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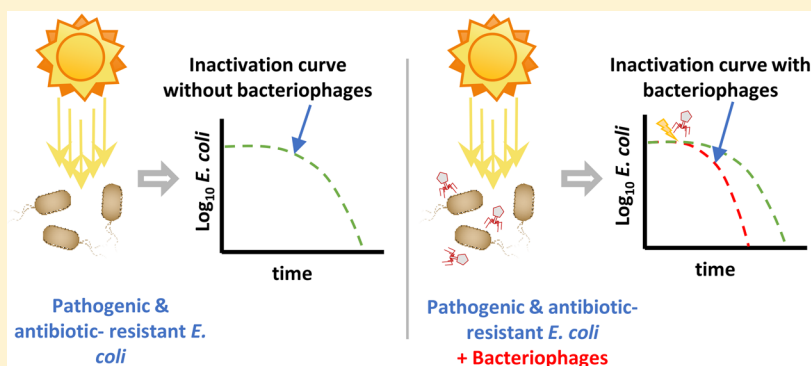
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## Bacteriophages To Sensitize a Pathogenic New Delhi Metallo $\beta$ -Lactamase-Positive *Escherichia coli* to Solar Disinfection

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### Supporting Information



**ABSTRACT:** Bacteriophages active against a New Delhi metallo beta lactamase (NDM)-positive *E. coli* PI-7 were isolated from municipal wastewater and tested for their lytic effect against the bacterial host. Bacteriophages were highly specific to *E. coli* PI-7 when tested for host-range. After determining host-specificity, bacteriophages were tested for their ability to sensitize *E. coli* PI-7 to solar irradiation. Solar irradiation coupled with bacteriophages successfully reduced the length of the lag-phase for *E. coli* PI-7 from 4 h to 2 h in buffer solution. The reduction of lag-phase length was also observed in filtered wastewater effluent and chlorinated effluent. Previously, we found through gene expression analysis that cell wall, oxidative stress, and DNA repair functions played a large role in protecting *E. coli* PI-7 against solar damage. Here, gene expression analysis of bacteriophage-supplemented solar-irradiated *E. coli* PI-7 revealed downregulation of cell wall functions. Downregulation of functions implicated in scavenging and detoxifying reactive oxygen species, as well as DNA repair genes, was also observed in bacteriophage-supplemented solar-irradiated *E. coli* PI-7. Moreover, solar irradiation activates *recA*, which can induce lytic activity of bacteriophages. Overall, the combined treatment led to gene responses that appeared to make *E. coli* PI-7 more susceptible to solar disinfection and bacteriophage infection. Our findings suggest that bacteriophages show good potential to be used as a biocontrol tool to complement solar irradiation in mitigating the persistence of antibiotic-resistant bacteria in reuse waters.

### 1. INTRODUCTION

Antibiotic-resistant bacteria (ARB) are among emerging microbial contaminants of increasing concern and challenge our current antimicrobial strategies. In fact, several reports have raised concerns that the development of new drugs alone will not adequately address the threat posed by the spread of antibiotic-resistant pathogens.<sup>1–3</sup> A major hotspot implicated in the creation of superbugs and the dissemination of these emerging contaminants is wastewater and its treatment facilities as abundance of nutrients, high bacterial numbers, and sublethal levels of antibiotics are considered favorable for both the survival ARB and the horizontal transfer of bacterial resistance genes.<sup>4,5</sup>

In particular, an earlier study has isolated an *E. coli* strain PI-7 from local wastewater influent in Jeddah, Saudi Arabia, and found it to be pathogenic, highly antibiotic-resistant, and, more alarmingly, carrying the New Delhi metallo- $\beta$ -lactamase

(NDM) antibiotic resistance gene, *bla*<sub>NDM</sub>.<sup>6</sup> Other studies have also independently reported the presence of *bla*<sub>NDM</sub> genes and NDM-harboring bacteria in urban wastewater<sup>6–8</sup> and in the final chlorinated secondary effluent despite having received treatment through a municipal wastewater treatment process.<sup>7</sup> The *bla*<sub>NDM</sub> gene codes for a carbapenemase, a class of enzymes that confer resistance to carbapenems and other  $\beta$ -lactam antibiotics that are normally reserved as a last line of defense against Gram-negative bacterial infections. Moreover, NDM-harboring bacteria typically carry resistance determinants against multiple other antibiotic classes, leaving very few treatment options available.<sup>8–11</sup> These findings highlight the

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risks of disseminating ARB and their genes through wastewater reuse,<sup>12,13</sup> with potential implications to public health. This is particularly relevant in places like Saudi Arabia, where pressure on water resources is high, and treated wastewater is being considered to alleviate considerable portions of this water-scarcity pressure.<sup>14</sup>

Solar irradiation can serve as a biocidal barrier to mitigate this problem. Many studies found rapid inactivation of fecal indicator organisms within a few hours of exposure to natural sunlight.<sup>15–19</sup> However, it was found that the NDM-positive *E. coli* PI-7 underwent a significantly longer lag-phase prior to decay and exhibited a longer inactivation half-life compared to another nonpathogenic and less antibiotic-resistant *E. coli* DSM1103 upon solar irradiation.<sup>20</sup> This suggests that a higher solar fluence or longer exposure time would be required to achieve the same log reduction for the NDM-positive *E. coli* compared to a nonresistant strain. Furthermore, *E. coli* PI-7 was observed to upregulate a larger suite of genes related to cell repair including upregulation of cell wall synthesis and extracellular polymeric substance genes that may have served as a physical barrier to solar damage. This is observed along with upregulation of oxidative stress response and ROS (reactive oxygen species) scavenger genes, which may have protected the cell from indirect mechanisms of solar damage. Collectively, these gene responses may have enabled longer survival. Moreover, the same study found upregulation of genes involved in persister cell formation, along with genes related to antibiotic resistance and virulence functions. These observations suggest that pathogenic antibiotic-resistant bacteria like *E. coli* PI-7 remain viable for longer period compared to an antibiotic-susceptible strain despite solar irradiation. There exists a need for an additional mitigation strategy besides solar irradiation to enhance biocidal effects against the *E. coli* PI-7.

A complementary treatment method to explore would be the use of lytic bacteriophages. Lytic bacteriophages are viruses that infect the bacterium host, replicate within and kill bacteria by lysing the host. In wastewater-related applications, bacteriophages have been applied to control biofilm formation on filtration membranes and to restore flux to fouled membranes.<sup>1,21</sup> Bacteriophage therapy has also been demonstrated to solve the problem of sludge bulking caused by filamentous bacteria.<sup>22–24</sup> However, those studies did not demonstrate the complementary use of bacteriophages along with solar irradiation to inactivate ARB like *E. coli* PI-7.

