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The absence or presence of a lytic coliphage affects the response of *Escherichia coli* to heat-, chlorine- or UV-exposure

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Abstract

Disinfection aims at maximal inactivation of target organisms and the sustainable suppression of their regrowth. Whereas many disinfection efforts achieve efficient inactivation when the effect is measured directly after treatment, there are questions about the sustainability of this effect. One aspect is that the treated bacteria might recover and regain the ability to grow. In an environmental context another question is how amenable surviving bacteria are to predation by omnipresent bacteriophages. Provisional data suggested that bacteria when subjected to sublethal heat stress might develop a phage-resistant phenotype. The result made us wonder about the susceptibility to phage-mediated lysis for bacteria exposed to a gradient of chlorine and UV-LED disinfection strengths. Whereas bacteria exposed to low sublethal chlorine doses still underwent phage-mediated lysis, the critical chlorine Ct of 0.5 mg min per L eliminated this susceptibility and induced phage resistance in the cells that survived treatment. In the case of UV, even the smallest tested dose of 2.8 mJ/cm^2 abolished phage lysis leading to direct regrowth. Results suggest that bacteria surviving disinfection might have higher environmental survival chances directly after treatment compared to non-treated cells. A reason could possibly lie in their compromised metabolism that is essential for phage replication.

Introduction

Whereas heat is undoubtedly one of human's best weapons for food preservation, treatment with chlorine and ultraviolet light in the UV-C range represent two of the most common disinfection procedures for water treatment (Hijnen et al. 2006; Munakata and Kuo 2016). Although the aim of disinfection is to maximally reduce counts of live target organisms, there are (in contrast to sterilization that utilizes harsher killing conditions) always surviving microbes (in a biofilm context referred to as 'persisters'). Factors known to reduce the efficiency of chlorination include the presence of chlorine demand (Haas and Karra 1984), the performance of disinfection at suboptimal pH (White 1999) or cold temperatures (Le Dantec et al. 2002). Factors reducing the efficiency of UV-C disinfection, on the other hand, include insufficient transmissivity of the treated water (Shin et al. 2001), the presence of substances with UV absorbing properties (Templeton et al. 2006) or shielding effects by particulates (Mamane-Gravetz and Linden 2004).

Microbiological assessment of the success of disinfection should focus both at measuring (1) the immediate efficiency directly after treatment and (2) the sustainability of the effect. The first can be determined relatively easily and depends on the chosen treatment conditions, the nature of the treated matrix and the target organism. Much more uncertainty is associated with the latter due to the fact that it needs more than a single diagnostic snapshot at a given time point to provide an answer. The sustainability of disinfection depends both on the innate capacity of the specific organism to repair the induced damage and the impact of disinfection on the organism's ability to survive in the surrounding environment. For bacteria, a critical biological factor consists in their susceptibility to bacteriophages that represent (together with protozoan grazers) the most important group of bacterial predators in real-world conditions (Clokie et al. 2011). Numbers of phages in the environment are estimated to exceed the ones of bacteria by up to 10-fold explaining a strong dynamics of bacterial

populations (Labrie et al. 2010; Brüssow and Hendrix 2002). Bacteria are however not equally susceptible to phage attack in every stage of their life cycle (McGrath and van Sinderen 2007) and phage replication depends on bacterial vitality (Said et al. 2010). In initial experiments we observed that bacteria exposed to sublethal heat stress were less susceptible to phage-mediated lysis. This led to the question whether a phage-resistant phenotype can also originate from other stresses. Chlorine and UV treatment were chosen as they are commonly applied water disinfection procedures and rely on different inactivation mechanisms.

For all three stresses, bacteria were subjected to stress gradients ranging from very mild to lethal. The research question was how amenable bacteria, which were subjected to sublethal disinfection stress, are to phage-mediated lysis (in comparison with controls without phage). Whereas the experiments with heat were produced by monitoring the optical densities of bacterial suspensions over time, experiments with chlorine and UV were performed in more depth with membrane filtration of samples at different time points after disinfection and subsequent phage challenge. *E. coli* and a lytic coliphage were chosen as models. The coliphage was isolated from an environmental brook and induced efficient lysis of its bacterial host within approx. 6 h.

Materials and methods

Bacterial growth conditions

Escherichia coli ATCC 25922 (a reference strain for antimicrobial susceptibility testing) was streaked from glycerol stock onto tryptone soya agar (TSA; CM0131; Oxoid Ltd, Basingstoke, Hampshire, UK) and grown for 24 h at 30 °C. Single colonies were subsequently transferred into 10 mL of dilute (strength: 10 %) tryptone soya broth (TSB; CM1016; Oxoid Ltd, Basingstoke, Hampshire, UK) contained in 50 mL Falcon tubes followed by overnight incubation at 20 °C (for heat experiments) or 25 °C (for chlorine and UV experiments) at 250 rpm in a temperature-controlled mini shaker (cat. number 980151, VWR, USA). Tubes were shaken in a 45^{o} angle. Cell density was measured in a spectrophotometer (JENWAY 6310, Camlab, England, UK) at 600 nm (absorbence A_{600}) and adjusted to an A_{600} = 1.0 by dilution with 10% TSB (equilibrated to room temperature). Aliquots of 1 mL of the density-adjusted bacterial culture were then transferred aseptically into sterile 1.5 mL microcentrifuge tubes and spun at 5,000 g for 5 min. The supernatants were removed carefully using a tip and the cell pellets were resuspended in equal volumes of 0.1 µm filtered mineral water (Evian, Evian-les-basin, France). This washing step was repeated two times to remove organic traces from the culture broth. Bacteria were finally resuspended in phosphate buffered saline (PBS, pH7) for the heat experiment or in filtered mineral water for the chlorine and UV experiment. Whereas the *E. coli* suspension was used undiluted (approximately 10^9 CFU/mL) for the heat experiment, cells were diluted to a final concentration of 10^5 CFU/mL for chlorine and UV disinfection.

