predation (**Fig. 2.1c**; **Table 2.4**). Correspondingly, the plaque counts for *B. bacteriovorus* PF13 increased by 5.01 logs to reach a maximum cell density at 48 h (**Table 2.5**). The GC of *P. fluorescens* ATCC 13525 also decreased significantly by 1.64 logs ($p < 0.0001$) after 120 h of co-culture with *B. bacteriovorus* PF13 in HEPES buffer (**Fig. 2.1d**; **Table 2.4**). In accordance with this decrease, the GC of *B. bacteriovorus* PF13 increased by 2.72 logs and reached the maximum concentration after 48 h of co-culture with *P. fluorescens* ATCC 13525 (**Table 2.5**).

For the predation assays performed in DNB using *P. aeruginosa* ATCC 27853 as prey, in comparison to the predator-free control group, the cell counts of the prey organism were significantly reduced by 1.85 logs ($p = 0.0081$) after 120 h of predation (**Fig. 2.1a**; **Table 2.4**). However, it should be noted that *B. bacteriovorus* PF13 did not produce visible plaques on the double-agar overlays when using *P. aeruginosa* ATCC 27853 as prey in either the DNB or HEPES buffer trials. In contrast to the culture-based enumeration of *P. aeruginosa* ATCC 27853 in co-culture with *B. bacteriovorus* PF13 in DNB, the EMA-qPCR results indicated that the *P. aeruginosa* ATCC 27853 GC only decreased by 0.159 logs after 120 h of predation (**Fig. 2.1b**; **Table 2.4**). In accordance with this decrease in the prey concentration, the EMA-qPCR results indicated that the GC of *B. bacteriovorus* PF13 increased by 0.744 logs and reached a maximum concentration after 48 h of co-culture (**Table 2.5**).

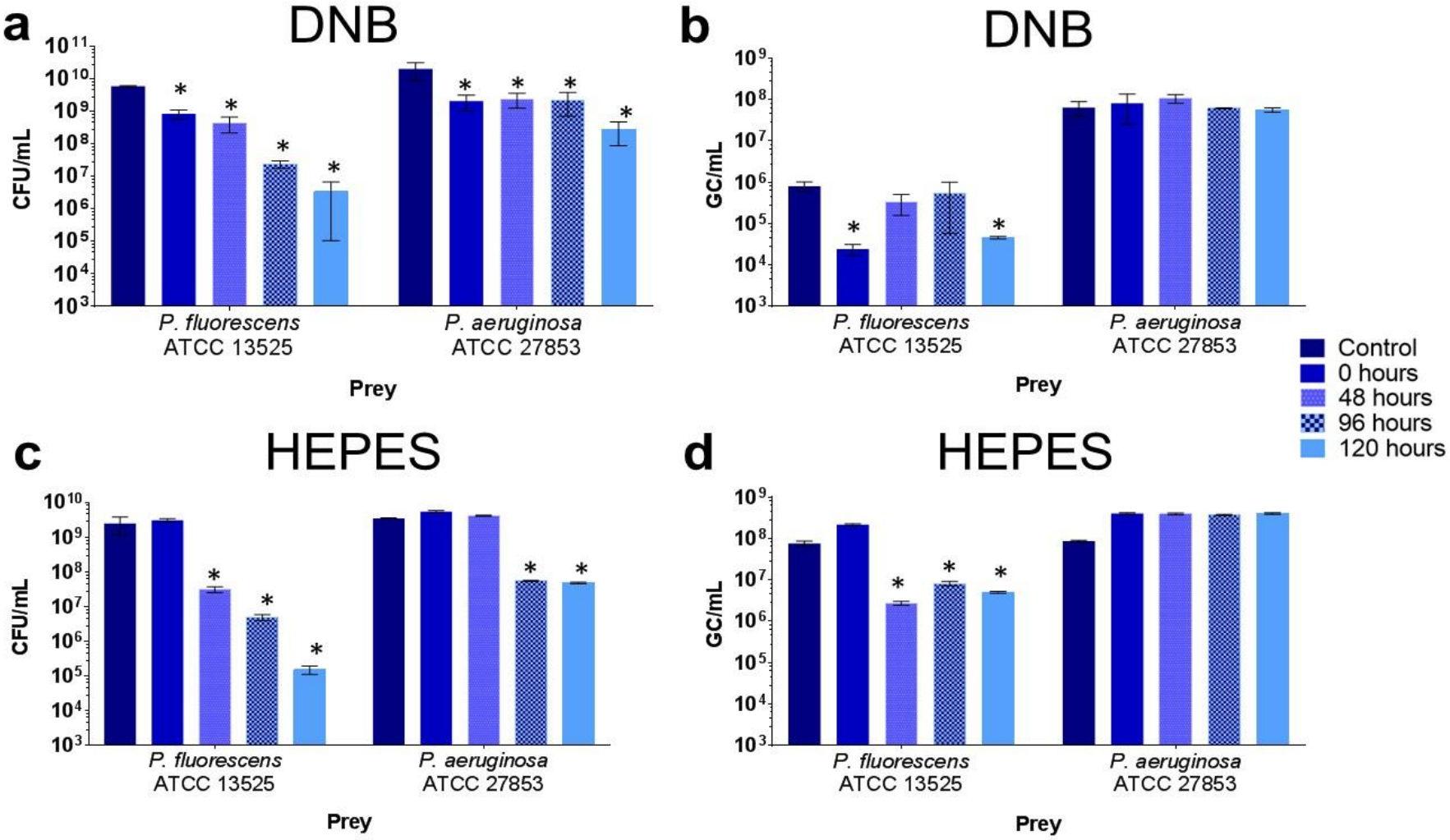

Fig. 2.1 Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *P. fluorescens* ATCC 13525 and *P. aeruginosa* ATCC 27853 co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration

Table 2.4 Summary of the results of the cell counts (CFU/mL) and gene copies (GC/mL) of the different prey cells after exposure to the predatory bacteria, *B. bacteriovorus* PF13, in DNB and HEPES buffer

Prey	DNB						HEPES Buffer					
	Culture-based Analysis			EMA-qPCR			Culture-based Analysis			EMA-qPCR		
	Control CFU/mL[a]	Predated CFU/mL	Log Change	Initial GC/mL[b]	Predated GC/mL	Log Change	Control CFU/mL[a]	Predated CFU/mL	Log Change	Initial GC/mL	Predated GC/mL	Log Change
Pseudomonas fluorescens	5.91×10^{9}	3.40×10^{6}	-3.24	8.06×10^{5} [c]	4.60×10^{4}	-1.24	2.48×10^{9}	1.51×10^{5}	-4.21	2.19×10^{8}	5.02×10^{6}	-1.64
Pseudomonas aeruginosa	2.02×10^{10}	2.83×10^{8}	-1.85	8.11×10^{7}	5.63×10^{7}	-0.159	3.53×10^{9}	4.80×10^{7}	-1.87	4.06×10^{8}	4.06×10^{8}	0.00
Klebsiella pneumoniae	1.44×10^{9}	3.80×10^{5}	-3.58	1.11×10^{8}	2.50×10^{4}	-3.65	2.72×10^{9}	2.00×10^{4}	-5.13	4.01×10^{8}	2.06×10^{5}	-3.29
Staphylococcus aureus	1.59×10^{9}	8.50×10^{8}	-0.272	1.18×10^{6}	4.22×10^{6}	+0.553	1.25×10^{9}	2.00×10^{7}	-1.80	4.96×10^{8}	2.64×10^{7}	-1.27
Enterococcus faecium	6.74×10^{9}	1.31×10^{7}	-2.71	5.17×10^{8}	1.18×10^{8}	-0.642	5.40×10^{8}	3.46×10^{8}	-0.193	1.43×10^{9}	6.88×10^{8}	-0.318

[a] Cell counts in CFU/mL of the non-predatory control samples after 120 h; [b] Initial inoculation concentration of the prey cells in GC/mL quantified using EMA-qPCR; [c] Concentration in GC/mL of *P. fluorescens* in the control sample after 120 h

Table 2.5 Summary of the results of the cell counts (PFU/mL) and gene copies (GC/mL) of *B. bacteriovorus* PF13 after co-culture with the different prey cells in DNB and HEPES buffer

Prey	DNB						HEPES Buffer					
	Culture-based Analysis			EMA-qPCR			Culture-based Analysis			EMA-qPCR		
	Initial PFU/mL	Maximum PFU/mL	Log Change	Initial GC/mL	Maximum GC/mL	Log Change	Initial PFU/mL	Maximum PFU/mL	Log Change	Initial GC/mL	Maximum GC/mL	Log Change
Pseudomonas fluorescens	2.53×10^{5}	6.95×10^{10}	+5.44	2.77×10^{3}	5.99×10^{7}	+4.33	1.80×10^{4}	1.83×10^{9}	+5.01	7.06×10^{3}	3.68×10^{6}	+2.72
Pseudomonas aeruginosa	ND	ND	ND	4.54×10^{3}	2.52×10^{4}	+0.744	ND	ND	ND	2.05×10^{3}	1.89×10^{3}	-0.035
Klebsiella pneumoniae	2.53×10^{5}	1.78×10^{9}	+3.85	1.24×10^{3}	5.95×10^{7}	+4.68	1.75×10^{4}	3.20×10^{9}	+5.26	3.22×10^{3}	5.32×10^{7}	+4.22
Staphylococcus aureus	ND	ND	ND	8.17×10^{2}	1.25×10^{7}	+4.18	ND	ND	ND	3.76×10^{3}	1.72×10^{6}	+2.66
Enterococcus faecium	2.53×10^{5}	9.45×10^{8}	+3.57	1.17×10^{3}	9.55×10^{6}	+3.92	ND	ND	ND	9.88×10^{6}	2.78×10^{7}	+0.449

ND – Not Determined as *B. bacteriovorus* PF13 did not produce visible plaques on the double-layer agar overlays for these prey cells

Comparatively, for the HEPES buffer predation assays, the cell counts of *P. aeruginosa* ATCC 27853 were also significantly reduced by 1.87 logs ($p = 0.0002$) after 120 h of predation (**Fig. 2.1c**; **Table 2.4**). However, for the HEPES buffer EMA-qPCR analysis of *P. aeruginosa* ATCC 27853 in co-culture with *B. bacteriovorus* PF13, after 120 h of predation, the GC of *P. aeruginosa* ATCC 27853 were equal to the initial concentration (4.06×10^8 GC/mL) (**Fig. 2.1d**; **Table 2.4**). Correspondingly, the GC of *B. bacteriovorus* PF13 did not increase, but decreased by 0.035 logs after 120 h of co-culture with *P. aeruginosa* ATCC 27853 in HEPES buffer (**Table 2.5**).

2.3.3 Predation by *B. bacteriovorus* PF13 on *Klebsiella pneumoniae*

The results showed that in DNB, the *K. pneumoniae* ATCC 333305 cell counts were significantly reduced by 3.58 logs ($p < 0.0001$) after 120 h of predation (**Fig. 2.2a**; **Table 2.4**). Correspondingly, the plaque counts of *B. bacteriovorus* PF13 increased by 3.85 logs to reach the maximum predator concentration after 48 h (**Table 2.5**). The EMA-qPCR analysis then indicated that the GC of *K. pneumoniae* ATCC 333305 also decreased significantly by 3.65 logs ($p = 0.0103$) after 120 h of predation in DNB (**Fig. 2.2b**; **Table 2.4**). In accordance with this decrease in the GC of *K. pneumoniae* ATCC 333305, the GC of *B. bacteriovorus* PF13 increased by 4.68 logs to the maximum GC concentration after 96 h of predation (**Table 2.5**).

In comparison, when *K. pneumoniae* ATCC 333305 was exposed to the predator in HEPES buffer, the cell counts were reduced significantly after 120 h of predation (5.13 log reduction; $p < 0.0001$) (**Fig. 2.2c**; **Table 2.4**). Accordingly, the plaque counts for *B. bacteriovorus* PF13 increased by 5.26 logs and reached the maximum cell density after 48 h of co-culture (**Table 2.5**). The molecular analysis also indicated that the GC of *K. pneumoniae* decreased significantly, by 3.29 logs ($p < 0.0001$) after 120 h of co-culture (**Fig. 2.2d**; **Table 2.4**). In accordance with this decrease, the GC of *B. bacteriovorus* PF13 increased by 4.22 logs and reached the maximum concentration after 96 h (**Table 2.5**).

2.3.4 Predation by *B. bacteriovorus* PF13 on *Staphylococcus aureus*

For the DNB trials using *S. aureus* ATCC 25925 as prey, the cell counts were reduced by 0.272 logs after 120 h of predation (**Fig. 2.3a**; **Table 2.4**). However, similar to the results observed for the *P. aeruginosa* trials, *B. bacteriovorus* PF13 did not produce plaques in the double-layer agar overlays when *S. aureus* was used as prey in either the DNB or HEPES buffer trials. In contrast to the culture-based enumeration of *S. aureus* ATCC 25925 in DNB, the EMA-qPCR results indicated that the GC of *S. aureus* ATCC 25925 increased (albeit not significantly) by 0.553 logs after 120 h of predation (**Fig. 2.3b**; **Table 2.4**).

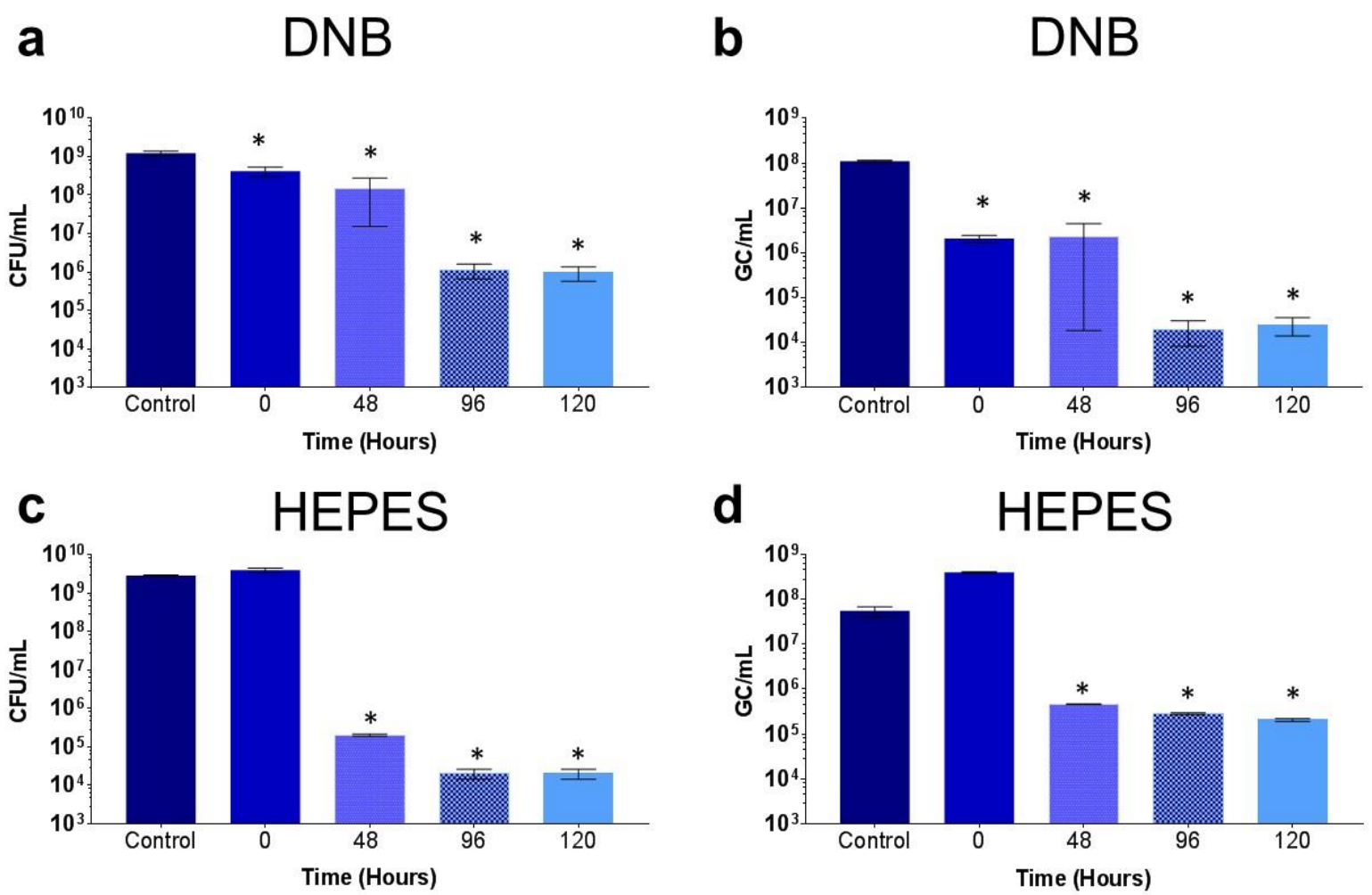

Fig. 2.2 Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *K. pneumoniae* ATCC 333305 co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration

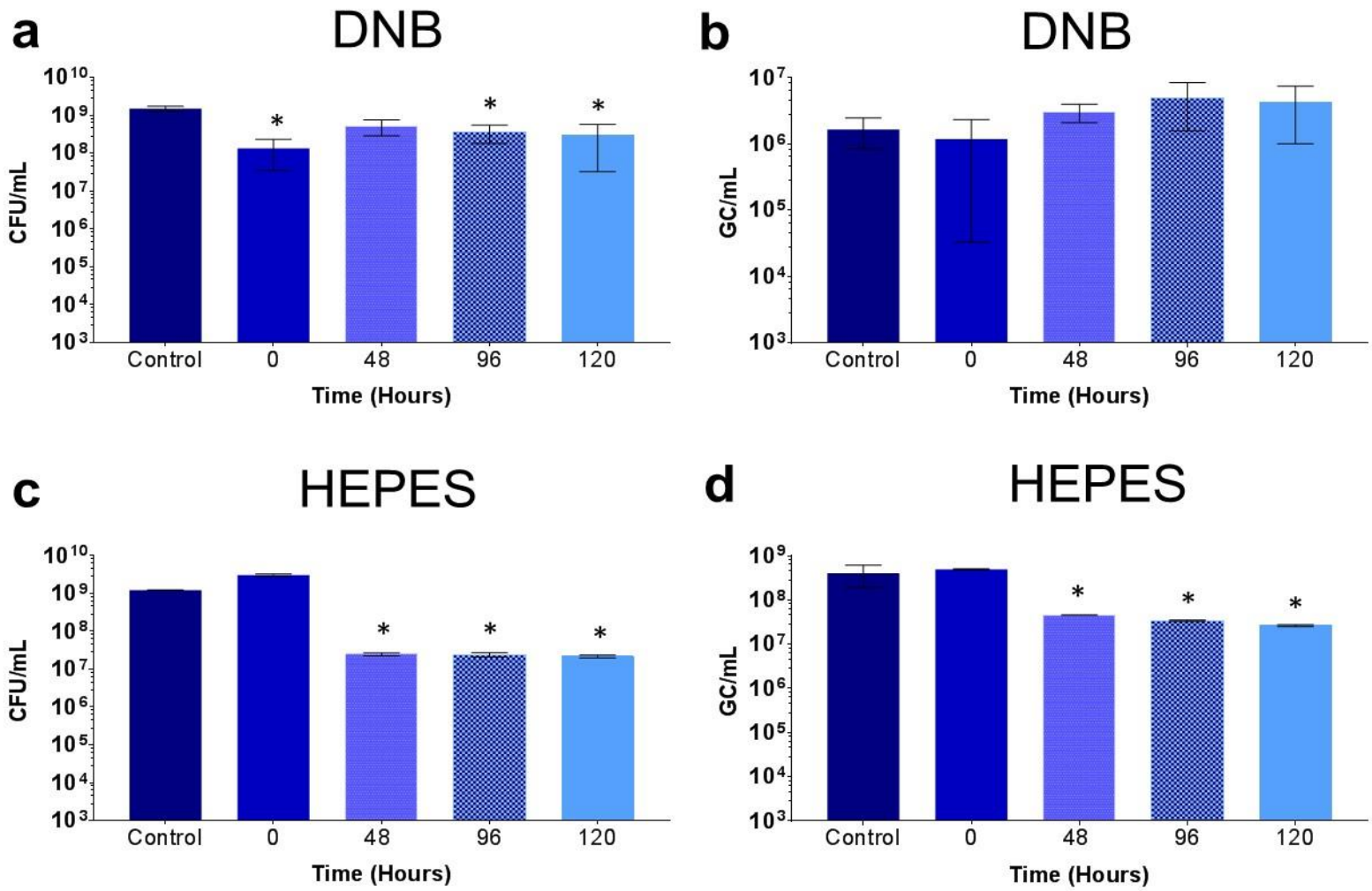

Fig. 2.3 Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *S. aureus* ATCC 25925 co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration

In addition, the GC of *B. bacteriovorus* PF13 increased significantly by 4.18 logs ($p < 0.0001$) to a maximum concentration after 96 h of predation (**Table 2.5**).

In comparison, when *S. aureus* ATCC 25925 was exposed to the predator in HEPES buffer, the cell counts were reduced significantly by 1.80 logs ($p < 0.0001$) after 120 h (**Fig. 2.3c**; **Table 2.4**). Accordingly, the EMA-qPCR analysis indicated that the concentration of *S. aureus* ATCC 25925 decreased by 1.27 logs ($p < 0.0001$) (**Fig. 2.3d**; **Table 2.4**), while the concentration of *B. bacteriovorus* PF13 increased by 2.66 logs after 120 h of predation (**Table 2.5**).

2.3.5 Predation by *B. bacteriovorus* PF13 on *Enterococcus faecium*

Upon exposure to the predator in DNB, the *E. faecium* cell counts were significantly reduced by 2.71 logs ($p = 0.0002$) after 120 h of predation (**Fig. 2.4a**; **Table 2.4**). In accordance with the reduction in cell counts of *E. faecium*, the *B. bacteriovorus* PF13 concentration increased by 3.57 logs to the highest concentration after 48 h of predation (**Table 2.5**). The results from the EMA-qPCR analysis also indicated that the concentration of *E. faecium* decreased (0.642 log) after 120 h of co-culture (**Fig. 2.4b**; **Table 2.4**), while the concentration of *B. bacteriovorus* PF13 increased by 3.92 logs after 120 h (**Table 2.5**).

For the HEPES buffer trials, however, the cell counts of *E. faecium* were only reduced by 0.193 logs after 120 h of predation (**Fig. 2.4c**; **Table 2.4**). Additionally, in contrast to the results obtained for the DNB trials, *B. bacteriovorus* PF13 did not produce any visible plaques on the double-layer agar overlays during the HEPES trials with *E. faecium* as prey (**Table 2.5**). The EMA-qPCR analysis then indicated that the concentration of *E. faecium* decreased (albeit not significantly) by 0.318 logs (**Fig. 2.4d**; **Table 2.4**) and correspondingly the concentration of *B. bacteriovorus* PF13 increased by 0.449 logs after 120 h of co-culture (**Table 2.5**).

2.4 Discussion

Pseudomonas fluorescens and *P. aeruginosa* were employed as prey cells in the current study as various studies have indicated that *Pseudomonas* spp. are sensitive to predation by *B. bacteriovorus* (Dashiff et al., 2011; Kadouri et al., 2013; Shanks et al., 2013). Based on the prey cell counts, predator plaque counts and GC for *P. fluorescens* and *B. bacteriovorus* in co-culture, it was evident that *B. bacteriovorus* PF13 was able to replicate at the expense of *P. fluorescens* ATCC 13525 in both the DNB and HEPES buffer. However, for *P. aeruginosa* variable results for the cell counts and GC were observed. The cell counts recorded for *P. aeruginosa* indicated that the concentration of this organism was significantly reduced ($p < 0.05$) after 120 h of co-culture with the predatory bacterium in DNB and HEPES buffer.