It is hypothesized that bacteriophages can aid in achieving further reduction of *E. coli* PI-7 numbers during disinfection by solar irradiation. This is inferred from the earlier study where downregulation of phage-shock functions as well as upregulation of phage integrase and DNA transfer genes were observed in *E. coli* PI-7 in response to solar irradiation.<sup>20</sup> The aim of this study is to investigate the use of bacteriophages as a biocidal tool in combination with solar irradiation for inactivating NDM-carrying *E. coli* PI-7. In this study, seven bacteriophages targeting *E. coli* PI-7 were isolated from wastewater. They were then tested for host-specificity and stability in controlling *E. coli* PI-7's growth under various conditions. Three bacteriophage candidates were used in combination with solar irradiation to test the possibility of hastening *E. coli* PI-7's inactivation. The response was assessed in terms of cell survival via quantification of culturable cell counts, and gene expression changes to reveal survival and damage responses at the molecular level.

## 2. MATERIALS AND METHODS

**2.1. Bacteriophage Isolation.** Bacteriophages specific to a NDM-positive pathogenic *E. coli* strain PI-7<sup>6</sup> were isolated from wastewater as follows. Fifty milliliters of wastewater influent was centrifuged at  $10\,000 \times g$  for 15 min, and the supernatant was filtered through a  $0.2\ \mu\text{m}$  filter. Then 50 mL of 2X LB broth was added to the filtrate as well as 50 mL of overnight PI-7 culture with  $8\ \mu\text{g}/\text{mL}$  Meropenem. The mixture was incubated at  $37\ ^\circ\text{C}$  and 200 rpm for 24 h. Chloroform was added in to a final concentration of 1%, and the mix was incubated for 1 h to lyse bacterial cells. Next, the mixture was centrifuged at  $10\,000 \times g$  for 30 min at  $4\ ^\circ\text{C}$ , and the supernatant was filtered through a  $0.2\ \mu\text{m}$  filter. The phage lysate filtrate was diluted in SM buffer (100 mM NaCl, 8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50 mM Tris buffer at pH 7.5). To isolate individual phages,  $10\ \mu\text{L}$  of phage lysate and  $100\ \mu\text{L}$  of overnight PI-7 culture were mixed together in soft LB agar supplemented with 2 mM  $\text{CaCl}_2$ , then poured over  $\text{CaCl}_2$ -supplemented LB (1.5% w/v) agar. After 24 h incubation at  $37\ ^\circ\text{C}$ , isolated plaques were individually picked, resuspended in SM buffer, and filtered through a  $0.2\ \mu\text{m}$  filter. Each plaque then went through seven rounds of plating and reisolation to isolate a total of seven individual bacteriophages.

**2.2. High-Titer Bacteriophage Stock Preparation.** Bacteriophage stocks containing high phage titer were prepared by first mixing  $50\ \mu\text{L}$  of original phage stock with  $100\ \mu\text{L}$  of fresh *E. coli* PI-7 in 3 mL of soft LB agar (0.3%) supplemented with 2 mM  $\text{CaCl}_2$ . The mixture was plated onto 1.5% w/v LB agar plates supplemented with 2 mM  $\text{CaCl}_2$  and incubated overnight at  $37\ ^\circ\text{C}$ . Then 3 mL of SM buffer was added onto each plate, and a sterile spreader was used to scrape off the top agar layer containing bacteriophages and bacterial cells. This mixture was then centrifuged at  $10\,000 \times g$  for 15 min, and the supernatant was filtered through  $0.2\ \mu\text{m}$  filters (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Afterward, polyethylene glycol 8000 was added to the filtrate to form 15% w/v, and the mixture was incubated overnight with stirring at  $4\ ^\circ\text{C}$ . Subsequently, the mixture was centrifuged at  $10\,000 \times g$  for 20 min, and the cell pellet containing the bacteriophage particles was resuspended in 1 mL of 1X PBS. Stocks were enumerated for titer concentration by plating serial dilutions of the stock with fresh *E. coli* PI-7 using the soft agar method described above, and quantifying PFUs (or plaque-forming units) after overnight incubation. Bacteriophages were characterized for their morphology and genome sizes as described in [Supporting Information 1](#).

**2.3. Bacteriophage Lytic Activity.** To check the lytic activity of the bacteriophage isolates against different bacteria, cultures of various bacteria from clinical and environmental origins (Table 1) were prepared overnight. One-hundred microliters of bacteria was plated in soft agar, mixed with the bacteriophages to perform a plaque assay, or followed by spotting of serial dilutions of different bacteriophages for spot test evaluation.<sup>25</sup>

On the basis of the lytic activity tests, bacteriophages were specific against *E. coli* PI-7 and further determination was done to monitor the impact on growth curve of *E. coli* PI-7 in the presence of bacteriophages. A fresh culture of *E. coli* PI-7 was prepared overnight, and then  $100\ \mu\text{L}$  was inoculated into 10 mL of 1:1 SM buffer mixed with LB broth in sterile transparent glass tubes. The  $\text{OD}_{600}$  of each tube was periodically measured

**Table 1. Lytic Activity Results for Bacteriophages Tested against Various Bacterial Isolates**

| bacterial species  | no. tested | activity <sup>a</sup>       | ref <sup>b</sup> |
|--|------------|-----------------------------|------------------|
| <i>Escherichia coli</i>  | 8          | only vs <i>E. coli</i> PI-7 | 6,56–58          |
| <i>Escherichia fergusonii</i>  | 3          | no activity                 | 56               |
| <i>S. sonnei</i> / <i>E. coli</i> O157:H7  | 6          | no activity                 | 56               |
| <i>Klebsiella</i> sp.  | 4          | no activity                 | 56,59            |
| <i>A. baumannii</i> , <i>Aeromonas</i> sp., <i>Citrobacter</i> sp., <i>Enterobacter</i> sp., <i>Enterococcus</i> sp. | 5 (1 each) | no activity                 | 56,59            |
| others <sup>c</sup>  | 4          | no activity                 | 56               |

<sup>a</sup>Activity results for all seven bacteriophage isolates are shown in one column only as they all showed the same result. Tests were performed using plaque and spotting assays in eight serial dilutions of the bacteriophages, with three replicates for each bacteriophage-bacteria pair. <sup>b</sup>Majority of bacterial isolates were environmental ones isolated from a local wastewater treatment plant in Jeddah, Saudi Arabia, while some were produce grown in local farms<sup>59</sup> or commercially available isolates. Two were commercially available isolates: *E. coli* DSM1103, originally a clinical commensal isolate,<sup>60</sup> and *Klebsiella quasipneumoniae* ATCC700603, formerly *K. pneumoniae* K6, also a clinical isolate, known for producing extended-spectrum  $\beta$ -lactamase (ESBL) enzymes.<sup>61</sup> Two were clinically isolated *E. coli* isolates that also carried the NDM antibiotic resistance genes.<sup>58</sup> The isolates were chosen to represent diverse antibiotic resistance profiles and represent close-relatives as well as distant species to the host used for page isolation, *E. coli* PI-7. <sup>c</sup>Others refers to one of each identified as *Azospirillum* sp., *Alcaligenes* sp., *Kluyvera ascorbate*, and *Leclercia adecarboxylata*.

to monitor *E. coli* PI-7's growth in presence or absence of bacteriophages at different temperatures (40, 37, 32, and 20 °C) and pH (6, 7, and 8).