Isolation of a lytic phage

A water sample was collected from Chicheley brook at a location receiving treated effluent discharge from the Cranfield University wastewater treatment plant (Cranfield, Bedforshire, UK). The sample was passed through a 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany). 10 mL of the filtered flow through was mixed in a 50 mL Falcon tube with an equal volume of double strength TSB and supplemented with 5 mmol/L $CaCl_2$. The mixture was inoculated with 0.4 mL of an overnight *E. coli* culture (grown at 20 ºC) followed by incubation (30ºC; 250 rpm) until clearance indicated bacteriophages-mediated cell lysis. Chloroform was added to a final concentration of 2% to eliminate remaining intact bacteria followed by centrifugation at 5,000 *g* for 5 min and passage of the supernatant through a 0.22 µm membrane filter (Millex GP, Merck Millipore, Darmstadt, Germany). The phage suspension was serially diluted in sodium magnesium buffer (SM buffer; 100 mmol/L NaCl, 8 mmol/L $MgS0_4.7H_20$, 50 mmol/L Tris-Cl, adjusted to pH 7.5). Aliquots of 100 µl were mixed with 150 µL of log-phase *E. coli* followed by incubation for 10 min to allow adsorption. The phage-bacterial mixture was added to 3 mL of molten 0.7% TSB agar (maintained at 48 ºC), mixed immediately by gentle vortexing and then distributed evenly over TSA agar plates supplemented with $CaCl_2$ to a final concentration of 5 mM. The soft agar was allowed to solidify for 20-30 min and plates were incubated overnight at 30 ºC (or indicated temperature) to allow for plaque formation. The plaque with the largest diameter was transferred into SM buffer and re-suspended. To ensure purity, the soft agar overlay method to obtain new plaques was repeated three times. To obtain a phage stock, isolated plaques were picked using sterile wooden toothpicks to inoculate 5 mL log-phase *E. coli* cultures followed by incubation at 30 ºC with shaking (250 rpm) for 8 h. Lysate from single plaques were treated with chloroform to a final concentration of 2%, mixed and centrifuged at 5,000 g for 5 min. The phages were recovered from the upper phase suspension and passed through a 0.22 µm filter (Millex GP, Merck Millipore, Darmstadt, Germany). Phage titers tended to be > 10^9 PFU/mL and stocks were stored at 4 ºC.

Transmission electron microscopy

For phage preparation, 3 μL of phage suspension (approx. 10^9 PFU/mL) were pipetted on to a negatively glow discharged 10 μm thick C-Flat™ carbon grid (400-mesh) with no dilution and allowed to sit for 1 min. The sample droplet was then partially blotted on Whatman ™ quantitative filter paper, Grade 1. Three μL of 2 % uranyl acetate stain solution was immediately applied. After 1 min of staining, all excess fluid was removed from the grid surface by positioning the filter in an angle and the sample was allowed to air-dry (approx. 3-5 min). The sample on each grid was then imaged in low dose conditions on a Tecnai 10 transmission electron microscope (FEI company, Oregon, USA) operating at 100 keV. Images were taken at 20 K magnification and captured using a Gatan Ultrascan 4000 4k × 4k CCD camera with an ultra-sensitive phosphor scintillator (Gatan, USA) to produce a final pixel sampling of 1.1 nm per pixel.

Heat experiments and monitoring of optical densities

Heat stress was performed by exposing ten aliquots (1 mL each) of *E. coli* suspensions (A_{600} = 1.0, in PBS) to different temperatures (4 °C, 20 °C, 37 °C, 41 °C, 44 °C or 50 °C) for 24 h. Cells were subsequently harvested by centrifugation (5,000 g for 5 min) and re-suspended in 1 mL TSB supplemented with 5 mmol/L $CaCl_2$. The ten aliquots exposed to the same temperature were pooled in 50 mL conical centrifuge tubes (Fisherbrand, Fisher Scientific UK Ltd., Loughborough) to obtain 10 mL suspensions for each temperature. Temperature-exposed bacteria were challenged with phage at a multiplicity of infection (MOI) of 1 by adding 10^{10} phage particles from the high titer phage stock followed by shaking at 250 rpm at 20 °C. Absorbances (600 nm) of 1 mL aliquots of cell suspensions were measured on a TECAN M200 Pro plate reader (Tecan UK Ltd, Reading, UK) in transparent 48 well tissue

culture plates (non-treated, flat bottom, cat. nr. TCP001048; Jet Biofil, Braine I'Alleud, Belgium). Readings were taken directly after addition of phage and after 6, 12, and 24 h. The experiment was repeated three times.