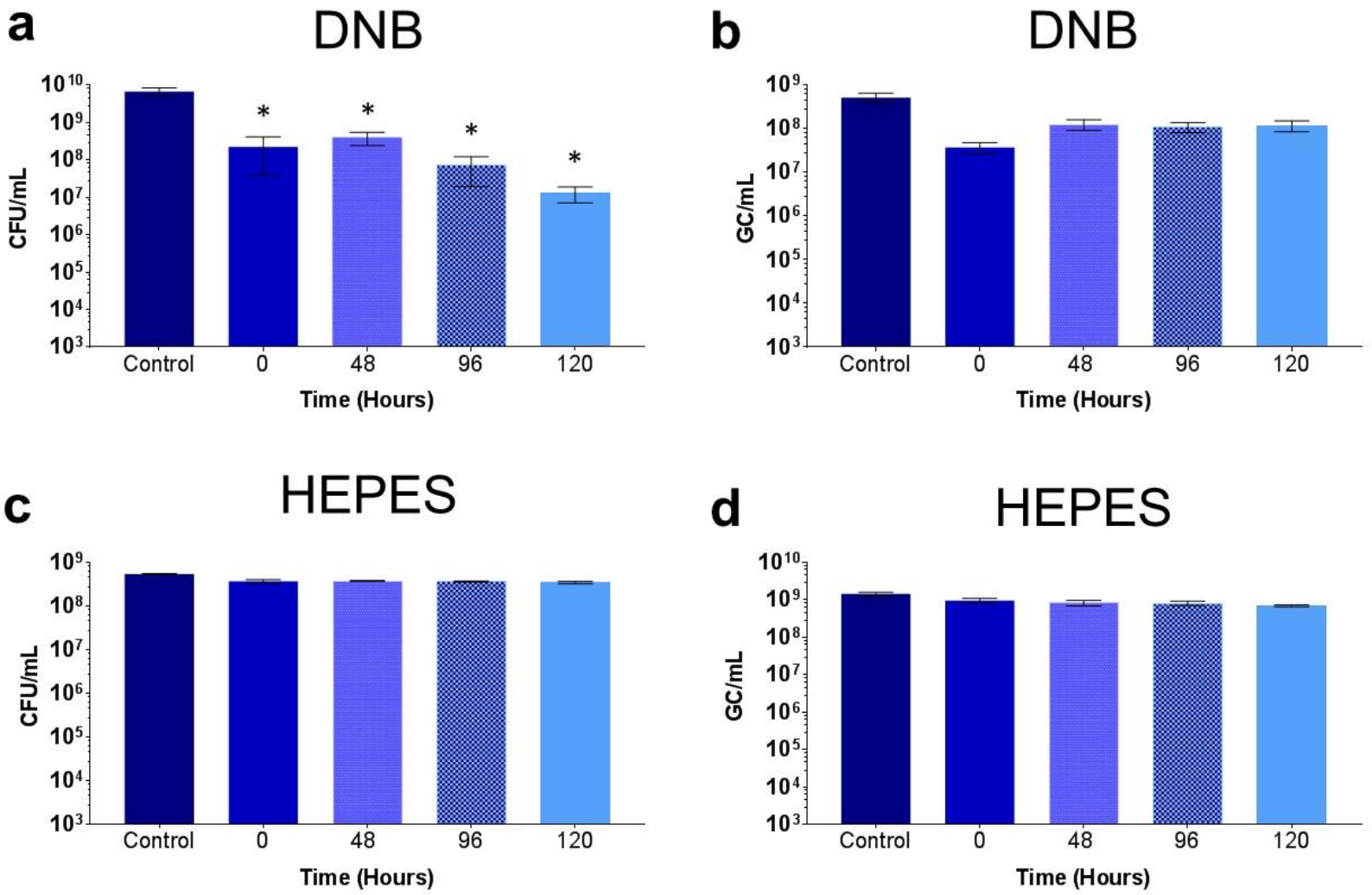

Fig. 2.4 Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *E. faecium* Clinical co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration

These findings were in accordance with a previous study by Dashiff et al. (2011) where *P. aeruginosa* ATCC BAA427 was sensitive to predation by *B. bacteriovorus* in DNB, with a 1.00 log reduction in the cell counts observed for co-culture experiments after 48 h of incubation. Kadouri et al. (2013) reported higher log reduction values, where the concentration of *P. aeruginosa* GB771 was reduced by 3.96 and 3.07 logs in DNB, using *B. bacteriovorus* 109J and HD100, respectively, indicating that different predator strains may have variable effects on different prey strains of the same species. While the EMA-qPCR analysis conducted in the current study confirmed that the GC of *P. aeruginosa* in co-culture with *B. bacteriovorus* PF13 in DNB were reduced, the reduction recorded (0.159 logs) was much lower than the log reduction observed for the cell counts (1.85 log reduction). Moreover, in HEPES buffer, the EMA-qPCR results indicated that the concentration of *P. aeruginosa* after 120 h of predation, was equal to the initial inoculum concentration. Accordingly, the *B. bacteriovorus* PF13 GC increased (0.744 logs) in the DNB, but decreased by a negligible margin (0.035 logs) in the HEPES buffer trials. For *P. aeruginosa*, the reductions recorded using the culture-based enumeration techniques did thus not correlate with the molecular analysis. It is well known that bacteria enter a VBNC state under unfavourable conditions, which can be induced if cells are stressed as a result of light, temperature, high or low salinity, pressure and low nutrient levels (Ramamurthy et al., 2014). Lambert et al. (2010) also showed that *B. bacteriovorus* can induce a stress response in *E. coli* with genes involved in osmotic stress, amino acid and carbon starvation and toxin efflux pumps significantly upregulated within 15 min of exposure to predatory bacteria. It is thus hypothesised that in the current study, a subpopulation of the *P. aeruginosa* cells entered a VBNC state upon exposure to the predator, which may account for the reduction in the cell count observed using the culture-based methods, while the GC, which enumerates cell viability, were not significantly reduced after 120 h of predation in the DNB or HEPES buffer. Moreover, the *P. aeruginosa* strain employed in the current study may not have been the ideal prey for the *B. bacteriovorus* strain isolated from wastewater. Pantanella et al. (2018) noted that mutants of *B. bacteriovorus* could arise in the absence of preferred or ideal prey as a potential survival strategy. These mutants could alter the secretion of hydrolytic enzymes such as proteases and access nutrients available in culture media or break down prey cells (releasing the cellular contents into the culture medium), to subsequently grow or survive axenically. The *B. bacteriovorus* strain employed in the current study may thus have altered its expression of lytic enzymes in order to survive, which in turn could have influenced the culturability of the *P. aeruginosa* prey. However, this hypothesis should be assessed in future studies by screening for mutations in the host-independent (*hit*) locus which has been associated with the host-independent phenotype of *Bdellovibrio* spp. (Capeness et al., 2013).

Based on the results obtained for the trials using *K. pneumoniae* as prey, it was evident that *B. bacteriovorus* PF13 could effectively utilise the *K. pneumoniae* cells as a nutrient source and

for replication as the cell counts and GC of *K. pneumoniae* were significantly reduced after 120 h of co-culture, in both the DNB and HEPES buffer trials. Correspondingly, the *B. bacteriovorus* PFU and GC significantly increased in the presence of *K. pneumoniae* in DNB and HEPES buffer. These results were in accordance with results reported by Dashiff et al. (2011), where planktonic cell counts in DNB were reduced by 2, 2 and 4 logs for *K. pneumoniae* ATCC 33495, ATCC BAA1705 and ATCC BAA1706, respectively, while the concentration of six different *K. pneumoniae* clinical isolates were each reduced by 5 logs in the presence of *B. bacteriovorus*. However, similar to the results observed for the *P. fluorescens* ATCC 13525, the *K. pneumoniae* cells were not completely eradicated, even after 120 h of co-culture with *B. bacteriovorus* PF13 in DNB and HEPES buffer, as cell counts and GC were still detected. This observation is in accordance with literature indicating that prey cell populations exhibit an inherent plastic phenotypic resistance towards predation (Shemesh & Jurkevitch, 2004; Kadouri et al., 2013; McNeely et al., 2017). This resistance mechanism is not specific towards a predator strain and is a general phenotypic adaptation observed in residual prey populations after exposure to a predatory bacterium (Shemesh & Jurkevitch, 2004). Once the predator is removed or the predator concentration is reduced, the prey population returns to a predation sensitive phenotype (Shemesh & Jurkevitch, 2004).

The results for *S. aureus* indicated that while a negligible decrease in cell counts was recorded after 120 h of co-culture with *B. bacteriovorus* PF13 in DNB, a significant decrease in prey cell counts was recorded for the co-culture experiments conducted in HEPES buffer. Accordingly, while the EMA-qPCR results indicated that the GC of *S. aureus* were reduced in HEPES buffer, the GC of *S. aureus* increased in DNB. It was however, interesting to note that the GC of *B. bacteriovorus* PF13 increased significantly in co-culture with *S. aureus* in DNB and the HEPES buffer, respectively. Iebba et al. (2014), reported that in the presence of Gram-positive prey such as *S. aureus* cells, *B. bacteriovorus* produces non-secreted hydrolytic enzymes which may include proteases, glycanases and DNases, which differ from the enzymes produced in the presence of Gram-negative prey, subsequently enabling the predator to utilise Gram-positive organisms as a nutrient source. Furthermore, Monnappa et al. (2014) confirmed that host-independent *B. bacteriovorus* produce a range of lytic enzymes such as proteases and nucleases that are effective in dispersing *S. aureus* and *S. epidermis* biofilms. *Bdellovibrio bacteriovorus* also switches from a periplasmic predation strategy in the presence of Gram-negative prey, to an epibiotic predation strategy in the presence of *S. aureus* cells (Iebba et al., 2014; Pantanella et al., 2018). Based on the results obtained for the *B. bacteriovorus* PF13 and *S. aureus* ATCC 25925 co-cultures in HEPES buffer; the predator used in the current study may thus have been able to produce hydrolytic enzymes or switch its predation strategy in order to utilise the *S. aureus* cells as a nutrient source and for replication. For the DNB trials however, in comparison to the cell counts obtained in the HEPES buffer, the *S. aureus* cell counts were not significantly reduced,

while the GC of *S. aureus* increased during the DNB trials. Thus, in DNB the predatory bacteria could have secreted lytic enzymes to access the nutrients suspended in the media in order to survive and grow, while they were not actively preying on the *S. aureus* cells. Additionally, the *S. aureus* cell counts were only reduced by 0.272 logs in DNB, compared to a 1.80 log recorded for the HEPES buffer trials, suggesting that *B. bacteriovorus* does not directly attack the *S. aureus* cells in the DNB and rather utilises the nutrients in this medium for growth.

Based on predominantly the culture-based analysis results obtained for the trials where *E. faecium* was utilised as prey, it is hypothesised that *B. bacteriovorus* PF13 benefitted from being co-cultured with *E. faecium* in DNB as the concentration of the predator increased, while the concentration of the prey decreased. In addition, plaque formation of *B. bacteriovorus* PF13 was observed when *E. faecium* was utilised as prey during the DNB trials, indicating that in DNB *B. bacteriovorus* PF13 preys on *E. faecium*. Contrastingly, the *E. faecium* concentration in HEPES buffer was marginally reduced with a corresponding increase in the predator concentration, albeit not as great as the increase observed for the DNB trials, while plaques were also not observed during the HEPES buffer trials. Limited information is however, available on the interaction of *B. bacteriovorus* with *Enterococcus* spp. (Jurkevitch, 2006). Dashiff et al. (2011) however, indicated that *E. faecalis* is not sensitive to predation by *B. bacteriovorus* or *M. aeruginosavorus*. Thus, in contrast to available literature, preliminary results (in DNB media) in the current study suggests that the *B. bacteriovorus* strain isolated from wastewater is able to interact with *E. faecium* as a potential nutrient source, however additional research is required to corroborate these results. This may include the use of gene expression analysis targeting predation specific genes (encoding for flagella, pili and various lytic enzymes) of *B. bacteriovorus,* to determine if these genes are similarly upregulated when *B. bacteriovorus* is exposed to *E. faecium* as compared to Gram-negative prey.

While both culture-based and molecular techniques can be used to assess viable microbial cells, molecular techniques are more accurate if VBNC cells are present (Delgado-Viscogliosi et al., 2009; Reyneke et al., 2017). Although limited information on the use of molecular techniques to assess the interaction of *B. bacteriovorus* with potential prey cells is available, qPCR has been used to assess the interaction of *B. bacteriovorus* HD100 with *P. aeruginosa* and *S. aureus* in DNB (Pantanella et al., 2018). The viability dye EMA was thus combined with qPCR in the current study in order to distinguish between live and dead microbial cells and was particularly beneficial during the predation trials, where *P. aeruginosa*, *S. aureus* and *E. faecium* were used as prey and *B. bacteriovorus* PF13 did not produce plaques on the double-layer agar overlays (with the exception of the trials conducted in DNB with *E. faecium* as prey). However, while EMA effectively suppresses the signal from extracellular DNA or DNA from cells with compromised membranes, it has been reported that this viability dye can penetrate the intact membrane of live cells, while high concentrations of EMA may also be cytotoxic to viable cells, which may influence the

accuracy with which live cells are quantified (Nocker et al., 2006; Fittipaldi et al., 2012). Conflicting conclusions have also been made regarding the effect of the membrane composition of bacterial cells (complex structure of the outer membrane of Gram-negative bacteria versus the thick peptidoglycan layer of Gram-positive bacteria) on the efficacy of viability dye treatment (Nocker et al., 2006; Flekna et al., 2007; Fittipaldi et al., 2012). However, an in-depth analysis by Reyneke et al. (2017) on the application of EMA, PMA and DNase in combination with qPCR to detect viable cells indicated that EMA-treatment was effective for certain Gram-positive (*E. faecalis*) and Gram-negative organisms (*P. aeruginosa* and *S. typhimurium*). Additionally, Reyneke et al. (2017) optimised the concentration of EMA to detect viable cells of Gram-negative and Gram-positive bacteria and found that a final concentration of 6 µM EMA (concentration of EMA applied in the current study) was optimal.

2.5 Conclusions

Corresponding results were generally obtained for the culture-based and EMA-qPCR analysis and it can be concluded that EMA-qPCR can be used to monitor the interaction of *B. bacteriovorus* with various prey cells in different media. Additionally, while variable results were obtained specifically with regards to the interaction of *B. bacteriovorus* with Gram-positive prey, these variations were dependent on the specific prey cells used and the media employed to assess these interactions.

HEPES buffer or DNB could also be employed to monitor the predation of *P. fluorescens* and *K. pneumoniae* by *B. bacteriovorus*, while predation on *E. faecium* can be monitored in DNB and predation on *S. aureus* can be monitored in HEPES buffer. Results from this study thus indicated that it may be noteworthy to assess the activity of newly isolated *B. bacteriovorus* strains on prey species in both DNB and HEPES buffer, in order to fully investigate the predator-prey interactions in nutrient poor (DNB) and nutrient deficient (HEPES buffer) conditions. However, for *P. aeruginosa*, conflicting results were obtained for the plate counts versus the EMA-qPCR results during the DNB and HEPES buffer trials. Further analysis is thus required to determine whether the *P. aeruginosa* strain employed in the current study entered a VBNC state during the co-culture trials and co-culture experiments with different *P. aeruginosa* strains may need to be conducted.

Importantly, *P. fluorescens*, *K. pneumoniae*, *E. faecium* (in DNB) and *S. aureus* (in HEPES buffer) were found to be sensitive to predation in varying degrees in the current study. This is significant as these organisms are associated with human disease, which supports the notion of employing predatory bacteria as live antimicrobials or biocontrol agents to combat pathogenic microbial species.

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Authors' contributions

Conceived and designed the experiments: MW WK. Performed experiments: MW. Analysed the data: MW WK. Contributed reagents/materials/analysis tools: WK. Compiled the manuscript: MW WK. Edited the manuscript: MW WK SK. All authors approved the final version of the manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

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Chapter 3:

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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* (*B. 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. For the biological *B. bacteriovorus* pre-treatment (72 h) experiments, synthetic rainwater was seeded with either *Klebsiella pneumoniae* (*K. pneumoniae*) or *Enterococcus faecium* (*E. faecium*). Hereafter, the *B. bacteriovorus* treated samples were exposed to solar disinfection or solar photocatalysis for 4 h. Additionally, synthetic rainwater samples were seeded with either *K. pneumoniae* or *E. faecium* and were exposed to only solar disinfection or solar photocatalysis for 4 h. The pre-treatment followed by solar photocatalysis for 120 min under natural sunlight reduced the viable counts of *K. 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 *E. 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 thus 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

3.1 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 diarrhoeal 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* (*E. 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 (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 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. Fernández-Ibáñez et al. (2015) and Cruz-Ortiz et al. (2017) have also previously reported on enhanced solar disinfection utilising TiO_2-reduced graphene oxide composites (TiO_2-rGO). 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 (Sockett, 2009). This group of predatory bacteria include species such as *Bdellovibrio bacteriovorus* (*B. bacteriovorus*) and *Micavibrio aeruginosavorus* (*M. 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 focusing 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; Özkan et al., 2018).

Based on the survival of pathogenic and opportunistic pathogenic organisms in treated rainwater, a need exists to investigate a combination of technologies that incorporate biocontrol, physical and chemical treatment. The aim of the study was thus to apply *B. bacteriovorus* in combination with solar-CPC reactors and solar-CPC with photocatalysis to disinfect harvested rainwater. This aim was achieved by designing and constructing small-scale solar-CPC systems. Environmental *Klebsiella pneumoniae* (*K. pneumoniae*) S1 43 and *Enterococcus faecium* (*E. faecium*) 8D

isolates were selected as test organisms. *Klebsiella pneumoniae* S1 43 was previously isolated by members of our research group from SOPAS treated rainwater at a treatment temperature above 70°C (Clements et al., 2019). Additionally, *E. faecium* 8D [isolated from untreated harvested rainwater (Dobrowsky et al., 2014)] was included as enterococci are frequently used as indicator organisms to monitor rainwater quality.

3.2 Materials and Methods

3.2.1 Design and Construction of a Small-Scale Solar-CPC Reactor

For the construction of the CPC, a frame consisting of an arch profile, two end plates and flat sheets was designed using the computer software AutoCAD® 2018 and the frame was constructed from 2 mm thick aluminium sheets (**Appendix A Fig. A.1 – A.5**). The CPC mirror was designed with a concentration factor of 1.00 and consisted of two identical reflective sheets of 304 grade stainless steel which was electropolished (**Appendix A Fig. A.2**). The reflectance of the electropolished stainless steel was measured with a Thorlabs CCS200 spectrometer (Thorlabs, Newton, New Jersey, USA) which indicated that the material had an average reflectivity of 57.1% in the UV light range (240 – 400 nm) and an average reflectivity of 48.3% in the visible light range (400 – 500 nm) (**Appendix A Fig. A.6**). The sheets were 2 mm thick, had a length of 250 mm, a width of 87 mm and were bent to obtain a variable radius of 31.5 to 78.0 mm (**Appendix A Fig. A.2**). These sheets were superimposed onto the arch profile (length of 183.6 mm and a height of 80 mm with the profile of the CPC mirror cut from the sheet) to form the CPC (**Appendix A Fig. A.1 – A.5**).

The transparent reactor consisted of a borosilicate tube with a glass thickness of 2.5 mm, an inner diameter of 50 mm, a length of 250 mm and a total volume of 490 mL. The ends of the glass tubes were fitted with polyvinyl chloride (PVC) plugs (**Appendix A Fig. A.4** and **A.5**). The top plug contained a screw cap top, which was utilised to fill the system with rainwater and also contained a valve to release pressure from the system (**Appendix A Fig. A.4**). The bottom plug contained a glass stopcock which was used for sample collection during treatment experiments as well as a valve where an oxygen pump (DARO single aquarium airpump, South Africa) was connected to allow for air sparging during the solar disinfection treatments. Each glass tube was positioned in the centre of the CPC where the two arches of the CPC mirror connect axially and the tubes rested on the two end plates of the solar-CPC frame (**Appendix A Fig. A.4** and **A.5**).

Each reactor tube was filled with either TiO_2-rGO coated raschig rings (Sigma-Aldrich, Germany) for the solar photocatalytic disinfection experiments or uncoated raschig rings for the solar disinfection experiments. The raschig rings displaced approximately 100 mL of water once the tubes were filled and therefore the total treatment volume in these reactors was approximately 390 mL (**Fig. 3.1** and **Appendix A Fig. A.7**).

3.2.2 Coating of Raschig Rings

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). Briefly, the GO and TiO_2 were dispersed in aqueous suspension by ultrasound, whereafter the suspension was irradiated with a UV source in the presence of methanol as a hole acceptor. The GO is reduced to rGO by the photo-excited TiO_2 to form TiO_2-rGO composites. 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 by submerging the rings in a solution of distilled water and ethanol (1:1 v/v) (Cunha et al., 2018). The rings were then sonicated for 60 min and dried in an oven at 100°C (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^{o}C for 2 h in air (with a heating rate of 2°C per min) (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.

3.2.3 Solar Disinfection Experiments

3.2.3.1 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). The test strains 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 cells were harvested by centrifugation at 8 000 rpm (11 305 x *g*) for 15 min. The bacterial pellets were 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 volume of the suspension 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).

3.2.3.2 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 outlined in Waso et al. (2019). The predatory isolate was stored as plaques on double-layer agar plates, with *P. fluorescens* ATCC 13525 used as prey cells at 4°C until further experimentation commenced. To apply *B. bacteriovorus* PF13 as a pre-treatment to SODIS, a predator stock lysate (which was utilised as the predatory inoculum) was prepared as described by Dashiff et al. (2011) in the presence of *P. fluorescens* ATCC 13525 as prey cells. Briefly, a plaque was selected from a double-layer agar plate and was added to 90 mL of HEPES buffer [25mM, pH 7.2; supplemented with 3 mM $MgCl_2$ (Oxoid) and 2 mM $CaCl_2$ (Biolab)] (Shemesh & Jurkevitch, 2004) and 10 mL of *Pseudomonas fluorescens* prey cells (OD_{600} = ~1.00). The suspension was incubated at 30°C with shaking at 200 rpm. Once the medium was cleared (OD_{600} < 0.20; corresponding to approximately 10^9 PFU/mL) (Im et al., 2014; Gupta et al., 2016), the suspension was filtered through a 1.2 µm cellulose nitrate filter (47 mm; Sartorius Biolab Products) to remove any debris from the suspension. Subsequently, the suspension was filtered through a 0.45 µm GN-6 Metricel® filter (47 mm; Pall Life Sciences, Separations, South Africa) three times to remove any residual prey cells, resulting in a purified predator stock lysate which was used as the predator inoculum in the pre-treatment experiments.

3.2.3.3 Experimental Set Up

Synthetic rainwater was used to ensure that the composition of the medium remained constant throughout the study and was prepared using the method reported by Jones and Edwards (1993). In total, two disinfection trials were conducted of which the first trial included synthetic rainwater samples seeded with *K. pneumoniae* S1 43 and the second trial included synthetic rainwater samples seeded with *E. faecium* 8D. For each test organism, 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. 3.1**). 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. 3.1**).

For the pre-treated samples, 800 mL of synthetic rainwater was seeded with 100 mL of *K. pneumoniae* S1 43 or *E. faecium* 8D (OD_{600} = 1.00). Subsequently, each sample was inoculated with 100 mL of the *B. bacteriovorus* PF13 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* PF13 on the respective prey cells. For the samples which were not subjected to *B. bacteriovorus* PF13 pre-treatment, 900 mL of synthetic rainwater was seeded with 100 mL of *K. pneumoniae* S1 43 or *E. faecium* 8D cells (OD_{600} = 1.00) (**Fig. 3.1**) on the day of solar treatment.

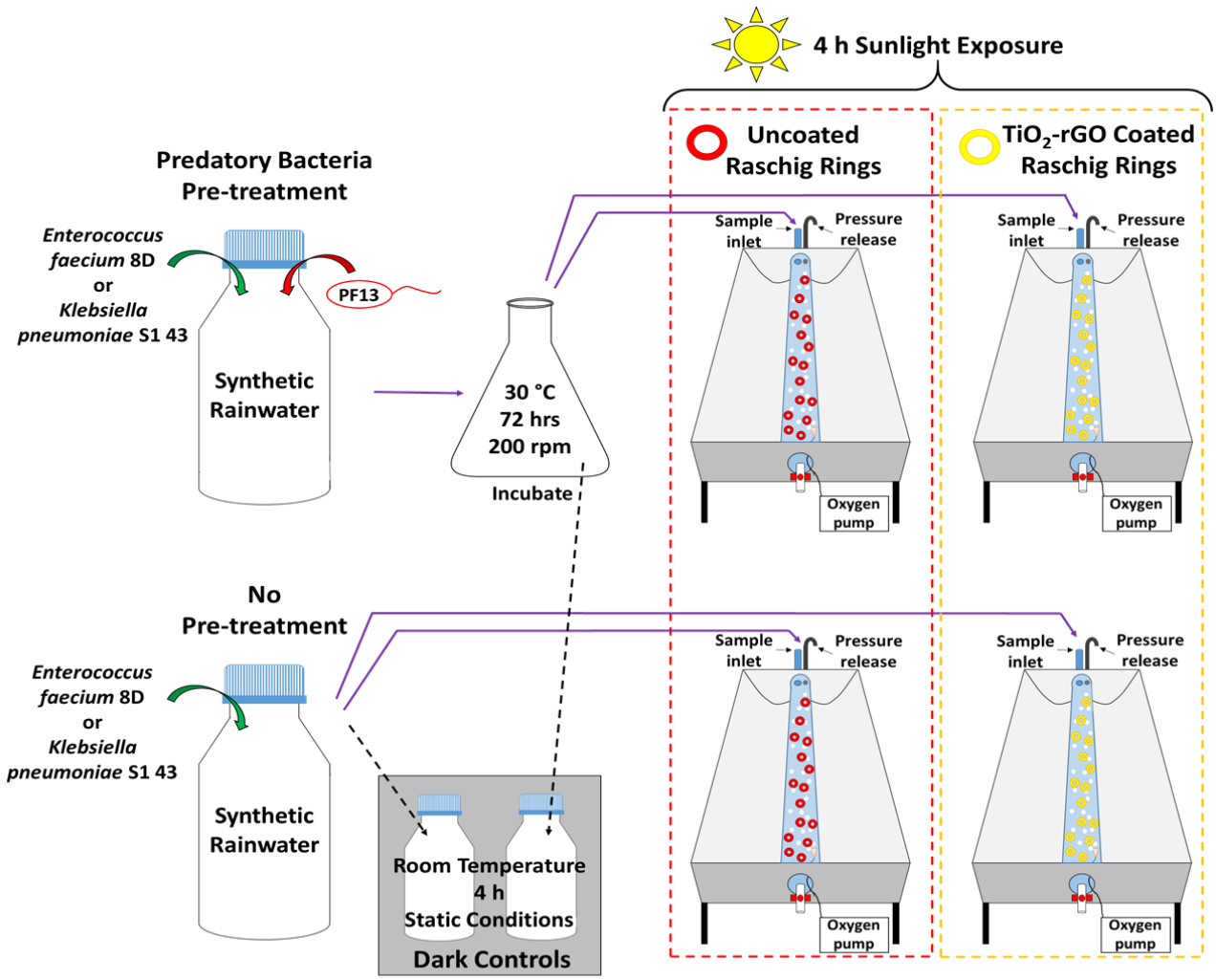

Fig. 3.1 The solar disinfection experimental set-up

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. 3.1**). The remaining volume of each sample was kept in the dark and served as dark controls (**Fig. 3.1**). 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, South Africa). 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.