**2.4. Solar Inactivation and Decay Kinetics.** Solar inactivation trials were conducted using an Atlas Suntest XLS+ photosimulator (Chicago, IL) equipped with a xenon arc lamp as described in our previous work.<sup>20</sup> The solar simulator's irradiance is described in Supporting Information 2 and shown in Figure S1. Briefly, prior to the experiments, overnight cultures of *E. coli* PI-7 were thoroughly washed in 1 mM NaHCO<sub>3</sub>, then resuspended in 80 mL of the final matrix of either 1X PBS, filtered tertiary-treated wastewater effluent (collected after treatment in an anoxic–oxic activated sludge tank followed by microfiltration) or filtered chlorinated effluent collected after microfiltration. All wastewater samples were filtered through 0.2  $\mu$ m polycarbonate membrane filters (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and stored at 4 °C in sterile glass bottles within 4 h of collection. No additional step was taken to quench residual chlorine in chlorinated effluent samples prior to inactivation experiment as no residual chlorine were detected by DPD method (Hach, Loveland, CO). Starting bacterial numbers for solar inactivation experiments were always approximately 1–9  $\times 10^8$  CFU/mL. For bacteriophage-supplemented experiments (SI+phage), 1 mL of high titer phage solution was added to the microcosms to a final PFU concentration similar to the bacterial concentration (i.e., MOI = 1). Microcosms were then either covered with foil (dark controls) or glass filters permitting wavelength 280 nm and above (Newport Corporation, Irvine, CA). A set of nonbacteriophage-

supplemented experiments (SI-alone), both dark and irradiated, was always included in parallel to bacteriophage-supplemented solar irradiation (SI+phage) trials, but SI-alone microcosms were inoculated with sterile buffer solution instead of phage solution. After inoculation with bacteria and bacteriophages where applicable, the microcosms were placed inside the Atlas Suntest and exposed to solar irradiation. SI+phage and SI-alone inactivation kinetics data presented in this study was obtained from four biological replicates.

A set of solar inactivation experiments with seven bacteriophages individually inoculated into buffer solution in separate microcosms was also performed without replication to denote the stability of these bacteriophages upon exposure to sunlight. This observation, along with that obtained from lytic activity tests and growth curve impediment experiments (described in section 2.3), was used to select for the most suitable bacteriophage isolates to form a bacteriophage mixture. Upon selection of the bacteriophage mix (i.e., bacteriophage P1, P4, and P5a), solar inactivation was performed on this mixture inoculated in sterile buffer solution and in the presence of *E. coli* PI-7. Three biological replicates were performed for this experiment to determine the bacteriophage mixture's decay kinetics.

The number of colony forming units (CFU/mL) or plaque forming units (PFU/mL) in the irradiated and dark experimental microcosms was quantified at the starting point and over irradiation time. Inactivation results were converted to log<sub>10</sub> and natural log (ln) curves of concentration values normalized as CFU<sub>t</sub>/CFU<sub>t=0</sub> for *E. coli* PI-7 or PFU/PFU<sub>t=0</sub> for bacteriophages and plotted against time to obtain the inactivation curves. The decay rate constants (*k*) of dark control and irradiated sample inactivation curves were calculated as the negative slopes of natural log inactivation curves, with the following caveat: in cases where there was a detectable lag-phase, the curve was considered bimodal and the lag-phase data points were excluded from the slope calculation. Further detail on lag-phase can be found in Supporting Information 3 and Figure S2. The decay rate constants were mathematically corrected for attenuation of light penetration caused by light screening (due to variable light absorbance) but not light scattering (due to turbidity) in the samples. The corrected decay rate constants were then used in statistical comparisons or half-life calculations. Because differences in light absorbance and screening can also occur due to sample variation or different water matrices (Figure S3), this adjustment was hence performed for each experimental microcosm by measuring each individual sample's absorbance at the start of the experiment as described previously,<sup>26,27</sup> and shown in Supporting Information 2. For inactivation by full spectrum irradiation (280–700 nm) as is shown in this study, correction factor for the decay rate constants was based on the concept that different wavelengths differ in their inactivation efficiencies. UV portions of the spectrum are generally the most important wavelengths that are most lethal to the microorganism compared to the visible range wavelengths. Mathematical calculations of correction factors therefore also weigh in the difference in contribution of UV and visible range to the decay.<sup>27</sup> The half-lives for each experiment, or the durations needed to reduce the bacterial or bacteriophage concentration by half (after and excluding lag-phase), were calculated using the first-order decay kinetics equation:

$$\ln(\text{CFU}_t/\text{CFU}_0) = -k * t$$



where  $k^*$  is the decay rate constant, corrected for light screening, of the natural log inactivation curve, and  $t$  is time.

Statistical analyses were performed using Minitab version 1.4.0. One-way ANOVA was performed to compare the lengths of lag-phases, the corrected decay rate constants, and the half-life values of the different experimental conditions and isolates. Linear regression analysis was performed to compare the slope of each experiments inactivation curve to 0 ( $\alpha = 0.05$ ) to determine if significant change/decay is observed (Supporting Information 3). Errors presented at various points in the text and tables, and as error bars in some charts, are standard deviation from the mean values reported. No error was propagated on  $C_0$  in  $(C/C_0)$ -based plots. This is because each sample had its own  $C_0$ . After samples were enumerated for  $C$ ,  $\text{Log}_{10}(C/C_0)$  or  $\text{Ln}(C/C_0)$  values were generated for each microcosm. Afterward the average  $\text{Log}_{10}(C/C_0)$  or  $\text{Ln}(C/C_0)$ , including all technical and biological replicates, for one experimental condition was calculated, and then the standard deviation error for that average was calculated. Lag-phase length and half-life were not analyzed for dark control samples due to the lack of significant decay in these samples, as determined by single linear regression analysis.

### 2.5. RNA-seq Sampling, Extraction, and Sequencing.

RNA-seq was performed to provide comparative analysis of gene expression between samples with and without bacteriophage addition upon solar irradiation. Samples were taken at various time points (at 0, 1, 2, 4, 6, 8, and 10 h) to capture the gene expression at different phases of the bacterial CFU inactivation curves. For bacteriophage-supplemented experiments (SI+phage), three solar inactivation experiments were conducted for RNA sampling to obtain three biological replicates, each with two dark and two irradiated technical replicate microcosms at every sampling point. For non-bacteriophage-supplemented (SI-alone) experiments, two solar inactivation experiments for RNA sampling were performed, also with two dark and two irradiated technical replicate microcosms at every sampling point.