Disinfection by chlorination

A chlorine solution was prepared by diluting 95 µL of sodium hypochlorite stock solution (10-15% available chlorine, Sigma-Aldrich, USA) in 50 mL of ultrapure water of 100 mL volume flask to a concentration of approximately 200 mg/L free chlorine. Volumes of 0.5, 1.25, 5, 12.5 and 25 µL of this chlorine solution were added to 50 mL aliquots of bacterial suspensions (10^5 CFU/mL in 0.22 µm filtered Evian mineral) and stirred at 150 rpm in chlorine demand-free beakers to obtain the following final free chlorine concentrations: 0.002, 0.005, 0.02, 0.05 and 0.1 mg/L. Cells were exposed to chlorine at room temperature (20 °C) for 10 min translating to a Ct value range between 0.02 and 1 mg min per L(free chlorine concentration x time). Chlorine disinfection was stopped by addition of 250 µL of 0.1 mol/L sodium thiosulphate ($Na_2S_2O_3.5H_2O$) (Acros Organics, Geel, Belgium). A bacterial suspension without added chlorine served as a control. After letting the samples stand for 10 min, TSB and $CaCl_2$ were added to final concentrations of 10% TSB and 5 mmol/L $CaCl_2$, respectively, to provide nutrients for the bacteria and to enable phage adsorption. To one series of samples, a small volume of concentrated phage stock was added to obtain a phage concentration of 10^5 PFU/mL (corresponding to a multiplicity of infection (MOI) of 1. For time point t = 0, samples were membrane-filtered directly after phage addition, no time for phage adsorption was provided. An identical series of bacterial suspensions was left without phage addition with one aliquot also being membrane-filtered at t=0 for colony enumeration. Suspensions (with and without phage) were shaken on an orbital shaker (250 rpm) at 20 °C for 4 days in total and colonies enumerated by membrane filtration after 6, 12, 24, 48, 72 and

96 h. All experiments were performed with chlorine demand-free glassware prepared according to Charnock and Kjønnø (2000) and carried out as three independent repeats.

Disinfection by LED-UV

UV disinfection was performed at room temperature (20 °C) using a UV-C-LED system consisting of a UVCLEAN lamp with multi-chip collections of UV LEDs from Sensor Electronic Technology (Columbia, South Carolina, USA). LEDs emitted UV light at 260 nm with a power output of 15 mW. Samples were placed at a distance of 1 cm to the UV source. The delivered dose was verified using a UV-C radiometer (VLX-3W from Vilber Lourmat) and determined to be 20 W/m^2. Bacterial suspensions (10^5 CFU/mL in filtered Evian water) were transferred in 20 mL aliquots into a 25 mL Pyrex petri dishes (Fisher Scientific, UK). During UV exposure suspensions were stirred constantly using a magnetic stir bar on a stirrer set to 400 rpm. Bacteria were exposed to the following UV doses: 2.8, 4.8, 10.5, 15.4, 20.3, and 39.9 mJ/cm^2. A bacterial suspension that was treated identically, but not exposed to UV served as a control. Following UV exposure, suspensions were supplemented with TSB and $CaCl_2$ to final concentrations of 10% and 5 mM $CaCl_2$, respectively. To one series of samples phages were added to achieve a multiplicity of infection (MOI) of 1. For time point t = 0 samples were membrane-filtered directly after phage addition, no time for phage adsorption was provided. An identical series of bacterial suspensions was left without phage addition with one aliquot also being membrane-filtered at t=0 for colony enumeration. Suspensions (with and without phage) were shaken at 250 rpm at 20 °C for 4 days in a laboratory incubator with a transparent lid receiving only normal laboratory light during the day. Colonies were enumerated by membrane filtration after 6, 12, 24, 48, 72 and 96 h. Experiments were carried out in three independent repeats with standard deviations being slightly greater than for the chlorine experiment.

Bacterial quantification by plate counting and flow cytometry

For enumeration of *E. coli*, the membrane filtration method was used. Filtered mineral water (~ 3 mL) was poured into the filter manifold (Combisart, Sartorius, UK) prior to addition of 100 µL of sample (10^5 CFU/mL) to ensure homogeneous distribution of bacteria on the filter. Samples were filtered onto 0.45 µm cellulose filters (47 mm, white gridded, Cat. No. HAWG047S6 Fisher Scientific, UK) and placed on 55 mm Petri dishes with membrane lactose glucuronide agar (MLGA: Oxoid, Fisher Scienitic, UK). MLGA gives rise to green *E. coli* colonies. Plates were incubated at 35 °C for 24 h before enumeration. Flow cytometry was performed as described previously (Nocker et al. 2017). Bacterial were stained with SYBR Green I and propidium iodide with final concentrations of 1 x and 3 µmol/L.

Results

E. coli was subjected to gradients of heat, chlorine or UV of increasing intensities with aliquots not undergoing treatment serving as controls. Bacteria were subsequently challenged with a lytic coliphage or not. A phage was isolated for this purpose from a brook receiving treated wastewater discharge. The phage was identified by transmission electron microscopy to belong to the group of Myoviridae, which have double-stranded DNA and contractile tails (Fig. 1A). The kinetics of the phage to lyse *E. coli* at a multiplicity of infection (MOI) of 1.0 was measured by flow cytometry. Time to maximal lysis at 20 ˚C was around 6 h under given conditions (Fig. 1B).