Throughout the text the term “solar disinfection” will refer to solar treatment using only the designed solar-CPC system (with uncoated raschig rings), while “solar photocatalytic disinfection”

or “solar photocatalysis” will refer to the solar treatment using the designed solar-CPC system in combination with the immobilised TiO_2-rGO. Furthermore, “solar treatment” will be used to refer simultaneously to both disinfection strategies.

3.2.3.3.1 Culture-based Analysis

To enumerate the *K. pneumoniae* and *E. faecium* cells during the solar treatments [in colony forming units per mL (CFU/mL)], samples (10 mL) were collected as described in section 3.2.3.3. In addition, for the seeded synthetic rainwater samples subjected to *B. bacteriovorus* pre-treatment, 10 mL samples were collected before (0 h) and after pre-treatment (72 h). A further 10 mL sample was collected from each of the dark control samples after 240 min (to confirm that the changes in viable organisms occurred as a result of solar or solar photocatalytic disinfection). Thus, a total of 40 samples were collected for trials conducted with *K. pneumoniae* and *E. faecium*, respectively. A 10-fold serial dilution was prepared (ranging from undiluted to 10^{-6}) for each sample (n = 40) and 100 µL of each dilution was spread plated onto LB agar in triplicate. The plates were incubated at 30°C for 12 to 18 h (overnight).

In order to verify that the solar treatment effectively removed the predatory bacteria from the pre-treated samples, double-layer agar overlays [as described by Yu et al. (2017)] were also prepared using the serial dilutions from the seeded synthetic samples (with *B. bacteriovorus* pre-treatment) collected at 0 (initial bacterial concentration), 30, 60, 90, 120, 150, 180, 210 and 240 min (after the solar treatment). The plates were incubated at 30°C for up to 7 days and the predatory bacteria were enumerated in plaque forming units per mL (PFU/mL).

3.2.3.3.2 Molecular Analysis

For the molecular analysis of the solar-CPC samples collected at each time point (0 to 240 min) as well as the samples collected before (0 h) and after (72 h) *B. bacteriovorus* pre-treatment, 500 µL of each sample was EMA treated as described by Reyneke et al. (2016). The EMA-treated aliquots were subjected to DNA extractions using the *Quick*-DNA™ Fecal/Soil Microbe Miniprep (Zymo Research, Inqaba Biotech, South Africa) kit 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, Mannheim, Germany). All the qPCR primers and cycling parameters are outlined in **Table 3.1**, while the qPCR mixture as outlined in Waso et al. (2018) was utilised.

For GC quantification, a standard curve was generated for each of the respective qPCR assays from genomic DNA of *K. pneumoniae* S1 43, *E. faecium* 8D and *B. bacteriovorus* PF13,

respectively. The genomic DNA was extracted using the *Quick*-DNA™ Fecal/Soil Microbe Miniprep (Zymo Research, Inqaba Biotech) as per manufacturer's instructions. The specific gene targets for the respective qPCR assays were first amplified using conventional PCR (primers and cycling parameters outlined in **Table 3.1**). The conventional PCR mixture for *Bdellovibrio* spp. consisted of a final concentration of 1X Green GoTaq® Reaction Buffer (Promega Corp, Madison, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.9 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega Corp, Madison, USA) and 5 µL of DNA in a final volume of 25 µL. For *Klebsiella* spp. the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.3 µM of each primer and 1.5 U GoTaq® Flexi DNA Polymerase was used, while for *Enterococcus* spp. 2 mM $MgCl_2$, 0.2 µM of each primer, 1.5 U GoTaq® Flexi DNA Polymerase and 2 µL of DNA was used.

The conventional PCR products were analysed by gel electrophoresis, whereafter the PCR products were cleaned and concentrated, and the concentrations of the PCR products were determined as outlined by Waso et al. (2018). These PCR products were diluted from 10^9 to 10^0 GC/µL and each dilution served as the DNA used for each of the standards included for the standard curve. The respective standard curves and the DNA samples from the treatments were analysed in duplicate per qPCR assay, while a template negative control (sterile milliQ) was also included for each qPCR assay.

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) was determined as the GC/µL consistently detected per qPCR assay for the standard with the lowest GC. Furthermore, the lower limit of quantification (LLOQ) was determined as the lowest number of GC/µL that could reliably be quantified with the respective qPCR assays. 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):

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

Table 3.1 The primers and cycling parameters utilised for the detection and quantification of *B. bacteriovorus*, *K. pneumoniae*, and *E. faecium*

Organisms	Primer	Primer Sequences (5' – 3')	qPCR Cycling Parameters	Conventional PCR Cycling Parameters	Gene (product size in bp)	Melting Peak (°C)	Reference
***Bdellovibrio* spp.**	Bd347F	GGAGGCAGCAGT AGGGAATA	2 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; final elongation of 10 min at 72°C	16S rRNA (202)	84.3± 1.00	Van Essche et al. (2009)
	Bd549R	GCTAGGATCCCT CGTCTTACC					
***Klebsiella* spp.**	gyrA-A	CGCGTACTATACG CCATGAACGTA	10 min at 95°C; 50 cycles of 60 s at 94°C, 30 s at 50°C and 30 s at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	3 min at 95°C; 35 cycles of 60 s at 94°C, 30 s at 50°C and 30 s at 72°C; final elongation of 10 min at 72°C	*Gyrase A* (383)	87.7 ± 1.00	Brisse & Verhoef (2001)
	gyrA-C	ACCGTTGATCACT TCGGTCAGG					
***Enterococcus* spp.**	ECST784F	AGAAATTCCAAAC GAACTTG	10 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	5 min at 95°C; 30 cycles of 30 s at 94°C, 60 s at 59°C and 60 s at 72°C; final elongation of 10 min at 72°C	23S rRNA (75)	79.2 ± 1.00	Frahm & Obst (2003)
	ENC854R	CAGTGCTCTACCT CCATCATT					

3.2.4 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 $p < 0.05$.

3.3 Results

3.3.1 Physico-chemical Parameters

The results for the pH, temperature, TDS, EC and DO collected for the different solar treatment time points (0 to 240 min) for each test organism and experimental design, are summarised in the **Appendix A Tables A.1** and **A.2**. Overall the pH, TDS, EC and DO of the individual samples did not change significantly during the experimental trials. The temperature of the treated water samples increased during the trials with an average initial temperature of 21.7°C recorded and an average temperature of 34°C recorded for the first (*K. pneumoniae* disinfection) and second trial (*E. faecium* disinfection), respectively, after 240 min of solar exposure. An average UV-A irradiance of 34.7 W/m^2 was recorded for the first trial and 35.6 W/m^2 was recorded for the second trial, while an average UV-B irradiance of 4.42 W/m^2 was recorded for the first trial and 4.59 W/m^2 was recorded for the second trial. Lastly, an average DNI of 979.5 W/m^2 was recorded for the first trial and 979.2 W/m^2 was recorded for the second trial. Lastly, for the *K. pneumoniae* trial, a solar UV-A dose of 25.83 J/cm^2 was obtained within 120 min of solar exposure. After 240 min of solar exposure, a solar UV-A dose of 51.66 J/cm^2 was recorded. Similarly, for the *E. faecium* trial, a solar UV-A dose of 51.66 J/cm^2 was obtained after 240 min of solar exposure.

3.3.2 Solar and Solar Photocatalytic Disinfection of *Klebsiella pneumoniae*

To assess the effect of the different treatments on the concentration of *K. pneumoniae*, the CFU at the different time intervals were enumerated using culture-based methods, while the GC from viable cells were quantified using EMA-qPCR. In addition, for the biological pre-treatment trial, *B. bacteriovorus* was enumerated at the different time intervals using double-layer agar overlays and GC were quantified using EMA-qPCR. This was done to determine whether solar disinfection or solar photocatalysis would reduce or remove the predator cells during treatment. All the EMA-qPCR characteristics are summarised in **Appendix A Table A.3**.

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

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. 3.2A**; **Table 3.2**). 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 (recorded prior to predation; **Table 3.2**). 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. 3.2B**; **Table 3.2**). 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 (**Appendix A 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 (**Appendix A 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. 3.2A**; **Table 3.2)**. 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 (recorded prior to predation; **Table 3.2**). 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. 3.2B**; **Table 3.2**). 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 (**Appendix A 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 (**Appendix A Fig. A.8B**).

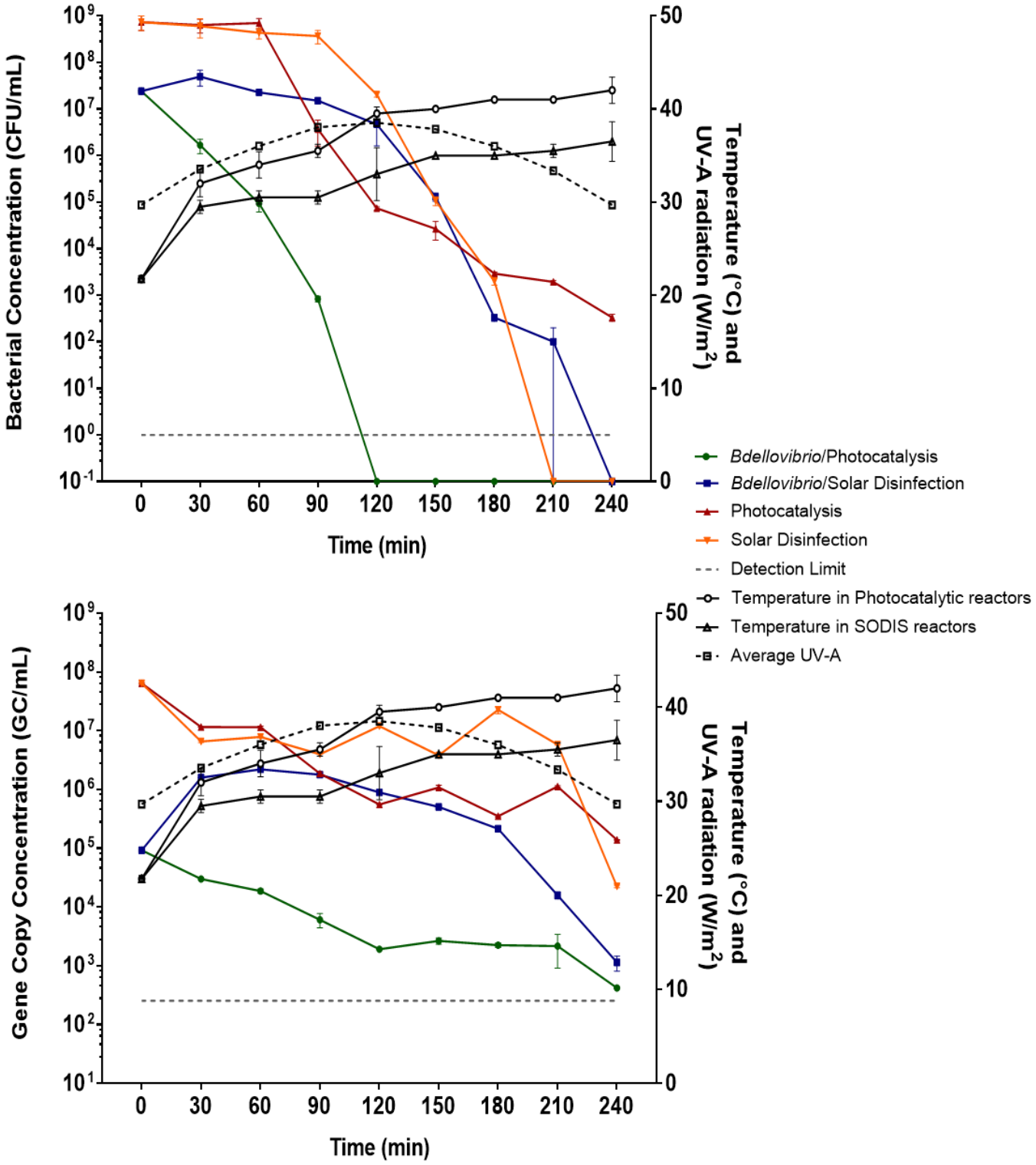

Fig. 3.2 The (A) cell counts (CFU/mL) and (B) gene copies (GC/mL) of *K. pneumoniae* recorded during the various combinations of solar treatments after 72 h of *B. bacteriovorus* pre-treatment

Table 3.2 Summary of the cell counts, gene copies and log reductions obtained during the various treatments for *K. pneumoniae* and *E. faecium*

Trial	Treatment Combinations	Cell Counts (CFU/mL)			EMA-qPCR (GC/mL)		
		Initial Concentration[a]	Final Concentration	Log Reduction	Initial Concentration[a]	Final Concentration	Log Reduction
***K. pneumoniae* Disinfection (72 h pre-treatment)**	*Bdellovibrio* pre-treatment + photocatalysis	2.00×10^9	BDL[b]	9.30	2.95×10^8	4.19×10^2	5.85
	Bdellovibrio pre-treatment + solar disinfection		BDL[b]	9.30		1.14×10^3	5.41
	Photocatalysis	7.33×10^8	3.33×10^2	6.34	6.41×10^7	1.39×10^5	2.67
	Solar disinfection		BDL[b]	8.87		2.24×10^4	3.46
***E. faecium* Disinfection (72 h pre-treatment)**	*Bdellovibrio* pre-treatment + photocatalysis	3.57×10^9	1.40×10^5	4.41	8.24×10^5	4.35×10^3	2.28
	Bdellovibrio pre-treatment + solar disinfection		1.67×10^2	7.33		1.29×10^3	2.81
	Photocatalysis	1.00×10^8	BDL[b]	8.00	2.09×10^6	8.74×10^2	3.38
	Solar disinfection		BDL[b]	8.00		8.53×10^2	3.39

[a] Initial concentration before predation for the pre-treated samples; [b] BDL - Below the Detection Limit; [c] ND – Not Determined

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. 3.2A**; **Table 3.2**), 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. 3.2B**; **Table 3.2**). Furthermore, for the non-pre-treated sample exposed to 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. 3.2A**; **Table 3.2**). 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 3.2**).

3.3.3 Solar and Solar Photocatalytic Disinfection of *Enterococcus faecium*

To assess the effect of the different combination treatments on the concentration of *E. faecium*, the CFU of *E. faecium* were also enumerated at the different time intervals using culture-based methods, while the GC from viable cells were quantified using EMA-qPCR. Additionally, the concentration of *B. bacteriovorus* was determined using double-layer agar overlays (PFU) and was quantified using EMA-qPCR (GC) in the various samples pre-treated with the predatory bacteria.

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

Subsequently, the cell counts of *E. faecium* recorded for the pre-treated sample exposed to solar photocatalysis were reduced by 3.81 logs from 9.00×10^8 CFU/mL (at 0 min) to 1.40×10^5 CFU/mL (at 240 min) ($p < 0.0001$) (**Fig. 3.3A**). Thus, the CFU/mL of *E. faecium* was reduced by a total of 4.41 logs from an initial concentration of 3.57×10^9 CFU/mL (recorded before the 72 h of predation) (**Table 3.2**). The EMA-qPCR analysis then indicated that the

E. faecium concentration was reduced by 1.57 logs from 1.60 × 10^5 GC/mL (at 0 min) to 4.35 × 10^3 GC/mL (at 240 min) (**Fig. 3.3B**), with an overall reduction of 2.28 logs recorded from an initial concentration of 8.24 × 10^5 GC/mL (recorded prior to the 72-hr predation pre-treatment) (**Table 3.2**). As mentioned previously, *B. bacteriovorus* did not produce any plaques on the double-layer agar overlays when *E. faecium* was utilised as prey and the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus* 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 log reduction; $p < 0.0001$) after 240 min of solar photocatalysis (**Appendix A Fig. A.9**).

The cell counts of *E. faecium* recorded for the pre-treated sample exposed to solar disinfection, were 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) ($p < 0.0001$) (**Fig. 3.3A**; **Table 3.2**). Therefore, an overall log reduction of 7.33 in *E. faecium* CFU/mL was recorded after the *B. bacteriovorus* pre-treatment and the solar disinfection (**Table 3.2**). The EMA-qPCR analysis indicated that the concentration of *E. faecium* was reduced by 2.09 logs from 1.60 × 10^5 GC/mL (at 0 min) to 1.29 × 10^3 GC/mL (at 240 min) ($p < 0.0001$) (**Fig. 3.3B**). Overall, the concentration of *E. faecium* was thus reduced by a total of 2.81 logs in the pre-treated sample exposed to solar disinfection, from an initial concentration of 8.24 × 10^5 GC/mL (recorded prior to the 72-hr predation pre-treatment) (**Table 3.2**). For the predatory bacteria, the EMA-qPCR analysis indicated that the concentration of *B. bacteriovorus* was 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 reduction; $p < 0.0001$) after 240 min of solar exposure (**Appendix A Fig. A.9**).

For the samples which were not pre-treated with *B. bacteriovorus* but were exposed to solar disinfection and solar photocatalytic disinfection, 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. 3.3A**; **Table 3.2**). 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. 3.3B**; **Table 3.2**). Similarly, for the sample exposed to solar photocatalytic disinfection, 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. 3.3B**; **Table 3.2**).

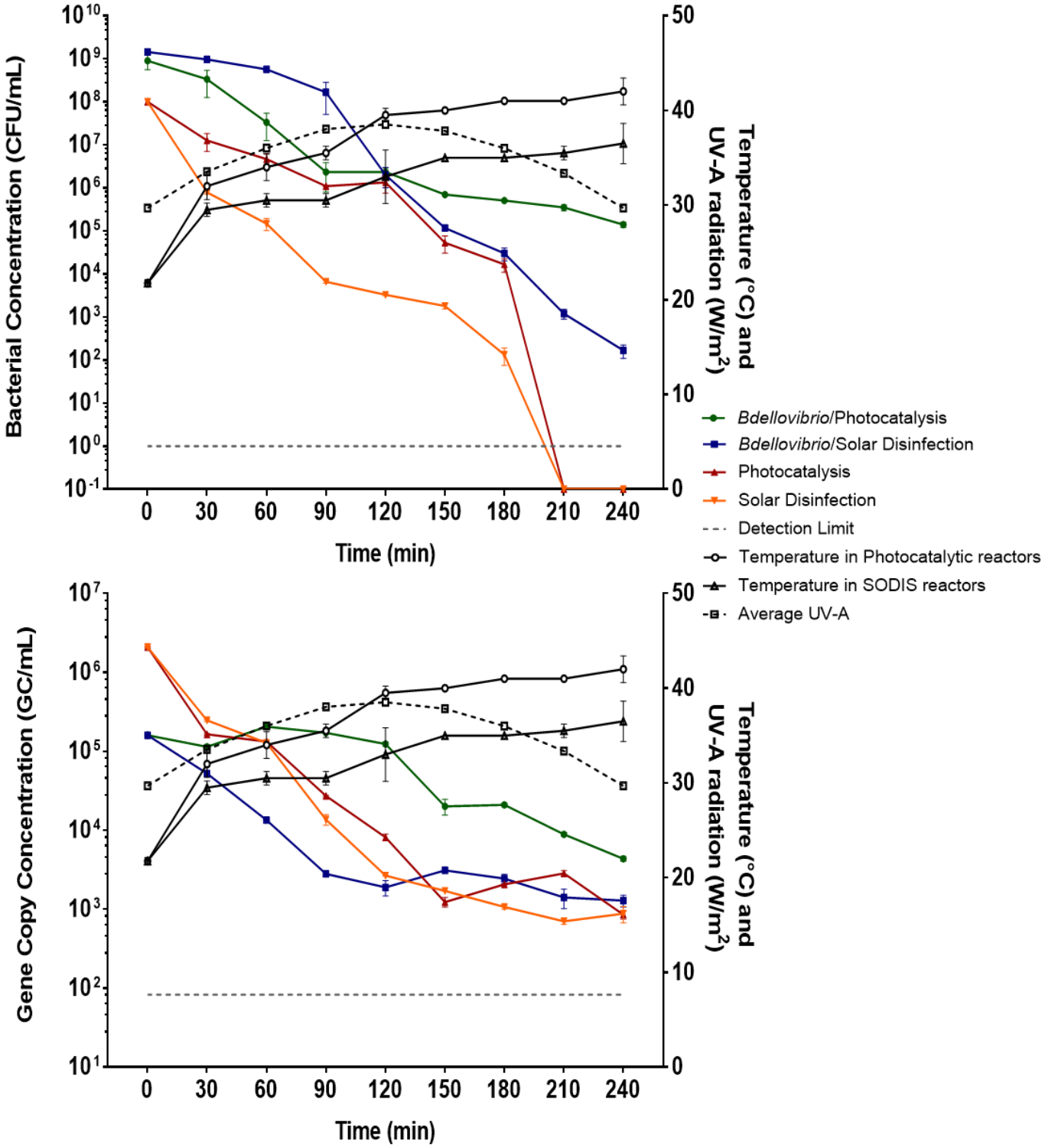

Fig. 3.3 The (A) cell counts (CFU/mL) and (B) gene copies (GC/mL) of *E. faecium* recorded during the various combinations of solar treatments after 72 h of *B. bacteriovorus* pre-treatment

3.4 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. Culture-based enumeration then indicated that the highest log reduction in *K. pneumoniae* CFU (9.30 logs; $p < 0.0001$) was obtained for the combination of *B. bacteriovorus* pre-treatment followed by solar disinfection and solar photocatalytic disinfection. This was confirmed by the molecular analysis where the highest log reductions were recorded for the *B. bacteriovorus* pre-treated samples exposed to solar photocatalysis (5.85 logs; $p < 0.0001$) and solar disinfection (5.41 logs; $p < 0.0001$). *Bdellovibrio bacteriovorus* is known to attach to the cell wall of Gram-negative prey through an unknown mechanism or receptor, whereafter the predator rotates to create a pore in the prey cell wall (Sockett, 2009). The predatory bacterium subsequently 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). Upon maturation of the progeny cells and depletion of the nutrients in the bdelloplast, the prey cell is lysed to release the *B. bacteriovorus* progeny, in search of new prey cells (Sockett, 2009). Correspondingly, as the Gram-negative bacterium *K. pneumoniae* is sensitive to predation (Dashiff et al., 2011), 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). It is well established that ROS significantly disrupts the cell membrane structures and damages DNA and RNA, ultimately leading to cell death (Byrne et al., 2011). Fernández-Ibáñez et al. (2015) and Cruz-Ortiz et al. (2017) previously investigated the mechanisms behind the antimicrobial activity of TiO_2-rGO in water using *E. coli* as the model organism. Cruz-Ortiz et al. (2017) found that overall, *E. coli* was reduced by 6 logs (within 90 min) and 5.3 logs (after 180 min) in the presence of UV-visible and visible light, respectively, while Fernández-Ibáñez et al. (2015) reported that *E. coli* was reduced by 6 logs (within 10 min) 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 it was 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 (Lin et al., 2014). 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 Cruz-Ortiz et al. (2017) and Lin et al. (2014) evaluated the photocatalyst in suspension, in the current study, the photocatalyst was immobilised onto glass raschig rings and exposed to natural 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 efficiency 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 (Sordo et al., 2010). 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 (Sordo et al., 2010). 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). It has previously been noted that mechano-osmotic stress can account for up to 99% of bacterial inactivation in recirculating systems (Sichel et al., 2007). The use of raschig rings as support material for the immobilisation of photocatalysts is thus advantageous as post-treatment removal of the photocatalytic material is not required, it generates a greater contact area for increased exposure of cells to the photocatalytic material and if a flow rate is applied, mechanical stress is exerted on the cells.