RNA preservation, extraction, and sequencing were performed as described previously.<sup>20</sup> Briefly, sample volumes varying between 1 to 4 mL were taken from the experimental microcosms, and an equal volume of RNAProtect reagent (Qiagen, Valencia, CA) was added. Samples were stored at 4 °C for less than a week until RNA extraction. Samples were centrifuged at  $5000 \times g$  for 10 min and the pellets used for total RNA extraction using the RNeasy Mini kit (Qiagen, Hilden, Germany) with the on-column DNase treatment. The RNA was eluted with RNase-free water, and RNA quality was determined using 2200 TapeStation Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples that passed the quality control were submitted to KAUST Genomics Core lab for RNA-Seq on Illumina HiSeq platform. Prior to sequencing, the bacterial rRNA was removed from 1  $\mu\text{g}$  of total RNA using the Ribo Zero rRNA removal kit (Illumina, San Diego, CA, USA). The enriched mRNA fraction was converted to RNA-seq libraries compatible for runs on Illumina HiSeq platforms based on protocols described by manufacturer. Fastq files were generated and demultiplexed by KAUST Bioinformatics core lab. All fastq files associated with *E. coli* PI-7 with and without bacteriophages are available on the ENA SRA public depository under accession numbers PRJEB25911 and 25812.

**2.6. RNA-seq Data Analysis.** The RNA-seq data were analyzed using CLC Genomics Workbench version 8.0.1 from CLC Bio (Cambridge, MA). For the reference genome

sequence, and to assign genes to specific categories, genomic DNA sequences annotated using RAST were used for *E. coli* PI-7.<sup>6</sup> RNA-seq reads were mapped onto the reference sequences using CLC. Reads were only assembled if the fraction of reads that aligned to the reference genome was greater than 0.9 and if the read matched other regions of the reference genome at fewer than 10 nucleotide positions. Summarized RNA-seq mapping results are shown in Tables S1 and S2 and Supporting Information 4.

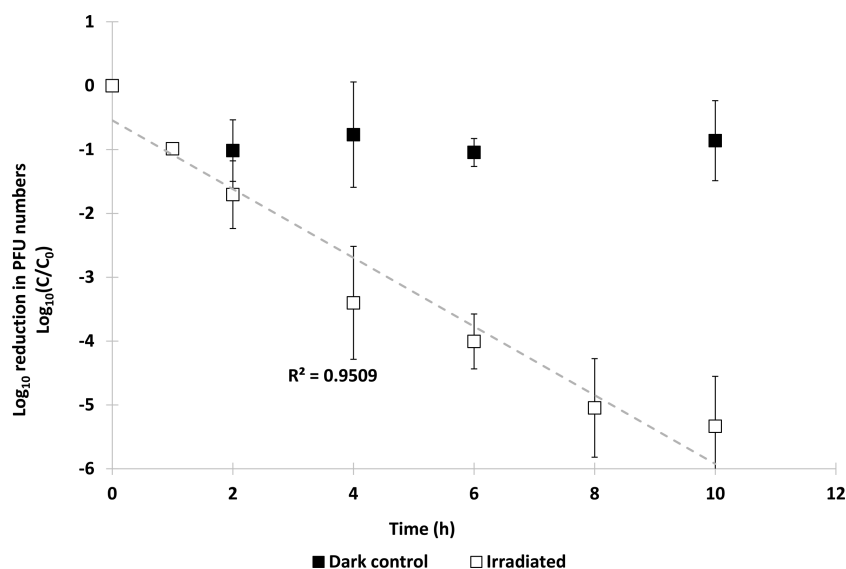
CLC was used to generate normalized mean expression values by applying a scaling correction to samples to facilitate cross-sample comparisons within and across biological batches. The gene expression results were then analyzed for statistical significance, separately for each of the biological experimental replicate batches. The statistical comparison was based on the proportion-based test described by Baggerly et al.<sup>28</sup> Mean gene expression values as normalized RPKM (reads per kilobase million) in irradiated samples were compared with the mean normalized RPKM values of their respective dark controls at each time point for each experimental batch. Then, for each condition, the fold-change in expression level was taken into consideration if it was statistically significant ( $p$ -value < 0.05) in a consistent trend in at least two of its biological replicate batches. More detail is provided in Supporting Information 5. The terms upregulation or downregulation are used to describe the fold-change in gene expression levels, and those up/downregulation terms describe the irradiated samples responses relative to their corresponding dark controls. Fold-changes in expression levels were calculated based on the normalized mean expression values. Additionally, gene expression levels are based on the number of transcript reads present, and hence the expression levels change according to transcription rates as well as transcript degradation rates. It should be noted that as a consequence of the way the analyses were performed, some of the results showed high yet statistically insignificant fold-changes in expression levels. This might have been caused by only one of the experimental batches and not the other(s) having a large and significant fold-changes.

Confirmation experiments using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were performed as described in Supporting Information 6 and 7, and Table S3. Briefly, two genes that showed significant fold-change responses at high expression levels in RNA-seq results were chosen and normalized against a housekeeping *uidA* gene, which is present at one copy per genome in *E. coli* PI-7.<sup>29</sup> Normalized copy numbers in irradiated samples were then compared to corresponding dark controls to verify RT-qPCR results against RNA-seq results.

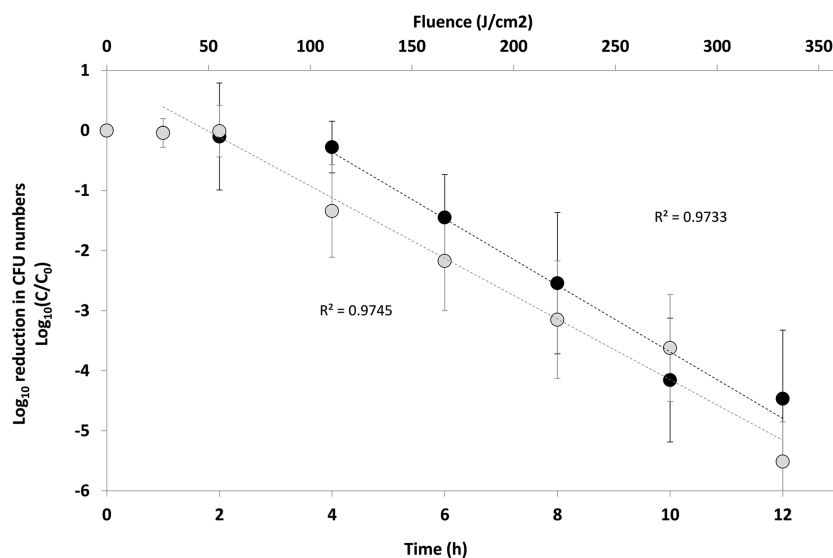
## 3. RESULTS

**3.1. Lytic Activity and Growth Curve.** All seven isolated bacteriophages showed strain-specific lytic activity against *E. coli* PI-7 but not the other *E. coli* strains (Table 1). The bacteriophages also showed no lytic activity against the other tested non-*E. coli* bacteria.