Fate of *E. coli* after heat stress

In an initial experiment we examined the effect of temperature on fully grown bacteria in regard to their susceptibility to phage-mediated lysis. *E. coli* was grown at 20 ˚C and subsequently suspended in physiological salt to prevent cell replication. Bacterial suspensions were exposed to either 4 ˚C, 20 ˚C, 37 ˚C, 41 ˚C, 44 ˚C or 50 ˚C for 24 h prior to phage challenge at 20 ˚C (in the presence of nutrients). Bacteria exposed to temperatures ≤ 37 ˚C were all found to be susceptible to phage lysis reflected by a decrease in A_{600} (Fig. 2A). Bacteria exposed to heat (41 and 44 ˚C) on the other hand were not lysed and the A_{600} slightly increased suggesting the presence of viable bacteria (especially when exposed to 41 °C). Exposure to 50 ˚C, on the other hand, can be considered lethal as no change in optical density was observed and no colonies were obtained after this treatment.

To unequivocally assign the temperature effect to phage susceptibility, bacterial aliquots with no added phage served as controls (Fig. 2B). Optical densities of bacteria exposed to 4, 20 or 37 ˚C increased rapidly suggesting healthy bacteria. Also optical densities of bacteria exposed to 41 and 44 ˚C showed an increase (although slow and moderate)

indicating that these aliquots contained viable cells and that bacteria have the ability to survive exposure to these temperatures. The slow increase was probably caused either by a longer lag time due to stress exposure or by the reduced number of viable cells in the bacterial population. Only aliquots exposed to 50 ˚C showed a straight line suggesting that this temperature was lethal.

The described trends are reflected in a slope analysis indicating the rates of change in optical densities (Fig. 2A and B). In case of bacterial suspensions exposed to 4, 20, 30 ˚C prior phage challenge, previously positive slopes (as seen in the control without phage) are turned negative by phage addition resulting in lysis and consequently a drop in optical densities. For *E. coli* suspensions treated at 41 and 44 ˚C, on the other hand, slopes were slightly positive in all samples independent of whether a phage was added or not. Obviously exposure to these temperatures prevented overall lysis and a dip in A_{600}. Although interesting in itself, the question which remained unsolved from the experiment was the distribution of viability states within the bacterial population. Only a small portion of the *E. coli* population might still have remained viable and been amenable to lysis. As measurement of optical density has limited sensitivity and does not reflect changes to a small proportion of bacteria, colony counting was applied for subsequent experiments.

Fate of *E. coli* after chlorine disinfection

Bacteria were exposed to increasing free chlorine doses up to 1 mg min per L (followed by addition of thiosulphate to neutralize the disinfectant). The increasing damage to the cell membrane inflicted by increasing chlorine doses was visualized by flow cytometry in combination with membrane integrity staining. Bacterial membrane damage started to be visible in dot blots in samples exposed to 0.5 mg min per L and bacteria in samples exposed to 1 mg min per L were readily stained with propidium iodide indicating substantial membrane damage (data not shown). After chlorine exposure bacteria were either mixed with

the lytic bacteriophage or not. The ability of the cells to form colonies was assessed over 4 days at time points 0, 6, 12, 24, 48, 72 and 96 h. Using selective MLGA agar, *E. coli* appear as green colonies or, in case of many bacteria, as a green lawn (supplementary Fig. 1S). Directly after disinfection (time point zero), strong growth was detected for bacterial suspensions exposed to up to 0.2 mg min per L free chlorine meaning that these doses had little effect on culturability. Exposure to 0.5 mg min per L chlorine on the other hand led to a substantial decrease in colonies on the filters (although bacterial membrane damage was only moderate as assessed by flow cytometry). Higher chlorine doses completely suppressed growth. This effect at time point zero was independent of whether phage was added or not. At later time points differences between the two sample series became visible.

The change in colony numbers within the first 6 h (representing the time to maximal lysis) is graphically shown in Fig. 3. In the absence of phage, the number of colonies on filters increased over time for all samples where growth was seen directly after disinfection. This is reflected in a positive log change in colony numbers within the first 6 h. For bacteria exposed to the highest chlorine dose (1 mg min per L), the chlorine effect was sustained with no colonies detected even after 96 h. In case, a phage was added directly after disinfection, the outcome was very different. Bacterial densities of samples exposed to chlorine doses up to 0.2 mg min per L were visibly reduced after 6 h with the log changes in CFU being negative for those samples (Fig. 3). The reduction in bacterial colonies was strongest for aliquots with no chlorine, however lytic activity was observed up to chlorine doses of 0.2 mg min per L. For bacterial suspensions exposed to 0.5 mg min per L chlorine, on the other hand, colony numbers did not decrease, but rather an increase in colonies was observed. Bacterial numbers on these filters were comparable to the ones in the corresponding sample where no phage had been added (Fig. 1S) and the log change in CFU within the first 6 h was identical for the two experimental series (independent of whether a phage was added or not; Fig. 3). The chlorine dose of to 0.5 mg min per L therefore seemed to erase the difference

between samples with and without phage, probably because such treated bacteria were not lysed by the phage. In other words, the phage appeared unable to lyse *E. coli* that were exposed to a chlorine dose of 0.5 mg min per L. No phage effect could be deducted for the highest chlorine dose (1 mg min per L) as this treatment sustainably eliminated all *E. coli* growth, independent of the presence of phage.