Apart from using photocatalytic material in two of the solar-CPC reactors in the current study, all the water samples (pre-treated with *B. bacteriovorus* and non-pre-treated) were exposed to solar treatment under CPC concentrated solar UV-A radiation. The CPC mirrors were used for the solar treatment reactors as it significantly enhances any kind of solar water treatment by improving the solar UV energy income by a concentration factor of 1 (Keane et al., 2014). Navntoft et al. (2008) demonstrated that the use of a CPC accelerated the reduction of 6-log *E. coli* K12 under solar disinfection by 90 minutes as compared to a PET plastic bottle. Based on the solar UV-A dose calculated for the *K. pneumoniae* trials (section 3.3.1), a similar UV-A dose was obtained within 120 min of solar exposure (25.83 J cm^{-2}), to the dose reported in literature (27 J/cm^2) to achieve a 5-log reduction in *E. coli* K12 by solar disinfection in a 2 L-PET bottle filled with clear transparent water (Castro-Alférez et al., 2018). Additionally, the dose obtained in the current study 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-log reduction of *E. coli* K 12 using the same catalyst (TiO_2-rGO) suspended as a slurry at a concentration of 500 mg/L (Fernández-Ibáñez et al., 2015). Similar solar dose values were obtained for the *E. faecium* trial. Thus, sufficient solar irradiation was obtained to reduce the concentration of *K. pneumoniae* and *E. faecium* during the current study.

Correspondingly, the most efficient treatment strategy for the reduction of *E. faecium* was the use of solar disinfection or solar photocatalytic disinfection without *B. bacteriovorus* pre-treatment. The culture-based enumeration of *E. faecium* indicated that the greatest log reduction (8.00 logs; $p < 0.0001$) was obtained within 210 min for the samples not pre-treated with *B. bacteriovorus* and only exposed to solar disinfection or solar photocatalytic disinfection. Similar reductions in the GC of *E. faecium* were observed using EMA-qPCR for the samples which were exposed to solar disinfection (3.39 logs; $p < 0.0001$) and solar photocatalytic disinfection (3.38 logs; $p < 0.0001$). While it is generally theorised that *B. bacteriovorus* does not prey on Gram-positive bacteria, recent studies have indicated that this predator can prey on *Staphylococcus aureus* (*S. aureus*) (Iebba et al., 2014; Pantanella et al., 2018). The lytic enzymes produced by *B. bacteriovorus* have also been shown to disrupt biofilms produced by Gram-positive bacteria, while proteases produced by *B. bacteriovorus* can decrease the efficiency of *S. aureus* invasion into human epithelial cells (Monnappa et al., 2014). Furthermore, 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. Results indicated that in all the systems analysed, *E. coli* was readily reduced to BDL, while *Enterococcus* spp. and *C. perfringens* were more resistant to disinfection (Gutiérrez-Alfaro et al., 2015). 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. perfringes* under natural sunlight (Gutiérrez-Alfaro et al., 2015). The authors ultimately concluded that Gram-positive organisms, which have more complex cell walls, are more resistant to disinfection in comparison to Gram-negative organisms. However, they found that recirculating the water in the solar photocatalytic systems, increased turbulence and contact between the catalyst and the bacteria, and significantly enhanced the disinfection

efficiency, especially for Gram-positive organisms (Gutiérrez-Alfaro et al., 2015). Venieri et al. (2014) also investigated the disinfection of *E. 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), the *E. faecalis* plate counts were reduced by 7 logs to BDL after approximately 40 min of treatment. However, 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 (Venieri et al., 2014). 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* (Venieri 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; Dobrowsky et al., 2014; Ahmed et al., 2018), 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* are rod-shaped cells, which readily form cell aggregates and have prominent capsules (Thornton et al., 2012; Dorman et al., 2018). These capsules are known to harbour important virulence factors, which protects the cells from phagocytosis and from the bactericidal effect of human blood serum (Struve & Krogfelt, 2004). The capsule and cell aggregation properties of *K. pneumoniae* have also been hypothesised to protect *K. pneumoniae* from bactericidal stressors such as UV irradiation and antibiotic agents (Thornton et al., 2012; Venieri et al., 2017). In contrast, the *E. faecium* strain (8D) employed was isolated from untreated harvested rainwater (Dobrowsky et al., 2014). *Enterococcus* spp. are known to be tolerant to a wide range of environmental conditions such as oxygen-rich and -poor environments, pH ranging from 4.5 to 10, high chloride concentrations and temperatures ranging from 10°C to 45°C. Moreover, they have been found to exhibit increased resistance to UV disinfection (McKinney & Pruden, 2012). This has been ascribed to the differences between Gram-positive and Gram-negative cell wall structures where the thick peptidoglycan layer in the Gram-positive cell wall has been hypothesised to provide protection against UV irradiation (McKinney & Pruden, 2012). Some strains of enterococci have also 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. 3.2B** and **3.3B**), further work may include extending the solar and solar photocatalytic treatment time. The recommended time for solar disinfection in PET bottles (SODIS) is 6 h under direct sunlight.

3.5 Conclusions

Based on the results obtained, *B. bacteriovorus* may be applied to decrease the concentration of Gram-negative bacteria, such as *K. pneumoniae* (which are sensitive to predation), prior to solar disinfection. This is crucial as many pathogenic Gram-negative organisms have been found to persist after the implementation of various disinfection strategies. Solar disinfection or solar photocatalytic disinfection 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 also 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. This may include pre-treating harvested rainwater with *B. bacteriovorus* and then exposing the rainwater to solar or solar photocatalytic disinfection treatment. Illumina next-generation sequencing in combination with viability dyes can then be used to monitor the effect on the whole microbial community and subsequently elucidate the effect of predatory bacteria pre-treatment on the disinfection of natural water sources.

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Declaration of Interest

The authors declare no conflict of interest.

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Expression of attack and growth phase genes of *Bdellovibrio bacteriovorus* in the presence of Gram-negative and Gram-positive prey

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Abstract

The expression of attack phase (AP) and growth phase (GP) genes of *Bdellovibrio bacteriovorus* (*B. bacteriovorus*) was compared in the presence of Gram-negative [*Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*)] and Gram-positive [*Enterococcus faecium* (*E. faecium*)] prey, using relative quantitative polymerase chain reaction (relative qPCR) assays. The genes *bd0108* (pili retraction/extrusion) and *merRNA* (massively expressed riboswitch RNA) were highly expressed in the AP cells [3.99- to 6.06-fold (*E. coli*), 3.91- to 7.05-fold (*K. pneumoniae*) and 2.91- to 7.30-fold (*E. faecium*)]. The *fliC1* gene (flagella filament) was also expressed at a high level in the AP cells however, after 240 min of co-culture with *E. faecium* the expression of *fliC1* remained low (at 0.759-fold), while in the presence of the Gram-negative prey *fliC1* expression increased. Additionally, the GP genes *bd0816* (peptidoglycan-modifying enzyme) and *groES1* (chaperone protein) were not induced in the presence of *E. faecium*. However, they were expressed in the early GP and GP of *B. bacteriovorus* after exposure to the Gram-negative prey. It can thus be concluded that *B. bacteriovorus* senses the presence of potential prey when exposed to Gram-positive and Gram-negative bacteria, however the GP genes are not induced in co-culture with *E. faecium*. The results from this study thus indicate that *B. bacteriovorus* does not actively grow in the presence of *E. faecium* and the second predatory cue (induces active growth of *B. bacteriovorus*) is lacking when *B. bacteriovorus* is co-cultured with the Gram-positive prey.

Keywords: *Bdellovibrio bacteriovorus*; Gram-positive prey; Gram-negative prey; gene expression; attack phase; growth phase

4.1 Introduction

Bdellovibrio bacteriovorus (*B. bacteriovorus*) is a predatory bacterium that utilises mainly Gram-negative organisms as prey. This predator exhibits a dimorphic and biphasic life cycle consisting of an attack phase (AP) and growth phase (GP) (Sockett, 2009; Rotem et al., 2015). Various authors have subsequently studied the genetic mechanisms involved in the predatory behaviour exhibited by *B. bacteriovorus* (Lerner et al., 2012; Karunker et al., 2013; Rotem et al., 2015), with most studies focusing on elucidating the genetic differences between host-dependent and host-independent growth of *B. bacteriovorus* strains (Lambert et al., 2006; Evans et al., 2007; Lambert et al., 2010; Avidan et al., 2017). Rendulic et al. (2004) sequenced the genome of *B. bacteriovorus* HD100, the type strain of *B. bacteriovorus* and found that this bacterium has a genome size of 3.85 Mb, which includes core genes similar to those found in non-predatory bacteria, with approximately 40% of the genome encoding for genes essential for predation. These genes were referred to as the potential “predatosome” of *B. bacteriovorus* and code for various hydrolytic enzymes (for prey degradation), for prey cell invasion and for regulating the growth of the predator in the bdelloplast (Rendulic et al., 2004).

Lambert et al. (2010) used oligonucleotide arrays and reverse transcription quantitative polymerase chain reaction (RT-qPCR) to investigate the genes that were up- and downregulated during the AP and 30 min into the predatory interaction (periplasm entry and bdelloplast formation). During the AP, 230 genes (6.4% of the genome) were found to be significantly upregulated. In comparison, 30 min into predation 479 genes (13% of the genome) were upregulated, while the 230 AP genes were downregulated. The upregulated AP genes were found to encode for transcriptional regulators, flagella and pili production, the synthesis of cell surface proteins and 99 hypothetical proteins, amongst others, while 30 min into predation, genes associated with peptidoglycan modification and metabolism, ATP synthase, hydrolytic enzymes (proteases, esterases, helicases and endonucleases), nucleic acid synthesis and 173 hypothetical proteins, amongst others, were found to be upregulated (Lambert et al., 2010). Karunker et al. (2013) used Illumina next-generation RNA sequencing to characterise expressed genes associated with the AP and the GP of *B. bacteriovorus*. The authors found that 353 genes (15% of the expressed genes) were exclusively upregulated during the AP, 1557 genes (67% of the expressed genes) were upregulated during the GP, while 180 genes (18% of the expressed genes) were expressed during both phases with at least a 5-fold change in gene expression. The authors concluded that these genes indicated a transcriptional switch between the AP and GP and that these phases are governed by almost mutually exclusive transcriptional programmes. Rotem et al. (2015) then proposed a model for the transcriptional regulation involved in the predation mechanisms of *B. bacteriovorus* and confirmed that the predator requires two predatory cues (which are prey related cues) to switch from an AP cell to an actively growing GP cell. The nature of the first cue is still unknown but it has been hypothesised that this cue may be associated

with the prey cell envelope and the subsequent physiological switching of the predator from the AP to the GP (Rotem et al., 2014; 2015). The second predatory cue is theorised to be a soluble cue associated with the prey cell cytoplasm, which prompts the predator to start actively growing within the bdelloplast by activating DNA synthesis (Rotem et al., 2014; 2015). In addition, the authors found that *B. bacteriovorus* enters a transitionary phase between the first and second predatory cues to assess whether the predator should start growing filamentously (Rotem et al., 2015).

Based on the model proposed by Rotem et al. (2015), which describes the molecular cascade involved in the predatory behaviour of *B. bacteriovorus*, the *bd0108*, *merRNA* and *fliC1* genes were selected for analysis in the current study as genes specific to the AP, while *bd0816* and *groES1* represented genes specific to the early GP and GP, respectively. In AP *B. bacteriovorus* cells, *bd0108* (regulates type IVa pili extrusion/retraction and plays a role in the signal transduction of the first predatory cue), *merRNA* (highly expressed putative cyclic di-GMP riboswitch) and genes associated with the motility of the predator [such as the *fliC* (*fliC1 – 5*) genes] are significantly upregulated as the free-living, fast-swimming cells hunt for prey cells (Karunker et al., 2013; Rotem et al., 2015). Once the predator encounters a prey cell, *bd0108* expression is reduced which results in the transduction of the first predatory cue signalling that a prey cell has been encountered (Rotem et al., 2015). This in turn results in the reduced expression of *merRNA*, which subsequently prompts the AP *B. bacteriovorus* cell to switch to a GP cell (Karunker et al., 2013; Rotem et al., 2015). The predatory cell then produces various enzymes to invade the prey cell to which it is attached. Amongst these enzymes are the peptidoglycan-modifying enzymes encoded for by *bd0816* and *bd3459*, which aids the predator in modifying the peptidoglycan layer of the prey cell it is infecting (Lerner et al., 2012; Rotem et al., 2015). These enzymes are specifically used to create a pore through which the predator enters the prey cell (to form the bdelloplast), which houses the actively growing predator (Lerner et al., 2012; Rotem et al., 2015). Once *B. bacteriovorus* starts growing within the bdelloplast, genes associated with DNA replication, protein synthesis (such as *groES1* encoding for a chaperone protein) and hydrolytic enzymes, utilised to digest the cellular components of the invaded prey cell, are upregulated (Lambert et al., 2012; Rotem et al., 2015).

Although progress has been made in elucidating the molecular regulatory cascade involved in the predatory behaviour of *B. bacteriovorus*, most studies have focused on the interaction between laboratory strains of *B. bacteriovorus* and *Escherichia coli* (*E. coli*), as a surrogate for Gram-negative prey (Evans et al., 2007; Lambert et al., 2010; Karunker et al., 2013; Rotem et al., 2015). In the natural environment, *B. bacteriovorus* will however, be exposed to bacterial populations comprised of Gram-negative and Gram-positive organisms (Im et al., 2018). Limited information on the interaction of *B. bacteriovorus* with Gram-positive prey is currently available, as it was originally hypothesised that these predatory bacteria are solely dependent on Gram-negative prey

for nutrients and replication (Im et al., 2018). Recent studies have however, indicated that under certain conditions *B. bacteriovorus* could utilise Gram-positive organisms such as *Staphylococcus aureus* (*S. aureus*) as prey (Iebba et al., 2014; Pantanella et al., 2018). Iebba et al. (2014) showed that *B. bacteriovorus* HD100 reduced *S. aureus* biofilms by 46 to 74%, while Field-Emission Scanning Electron Microscopy (FESEM) confirmed that *B. bacteriovorus* switches its predation strategy and preys on *S. aureus* in an epibiotic manner. Pantanella et al. (2018) hypothesised that when natural or ideal prey (i.e. Gram-negative prey) are not available, *B. bacteriovorus* would engage with Gram-positive prey as a survival strategy. Using qPCR, Pantanella et al. (2018) also showed that the concentration of *B. bacteriovorus* increased with a corresponding decrease in the concentration of *S. aureus*, while Scanning Transmission Electron Microscopy (STEM) confirmed that *B. bacteriovorus* directly interacts with *S. aureus* by attaching to the outside of the cells and disrupting the cytoplasmic membrane.

The aim of this study was thus to monitor the expression of AP (*bd0108*, *merRNA* and *fliC1*) and GP (*bd0816* and *groES1*) genes of *B. bacteriovorus* exposed to Gram-negative prey and compare the gene expression profile to the exposure of *B. bacteriovorus* to Gram-positive prey. This aim was achieved by exposing *B. bacteriovorus* PF13 to Gram-negative [*E. coli* ATCC 417373 and *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 333305] and Gram-positive [a clinical *Enterococcus faecium* (*E. faecium*) isolate] prey, whereafter relative qPCR was employed to monitor the gene expression levels.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Growth Conditions

Bdellovibrio bacteriovorus PF13 was isolated from wastewater collected from the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: 33° 59' 21.13"S 18° 47' 47.75"E) as previously described by Waso et al. (2019). *Escherichia coli* ATCC 417373 (Gram-negative prey) was used as a control to compare the gene expression analysis to previously published data (Lambert et al., 2006; 2010; Karunker et al., 2013; Rotem et al., 2015). *Klebsiella pneumoniae* ATCC 333305 was used as the Gram-negative prey, while a clinical *E. faecium* isolate was utilised as Gram-positive prey. All the bacterial isolates were obtained from the Water Resource Laboratory Culture Collection at Stellenbosch University (Department of Microbiology).

For the co-culture experiments, the test prey strains (*E. coli*, *K. pneumoniae* and *E. faecium)* were inoculated into 500 mL Luria Bertani (LB) broth (Biolab, Merck, South Africa) and were incubated for 24 h at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 8 000 rpm for 20 min. The obtained bacterial pellets were washed and re-suspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer [25mM, pH 7.2; supplemented with 3 mM $MgCl_2$ (Oxoid) and 2 mM $CaCl_2$ (Biolab)] (Shemesh & Jurkevitch, 2004) and the concentration of the

bacterial cells was adjusted with HEPES buffer to obtain a final optical density (OD) of 1.00 (which corresponded to approximately 10^9 cells/mL). The OD was measured using the T60 UV-Visible Spectrophotometer (PG Instruments Limited, Thermo Fisher Scientific, South Africa) at 600 nm (OD_{600}).

To apply *B. bacteriovorus* PF13 as the predator in the co-culture experiments, a plaque was excised from a stock culture plate where *B. bacteriovorus* PF13 was maintained with *E. coli* ATCC 417373 as prey. The plaque was added to 1 L HEPES buffer with 10 mL of *E. coli* ATCC 417373 (OD_{600} = 1.00) and the suspension was incubated at 30°C with shaking at 200 rpm for 48 to 72 h to obtain a predator stock lysate (Lambert et al., 2010).

4.2.2 Synchronisation of Predatory Cultures

Prior to initiating the experimental co-cultures between *B. bacteriovorus* and *E. coli*, *K. pneumoniae* and *E. faecium*, respectively, semi-synchronised predatory cultures were established (Lambert et al., 2010). To obtain the semi-synchronised predatory cultures, 5 mL of the stock lysate obtained as described in section 4.2.1 was filtered through a 0.45 µm GN-6 Metricel® filter (47 mm; Pall Life Sciences, Separations, South Africa) three times to remove any remaining prey cells. The filtered stock lysate (5 mL) was subsequently added to 50 mL HEPES buffer with 5 mL of *E. coli* ATCC 417373 (OD_{600} = 1.00) (Lambert et al., 2010). This predatory co-culture was sub-cultured every 24 h for a period of 3 days by transferring 5 mL of the co-culture to 50 mL of HEPES buffer with 5 mL fresh *E. coli* cells (Lambert et al., 2010). The sub-culture (60 mL) obtained after 3 days was utilised to inoculate 1 L of HEPES buffer containing 60 mL of *E. coli* ATCC 417373 cells (OD_{600} = 1.00) (Lambert et al., 2010). This suspension was incubated for 24 h at 30°C with shaking at 200 rpm. The AP *B. bacteriovorus* cells were harvested by centrifugation at 10 000 rpm for 30 min and were suspended in 100 mL HEPES buffer (Lambert et al., 2010). The concentrated *B. bacteriovorus* cells were subsequently mixed with 100 mL *E. coli* ATCC 417373 (OD_{600} = 1.00) prey (Lambert et al., 2010). This suspension was incubated at 30°C for 3 h to stabilise the gene expression of *B. bacteriovorus* (Lambert et al., 2010).

4.2.3 Experimental Co-cultures

To initiate the experimental co-cultures between *B. bacteriovorus* and *E. coli*, *K. pneumoniae* and *E. faecium*, respectively, 50 mL of the semi-synchronised *B. bacteriovorus* culture obtained in section 4.2.2 was filtered through a 0.45 µm GN-6 Metricel® filter three times. The filtered *B. bacteriovorus* culture (50 mL) was mixed with 40 mL of the respective prey cells (OD_{600} = 1.00) and 30 mL HEPES buffer (Lambert et al., 2010). Control cultures of *B. bacteriovorus* only (50 mL filtered *B. bacteriovorus* culture and 70 mL HEPES buffer) and prey cells only (40 mL *E. coli*, *K. pneumoniae* or *E. faecium* and 80 mL HEPES buffer) were included for all the co-culture experiments (Lambert et al., 2010). All the co-culture experiments were conducted in duplicate.

These cultures were incubated at 30°C with shaking at 200 rpm for 4 h. Samples (10 mL) were collected from each culture (the co-cultures and the control cultures) at 0, 15, 30 and 240 min post-infection. Each 10 mL sample was added to a 2% phenol: 38% ethanol solution (final concentration of 1% phenol: 19% ethanol) and was incubated at 4°C for 45 min (Lambert et al., 2010). The samples were then centrifuged at 3000 rpm for 10 min at 4°C and the obtained pellets were stored at -80°C prior to RNA extractions (Lambert et al., 2010).

4.2.4 RNA Isolation and cDNA Synthesis

Ribonucleic acids (RNA) were extracted from the frozen bacterial pellets using the TRI Reagent® (Sigma-Aldrich, Mannheim, Germany) as per the manufacturer's instructions. The purity and concentration of each RNA extract was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, USA) in triplicate. Subsequently, 1 µg of total RNA was treated with the TURBO DNA-*free*™ kit to remove any remaining DNA from the RNA samples. Each DNase treated RNA sample was screened with the respective PCR assays (section 2.5) to confirm that the RNA samples were free of DNA prior to cDNA synthesis. The RNA from each sample was subsequently transcribed to cDNA (0.1 µg RNA used per reverse transcription reaction) using the ImProm-II™ Reverse Transcriptase system and the Oligo(dT)$_{15}$ primer set as per the manufacturer's instructions. The cDNA was diluted 1:10 prior to qPCR and each cDNA sample was analysed in duplicate using qPCR for each gene as outlined in section 4.2.5.

4.2.5 Gene Expression Analysis using Relative qPCR

The relative qPCR assays were conducted using the LightCycler® 96 Instrument (Roche Diagnostics, Mannheim, Germany) and the FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany). To monitor the AP of *B. bacteriovorus*, primer sets targeting the genes encoding for the host-independent locus involved in prey sensing and pili retraction (*bd0108*), a massively expressed riboswitch RNA (*merRNA*) and flagella filament (*fliC1*) were utilised. To monitor prey invasion and bdelloplast formation (early GP), a primer set targeting a transcript of the gene which encodes a peptidoglycan-modifying enzyme (*bd0816*) was utilised, while the GP was monitored using a primer set specific to a gene encoding for a chaperone protein [*groES1* (*bd0097*)]. In addition, the expression of these genes was compared and normalised to the expression of three housekeeping/reference genes which were identified from literature to be expressed constitutively throughout the life cycle of *B. bacteriovorus* and included the *16S rRNA*, *lon* (*bd3749*; ATP-dependent protease) and *bd3244* (endopeptidase) genes (Lerner et al., 2012; Karunker et al., 2013; Rotem et al., 2015). The qPCR cycling parameters for all genes are summarised in **Table 4.1**, while the qPCR mastermix as outlined by Waso et al. (2018) was utilised.