The lytic effect of bacteriophages against *E. coli* PI-7 was also assessed by examining the growth duration required to reach a specific optical density in the presence of bacteriophages. Results showed that bacteriophages maintained a slowing effect on PI-7's growth under all tested temperatures and pH (Figure S4).



**Figure 1.**  $\text{Log}_{10}$  bacteriophage decay kinetics under solar irradiation in the presence of their host *E. coli* PI-7 in PBS buffer solution. Fluence presented in charts is from visible + UV light and was  $27.8 \text{ J/cm}^2$  per hour. Fluence in the UV portion alone was  $8.1 \text{ J/cm}^2$  per hour. See Supporting Information 2 for more detail on fluence. The average decay rate constant of the irradiated samples was  $-2 \pm 0.3 \text{ h}^{-1}$ , and half-life  $21 \pm 3.3 \text{ min}$  (equating to a visible + UV fluence half-life of  $9.7 \pm 1.5 \text{ J/cm}^2$ ). Inactivation curves are presented as  $\text{log}_{10}$  curves for ease of interpretation, but decay rate constants and half-lives were calculated from the natural log inactivation curves. See Figure S7 for the natural log inactivation curves and more detail on inactivation kinetics. Error bars represent standard deviation from the mean  $\text{log}_{10}$  reduction value.



**Figure 2.** Inactivation curves of *E. coli* PI-7 with (gray circles) and without (black circles) bacteriophages under simulated solar irradiation in PBS buffer solution. Fluence presented in charts is from visible + UV light and was  $27.8 \text{ J/cm}^2$  per hour. Fluence in the UV portion alone was  $8.1 \text{ J/cm}^2$  per hour. See Supporting Information 2 for more detail on fluence. Inactivation curves are presented as  $\text{log}_{10}$  curves for ease of interpretation, but decay rate constants and half-lives are calculated from natural log inactivation curves. See Figure S8 for the natural log inactivation curve. Error bars represent standard deviation from the mean  $\text{log}_{10}$  reduction value.

**3.2. Stability of Bacteriophages in Sunlight.** Simulated solar inactivation trials were first conducted on the seven bacteriophage isolates in absence of *E. coli* PI-7 to determine their stability upon exposure to solar irradiation. On the basis of the log reduction curves, the three bacteriophages P1, P4, and P5a remained relatively stable for the first 2 h, while all other bacteriophages decayed almost immediately upon solar irradiation. Upon decay, bacteriophages P1, P4, and P5a demonstrated longer half-lives than the other bacteriophages (Figure S5).

**3.3. Solar Inactivation in the Presence of Bacteriophages.** The three bacteriophages (P1, P4, and P5a)

demonstrated optimal lytic efficacy over a wide range of pH and temperature and exhibited relatively slower decay in the presence of sunlight. Hence, they were selected for application to *E. coli* PI-7 under simulated solar irradiation. When these bacteriophages were characterized for their morphology and genome sizes, it was found that P1 had a genome fragment size of fragment size of 65042 bp and did not show any contractile tail (Figure S6A). It was therefore inferred that P1 likely belongs to Podoviridae family. Bacteriophages P4 and P5a had genome fragment sizes of 54516 bp and 56020 bp, respectively, and both were observed to have contractile tails, with P5a having a longer tail than P4 (Figure S6B,C). It was therefore

inferred that they are both likely to belong to Siphoviridae family. The bacteriophages were administered in solar irradiation experiments as a mixture since previous studies showed that bacteria are less likely to develop bacteriophage-insensitive mutants to a mixture of viruses rather than a single bacteriophage isolate.<sup>30–33</sup> Bacteriophage decay kinetics in bacteriophage-supplemented *E. coli* PI-7 microcosms showed a half-life of  $21 \pm 3.3$  min equating to a fluence half-life of  $9.7 \pm 1.5$  J/cm<sup>2</sup> (Figures 1 and S7). This half-life was shorter than that observed when bacteriophage were irradiated without their bacterial hosts, which ranged between 33 and 35 min for the three selected bacteriophage candidates (Figure S5).

In the absence of bacteriophages, *E. coli* PI-7 showed a lag phase of  $4 \pm 0.8$  h (or  $114 \pm 22$  J/cm<sup>2</sup>) during which there was no detectable reduction in viable cell numbers, while the addition of bacteriophages reduced this lag-phase length to  $2 \pm 0.6$  h (or  $51 \pm 17$  J/cm<sup>2</sup>;  $p \leq 0.0001$ ) (Figures 2 and S8, and Table 2). Upon the onset of inactivation, there was no significant difference in corrected decay rate constants ( $k^*$ ) and therefore the half-life of *E. coli* PI-7 in presence ( $12 \pm 3.9$  min;  $5.6 \pm 1.8$  J/cm<sup>2</sup>) or absence ( $8.6 \pm 2.8$  min;  $3.9 \pm 1.3$  J/cm<sup>2</sup>) of bacteriophages ( $p$ -value = 0.20; Table 2).

**Table 2. Inactivation Kinetics of *E. coli* PI-7 with and without Bacteriophages in PBS Buffer, and Filtered Wastewater Effluent and Chlorinated Effluent Waters<sup>a,d,e</sup>**

| (A) <i>E. coli</i> PI-7                  |  | PBS            | effluent       | chlorinated effluent |
|--|--|----------------|----------------|----------------------|
| irradiated                               | sample size                                  | 8              | 4              | 4                    |
|  | lag phase length (h)                         | $4 \pm 0.8$    | $4^b$          | $4^b$                |
|  | lag phase fluence (J/cm <sup>2</sup> )       | $114 \pm 22$   | $111^b$        | $111^b$              |
|  | decay rate constant $k^c$ (h <sup>-1</sup> ) | $-5.3 \pm 1.9$ | $-6.3 \pm 0.1$ | $-4.1 \pm 0.1$       |
|  | half-life length (min)                       | $8.4 \pm 2.8$  | $6.7 \pm 0.1$  | $10 \pm 0.2$         |
|  | half-life fluence (J/cm <sup>2</sup> )       | $3.9 \pm 1.3$  | $3.1 \pm 0.1$  | $4.8 \pm 0.1$        |
| (B) <i>E. coli</i> PI-7 + bacteriophages |  | PBS            | effluent       | chlorinated effluent |
| irradiated                               | sample size                                  | 9              | 4              | 4                    |
|  | lag phase length (h)                         | $2 \pm 0.6$    | $2^b$          | $3 \pm 1$            |
|  | lag phase fluence (J/cm <sup>2</sup> )       | $51 \pm 17$    | $56^b$         | $70 \pm 28$          |
|  | decay rate constant $k^c$ (h <sup>-1</sup> ) | $-3.8 \pm 1.2$ | $-4.8 \pm 1.8$ | $-3.1 \pm 0.4$       |
|  | half-life length (min)                       | $12 \pm 3.9$   | $9.6 \pm 3.6$  | $14 \pm 0.8$         |
|  | half-life fluence (J/cm <sup>2</sup> )       | $5.6 \pm 1.8$  | $4.5 \pm 1.7$  | $6.4 \pm 0.4$        |

<sup>a</sup>Errors after the  $\pm$  sign represent standard deviation from the mean.