Fate of *E. coli* after UV disinfection

E. coli suspensions were exposed to different UV-C doses up to approx. 40 mJ/cm^2 (which is the minimal dose that has be applied for disinfection of drinking water in a number of countries; DVGW 2006). In contrast to chlorine, the killing by UV left bacterial membranes intact as assessed by flow cytometry (data not shown). As for the chlorine experiment, bacteria were subsequently either mixed with the lytic bacteriophage or not. Bacterial densities on filters at time point zero (directly after disinfection) progressively decreased with increasing UV doses (both for samples where phage was absent or present; supplementary Fig. 2S). No colonies were observed in the sample experiencing the highest radiation. Like for chlorine disinfection, monitoring was performed over 4 consecutive days with samples being membrane-filtered and cultured after 0, 6, 12, 24, 48, 72 and 96 h. Also in line with chlorination, the change in colony numbers within the first 6 h was numerically assessed (Fig. 4).

In the absence of a phage, colony numbers on filters increased over time for all samples. This is reflected in a positive log change in colony numbers within the first 6 h (Fig. 4). The higher the applied UV dose, the longer regrowth was delayed translating into decreasing log changes in CFU within the first 6 h. After 12 h, regrowth was also observed for the sample exposed to the highest dose and that did not show any growth directly after UV disinfection (supplementary Fig. 2S). The reappearance of colonies might be due to

repair of UV damage over time. Another possibility is the replication of bacteria that survived UV treatment (e.g. due to shading effects during stirring).

The same outcome applied to all samples where a phage had been added after UV disinfection with the non-irradiated control being the only exception. Whereas all bacterial suspensions undergoing UV treatment showed increasing colony numbers and positive log changes in CFU within the first 6 h (as in the samples without phage), only the non-irradiated control showed a decrease in bacterial numbers in the first h after phage addition by > 3 log units (Fig. 4). It seemed that only those bacteria that were not exposed to UV were lysed by the phage, whereas even low UV doses efficiently prevented lysis. The fact that the dose of 2.8 mJ/cm^2 extinguished the phage effect suggests that UV exposure inflicted changes to the bacteria that prevented phage-mediated lysis, but did not suppress growth.

Discussion

We investigated in this study the sustainability of the effect of different disinfection intensities on the culturability of *E. coli* in the absence and presence of a lytic bacteriophage that exerted maximal lytic activity within several hours. Apart from the universally relevant thermal disinfection, chlorine and UV were chosen not only because they are frequently applied in water treatment, but also because they differ in their mechanisms of action. Whereas wet heat is assumed to kill primarily through inducing enzyme inactivation and protein denaturation (Mackey et al. 1991), chlorine as an unspecific oxidant inflicts damage to any cellular component it reacts with (including the cell envelope, nucleic acids and enzymes (Camper and McFeters 1979; du Preez et al. 1995). UV on the other hand inactivates microbes by damaging their nucleic acids (Cutler and Zimmerman 2011).

Independent of phage addition, results of the disinfection experiments demonstrated differences in regard to the sustainabilities of the different treatments when comparing conditions that abolished growth directly after disinfection. No regrowth was measured in aliquots subjected to 50 ˚C or a chlorine dose of 1 mg min per L, whereas a UV dose of 40 mJ/cm^2 (the minimal UV dose recommended for disinfecting drinking water in a number of countries) did not prevent re-appearance of colonies after 12 h. Full regrowth was obtained after 72-96 h. It should be noted in this context that all samples were supplemented with nutrient broth after disinfection meaning that conditions allowed for rapid growth. This observation is in line with a study from Kollu and Örmeci (2014) reporting regrowth of *E. coli* after UV irradiation at 40 mJ/cm^2**.** No conclusion was however possible on the origin of the regrowing *E. coli*. It is not clear to date, whether the majority of regrowth comes from disinfection survivors (e.g. through shading events) or from recovery after repair. Both scenarios have support in the literature **(**Bohrerova et al. 2014; Hu et al. 2005). It should be noted however that a relatively large volume (1 mL, representing 5% of the total volume) was

filtered directly after UV disinfection. As no single colony appeared directly after UV disinfection, repair appears to be more likely.