Table 4.1 Conventional PCR and qPCR primers and cycling parameters utilised to monitor the gene expression of *B. bacteriovorus* in co-culture with *E. coli*, *K. pneumoniae* and *E. faecium*

Gene	Primer	Primer Sequence (5' – 3')	qPCR Cycling Parameters	Conventional PCR Cycling Parameters	Gene product size in bp	Reference
16S rRNA	Bd347F	GGAGGCAGCAGTAGGGAATA	2 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 50 cycles of 15 s at 95°C and 60 s at 60°C; final elongation of 10 min at 72°C	202	Van Essche et al., 2009
	Bd549R	GCTAGGATCCCTCGTCTTACC				
lon	F_sq_lon	CTTTGCAATCGGAACAGT	2 min at 95°C; 50 cycles of 60 s at 95°C, 30 s at 51°C and 30 s at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 50 cycles of 60 s at 95°C, 30 s at 51°C and 30 s at 72°C; final elongation of 5 min at 72°C	172	Rotem et al., 2015
	R_sq_lon	AACGATGTTGGTAGGATCTT				
Bd3244	BD3244RT-F	CTTTAAGAACATCACGCAGC	2 min at 95°C; 50 cycles of 60 s at 94°C, 60 s at 48°C and 1 min at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	15 min at 95°C; 30 cycles of 60 s at 94°C, 60 s at 48°C and 2 min at 72°C; final elongation of 10 min at 72°C	87	Lerner et al., 2012
	BD3244RT-R	GTACCGGTGAGTTGAATTCC				
Bd0108	F_sq_bd0108	GCTTCTCCTTTGCGGGAACAG	2 min at 94°C; 50 cycles of 60 s at 94°C, 30 s at 48°C and 1 min at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 94°C; 25 cycles of 60 s at 94°C, 30 s at 48°C and 1 min at 72°C; final elongation of 10 min at 72°C	126	Rotem et al., 2015
	R_sq_bd0108	AGTCGCCCAAAGCCGATGTC				
merRNA	F_sq_merRNA	GGAGGTTCCTAGGGTTATA	2 min at 94°C; 50 cycles of 60 s at 94°C, 60 s at 44°C and 2 min at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 25 cycles of 60 s at 94°C, 60 s at 44°C and 2 min at 72°C; final elongation of 10 min at 72°C	147	Rotem et al., 2015
	R_sq_merRNA	TTCCTCAGGTGACTCATGA				

Table 4.1 (Continued) Conventional PCR and qPCR primers and cycling parameters utilised to monitor the gene expression of *B. bacteriovorus* in co-culture with *E. coli*, *K. pneumoniae* and *E. faecium*

Gene	Primer	Primer Sequence (5' – 3')	qPCR Cycling Parameters	Conventional PCR Cycling Parameters	Gene product size in bp	Reference
FliC1	F_sq_fliC1	CAACAAACACCGC ATCTAT	2 min at 95°C; 50 cycles of 60 s at 94°C, 60 s at 42°C and 2 min at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 25 cycles of 60 s at 94°C, 60 s at 42°C and 2 min at 72°C; final elongation of 10 min at 72°C	152	Rotem et al., 2015
	R_sq_fliC1	TCCTTTGAAGTTCT CAGAAAT				
Bd0816	BD0816RT-F	TCTGCACCTGACT CCAACAG	2 min at 95°C; 50 cycles of 60 s at 94°C, 60 s at 48°C and 2 min at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	15 min at 95°C; 30 cycles of 60 s at 94°C, 60 s at 48°C and 2 min at 72°C; final elongation of 10 min at 72°C	120	Lerner et al., 2012
	BD0816RT-R	ACCTTCATGCGGT TCAGTTC				
GroES1	F_sq_groES1	GCCCACTTCATGA CAGAA	2 min at 95°C; 50 cycles of 60 s at 94°C, 60 s at 42°C and 2 min at 72°C; high resolution melting of 60 s at 95°C, 60 s at 40°C, 1 s at 65°C and 1 s at 97°C	2 min at 95°C; 50 cycles of 60 s at 94°C, 60 s at 42°C and 2 min at 72°C; final elongation of 10 min at 72°C	183	Rotem et al., 2015
	R_sq_groES1	CTTTATCGCCAACT TTAACT				

To monitor the efficiency (*E*) of the various primer sets, a standard curve was included for each qPCR assay. Each standard curve was generated using conventional PCR products obtained using the primer sets and cycling parameters as outlined in **Table 4.1** and genomic DNA of *B. bacteriovorus* PF13 [extracted using the *Quick*-DNA™ Fecal/Soil Microbe Miniprep (Zymo Research, Inqaba Biotech) as per manufacturer's instructions]. The conventional PCR products were cleaned, concentrated and serially diluted as outlined by Waso et al. (2018). The conventional PCR mixture for the *Ion* gene consisted of a final concentration of 1X Green GoTaq® Reaction Buffer (Promega Corp, Madison, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 1.25 U GoTaq® Flexi DNA Polymerase (Promega Corp, Madison, USA) and 4 µL of DNA in a final volume of 25 µL. For the *16S rRNA* gene, the same PCR mixture was utilised with 0.9 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase and 5 µL of DNA. For *bd3244* and *bd0816*, the same PCR mixture was utilised with 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.6 µM of each primer and 2 µL of DNA. For *merRNA*, *fliC1* and *groES1*, the same PCR mixture was utilised with 2 mM $MgCl_2$ and 2.5 µL of DNA. For *bd0108*, the same PCR mixture was utilised with 2 mM $MgCl_2$, 0.4 mM of each dNTP, 0.6 µM of each primer and 4 µL of DNA. Additionally, a no-reverse transcription control and a no template control (negative control) was included for each qPCR assay.

4.2.6 Data Analysis

The efficiency of each qPCR assay and the threshold cycle (Cq) of each sample was determined using the Roche LightCycler® 96 Software Version 1.1. All subsequent analyses were conducted in Microsoft Excel 2016. To calculate the relative gene expression of the target genes (*bd0108*, *merRNA*, *fliC1*, *bd0816* and *groES1*) to the housekeeping genes (*16S rRNA*, *bd3244* and *Ion*), the ΔΔCq method with efficiency correction (also known as the Pfaffl method) as outlined by equation (Eq. 1) was utilised (Pfaffl, 2004):

$$\text{relative gene expression ratio (R)} = \frac{(E_{\text{target}})^{\,\Delta \text{Cq target}}}{(E_{\text{reference index}})^{\,\Delta \text{Cq reference index}}} \qquad (1)$$

To determine if the gene expression changed significantly in co-culture with the various prey cells over time, t-tests were conducted using Microsoft Excel 2016.

4.3 Results

4.3.1 Relative Gene Expression of the Attack Phase Genes

In co-culture with all three test strains, the *bd0108* gene was expressed at a high level [3.99 (*E. coli*), 3.91 (*K. pneumoniae*) and 2.91-fold (*E. faecium*)] in the AP *B. bacteriovorus* cells at 0 min (**Fig. 4.1**).

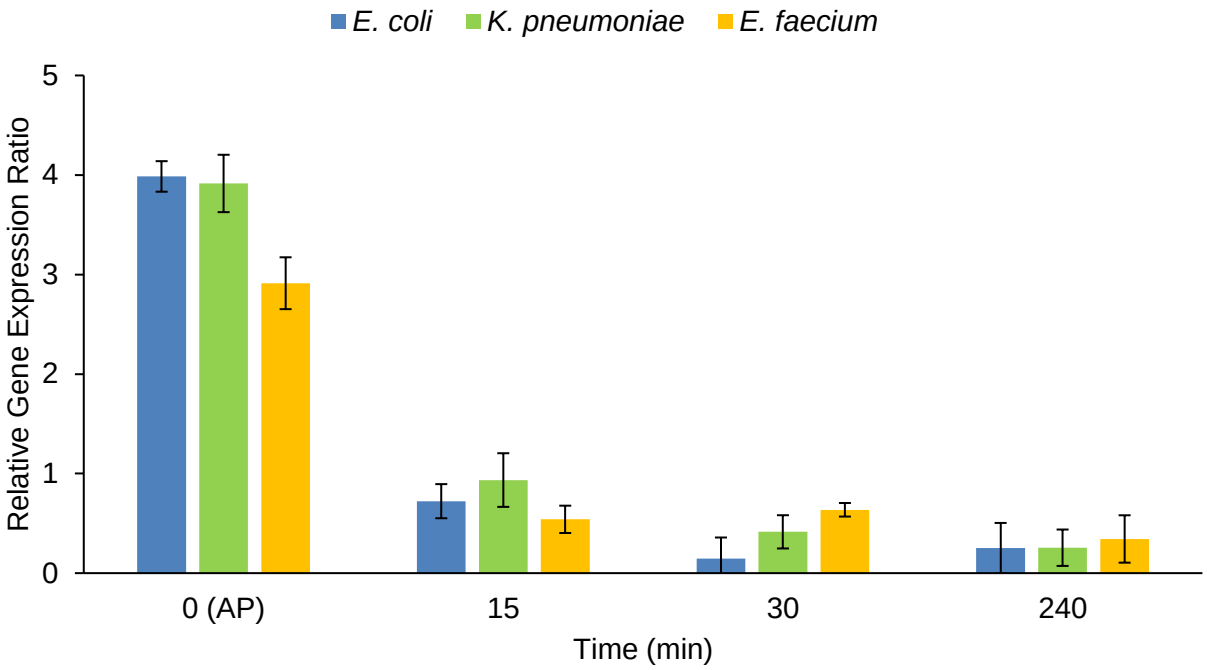

Fig. 4.1 The relative gene expression of *bd0108* in the presence of *E. coli*, *K. pneumoniae* and *E. faecium* as prey

In contrast, after 15, 30 and 240 min of co-culture with *E. coli*, the expression of *bd0108* was significantly reduced to 0.720 ($p < 0.0001$), 0.150 ($p < 0.0001$) and 0.250-fold ($p < 0.0001$), respectively (**Fig. 4.1**). Similarly, in co-culture with *K. pneumoniae*, the *bd0108* gene was significantly reduced to 0.935 ($p = 0.0056$), 0.415 ($p = 0.011$) and 0.255-fold ($p = 0.0066$) after 15, 30 and 240 min, respectively (**Fig. 4.1**), with the expression of *bd0108* reduced to 0.541 ($p = 0.0033$), 0.637 ($p = 0.011$) and 0.343-fold ($p = 0.0039$) in co-culture with *E. faecium* after 15, 30 and 240 min, respectively (**Fig. 4.1**).

For the *merRNA* gene, expression was high in the AP cells at 0 min for *B. bacteriovorus* in co-culture with *E. coli* (6.06-fold), *K. pneumoniae* (7.05-fold) and *E. faecium* (7.30-fold) (**Fig. 4.2**). At 15, 30 and 240 min after exposure to the *E. coli* prey, *merRNA* expression was significantly reduced to 1.31 ($p = 0.0283$), 1.47 ($p = 0.00692$) and 0.165-fold ($p = 0.0189$), respectively (**Fig. 4.2**). Similarly, after 15, 30 and 240 min of exposure to the *K. pneumoniae* prey, *merRNA* expression was reduced to 2.52 ($p = 0.0312$), 3.39 ($p = 0.0431$) and 0.141-fold ($p = 0.0220$), respectively (**Fig. 4.2**), with *merRNA* expression reduced to 2.21 ($p = 0.0203$), 3.40 ($p = 0.0234$) and 0.136-fold ($p = 0.0241$) after 15, 30 and 240 min, respectively, of exposure to the *E. faecium* prey (**Fig. 4.2**).

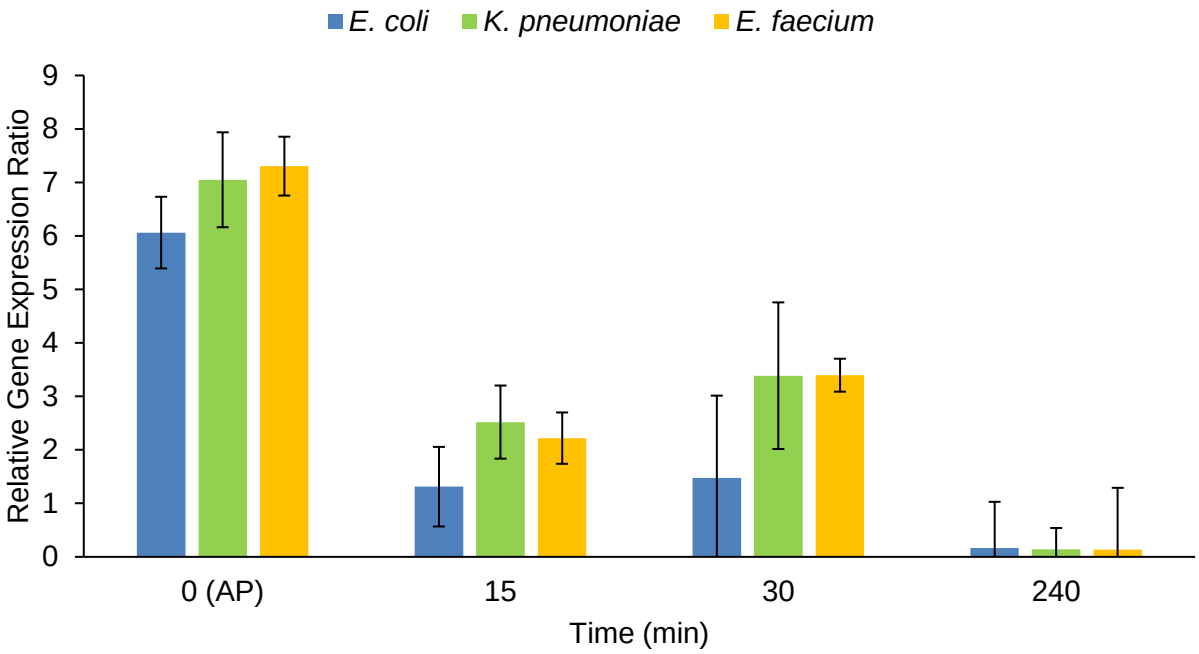

Fig. 4.2 The relative gene expression of *merRNA* in the presence of *E. coli*, *K. pneumoniae* and *E. faecium* as prey

For the *B. bacteriovorus* cells in co-culture with all the test strains, *fliC1* was expressed at 3.57 (*E. coli*), 5.66 (*K. pneumoniae*) and 3.74-fold (*E. faecium*) in the AP cells (at 0 min) (**Fig. 4.3**). At 15 and 30 min after exposure to the *E. coli* prey, *fliC1* expression was reduced to 1.49 ($p = 0.0143$) and 0.278-fold ($p = 0.0291$), respectively (**Fig. 4.3**).

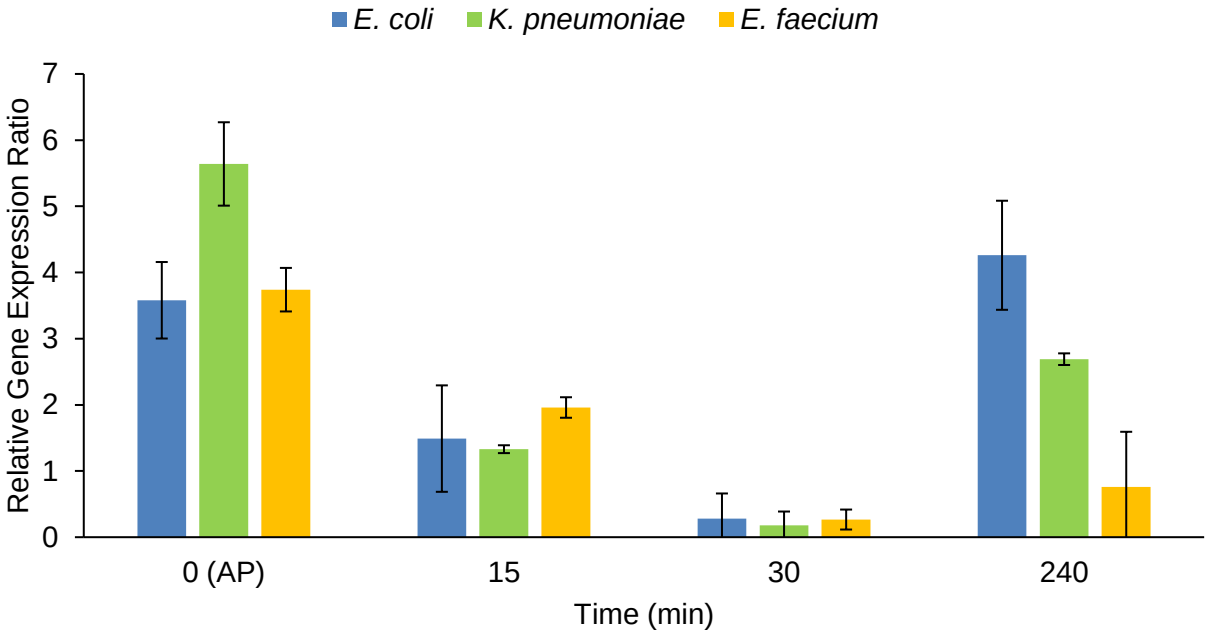

Fig. 4.3 The relative gene expression of *fliC* in the presence of *E. coli* (A), *K. pneumoniae* (B) and *E. faecium* (C) as prey

After 240 min of co-culture, the expression of *fliC1* increased to 4.62-fold ($p < 0.0001$) (**Fig. 4.3**). A similar trend was observed when *B. bacteriovorus* was co-cultured with *K. pneumoniae*, where *fliC1* expression was reduced to 1.33 ($p = 0.0178$) and 0.176-fold ($p = 0.0134$), after 15 and 30 min, respectively and after 240 min of co-culture, the expression of *fliC1* increased to 2.69-fold ($p < 0.0001$) (**Fig. 4.3**). In co-culture with *E. faecium*, at 15 and 30 min after exposure to the *E. faecium* prey, *fliC1* expression was reduced to 1.96 ($p = 0.943$) and 0.267-fold ($p = 0.00885$), respectively (**Fig. 4.3**). However, in contrast to the trend observed for the Gram-negative prey, after 240 min of co-culture with *E. faecium*, the expression of *fliC1* remained low and was expressed at 0.759-fold (**Fig. 4.3**).

4.3.2 Relative Gene Expression of the Growth Phase Genes

In co-culture with *E. coli*, the *bd0816* gene was expressed at 0.917-fold in the AP *B. bacteriovorus* cells (**Fig. 4.4**). After 15 and 30 min, the expression of *bd0816* increased to 4.22 ($p = 0.039$) and 10.7-fold ($p = 0.033$), respectively (Fig. 4A). However, after 240 min of co-culture with *E. coli*, the expression of *bd0816* decreased to 1.09-fold (Fig. 4A). Similarly, in co-culture with *K. pneumoniae*, the *bd0816* gene was expressed at 1.78-fold in the AP cells (**Fig. 4.4**), whereafter the expression of *bd0816* increased to 5.24 ($p = 0.030$) and 13.1-fold ($p = 0.007$) after 15 and 30 min, respectively (**Fig. 4.4**). After 240 min of co-culture with *K. pneumoniae*, the expression of *bd0816* was however, reduced to 0.560-fold (**Fig. 4.4**).

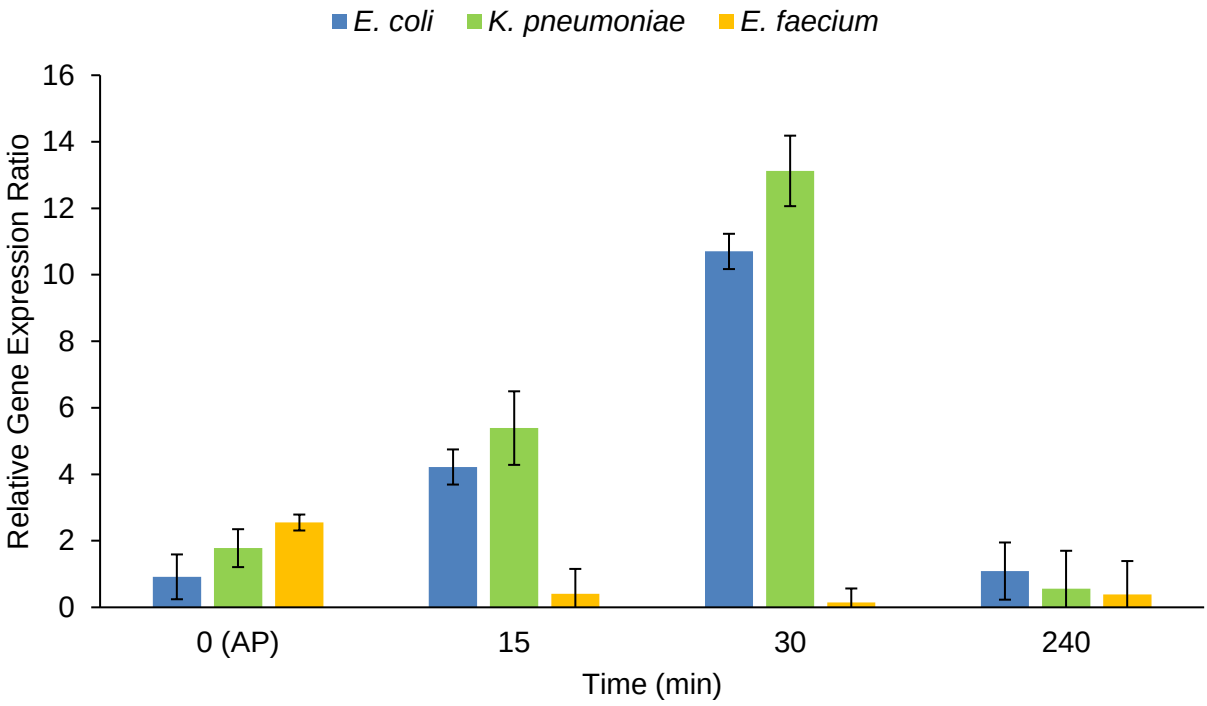

Fig. 4.4 The relative gene expression of *bd0816* in the presence of *E. coli*, *K. pneumoniae* and *E. faecium* as prey

In contrast, in co-culture with *E. faecium*, the *bd0816* gene was expressed at 2.55-fold in the AP cells (**Fig. 4.4**), whereafter the expression was reduced 0.405- (p = 0.241), 0.142- (p = 0.072) and 0.392-fold (p = 0.068) after 15, 30 and 240 min, respectively (**Fig. 4.4**).

For the *B. bacteriovorus* cells in co-culture with *E. coli*, *groES1* was expressed at 0.561-fold in the AP cells (**Fig. 4.5**). After exposure to the *E. coli* prey, the expression of *groES1* increased to 4.26 (p < 0.0001), 5.50 (p = 0.0013) and 12.0-fold (p < 0.0001) at 15, 30 and 240 min, respectively (**Fig. 4.5**). Similarly, in co-culture with *K. pneumoniae*, *groES1* was expressed at 0.461-fold in the AP cells (**Fig. 4.5**), whereafter the expression of *groES1* increased to 1.67 (p = 0.033), 7.24 (p < 0.0001) and 20.1-fold (p = 0.013) at 15, 30 and 240 min, respectively (**Fig. 4.5B**).

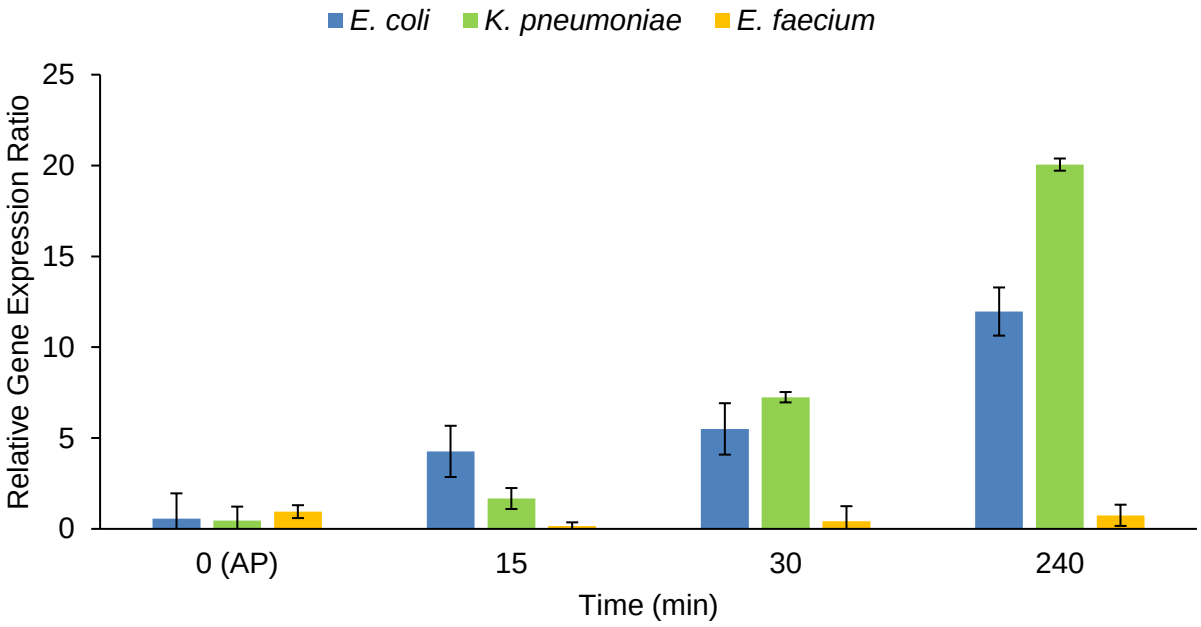

Fig. 4.5 The relative gene expression of *groES1* in the presence of *E. coli*, *K. pneumoniae* and *E. faecium* as prey

In co-culture with *E. faecium*, *groES1* was expressed at 0.949-fold in the AP cells (**Fig. 4.5**). In contrast to the results obtained for the Gram-negative prey, the expression of *groES1* subsequently decreased to 0.149 (p = 0.020), 0.425 (p = 0.001) and 0.747-fold (p = 0.0573) after 15, 30 and 240 min, respectively (**Fig. 4.5**).

4.4 Discussion

Research has indicated that in the presence of Gram-negative prey, during the AP of *B. bacteriovorus*, the *bd0108* gene is upregulated as the Bd0108 protein controls the extrusion/retraction of the predator's type IVa pili (Capeness et al., 2013; Rotem et al., 2015). Once a prey cell is encountered and sensed via the type IVa pili, *bd0108* expression is downregulated and the first predatory cue is transduced (Rotem et al., 2015). In the current study,

a similar trend was observed for the co-culture of *B. bacteriovorus* PF13 with *E. coli*, *K. pneumoniae* and *E. faecium*, where *bd0108* was found to be highly expressed in the AP cells, whereafter the expression was reduced after 15, 30 and 240 min of co-culture with the respective prey. Thus, even in the presence of Gram-positive prey cells, *B. bacteriovorus* senses that it has encountered a cell, which could potentially be used as a nutrient source and for replication (Karunker et al., 2013; Rotem et al., 2015; Avidan et al., 2017).