<sup>b</sup>No standard deviation is shown because all replicates resulted in the same value due to the low sampling resolution of hourly intervals.

<sup>c</sup>Decay rate constants (negative slopes of natural log inactivation over time curves) were mathematically corrected for light attenuation.

<sup>d</sup>Each set of inactivation kinetics data was based on at least three biological replicates. <sup>e</sup>Fluence values presented were for visible + UV light. Fluence from visible + UV light was 27.8 J/cm<sup>2</sup> per hour. Fluence in the UV portion alone was 8.1 J/cm<sup>2</sup> per hour. See Supporting Information 2 for more detail on fluence.

The inactivation kinetics was also examined in treated wastewater. The bacteriophage mix maintained its effect in shortening lag phase length from 4 to 2 h in wastewater effluent ( $p$ -value = 0.002), and from 4 to 3 h in chlorinated effluent ( $p$ -value = 0.02, Table 2). The corrected decay rate constant  $k^*$  and half-life length did not change statistically significantly with bacteriophage addition in the effluent ( $9.6 \pm 3.6$  min,  $p$ -value = 0.34) or in the chlorinated effluent ( $14 \pm 0.8$ ,  $p$ -value  $\geq 0.20$ ) compared to their respective no-bacteriophage SI-alone control (Table 2).

#### 3.4. Overview of Transcriptomic Response under Phage-Supplemented Solar Inactivation.

RNA-seq analysis was performed to compare the response of *E. coli* PI-7 to only solar irradiation (referred to as SI-alone) and those supplemented with bacteriophages (SI+Phages). Gene expression for each of the two experimental conditions was analyzed independently by comparing the gene response of the irradiated sample to that of its corresponding dark control at each time point. Overall, a total of 1601 genes were significantly up/down-regulated in SI+Phages experiments and 800 genes in SI-alone experiments (Table S4). Specifically, the response for both SI-alone and SI+Phages samples was dominated by downregulation at the initial time points, with 80% and 87% of responding genes being downregulated at  $t = 1$  h in SI+Phages and SI-alone samples, respectively (Figure S9A,B). However, the proportion of gene upregulation increased with the duration of exposure to solar irradiance, with 65% and 67% of responding genes being upregulated at  $t = 10$  h in SI+Phages and SI-alone samples, respectively. The trend and proportion of up/down-regulation observed in midlag and mid-decay phase of *E. coli* PI7 in this study, where downregulation is the dominant initial response in most genes and more genes gradually show upregulation, were similar to those observed in an earlier study (Figure S9C, Table S4).<sup>20</sup>

Out of all genes that were significantly up/down-regulated, 735 were shared between irradiated *E. coli* PI-7 samples with and without bacteriophage (Table S4). This meant that 866 genes were uniquely up/down-regulated for SI+Phages samples, higher than the 65 genes uniquely up/down-regulated for SI-alone samples. Among these genes that were unique in SI+Phages samples, there was higher percentage of genes that were downregulated at all time points except at  $t = 10$  h (Figure S9D,E and Table S4).

#### 3.5. Transcriptomic Response Induced Due to Direct Killing Mechanism by Bacteriophages.

Lytic bacteriophages employ direct killing mechanisms on bacterial hosts by traversing through the cell wall structures.<sup>34</sup> Moreover, the cell wall is the first site of damage by sunlight, and our previous work found increased expression in cell-wall component synthesis, cell capsule and extracellular polymeric substances (EPS) that contributed to extended survival of *E. coli* PI-7.<sup>20</sup> Therefore, emphasis was made to compare expression profiles of the genes related to cell-wall structures, cell capsule and EPS (see Table S5). Additionally, the focus was the early time points before  $t = 4$  h due to the main difference in the length of the lag phase between SI+Phages and SI-alone samples and not during the decay phase. Hence, underlying transcriptomic response differences were assumed to take place before  $t = 4$  h.

It was observed that several transmembrane and peripheral membrane proteins were downregulated in the presence of bacteriophages compared to SI-alone. For example, inner membrane transport protein YbaT gene was upregulated to higher fold-change levels in SI-alone, although it was



statistically significant only at  $t = 8$  h, while SI+Phages samples significantly downregulated it at  $t = 1$  and 2 h (Table S5:1.2). The sulfate and thiosulfate import protein CysA gene and a gene for an membrane lipoprotein clustered with the transmembrane ion transporters TehA/TehB were also downregulated in SI+Phages samples at  $t = 1$  and 2 h, and  $t = 4$  h, respectively (Table S5:1.2). Survival protein SurA precursor gene, a chaperone involved in the correct folding and assembly of outer membrane proteins, was downregulated at  $t = 1$  to 6 h in SI+Phages samples (Table S5:1.6). However, the above-mentioned genes and their respective changes in expressions were only observed in two out of the three biological replicates of SI+phage samples sent for transcriptomic profiling. Regardless, multiple components for cell-wall and capsule synthesis (e.g., L,D-transpeptidase that catalyzes the formation of peptidoglycan cross-linkages and outer membrane protein Imp required for envelope biogenesis) were consistently downregulated in the presence of bacteriophages across all three biological replicates of SI+Phages samples (Table S5:5–5.1). The downregulation of these associated genes likely compromised the cell wall structure of *E. coli* PI-7.

**3.6. Transcriptomic Responses Related to *E. coli* PI-7's Susceptibility to Solar Irradiation.** In all three biological replicates of SI+phage samples, solar irradiation upregulated the recA gene that triggers the activation of LexA, as evidenced at  $t = 2$  h (Table S5:2.3) and subsequently activates DNA repair system to repair solar-induced damages. Similar to an earlier study on solar-irradiated *E. coli* PI-7 (without bacteriophage addition),<sup>20</sup> SI-alone samples in this current study upregulated DNA metabolism and repair functions like cytosine-specific DNA methyltransferase, DNA-binding protein HU-beta, Holliday junction DNA helicase RuvB, and DNA-damage-inducible protein D genes (Table S5:2–2.3). In contrast, 52% of the genes associated with DNA repair category were downregulated in at least two out of the three biological sets of SI+Phages samples (compared with 26% in SI-alone). These include methylated-DNA-protein-cysteine methyltransferase, exodeoxyribonuclease VII, formamidopyrimidine-DNA glycosylase, DNA polymerases, and recR (Table S5:2–2.3).