The main focus of this study was however the investigation of the susceptibility of stressed bacteria to lytic phages. A typical reason for the loss in susceptibility to bacteriophage attack is the destruction of surface antigens on the bacterial envelope and the resulting inability of phages to adsorb (Abedon 2012). This possibility could especially apply to bacteria exposed to heat or chlorine with their effects on surface proteins through denaturation or oxidative damage. In the case of UV exposure, the destruction of surface antigens appears less likely as the integrity of the membrane was not compromised. A more probable hypothesis might be that the biosynthetic and metabolic capabilities of the UV-irradiated bacteria are affected to an extent where phage replication is no longer supported within the host cell. The overall viability and vitality of the bacterial host can be seen as an important determinant for the success of phage propagation. For *E. coli* 0157:H7, Awais et al. (2006) reported an elegant recombinant phage-based assay that allowed visualization of infected bacteria that supported phage proliferation. The phage was devoid of its lytic capability, but induced the expression of green fluorescence within host cells. It was shown with this assay that only culturable cells allowed phage replication, whereas viable but non-culturable (VBNC; produced by starvation at 4 °C) and dead cells merely allowed phage adsorption, but not biosynthesis. The observation was employed later in an assay to detect VBNC cells by Said et al. (2010). UV-exposed *E. coli* that had lost culturability were reported to allow adsorption, but did not support lysis. Further support came from subsequent results by Fernandes et al. (2014) for *Salmonella enteriditis*. When applying a phage-based system to assess the viability of the bacteria, VBNC cells (interestingly produced by treatment with a low chlorine dose) were reported to be recognized by the phage, but no lysis was induced. A different underlying mechanism was suggested for heat-treated cells that

were recognized by the phage only to a minor extent, possibly as phage receptors on the cell envelope had been destroyed by the heat.

In broad consent with these authors, our study corroborates that lytic phages can sense the viability or vitality of their bacterial hosts, which consequently determines the bacteria's fate. It is well known that viral proliferation requires a 'significant commitment of host resources to reproduce viral particles' (Maynard et al. 2011). The lytic success is thus dependent on the existence of a functional and energized metabolic machinery capable of production of nucleic acids, proteins and lipids that represent the building blocks of phage particles. A study using a marine bacterium model estimated that ~75% of carbon and nitrogen resources were redirected into the production of virions (Ankrah et al. 2014). Other researchers reported a strong depletion of bacterial transcripts upon phage infection (Chevallereau et al. 2016) and a strong perturbation to the interconnected network of metabolic pathways within the bacterial host (Birch et al. 2012). It is well conceivable that the metabolism of injured bacteria with compromised cellular functions might not be able to support this metabolic burden and therefore not undergo lysis. This does not answer on the other hand, how these bacteria would recover over time and why they would not be amenable at a later stage to phage attack. Instead of working with bacterial populations with undefined distribution of bacteria in different viability states, future experiments would profit from single cell techniques. It also will need to be shown whether the observations hold true for other lytic phages. Despite many open questions, results presented here suggest that sublethally stressed bacteria that survive mild disinfection conditions might show less susceptibility to lytic phages. For a limited time window, such bacteria might in consequence have an increased survival chance in the environment where phages are one of the major causes of bacterial mortality.

In conclusion, the role of bacteriophages in influencing the bacteria's fate after disinfection is typically an overlooked parameter. We report here in a laboratory-based study the possibility that stresses with different modes of action can modulate the bacteria's susceptibility to phage-mediated lysis. Although more bacteria-phage interactions need to be studies for generalization, sublethal disinfection could potentially rescue bacteria from phage-mediated lysis. The stress intensity threshold where such resistance is observed appears different between different stresses. We hypothesize that injury by mild disinfection might compromise the bacterial metabolic and biosynthetic machinery to an extent that it does not meet the high demand for phage replication.

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Figure legends

Fig. 1 Electron micrographic (EM) picture of bacteriophage isolated from wastewater effluent discharge and kinetics of bacterial lysis. (A) Phage with prolate head belonging to the group of Myoviridae, which are double-stranded DNA phages with contractile tails. Dimensions of the phage are shown in nm. (B) Kinetics of *E. coli* lysis as measured by flow cytometry. Changes in intact cell concentrations are shown for a bacterial suspension with the phage added at a multiplicity of infection (MOI) of 1. A bacterial suspension without phage served as a control. Error bars represent standard deviations from three independent repeats.

Fig. 2 Effect of temperature history of fully grown *E. coli* on susceptibility to phage lysis (A) in comparison with control without phage (B) and corresponding rates of change of A_{600} (slope analyses). Bacteria (grown at 20 ˚C; A_{600} = 1) were suspended in PBS and exposed to different temperatures (4, 20, 37, 41, 44 or 50 ˚C) for 24 h prior to resuspension in TSB and phage challenge at 20˚C. Changes in optical densities were monitored using a microplate reader. Error bars show standard deviations from three independent experiments. Rates of change in A_{600} of *E. coli* were determined by slope analysis and refer to 6 h after addition of phage as well as for the control without phage.

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Fig. 3 Change in colony forming units (CFUs) of *E.coli* on MLGA agar within 6 h after exposure to different chlorine disinfection strengths and absence of presence of a

bacteriophage. Samples without chlorine addition served as controls. Error bars show standard deviations from three independent repeats.

Fig. 4 Change in colony forming units (CFUs) of *E.coli* on MLGA agar within 6 h after exposure to different UV doses and absence of presence of a bacteriophage. Samples without UV irradiation served as controls. Error bars show standard deviations from three independent repeats.

Supplementary Fig. S1 Effect of increasing chlorine disinfection strengths on the culturability of *E.coli* in the absence or presence of a lytic bacteriophage over a time course of 96 h. Phage (if applicable) was added directly after disinfection and dechlorination at an MOI = 1. Samples were taken after indicated times and bacteria were collected by membrane filtration. Filters were placed on MLGA agar, where *E. coli* forms green colonies. Plates were incubated at 35 °C for 24 h. Samples without chlorine addition served as controls. Representative results from three independent repeats are shown.