In bacteria, cyclic di-GMP's then act as secondary messengers and in many proteobacteria, they control the transition from motile cells to biofilm associated cells or they control the transition of free-living cells to invading pathogenic cells (Tamayo et al., 2007; Hengge, 2009; Karunker et al., 2013). Karunker et al. (2013) proposed that *merRNA* (putative cyclic di-GMP riboswitch) is essential in controlling the switch of AP *B. bacteriovorus* cells to GP *B. bacteriovorus* cells. In the current study, *merRNA* was found to be highly expressed in the AP cells of *B. bacteriovorus* PF13 exposed to *E. coli*, *K. pneumoniae* and *E. faecium*. In the presence of all three prey cultures, the expression of *merRNA* was subsequently reduced from 15 to 240 min. These results correspond to the results reported by Karunker et al. (2013) where *merRNA* was upregulated in AP *B. bacteriovorus* cells and expression was reduced at 30, 60 and 180 min after exposure to *E. coli* prey. Moreover, in the current study, *merRNA* expression followed the same trend in the presence of Gram-negative and Gram-positive prey, indicating that the *B. bacteriovorus* cells are prompted to switch from AP to GP cells in the presence of both types of prey cells.

Research has also indicated that the flagella of *B. bacteriovorus* plays a pivotal role in prey cell invasion by providing the predator with the necessary force to enter the periplasmic space of a prey cell (Lambert et al., 2006). In the current study, *fliC1* (flagella filament gene) was found to be expressed at a high level in the AP cells for *B. bacteriovorus* PF13 in co-culture with *E. coli*, *K. pneumoniae* and *E. faecium*. In the presence of all three prey cultures, the expression of *fliC1* was subsequently reduced at 15 min and 30 min. At 240 min, however, the expression of *fliC1* increased significantly in the presence of the Gram-negative prey (in comparison to the 30 min expression), while the expression remained low in the presence of the Gram-positive *E. faecium*. The results obtained in the current study for the Gram-negative co-cultures corresponds to the results reported by Lambert et al. (2006) where *fliC* (*fliC1 – fliC5*) expression was high in the AP *B. bacteriovorus* cells, while 30 min and 1 h after co-culture with *E. coli*, the expression of *fliC* was reduced. It is hypothesised that after *B. bacteriovorus* attaches to a prey cell and enters the bdelloplast (15 to 30 min after exposure to a prey cell), the predator sheds its flagella, is no longer motile and thus *fliC* expression is reduced or completely silenced (Lambert et al., 2006). Lambert et al. (2006) also showed that after 4 h of co-culture with *E. coli* the expression of *fliC* returned to a high level of expression, as progeny cells are released from the bdelloplast and once again enter the AP. However, in the current study, after 4 h in the presence of *E. faecium*, *fliC1* expression did not increase significantly, which indicates that progeny *B. bacteriovorus* cells may

not have been produced in the presence of the Gram-positive prey. This corresponds to results reported by Waso et al. (2019) where the concentration of *B. bacteriovorus* PF13 did not increase significantly in the presence of *E. faecium* in HEPES buffer after 120 h of co-culture, although the predator remained viable based on the ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) analysis. *Bdellovibrio bacteriovorus* may thus acquire nutrients from the Gram-positive bacterium in order to survive, however, based on the results obtained, it does not actively replicate in the presence of the *E. faecium* cells (Im et al., 2018; Waso et al., 2019).

Subsequently, upon prey cell invasion (15 to 30 min after exposure to the prey), *B. bacteriovorus* has been shown to produce peptidoglycan-modifying enzymes, which aids in invading the prey cell and alters the prey cell wall to form the bdelloplast (Lerner et al., 2012). Specifically, *bd0816*, a *dacB*-like gene, encodes for a penicillin-binding-protein class 4 (PBP4) DD-endopeptidase. This enzyme is utilised to change the prey cell shape (from rod-shaped to spherical) in order to produce the bdelloplast and alter the prey cell wall, to prevent infection by a second predatory bacterium (Lerner et al., 2012). In the current study, in comparison to the AP cells (0 min), *bd0816* was expressed at significantly higher levels at 15 and 30 min, after *B. bacteriovorus* was co-cultured with *E. coli* and *K. pneumoniae*, respectively. After 240 min, the predator enters the active GP and as such the expression of *bd0816* is reduced. These results correspond to the results reported by Lerner et al. (2012) indicating that at 15 and 30 min into the predatory life cycle, *bd0816* and another DD-endopeptidase, *bd3459*, are significantly upregulated in the presence of the Gram-negative *E. coli* and after 240 min of co-culture the expression of these genes is significantly reduced. However, in the current study, in the presence of *E. faecium*, *bd0816* expression was reduced after 15, 30 and 240 min relative to the AP. Thus, while the AP genes were expressed in a similar fashion for all the prey cells analysed in the current study (with the exception of the *fliC1* gene expression in the presence of *E. faecium* at 240 min), which indicates that *B. bacteriovorus* receives a signal that a potential prey cell has been encountered, *B. bacteriovorus* is not prompted to proceed with modifying the peptidoglycan layer of *E. faecium* in order to invade the cell and subsequently form the bdelloplast. Based on this observation, it can be postulated that *B. bacteriovorus* does not enter the *E. faecium* cells. This corresponds to observations by Iebba et al. (2014) and Pantanella et al. (2018) where it was shown that *B. bacteriovorus* interacts with Gram-positive *S. aureus* cells in an epibiotic manner and *B. bacteriovorus* could interact with *E. faecium* in a similar manner. These results were corroborated by the reduced expression of the *groES1* gene from 15 to 240 min (relative to the AP) when *B. bacteriovorus* PF13 was co-cultured with *E. faecium* cells. Chaperone proteins such as GroES play an important role in protein folding in bacteria and Rotem et al. (2015) indicated that the *groES1* gene is significantly upregulated during the GP of *B. bacteriovorus* (in the presence of Gram-negative prey). It could thus be speculated that *B. bacteriovorus* does not actively grow in the presence of *E. faecium* in HEPES buffer as it does not receive the second

predatory cue which induces active growth. The second predatory cue is essential for *B. bacteriovorus* to actively grow in the presence of prey cells (Rotem et al., 2014; 2015). In contrast, in the presence of the Gram-negative prey, *groES1* expression was relatively low in the AP cells (at 0 min) and upon exposure to the Gram-negative prey cells *groES1* was induced and increasingly expressed from 15 to 240 min. These results also correspond to the results reported by Lambert et al. (2012), where the *groES1* gene was similarly upregulated as early as 15 min after exposure to *E. coli* cells and the gene was expressed up to 4 h after co-culture with *E. coli*.

4.5 Conclusions

Based on the expression profiles observed for the AP genes, *bd0108* and *merRNA*, in the presence of both Gram-positive and Gram-negative bacteria, the predator *B. bacteriovorus* is able to sense potential prey. However, the genes involved in prey cell invasion (*bd0816*) and predator growth (*groES1*) are not induced upon exposure to the Gram-positive cells. Furthermore, while the expression of *fliC1* was comparable when *B. bacteriovorus* was co-cultured with the Gram-negative and Gram-positive cells from 0 to 30 min, after 240 min expression increased in the presence of the Gram-negative prey (as compared to the expression recorded at 30 min) yet remained low in the presence of the Gram-positive prey. These results indicate that progeny *B. bacteriovorus* cells were not produced when *E. faecium* was used as prey in the co-culture experiments. Correspondingly, it could be concluded that a cue prompting the predator to actively grow in the presence of *E. faecium* is lacking under these conditions. Future studies should investigate the nature of the second predatory cue to elucidate the mechanisms involved in prompting the predator to actively grow within a prey cell. In addition, Illumina next-generation RNA sequencing technologies could be employed to investigate all the changes in the gene expression profile across the genome of *B. bacteriovorus* in co-culture with Gram-positive organisms to further elucidate the genetic mechanisms this predator employs to survive in the presence of these atypical prey.

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Authors' contributions

Conceived and designed the experiments: MW WK. Performed experiments: MW. Analysed the data: MW WK. Contributed reagents/materials/analysis tools: WK SK. Compiled the manuscript: MW WK. Edited the manuscript: MW WK SK WA. All authors approved the final version of the manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

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Chapter 5:

General Conclusions and Recommendations

(UK spelling is employed)

General Conclusions and Recommendations

Although 70.9% of households in South Africa have access to a basic water supply, the Northern Cape, Western Cape and Eastern Cape provinces were recently declared national disaster areas as the country experienced its worst drought in 23 years (Reuters, 2018). This placed severe pressure on municipal water supplies and in the Western Cape, strategies to supplement existing water sources were investigated as a priority by governing authorities. These interventions included implementing stringent water restrictions, extracting groundwater from the Table Mountain and Cape Flats aquifers and implementing pilot-scale desalination plants (GreenCape, 2017; 2018; City of Cape Town, 2018a; 2018b; Ndiritu et al., 2018). Rainwater harvesting was also identified as an alternative water source that could be utilised at the household level to reduce the consumer's use of municipal supplies (City of Cape Town, 2018b). In addition, harvesting rainwater can serve as a stormwater management strategy which may be beneficial in areas lacking stormwater infrastructure (Ward et al., 2012; Steffen et al., 2013). Despite these benefits, many studies have detected chemical and microbial contaminants in harvested rainwater (De Kwaadsteniet et al., 2013; Hamilton et al., 2017). While the chemical parameters generally adhere to drinking water standards (De Kwaadsteniet et al., 2013), opportunistic and pathogenic bacteria such as *Salmonella*, *Klebsiella*, *Pseudomonas* and *Legionella* spp., amongst others, have been detected in this water source (Ahmed et al., 2008; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014; Hamilton et al., 2017; Ahmed et al., 2018).

The use of harvested rainwater for domestic and potable purposes may thus pose a significant human health threat and methods such as filtration, chlorine- and ozone-based disinfection, solar disinfection (SODIS) and solar pasteurization (SOPAS), have been investigated to reduce the level of microbial contamination (Amin & Han, 2009; Moreira Neto et al., 2012; Nawaz et al., 2012; Ha et al., 2013; Lopez, 2014; Dobrowsky et al., 2015a; 2015b; Sánchez et al., 2015; Strauss et al., 2016; 2018). While numerous advantages and disadvantages are associated with the implementation of these treatment strategies, SODIS has been earmarked as an easy and cost-effective treatment which may be used to prevent diarrhoeal outbreaks in developing countries (Byrne et al., 2011; McGuigan et al., 2012). Solar disinfection relies on the combined effect of ultraviolet (UV) radiation and solar mild heat to disinfect contaminated water. Using culture-based methods, various studies have shown that after a 6 to 8 hour natural sunlight exposure period, bacterial counts can be reduced to below the detection limit using SODIS, which indicates that the water complies with drinking water standards (Ubomba-Jaswa et al., 2009; McGuigan et al., 2012; Keogh et al., 2015). However, recent studies have used molecular-based methods [such as ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) and EMA-Illumina next-generation sequencing] and highlighted that certain bacterial spp. (such as *Legionella* and *Pseudomonas*) may persist during SODIS treatment (Strauss et al., 2016; 2018; 2019). It is hypothesised that these bacteria are able to survive the UV irradiation and solar mild heat they

encounter in SODIS systems by employing photolyase enzymes (such as MutS and MutH), recombination repair mechanisms (such as RecA) and the SOS response mechanism. Many of these bacterial species can also form associations with protozoa which offers them a protective niche from environmental stressors (Murga et al., 2001; Fields et al., 2002; Willey et al., 2011; Dobrowsky et al., 2016; Strauss et al., 2016).

It is thus of the utmost importance to investigate treatment strategies that can target these persistent bacteria directly and overcome the resistance mechanisms they employ to evade disinfection. Consequently, various biocontrol methods are being investigated for water purification (Mathieu et al., 2019) and in the current dissertation, the use of *Bdellovibrio bacteriovorus* (*B. bacteriovorus*) as a potential biocontrol agent was investigated. *Bdellovibrio*-and-like-organisms (BALOs) are Gram-negative bacteria that predominantly attack and feed on other Gram-negative organisms (Sockett, 2009; Allen et al., 2014). The BALOs include *B. bacteriovorus*, *Bdellovibrio exovorus* (*B. exovorus*), *Micavibrio aeruginosavorus* (*M. aeruginosavorus*), *Bacteriovorax stolpii* (*B. stolpii*) and *Peredibacter starrii* (*P. starrii*). These organisms have been investigated as biocontrol agents in a variety of fields, including agriculture (Scherff, 1973; McNeely et al., 2017), aquaculture (Chu & Zhu, 2010; Cao et al., 2012; Willis et al., 2016; Kongrueng et al., 2017), water and wastewater treatment (Kim et al., 2013; Feng et al., 2016; 2017; Yu et al., 2017; Özkan et al., 2018), and they have been proposed as living antimicrobial agents in the medical field (Sockett, 2009; Kadouri et al., 2013; Shanks et al., 2013; Allen et al., 2014; Sun et al., 2017; Dharani et al., 2018).

The utilisation of *B. bacteriovorus* as a biocontrol agent in the current study was significant as various Gram-negative opportunistic pathogens, such as *Klebsiella* spp., *Legionella* spp. and *Pseudomonas* spp. amongst others, are frequently detected in rainwater and have been found to persist after treatment (Ahmed et al., 2008; De Kwaadsteniet et al., 2013; Dobrowsky et al., 2014; Hamilton et al., 2017; Clements et al., 2019). It has however, been highlighted that culture-based methods employed to monitor the interactions between *B. bacteriovorus* and different prey cells may limit the discovery of unique predator-prey interactions (Williams & Piñeiro, 2006; Zheng et al., 2008; Rotem et al., 2014). This may account for the fact that interactions with Gram-positive organisms are rarely observed using the traditional culture-based methods employed to study these predatory bacteria. However, using quantitative polymerase chain reaction (qPCR) and electron microscopy it has been found that under certain conditions (such as the absence of Gram-negative prey), *B. bacteriovorus* can adapt and interact with Gram-positive prey in order to survive and replicate (Iebba et al., 2014; Pantanella et al., 2018). It is thus not only important to investigate *B. bacteriovorus* as a biocontrol agent by screening for its predation activity against various Gram-negative prey using the conventional culture-based methods; but it is also important to consider the interaction of *B. bacteriovorus* with ideal or preferred prey (i.e. Gram-negative prey) and non-ideal prey (i.e. Gram-positive prey) under various conditions (such nutrient poor

versus nutrient deficient conditions) using molecular methods (such as EMA-qPCR and gene expression assays) in conjunction with culture-based methods in order to fully elucidate the predator-prey interactions.

The primary aim of **Chapter 2** (published in Microbiological Research, 2019) was thus to isolate *B. bacteriovorus* from wastewater and investigate the interaction of this predator with Gram-negative and Gram-positive prey using culture-based and molecular methods (EMA-qPCR). Secondary to this aim, the predation activity of *B. bacteriovorus* on the different prey cells was assessed and compared in a nutrient poor [diluted nutrient broth (DNB)] and nutrient deficient medium (HEPES buffer). To achieve these aims, wastewater was collected from the Stellenbosch Wastewater Treatment Plant and *B. bacteriovorus* was isolated from the wastewater using culture-based methods. Thereafter, the *B. bacteriovorus* isolate (PF13) was co-cultured with *Pseudomonas fluorescens* (*P. fluorescens*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecium* (*E. faecium*) in DNB and HEPES buffer, respectively. Contradictory results were then obtained when *P. aeruginosa* was utilised as prey, as the culture-based analysis indicated that the concentration of the prey was reduced, while the EMA-qPCR analysis indicated that the concentration of *P. aeruginosa* was only marginally reduced in DNB and the gene copies (GC) remained the same in the HEPES buffer. In contrast, results from the predation assays showed that *P. fluorescens* and *K. pneumoniae* were sensitive to predation in DNB and HEPES buffer, *E. faecium* was sensitive to predation in DNB, while the concentration of *S. aureus* was reduced after co-culture with *B. bacteriovorus* PF13 in HEPES buffer. These findings are significant as these organisms have all been associated with human disease, highlighting the value of employing *B. bacteriovorus* as a biocontrol agent to reduce the concentration of *P. fluorescens*, *K. pneumoniae, E. faecium* and *S. aureus* in environmental waters such as harvested rainwater. Dwidar et al. (2017) noted that nutrients present in a medium utilised to assess predator-prey interactions may influence the predation activity of the predator. This was specifically evident for the Gram-positive prey as in the current study, when nutrients were present in the medium (in DNB) the predation activity on *E. faecium* was enhanced, while when nutrients were absent (HEPES buffer) the predation activity on *S. aureus* was enhanced. It was thus postulated that under certain conditions *B. bacteriovorus* adapts to survive in the presence of Gram-positive prey.

As mentioned earlier, pitfalls have been associated with the accuracy of the methods employed to investigate predator-prey interactions, especially with regards to the culture-based methods (Williams & Piñeiro, 2006). These pitfalls include the fact that not all environmental prey bacteria are amenable to laboratory culture conditions, some BALOs do not readily form plaques on double-layer agar overlays and often plaques are only visible or formed after 7 days (Koval, 2006; Williams & Piñeiro, 2006; Zheng et al., 2008; Rotem et al., 2014). Therefore, alternative methods are required to study predator-prey interactions, which could provide additional information on the

adaptations BALOs employ to survive in the presence of their preferred prey (i.e. Gram-negative organisms) in comparison to non-ideal prey (i.e. Gram-positive organisms). In this regard, the use of molecular-based techniques, specifically viability qPCR (such as EMA-qPCR) may be advantageous. The use of viability dyes such as EMA effectively reduce the amplification of DNA from cells with compromised membranes (presumed non-viable) or extracellular DNA and can therefore be utilised to monitor the GC from predominantly viable cells (Cenciarini-Borde et al., 2009; Seinige et al., 2014). This was corroborated by the results obtained in the current study for *P. aeruginosa*, where the concentration of *P. aeruginosa* was reduced using plate counts, however, using the EMA-qPCR analysis, the concentration of this organism was not significantly reduced in both DNB and HEPES buffer. It can thus be speculated that the interaction between *B. bacteriovorus* and this opportunistic pathogen influenced the culturability of *P. aeruginosa*, but it did not kill *P. aeruginosa*. Future research should thus investigate the detailed interaction between *P. aeruginosa* and *B. bacteriovorus* PF13. In addition, *P. aeruginosa* could be utilised as prey to isolate new *B. bacteriovorus* strains from environmental sources such as soil, river water or wastewater, in order to obtain predatory strains that are more effective in reducing *P. aeruginosa* in co-culture. In the current study, the use of EMA-qPCR was also particularly useful during the predation trials using *P. aeruginosa*, *S. aureus* and *E. faecium* (HEPES buffer) as prey, as *B. bacteriovorus* PF13 did not produce plaques on the double-layer agar overlays. The EMA-qPCR technique could thus be used to quantify and assess the potential viability of the predator in these co-cultures and can aid in accurately monitoring and quantifying both predator and prey cells during co-culture experiments in a time-effective manner as results can be generated within 24 hours (Cenciarini-Borde et al., 2009; Seinige et al., 2014). In addition, these interactions should be assessed in different media or in the natural matrix where this predator is to be applied (such as a specific water source). This will aid in fully elucidating and understanding the effect the predator will have as a biocontrol agent.

The aim of **Chapter 3** (published in Water Research, 2020) was then to apply *B. bacteriovorus* PF13 as a pre-treatment to SODIS and solar photocatalytic disinfection. This chapter was conducted in collaboration with the Nanotechnology and Integrated Bioengineering Centre (NIBEC) at Ulster University (Northern Ireland, United Kingdom) where the PhD candidate was based for 10 weeks during the course of the study. Four small-scale SODIS systems with compound parabolic collectors (CPCs) were designed by the collaborators at NIBEC and the systems were constructed from locally sourced materials in South Africa. In addition, titanium dioxide-reduced graphene oxide (TiO_2-rGO) was synthesised and immobilised onto glass raschig rings by the PhD candidate at NIBEC. To test the efficiency of the various combination treatments of SODIS, solar photocatalytic disinfection and predatory bacteria pre-treatment, synthetic rainwater was seeded with *K. pneumoniae* [S1 43 – environmental isolate from pasteurised rainwater (Clements et al., 2019)] and *E. faecium* [8D - environmental isolate from untreated

harvested rainwater (Dobrowsky et al., 2014)] as the test organisms. The concentration of the predator, *K. pneumoniae* and *E. faecium* was subsequently monitored using culture-based methods (spread plating and double-layer agar overlays) and EMA-qPCR. Overall, the use of *B. bacteriovorus* pre-treatment in combination with photocatalysis resulted in the greatest reduction in the *K. pneumoniae* concentrations in the shortest treatment period, as the *K. pneumoniae* cell counts were reduced to below the detection limit within 120 minutes. In contrast, for *E. faecium* the most effective treatment was solar photocatalysis or SODIS without the *B. bacteriovorus* pre-treatment, as the cell counts of *E. faecium* were reduced to below the detection limit in both systems within 210 minutes, while the GC of *E. faecium* were reduced by 3.38 and 3.39 logs in the photocatalytic and SODIS systems after 4 hours, respectively. Based on these results it was evident that the application of *B. bacteriovorus* may specifically enhance the disinfection of Gram-negative bacteria which are sensitive to predation. This was expected as it is well known that *B. bacteriovorus* preys on Gram-negative bacteria (Dashiff et al., 2011). However, in **Chapter 2**, we showed that *B. bacteriovorus* PF13 can reduce the concentration of *E. faecium* and *S. aureus* in co-culture experiments and while the predatory pre-treatment did not enhance the disinfection of *E. faecium* in this study, investigating the effect of predatory bacteria pre-treatment on water contaminated with Gram-positive organisms is thus still warranted. Importantly, the interaction of *B. bacteriovorus* with prey cells may be dependent on the composition of the medium in which the predator is exposed to prey cells and therefore the predation efficiency of *B. bacteriovorus* on prey cells (including Gram-positive prey) should be assessed in different media (such as DNB versus HEPES buffer) or in different water sources (with varying concentrations of nutrients).

The use of the photocatalyst also enhanced the disinfection of *K. pneumoniae* (by reducing the treatment time of 240 minutes obtained for SODIS to 120 minutes for solar photocatalysis), however the same trend was not observed for *E. faecium*. This was theorised to be due to the fact that Gram-positive organisms have more complex cell walls and as such may be more resistant to disinfection. To counteract this phenomenon, it has been highlighted in literature that the use of flow or forced convection which creates turbulence within SODIS systems, may enhance the disinfection of Gram-positive organisms (Gutiérrez-Alfaro et al., 2015). Turbulence exerts mechano-osmotic stress on the bacterial cells, which has been shown to account for up to 99% of bacterial inactivation (Sichel et al., 2007; Gutiérrez-Alfaro et al., 2015). However, research has indicated that when water is recirculated within a SODIS system using piping which protect the bacteria from UV irradiation, the bacteria can recover from UV stress and may thus survive SODIS as a result of dark zones generated in the system (McGuigan et al., 2012; Gutiérrez-Alfaro et al., 2015). Therefore, future studies should investigate how turbulence can be generated within the SODIS vessel, without forming dark zones in the SODIS system, which may promote the survival of persistent bacterial species.

It is also important to consider the water source to be treated using predatory bacteria pre-treatment. In the current study, synthetic rainwater was seeded with a monoculture of prey cells, however, in natural water sources *B. bacteriovorus* will be exposed to a mixture of various Gram-negative and Gram-positive bacteria in varying concentrations. Wilkinson (2001) and Hobley et al. (2006) noted that the predation efficiency of *B. bacteriovorus* may decrease in the presence of decoys (non-ideal prey such as Gram-positive organisms) even if the preferred prey (Gram-negative prey) are present in high concentrations. It would therefore be interesting to investigate the effect *B. bacteriovorus* pre-treatment would have on natural water sources (such as harvested rainwater) prior to SODIS or solar photocatalytic disinfection. Future studies could thus employ Illumina next-generation sequencing technologies to investigate how the community composition in harvested rainwater (or any other environmental water source) changes after the water source has been pre-treated with predatory bacteria and the subsequent effect the pre-treatment may have on SODIS or solar photocatalytic disinfection.