Next, oxidative stress functions were examined as they play an important role in coping with damage from solar irradiation and its associated reactive oxygen species (ROS). SI-alone samples upregulated genes associated with oxidative stress response including iron superoxide dismutase (Table S5:1.5). In SI+Phages samples, 82% of genes associated with oxidative stress response were downregulated. The downregulated genes of this category include catalase, glutaredoxin 2, and glutathione-related genes in all three biological replicates of SI+phage samples (Table S5:1.5). Furthermore, stress-coping mechanism including biofilm formation was also investigated. The biofilm regulator BssR gene was upregulated in both sample types upon solar irradiation (Table S5:4).

Confirmation experiments using RT-qPCR were performed on 23 RNA samples after cDNA synthesis. Results are shown in Supporting Information Figure S10. For 26 out of 36 analyzed sample pairs (dark-irradiated), the results of RT-qPCR were in good agreement with RNA-seq. Most of the contradictions occur in sample pairs where the RNA-seq fold-change was statistically insignificant or was close to 1 (i.e., no fold-change).

## 4. DISCUSSION

Survival of ARB in treated wastewater poses a risk upon release of these effluents to the environment, particularly during wastewater reuse events. Solar irradiation can serve as a low-cost natural biocidal strategy to mitigate this potential concern. Although solar irradiation was demonstrated to reduce the bacterial cell numbers, including ARB, by up to 5-log over a 24 h duration,<sup>15–20</sup> gene expression response analysis revealed that ARB like the NDM-positive and pathogenic strain *E. coli* PI-7 upregulated a large number of stress response and cellular repair functions to enable a subpopulation of its cells to survive solar irradiation.<sup>20</sup> Downregulation of phage shock protein genes as well as upregulation of genes related to phage DNA transfer, phage component synthesis, and phage stability was also observed. Furthermore, the ultraviolet portion of the light spectrum has been demonstrated to induce lytic activity of bacteriophages through the activation of RecA and LexA.<sup>35</sup> These observations suggested that *E. coli* might be more vulnerable to bacteriophage infection under solar irradiation. This work aimed to make use of that phenomenon, hypothesizing that adding in bacteriophages specific to ARB such as *E. coli* PI-7 alongside exposure to sunlight could sensitize the bacterial host to solar irradiation and help achieve a faster die-off.

Before coupling bacteriophages with solar irradiation to mitigate the threat of NDM-positive *E. coli* PI-7, these bacteriophages were assessed for their host specificities. This is to avoid using bacteriophages that would cause undesirable ecological impacts through unintended target activity. All bacteriophages isolated in this study showed lytic activity only against *E. coli* PI-7, suggesting that they would be a suitable biocontrol tool for *E. coli* PI-7, and that natural environmental microbiota would not be affected by the application of these bacteriophages. The bacteriophages were able to slow down *E. coli* PI-7's growth in vitro by at least 4 h and up to 14 h under different temperature and pH, showing that infectivity was maintained at a wide range of environmental conditions. Although the subsequent growth of *E. coli* PI-7 in the presence of its bacteriophages seems counterintuitive, this is a common observation in literature.<sup>36–42</sup> Some authors have attributed this to the development of resistance to phage infection.<sup>39,41</sup> Other interpretations attribute this regrowth to the phage density balance, which cannot maintain effective infectivity when below a certain threshold relative to host density.<sup>40</sup> In this study, we aimed to achieve an MOI of 1 at the start of these experiments. However, cycles of phage infection, cell death, and survival can affect the densities of bacteriophages or bacteria over time. Other explanations may also include host bacteria entering less active physiological states after several hours of incubation, perhaps accompanied by biofilm formation, hence inhibiting phage-induced cell lysis.<sup>43,44</sup>

Next, these bacteriophages were also assessed for their stability in response to solar irradiation by comparing their natural log first order decay kinetics. This was to select for bacteriophages that are best able to remain viable in sufficient abundance to infect the intended bacterial host. Generally, the observed bacteriophage inactivation kinetics were linear, unlike the shouldered inactivation curves observed from *E. coli* in our current and previous solar inactivation studies.<sup>20</sup> These observations are in agreement with what has been observed in other works for viral and bacterial inactivation curves, respectively.<sup>18</sup> Differences in the decay rate constants between



individual bacteriophage isolates were not significant. However, three bacteriophage isolates, P1, P4, and P5a, were selected as the best candidates based on their stability under solar irradiation in terms of their decay rate constant and half-life, and based on their effect in slowing down *E. coli* PI-7's growth under different temperatures and pH in the OD-based experiments. When these three bacteriophages were inoculated with *E. coli* PI-7 and exposed to solar irradiation, the bacteriophages demonstrated a slower decay half-life than their bacterial host. This indicates that the bacteriophages are persisting sufficiently with their hosts and theoretically imposing infectivity on *E. coli* PI-7 throughout the solar irradiation period.

Bacteriophages were able to reduce the length of lag phase shown by the *E. coli* PI-7 before onset of inactivation in PBS and filtered wastewater. The presence of bacteriophages led to faster overall inactivation, which would equate to a lower solar fluence required to inactivate this ARB. On the other hand, the half-life length of *E. coli* PI-7 under solar irradiation was not significantly affected by bacteriophage addition. Bacteriophage infectivity is dependent on the metabolic activity of the bacterial host, and bacteriophage proliferation is also determined by the productivity and abundance of the host population.<sup>45</sup> Therefore, since bacterial numbers began to significantly decrease after the lag phase, it is likely that the bacteriophages' ability to be infectious and effective against *E. coli* PI-7 was reduced by that point. Moreover, decreasing bacteriophage numbers upon solar exposure may have interfered with the ability of bacteriophages to infect *E. coli* PI-7.

Gene expression was analyzed using RNA-seq to elucidate the response of *E. coli* PI-7 under bacteriophage-supplemented solar irradiation. The response at initial time points was dominated by downregulation of gene functions in both SI-alone and SI+Phages experiments. The same was observed in our previous work analyzing *E. coli* PI-7's response to solar irradiation (Table S4), and it appears that bacterial cells may adopt this strategy in response to solar irradiation stress and other types of stress in general.<sup>20,46,47</sup> However, SI+Phages experiments showed a response in higher numbers of total and unique genes at each time point. This different response compared to the SI-alone treatment showed how *E. coli* PI-7 cells attempt to survive the combined treatment by controlling additional functions.