Supplementary Fig. S2 Effect of increasing UV irradiation doses on the culturability of *E.coli* in the absence or presence of a lytic bacteriophage over a time course of 96 h. Phage (if applicable) was added directly after disinfection at an MOI = 1. Samples were taken after indicated times and bacteria were collected by membrane filtration. Filters were placed on MLGA agar, where *E. coli* forms green colonies. Plates were incubated at 35 °C for 24 h. Samples without UV irradiation served as controls. Representative results from three independent repeats are shown.

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Figures

Figure 1

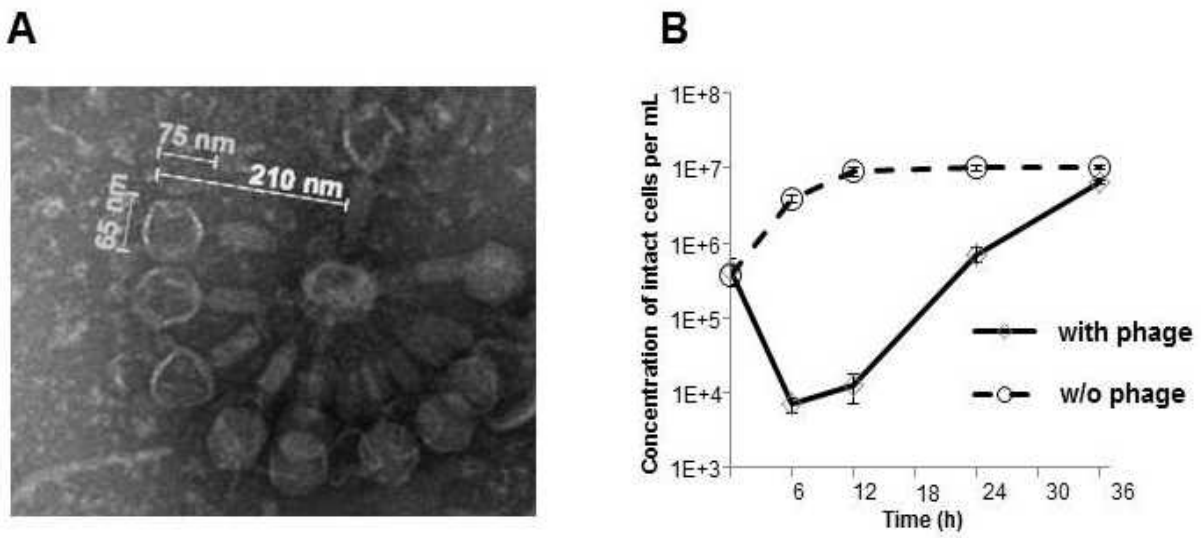


Fig. 1 Electron micrographic (EM) picture of bacteriophage isolated from wastewater effluent discharge and kinetics of bacterial lysis. (A) Phage with prolate head belonging to the group of Myoviridae, which are double-stranded DNA phages with contractile tails. Dimensions of the phage are shown in nm. (B) Kinetics of *E. coli* lysis as measured by flow cytometry. Changes in intact cell concentrations are shown for a bacterial suspension with the phage added at a multiplicity of infection (MOI) of 1. A bacterial suspension without phage served as a control. Error bars represent standard deviations from three independent repeats.