Based on the unique biphasic life cycle of *B. bacteriovorus*, various studies have investigated the genetic mechanisms employed by this predator to invade a prey cell and produce progeny (Lambert et al., 2006; Evans et al., 2007; Lambert et al., 2010; Lerner et al., 2012; Karunker et al., 2013; Rotem et al., 2015; Avidan et al., 2017). However, because it has long been hypothesised that the interaction with prey cells is exclusive to Gram-negative prey, most studies have focused on the interaction between *B. bacteriovorus* and *Escherichia coli* (*E. coli*) (Lambert et al., 2006; Evans et al., 2007; Lambert et al., 2010; Avidan et al., 2017), with limited literature available on the genetic mechanisms involved in the interaction between *B. bacteriovorus* and Gram-positive prey. In the last decade, studies have indicated that *B. bacteriovorus* can in fact prey on and proliferate in the presence of *S. aureus* cells (Iebba et al., 2014; Pantanella et al., 2018). Iebba et al. (2014) and Pantanella et al. (2018) also showed that *B. bacteriovorus* changes its predation strategy from a periplasmic strategy to an epibiotic strategy and alters the cocktail of hydrolytic enzymes to survive and grow in the presence of *S. aureus* cells. In addition, Monnappa et al. (2014) indicated that the hydrolytic enzymes produced by *B. bacteriovorus*, could reduce *S. aureus* biofilms and reduce this pathogen's virulence. Furthermore, we have confirmed that the interaction of *B. bacteriovorus* with Gram-positive prey is not limited to *S. aureus*, but *E. faecium* may also be sensitive to predation (**Chapter 2**). Therefore, there is a need to investigate the interaction of *B. bacteriovorus* with Gram-positive prey on a genetic level, by investigating the expression of predation-specific genes in the presence of Gram-positive prey in comparison to Gram-negative prey. This will allow for the identification of potential adaptation strategies employed by *B. bacteriovorus* to survive in the presence of Gram-positive prey.

The aim of **Chapter 4** (published in Microbiological Research, 2020) was therefore to monitor the expression of attack phase (AP) and growth phase (GP) genes when *B. bacteriovorus* was co-cultured with Gram-positive prey, and compare the expression profile to the profile obtained when

B. bacteriovorus was co-cultured with Gram-negative prey. To achieve this aim, semi-synchronised *B. bacteriovorus* PF13 cultures were established, whereafter *B. bacteriovorus* PF13 was co-cultured with *E. coli*, *K. pneumoniae* and *E. faecium*. Subsequently, relative qPCR was utilised to monitor the expression of *bd0108* (encoding for Bd0108 which controls type IVa pili retraction/extrusion), *merRNA* (encoding for a massively expressed riboswitch RNA) and *fliC1* (encoding for the flagellar filament) which are expressed at high levels specifically during the AP of the *B. bacteriovorus* life cycle. Additionally, the genes *bd0816* (encoding for a peptidoglycan-modifying enzyme) and *groES1* (encoding for a chaperone protein) were utilised to monitor the early GP and GP of the *B. bacteriovorus* life cycle, respectively. The results indicated that the *bd0108* and *merRNA* genes were highly expressed in the AP *B. bacteriovorus* cells exposed to *E. coli*, *K. pneumoniae* and *E. faecium*, whereafter the expression in co-culture with all the prey strains was reduced in the early GP (15 to 30 min) and GP (at 240 min). The *fliC1* gene was also expressed at relatively high levels in the AP cells exposed to *E. coli*, *K. pneumoniae* and *E. faecium* and after 15 and 30 minutes of co-culture with the prey cells, the expression of *fliC1* was reduced. However, after 240 minutes of co-culture with *E. coli* and *K. pneumoniae*, the expression of *fliC1* increased significantly as compared to the expression obtained at 30 minutes, while in the presence of *E. faecium* the expression of *fliC1* remained low. For the *bd0816* gene, the expression was relatively low in the AP *B. bacteriovorus* cells. After 15 and 30 minutes, the expression of *bd0816* increased significantly in the presence of *E. coli* and *K. pneumoniae*, respectively and after 240 minutes, the *bd0816* expression decreased. In contrast, in co-culture with *E. faecium*, the expression of *bd0816* remained low throughout the life cycle of *B. bacteriovorus*. Furthermore, *groES1* expression was relatively low in the AP *B. bacteriovorus* cells. After exposure to the *E. coli* and *K. pneumoniae* prey, the expression of *groES1* gradually increased from 15 to 240 minutes, while in co-culture with *E. faecium*, *groES1* expression was reduced after 15, 30 and 240 minutes, respectively.

Based on the expression of *bd0108* and *merRNA*, in the presence of both Gram-positive and Gram-negative bacteria, the predator is able to sense that it has encountered potential prey. However, the genes involved in prey cell invasion (*bd0816*) and predator growth (*groES1*) are not induced upon exposure to the Gram-positive cells. Furthermore, the expression of *fliC1* was comparable when *B. bacteriovorus* was co-cultured with the Gram-negative and Gram-positive cells from 0 to 30 minutes (AP and early GP). However, after 240 minutes (GP), expression increased in the presence of the Gram-negative prey, but remained low in the presence of the Gram-positive prey. It was thus concluded that under the conditions employed in this study, *B. bacteriovorus* does not invade the *E. faecium* cells and predatory progeny are not produced. It could be hypothesised that the second predatory cue, which induces active growth of *B. bacteriovorus* in the presence of these prey cells (Rotem et al., 2015), is absent and therefore the predator does not replicate in the presence of *E. faecium*. It is also proposed that

B. bacteriovorus requires more time to synthesise the necessary enzymes to break down the cell wall of Gram-positive organisms in order to access the intracellular contents of the prey and therefore growth with *E. faecium* prey was delayed. This was observed by Pantanella et al. (2018) where *B. bacteriovorus* required 20 hours to adapt in order to prey on *S. aureus* in co-culture experiments as compared to 10 hours required to predate Gram-negative prey cells. Using (semi-)synchronised *B. bacteriovorus* cultures, *B. bacteriovorus* requires 4 hours to complete a single life cycle in the presence of Gram-negative prey. Therefore, future studies could investigate the expression of the predatory genes of *B. bacteriovorus* over an extended time period in co-culture with Gram-positive prey, to allow the predator to adapt its predation strategy to use the Gram-positive prey. Alternatively, the *B. bacteriovorus* cultures could be synchronised in the presence of Gram-positive prey (which have been shown to be sensitive to predation), prior to gene expression assays. In addition, Illumina next-generation RNA sequencing technologies could be employed to investigate all the changes in the gene expression profile across the genome of *B. bacteriovorus* in co-culture with Gram-positive organisms to further elucidate the genetic mechanisms this predator employs to survive in the presence of these atypical prey.

In conclusion, limited research on the application of *B. bacteriovorus* as a biocontrol agent in the water industry is available: one study has indicated that *B. bacteriovorus* could be used as a biolysis agent in wastewater treatment (Yu et al., 2017); two studies have indicated that *B. bacteriovorus* could reduce membrane fouling in potable water and wastewater treatment systems (Kim et al., 2013; Özkan et al., 2018) and Feng et al. (2016; 2017) indicated that *B. bacteriovorus* can prey on Gram-negative bacteria found in wastewater and can alter the microbial community composition of floccular and granulated sludge in a wastewater treatment plant. This dissertation thus added valuable knowledge in terms of the potential application of *B. bacteriovorus* in the water treatment industry. It is however, recommended that future studies investigate applying *B. bacteriovorus* as a biocontrol agent on a larger scale by optimising the growth conditions to produce sufficient predatory inocula in order to treat increased volumes of contaminated water. Various environmental sources should also be prospected for new and unique BALOs using both Gram-negative and Gram-positive strains as prey. This may allow for the discovery of BALOs which are able to effectively prey on Gram-negative and Gram-positive bacteria, which may be beneficial in industries such as those aiming to purify water which contains mixed bacterial populations. In addition, *B. bacteriovorus* should be screened against a panel of Gram-positive organisms which are important in the medical, food and water industries such as *Listeria monocytogenes*, *Enterococcus faecalis*, *Corynebacterium* spp., *Streptococcus* spp., *Bacillus anthracis* and *Bacillus cereus* and *Clostridium* spp., to elucidate which Gram-positive organisms are potentially sensitive to predatory bacteria.

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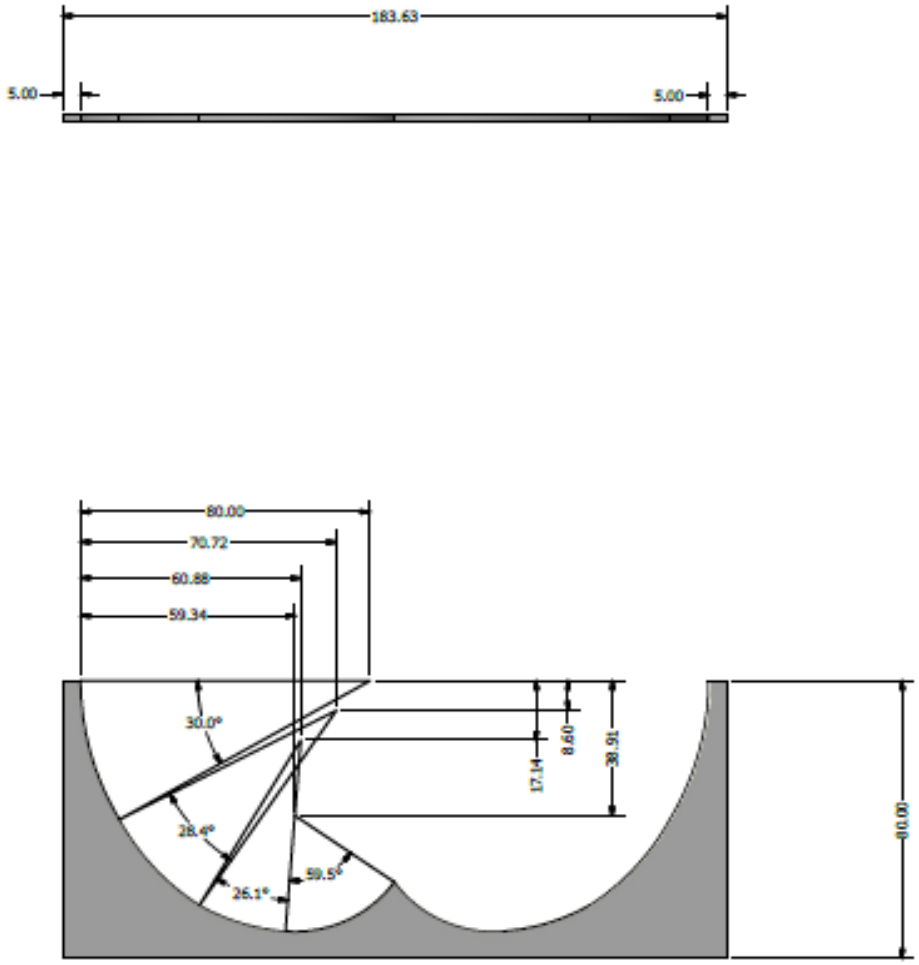
Appendix A

(UK spelling is employed)



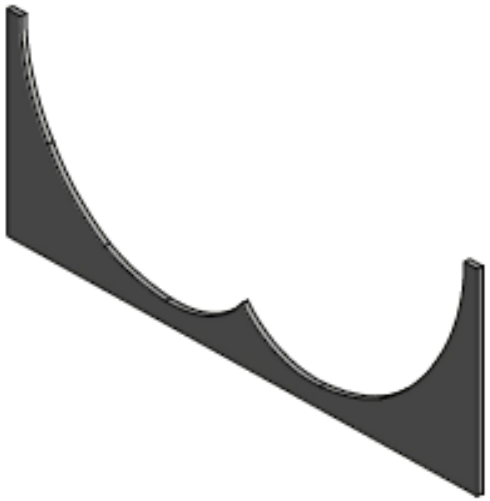
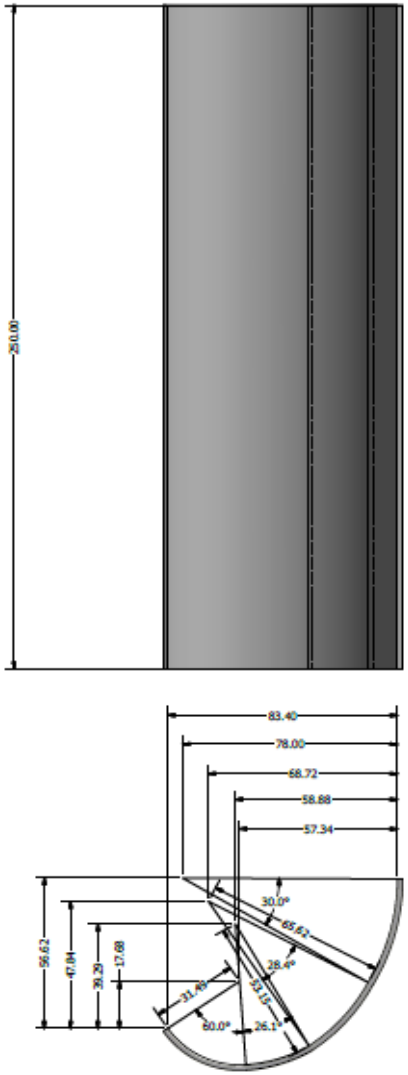


Fig. A.1 Diagram of the arch profile on which the electropolished stainless steel sheets for the CPC mirror was superimposed



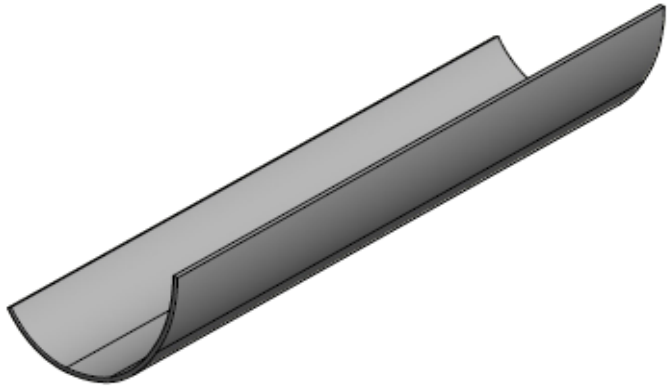


Fig. A.2 Diagram of the bent electropolished stainless steel sheets used for the CPC mirror

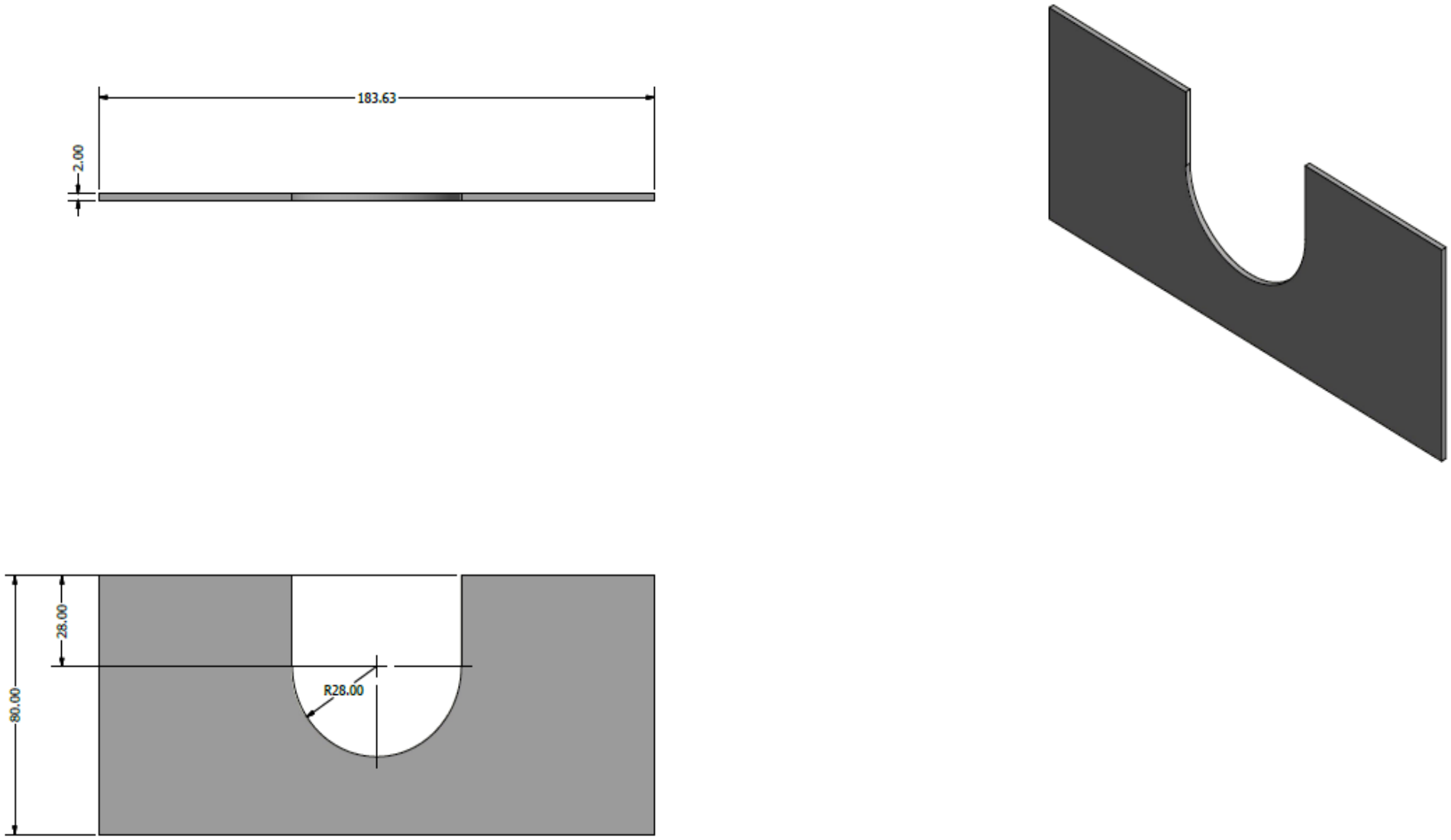


Fig. A.3 Diagram of the end plates used as supports for the borosilicate glass tube reactor of the SODIS-CPC

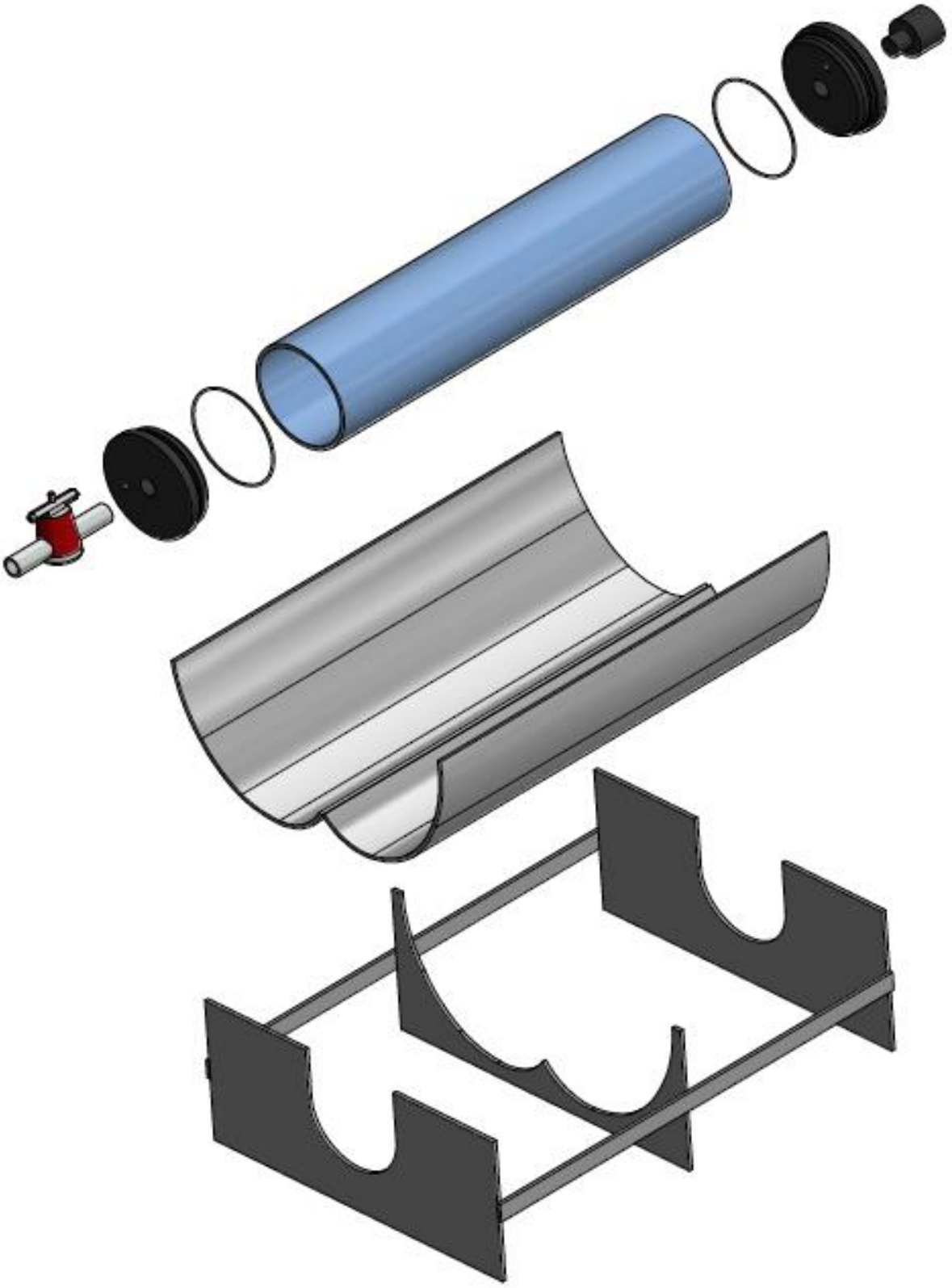


Fig. A.4 Deconstructed assembly of the small-scale solar disinfection systems utilised in this study

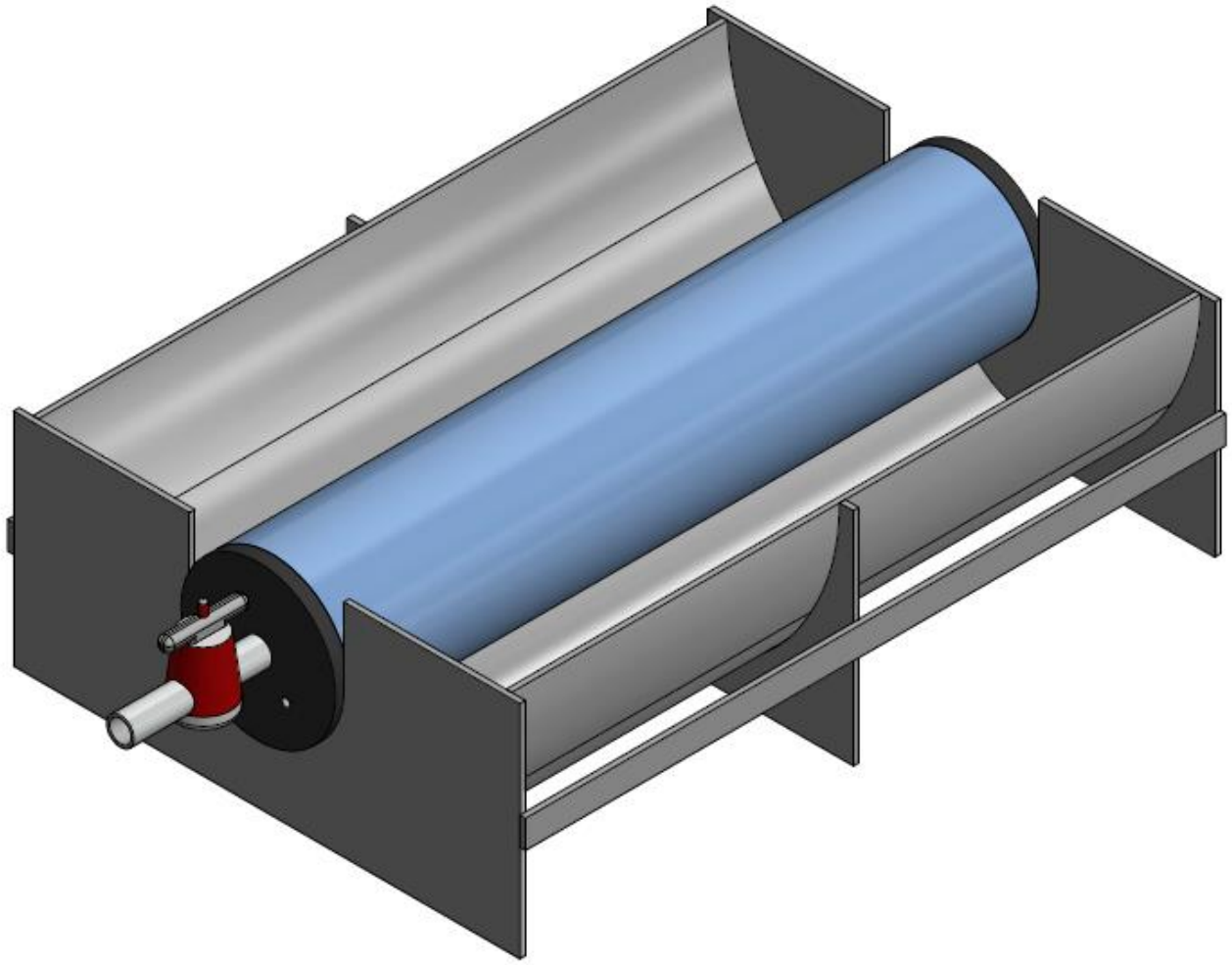


Fig. A.5 Assembly of the constructed small-scale solar disinfection systems utilised in this study