Specific genes and gene categories were therefore further examined to explain the response of *E. coli* PI-7 to combined bacteriophage infection and solar irradiation. In this work, the *recA* gene of *E. coli* PI-7 was upregulated in response to solar irradiation in both SI-alone and SI+Phages experiments. In a similar context, bacteriophages exposed to UV light have been shown to enter into the lytic phase of their cycle and induce bacterial killing mechanisms when activated RecA of the bacterial host interacts with CI repressor of bacteriophages, resulting in cleaved CI that stimulates lytic phase.<sup>48</sup> That is, solar irradiation can trigger bacteriophages to enter into the lytic cycle through *recA*-mediated response to increase damage to *E. coli* PI-7. This further indicates that the combination of solar irradiation along with bacteriophages provided an additive sensitization effect on *E. coli* PI-7 than just solar irradiation alone.

With the cell wall being the first site of solar damage and the barrier for bacteriophage infection, cell wall, cell capsule, and EPS synthesis and transport functions were examined. Our

previous study on solar irradiated *E. coli* PI-7 (without bacteriophages) found upregulation of many cell capsule and EPS functions.<sup>20</sup> In this work, SI+Phages experiments showed downregulation in several cell wall and EPS-related functions in two of the three biological data sets. Earlier studies determined that bacteriophages encode lytic and holin proteins as ways of compromising the cell wall to traverse their viral DNA into the host cytoplasm.<sup>34</sup> No literature is available to suggest that bacteriophages are able to induce downregulation in bacterial host's cell wall functions. It is however inferred here that combined bacteriophage and solar irradiation stress interfered with *E. coli* PI-7's ability to mount defenses, overwhelming them and leading to the downregulation of cell wall functions. This may have compromised *E. coli* PI-7 to bacteriophages by allowing improved penetration of bacteriophages through the weakened physical barriers surrounding the cell.

The gene expression results also suggest that the combined SI+Phages treatment increased the susceptibility of *E. coli* PI-7 toward solar irradiation by downregulating responses in oxidative stress and DNA repair functions, both of which are gene categories implicated in prolonged survival to UV and solar irradiation.<sup>20</sup> Our earlier study found an upregulation of catalases that scavenge hydrogen peroxides in *E. coli* PI-7 exposed to sunlight (without bacteriophage addition), suggesting that hydrogen peroxide may be the predominant reactive oxygen species prevalent during solar irradiation.<sup>20</sup> In SI+Phages experiments in this study, genes related to the synthesis of glutathione and its precursors were downregulated in *E. coli* PI-7 across all three biological replicates. Glutathione also functions to reduce hydrogen peroxide to harmless water molecules,<sup>49</sup> and the downregulation of its related genes in the presence of bacteriophages could have made *E. coli* PI-7 more susceptible to the ROS induced by solar irradiation, explaining shortened persistence under the combined SI+Phage.

Furthermore, the gene associated with methylated-DNA-protein-cysteine-methyl-transferase was downregulated in *E. coli* PI-7 in all three biological replicates of SI+Phage experiments. The methyltransferase is primarily involved in cellular defense against the mutagenic effects of O6-methylguanine and O4-methylthymine (i.e., methylated DN).<sup>50–53</sup> Sunlight has been shown to alter DNA methylation patterns in mammalian systems.<sup>54</sup> Although the same detrimental effect has not been studied in bacterial cells, it is possible that a mutagenic effect was induced due to DNA methylation by sunlight. Moreover, bacteria have been shown to utilize restriction-modification systems to distinguish self- and foreign DNA.<sup>55</sup> They do this by expressing DNA methyltransferases that methylate specific adenine or cytosine residues in their genome and protect the host genome from restriction enzyme cleavage, which may protect them from bacteriophages' genomic materials.<sup>55</sup> Therefore, the reduced ability to transfer the methyl group to a cysteine residue of the methyltransferase enzyme may have contributed to accelerating *E. coli* PI-7's decay in the presence of both bacteriophages and sunlight.

Overall, this study provided the proof-of-concept in utilizing bacteriophages in combination with solar irradiation to hasten the inactivation of *E. coli* PI-7. Specifically, coupling bacteriophages with solar irradiation shortened the time and solar fluence needed to trigger decay of *E. coli* PI-7. Our findings further illustrate the mechanisms involved in responses to bacteriophage-supplemented solar irradiation,

showing how the combined treatment is able to overwhelm *E. coli* PI-7's extended persistence. Sunlight acted as a stressor that can induce the infective bacteriophages to enter into lytic mode, which adds more stress on the bacterial host and leads to cell lysis. Bacteriophage-supplemented *E. coli* PI-7 was also more susceptible to solar irradiation due to impeded gene expression of functions related to defense mechanisms. It is envisioned that bacteriophages can be used in this manner in the last stage of the wastewater treatment process. For example, bacteriophages could be applied in open-air holding tanks of postchlorinated effluent, and subsequently exposed to solar irradiation for approximately 4 h to aid in inactivation of remaining ARB prior to reuse or discharge of treated waters. This short exposure time would not provide sufficient time for the bacterial hosts to develop resistance to bacteriophages since their metabolic activity and proliferation efficiency would be impeded by the presence of sunlight. However, future studies should aim to first demonstrate the efficacy of combining the use of solar disinfection and bacteriophages against an ARB host present within a mixed community. There is also a need to perform in-depth characterization of the bacteriophages in terms of their growth curve, burst sizes, latent and eclipse periods, as well as mutation rates so as to facilitate future application process. The potential of phage-resistance arising over long-term continuous cycles of application should also be determined. Host-range characterization of the bacteriophages in this study showed high specificity to *E. coli* PI-7, which can also mean a possible limitation on the potential efficacy of bacteriophages to infect a multitude of different undesirable bacterial targets. Future research efforts should therefore aim to widen the suite of bacteriophages that are available to better infect concerning emerging contaminants, possibly through exploring the use of polyvalent phages which have broader host ranges, engineered phages designed to target desired bacteria, or mixture of bacteriophages that target a variety of microbial threats.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.est.8b04501](https://doi.org/10.1021/acs.est.8b04501).

Bacteriophage morphology and genome size characterization, instrument irradiance and fluence, sample light absorbance correction, lag-phase concept, RNA-seq read mapping quality, RNA-seq significance criteria, cDNA synthesis, RT-qPCR confirmation experiments, RNA-seq mapping quality for solar inactivation experiments with and without bacteriophages, list of primers used for RT-qPCR, summary of transcriptomic results, irradiance profile of solar simulator, inactivation curve to illustrate lag-phase concept, growth curve analysis results, inactivation of bacteriophages under solar irradiation, bacteriophage characterization using TEM, natural log bacteriophage decay with *E. coli* PI-7 under solar irradiation, natural log inactivation curves of *E. coli* PI-7 with and without bacteriophages under simulated solar irradiation, summary of transcriptomic results, RNA-seq and RT-qPCR fold change for PI-7 with and without bacteriophages (PDF)

Fold-change in responding genes (XLSX)

Wastewater data, absorbance profiles of different water matrices (XLSX)

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### Notes

The authors declare no competing financial interest.

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