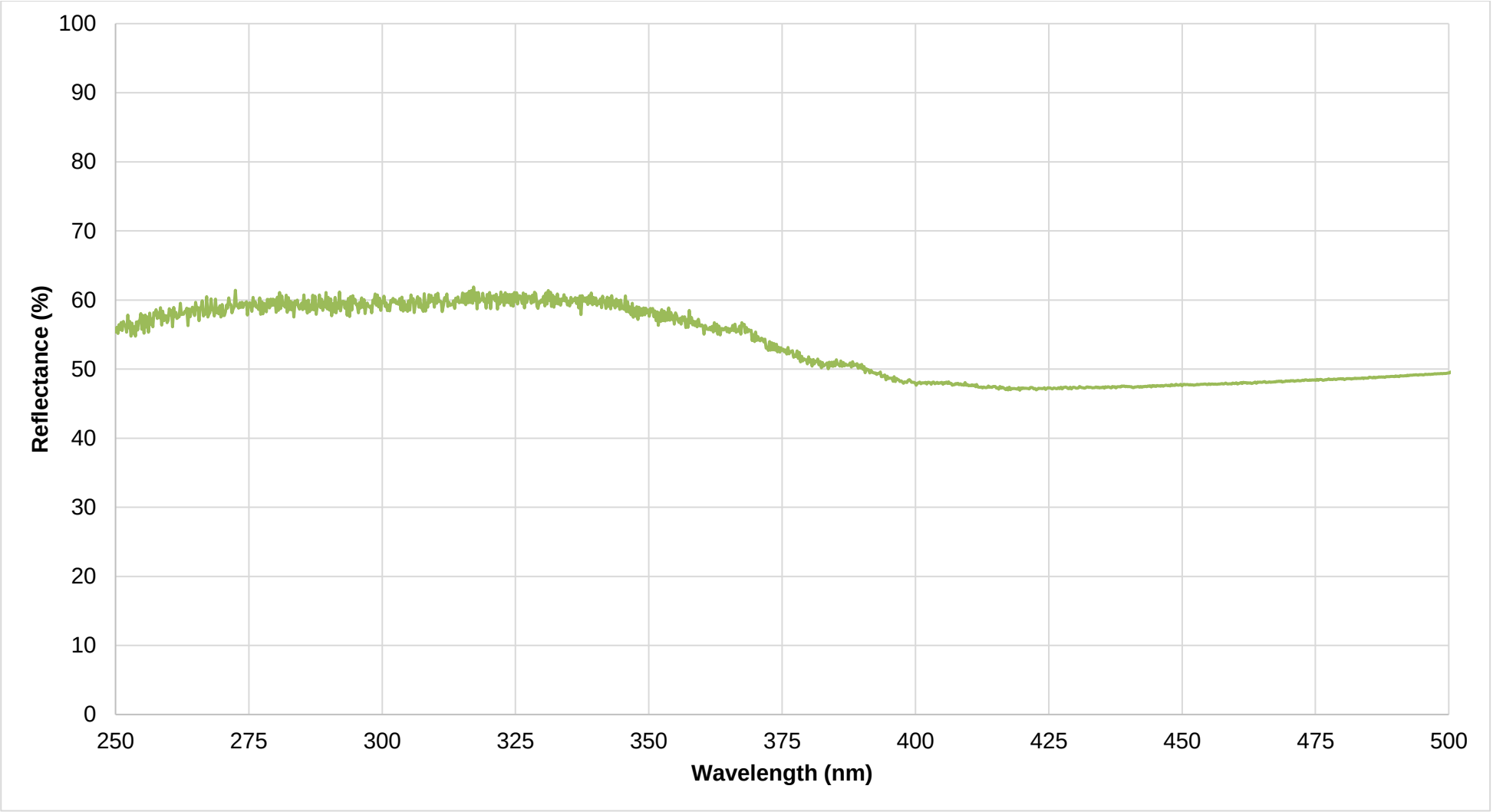


Fig. A.6 Percentage reflectance measured from 250 nm to 500 nm for the electropolished stainless steel used to construct the compound parabolic collected for the solar disinfection system

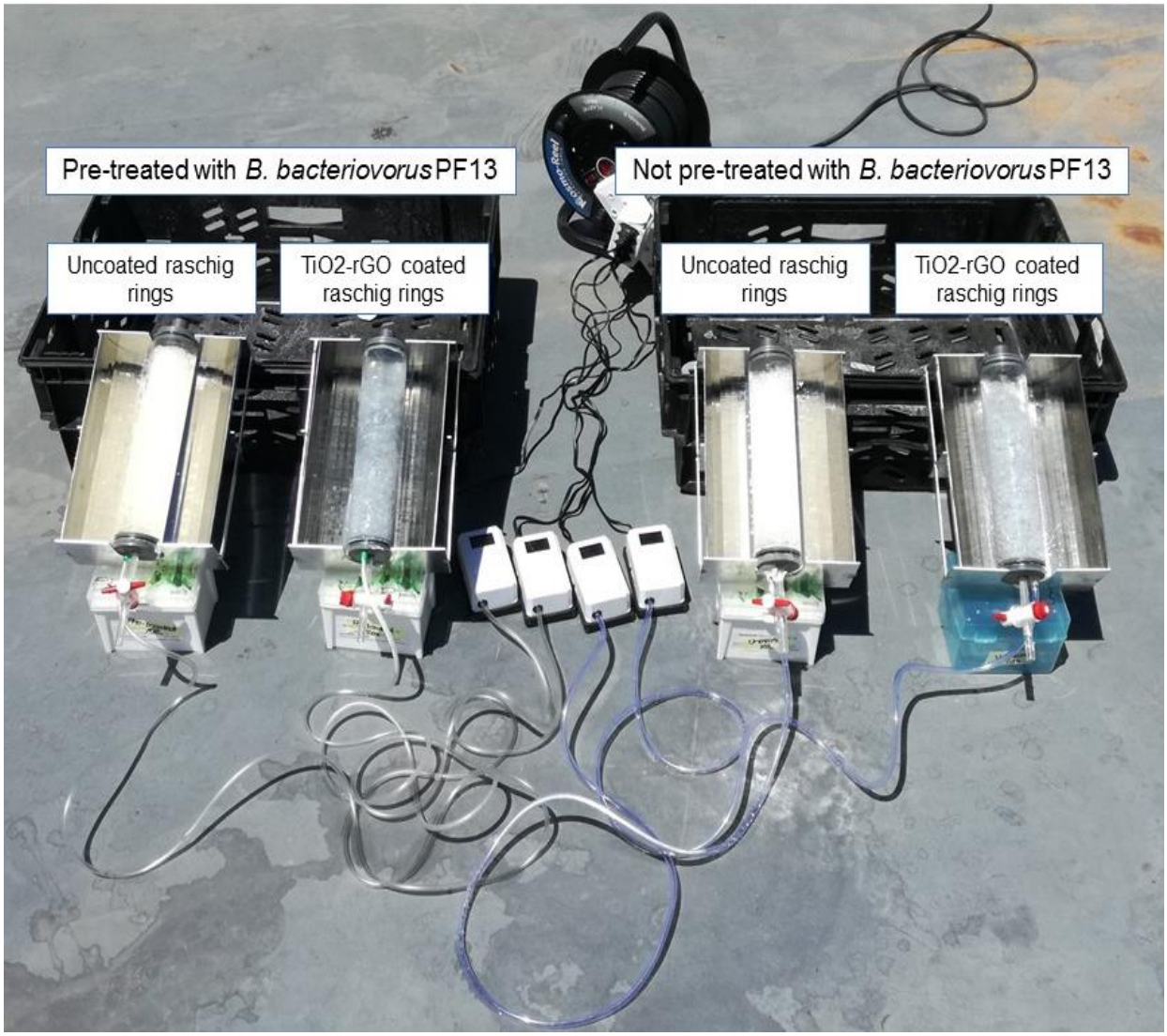


Fig. A.7 The small-scale solar disinfection systems used in the current study were divided into two groups; one group pre-treated with *B. bacteriovorus* for 72 h and one group not receiving any pre-treatment. These groups were then subdivided where one system contained TiO_2-rGO coated raschig rings and one system contained uncoated raschig rings

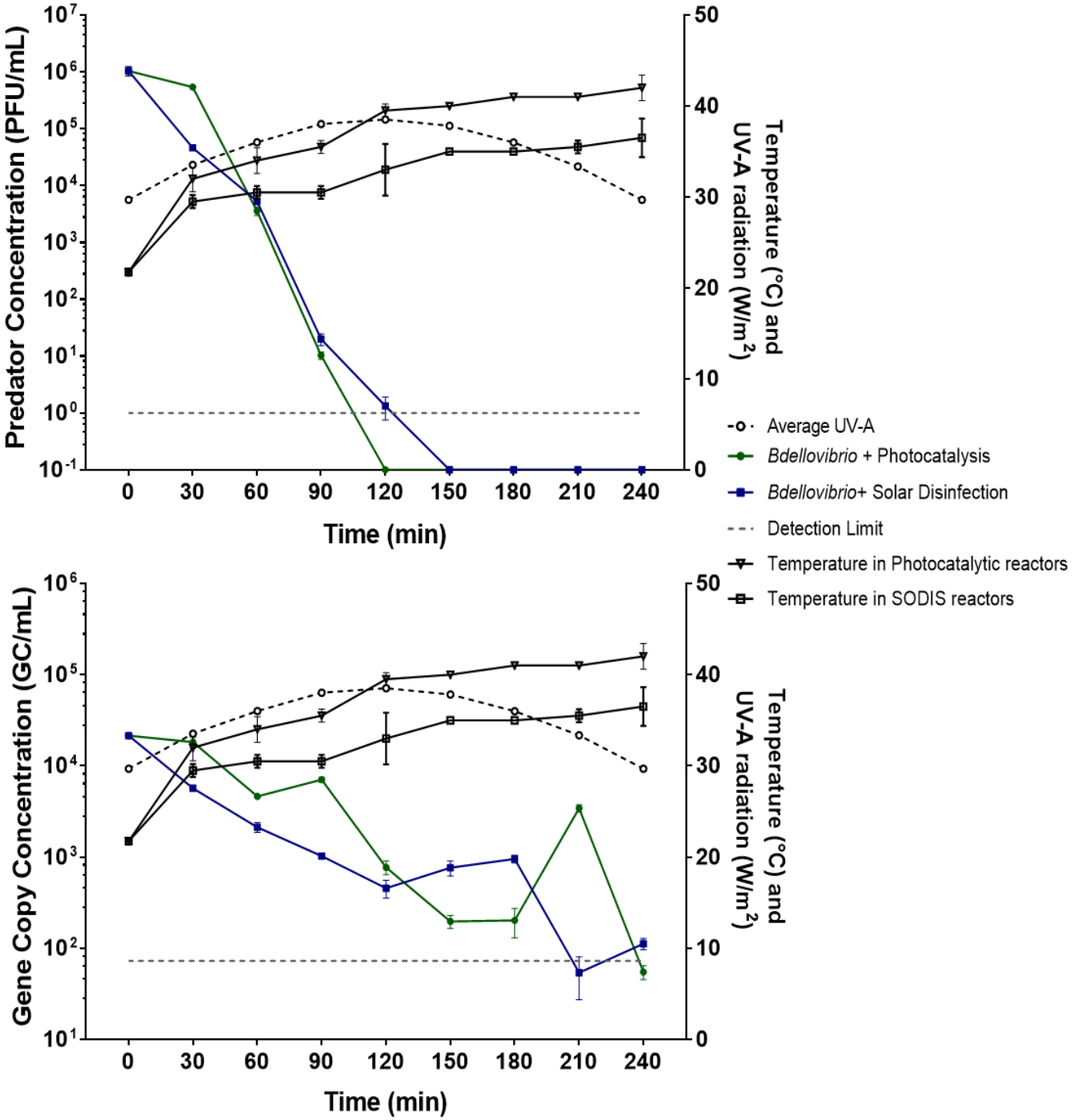


Fig. A.8 The concentration of *B. bacteriovorus* in (A) PFU/mL and (B) GC/mL during the solar disinfection experiments after *K. pneumoniae* was pre-treated with the predatory bacteria for 72 h

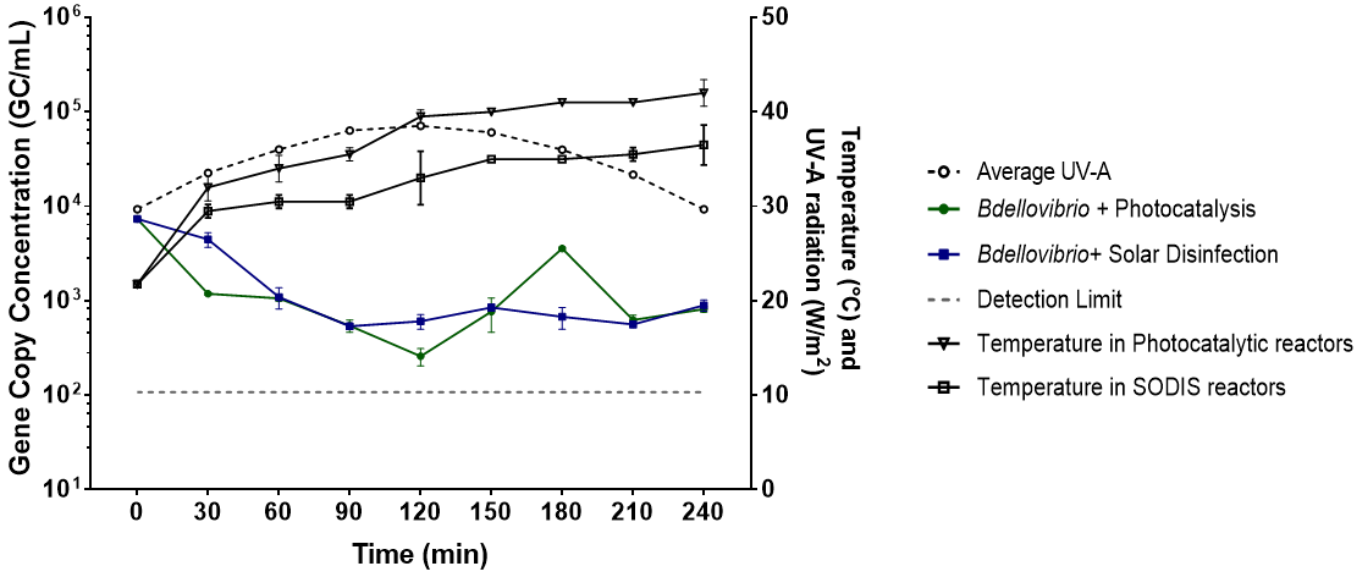


Fig. A.9 The concentration of *B. bacteriovorus* in GC/mL during the solar disinfection experiments after *E. faecium* was pre-treated with the predatory bacteria for 72 h

Table A.1 Summary of the physico-chemical parameters recorded for the *K. pneumoniae* trial during which *B. bacteriovorus* was applied as a pre-treatment for 72 h

Time solar exposure (min)	Dissolved Oxygen (mg/L)	pH	Electrical Conductivity (mS/cm)	Total Dissolved Solids (TDS; ppt)	Temperature of Treated Water (°C)	Ambient Temperature (°C)	Average UV-A (W/m2)	Average UV-B (W/m2)	Direct Normal Irradiance (W/m2)
***Bdellovibrio* + Photocatalysis**									
0	7.1	7.59	12.41	0.63	21.5	29.2	29.688	4.059	902.576
30	7.1	8.10	12.33	0.63	31		33.525	4.337	959.64
60	6.8	7.81	11.98	0.65	33	31.0	36.019	4.496	995.066
90	6.9	7.61	12.13	0.63	36		38.02	4.649	1029.22
120	5.9	7.53	11.89	0.64	40	31.6	38.528	4.69	1036.81
150	6.6	7.77	11.95	0.66	40		37.835	4.654	1030.84
180	6.6	7.98	11.93	0.62	41	31.2	36.006	4.529	1001.8
210	6.5	8.13	11.88	0.62	41		33.357	4.322	959.316
240	6.1	7.91	12.03	0.63	43	31.3	29.6974	4.032	900.104
***Bdellovibrio* + Solar Disinfection**									
0	6.9	7.84	12.44	0.65	21.5	29.2	29.688	4.059	902.576
30	6.9	8.29	12.33	0.66	30		33.525	4.337	959.64
60	6.8	8.18	12.39	0.62	31	31.0	36.019	4.496	995.066
90	6.8	8.12	12.51	0.63	31		38.02	4.649	1029.22
120	6.8	7.96	12.49	0.67	35	31.6	38.528	4.69	1036.81
150	6.7	8.21	12.44	0.71	35		37.835	4.654	1030.84
180	6.3	8.35	12.31	0.71	35	31.2	36.006	4.529	1001.8
210	5.9	7.96	12.31	0.63	36		33.357	4.322	959.316
240	5.9	8.69	12.32	0.59	38	31.3	29.6974	4.032	900.104

Table A.1 (Continued) Summary of the physico-chemical parameters recorded for the *K. pneumoniae* trial during which *B. bacteriovorus* was applied as a pre-treatment for 72 h

Time solar exposure (min)	Dissolved Oxygen (mg/L)	pH	Electrical Conductivity (mS/cm)	Total Dissolved Solids (TDS; ppt)	Temperature of Treated Water (°C)	Ambient Temperature (°C)	Average UV-A (W/m2)	Average UV-B (W/m2)	Direct Normal Irradiance (W/m2)
Photocatalysis									
0	4.3	7.10	13.01	5.16	22	29.2	29.688	4.059	902.576
30	6.9	7.11	12.96	5.96	33		33.525	4.337	959.64
60	6.7	7.30	12.96	5.46	35	31.0	36.019	4.496	995.066
90	6.8	6.90	12.18	5.53	35		38.02	4.649	1029.22
120	6.6	6.80	12.23	5.51	39	31.6	38.528	4.69	1036.81
150	6.6	6.88	11.9	6.13	40		37.835	4.654	1030.84
180	6.9	6.93	11.87	6.11	41	31.2	36.006	4.529	1001.8
210	7.1	6.69	11.56	6.12	41		33.357	4.322	959.316
240	7.2	6.65	12.39	6.21	41	31.3	29.6974	4.032	900.104
Solar Disinfection									
0	4.2	7.11	13.16	5.22	22	29.2	29.688	4.059	902.576
30	4.6	7.12	13.33	5.23	29		33.525	4.337	959.64
60	5.6	7.06	12.96	5.33	30	31.0	36.019	4.496	995.066
90	6.3	6.96	12.45	4.98	30		38.02	4.649	1029.22
120	6.4	6.89	12.98	3.99	31	31.6	38.528	4.69	1036.81
150	6.3	6.88	12.58	3.86	35		37.835	4.654	1030.84
180	6.8	6.89	12.61	3.94	35	31.2	36.006	4.529	1001.8
210	6.8	6.67	12.41	2.84	35		33.357	4.322	959.316
240	6.8	6.85	12.99	3.01	35	31.3	29.6974	4.032	900.104

Table A.2 Summary of the physico-chemical parameters recorded for the *E. faecium* trial during which *B. bacteriovorus* was applied as a pre-treatment for 72 h

Time solar exposure (min)	Dissolved Oxygen (mg/L)	pH	Electrical Conductivity (mS/cm)	Total Dissolved Solids (TDS; ppt)	Temperature of Treated Water (°C)	Ambient Temperature (°C)	Average UV-A (W/m2)	Average UV-B (W/m2)	Direct Normal Irradiance (W/m2)
***Bdellovibrio* + Photocatalysis**									
0	5.9	7.22	12.34	0.96	22	25.9	30.606	4.217	896.391
30	7.3	7.63	12.89	0.88	31		34.235	4.501	953.045
60	7.1	6.99	13.01	0.78	35	25.6	36.922	4.702	993.016
90	7.1	6.87	13.06	0.88	36		38.781	4.835	1016.04
120	7.6	6.79	13.14	0.84	40	26.5	39.441	4.873	1027.14
150	7.1	6.81	12.96	0.9	41		38.682	4.823	1023.37
180	7.1	6.69	12.01	0.91	41	27.9	36.861	4.681	1022.99
210	6.9	6.59	12.87	0.77	43		34.131	4.474	988.417
240	6.7	6.56	11.97	0.76	43	26.6	30.331	4.174	892.355
***Bdellovibrio* + Solar Disinfection**									
0	6.8	7.33	12.51	1.21	22	25.9	30.606	4.217	896.391
30	6.9	8.67	13.12	0.98	29		34.235	4.501	953.045
60	6.7	8.48	13.11	0.96	31	25.6	36.922	4.702	993.016
90	6.7	8.21	13.09	0.98	33		38.781	4.835	1016.04
120	6.5	7.68	12.84	1.18	33	26.5	39.441	4.873	1027.14
150	6.7	7.77	12.13	0.87	35		38.682	4.823	1023.37
180	7.1	7.32	12.77	0.88	35	27.9	36.861	4.681	1022.99
210	6.8	7.14	12.64	0.76	38		34.131	4.474	988.417
240	6.6	6.99	11.98	0.79	38	26.6	30.331	4.174	892.355

Table A.2 (Continued) Summary of the physico-chemical parameters recorded for the *E. faecium* trial during which *B. bacteriovorus* was applied as a pre-treatment for 72 h

Time solar exposure (min)	Dissolved Oxygen (mg/L)	pH	Electrical Conductivity (mS/cm)	Total Dissolved Solids (TDS; ppt)	Temperature of Treated Water (°C)	Ambient Temperature (°C)	Average UV-A (W/m2)	Average UV-B (W/m2)	Direct Normal Irradiance (W/m2)
Photocatalysis									
0	3.2	6.79	12.35	6.98	21	25.9	30.606	4.217	896.391
30	3.9	6.63	11.96	6.77	30		34.235	4.501	953.045
60	6.7	6.81	11.98	6.35	33	25.6	36.922	4.702	993.016
90	6	5.99	11.98	6.71	38		38.781	4.835	1016.04
120	6.3	6.63	12.36	6.49	41	26.5	39.441	4.873	1027.14
150	6.2	6.94	12.48	6.23	41		38.682	4.823	1023.37
180	6.3	6.77	12.45	5.93	42	27.9	36.861	4.681	1022.99
210	6.4	6.44	12.58	5.54	42		34.131	4.474	988.417
240	6.3	6.59	12.45	5.35	42	26.6	30.331	4.174	892.355
Solar Disinfection									
0	4.5	6.91	11.99	6.97	21	25.9	30.606	4.217	896.391
30	6.8	6.62	12.31	6.55	27		34.235	4.501	953.045
60	6.6	6.63	12.64	6.49	30	25.6	36.922	4.702	993.016
90	6.5	6.32	12.33	6.41	31		38.781	4.835	1016.04
120	6.7	6.22	12.22	6.35	31	26.5	39.441	4.873	1027.14
150	6.9	5.96	12.21	5.96	32		38.682	4.823	1023.37
180	5.9	5.88	11.96	5.34	34	27.9	36.861	4.681	1022.99
210	6.8	5.71	12.65	4.21	38		34.131	4.474	988.417
240	6.3	5.6	12.01	4.22	39	26.6	30.331	4.174	892.355

Table A.3 Performance characteristics for the EMA-qPCR assays utilised to quantify *K. pneumoniae*. *E. faecium and B. bacteriovorus*

qPCR Performance Characteristics	*K. pneumoniae*	*E. faecium*	*B. bacteriovorus*
Efficiency (*E*)	95.17 ± 2.93	101.3 ± 1.26	101 ± 3.56
y-intercept	35.12 ± 1.61	33.70 ± 1.68	33.27 ± 0.70
Correlation Coefficient (r^2)	0.99 ± 0.00	1.00 ± 0.00	0.99 ± 0.01
Lower Limit of Detection (LLOD) (GC/µL)	5.39 ± 1.70	2.55 ± 0.45	6.69 ± 5.16
Lower Limit of Quantification (LLOQ) (GC/µL)	40.19 ± 13.2	43.11 ± 28.45	32.14 ± 36.71
Slope	-3.59 ± 0.169	-3.26 ± 0.059	-3.22 ± 0.14