Figure 2

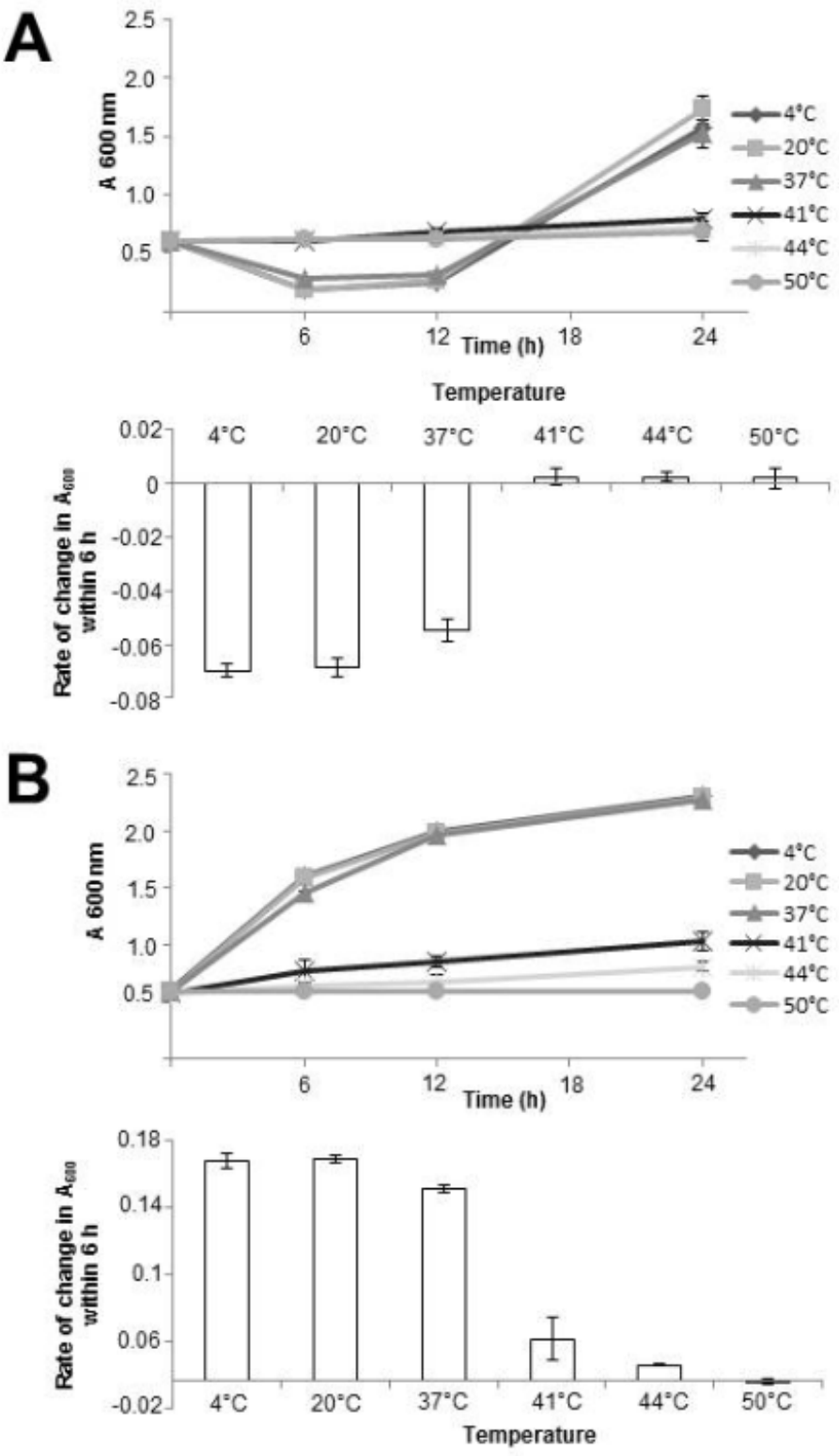


Fig. 2 Effect of temperature history of fully grown *E. coli* on susceptibility to phage lysis (A) in comparison with control without phage (B) and corresponding rates of change of A_{600} (slope analyses). Bacteria (grown at 20 °C; A_{600} = 1) were suspended in PBS and exposed to different temperatures (4, 20, 37, 41, 44 or 50 °C) for 24 h prior to resuspension in TSB and phage challenge at 20°C. Changes in optical densities were monitored using a microplate reader. Error bars show standard deviations from three independent experiments. Rates of change in A_{600} of *E. coli* were determined by slope analysis and refer to 6 h after addition of phage as well as for the control without phage.

Figure 3

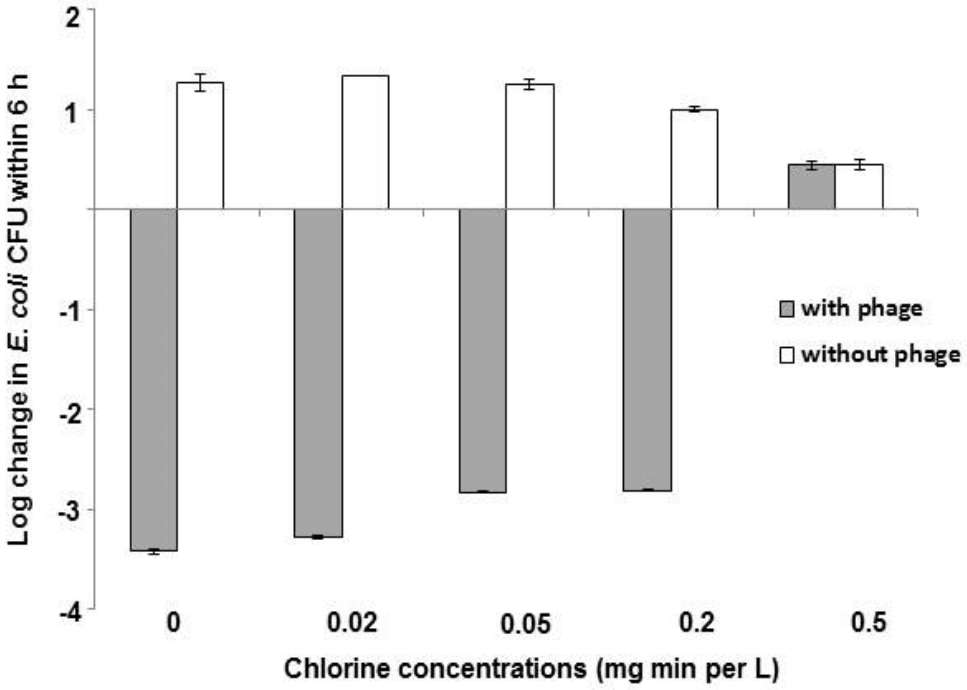


Figure 3. Change in colony forming units (CFUs) of *E.coli* on MLGA agar within 6 h after exposure to different chlorine disinfection strengths and absence of presence of a bacteriophage. Samples without chlorine addition served as controls. Error bars show standard deviations from three independent repeats.

Figure 4

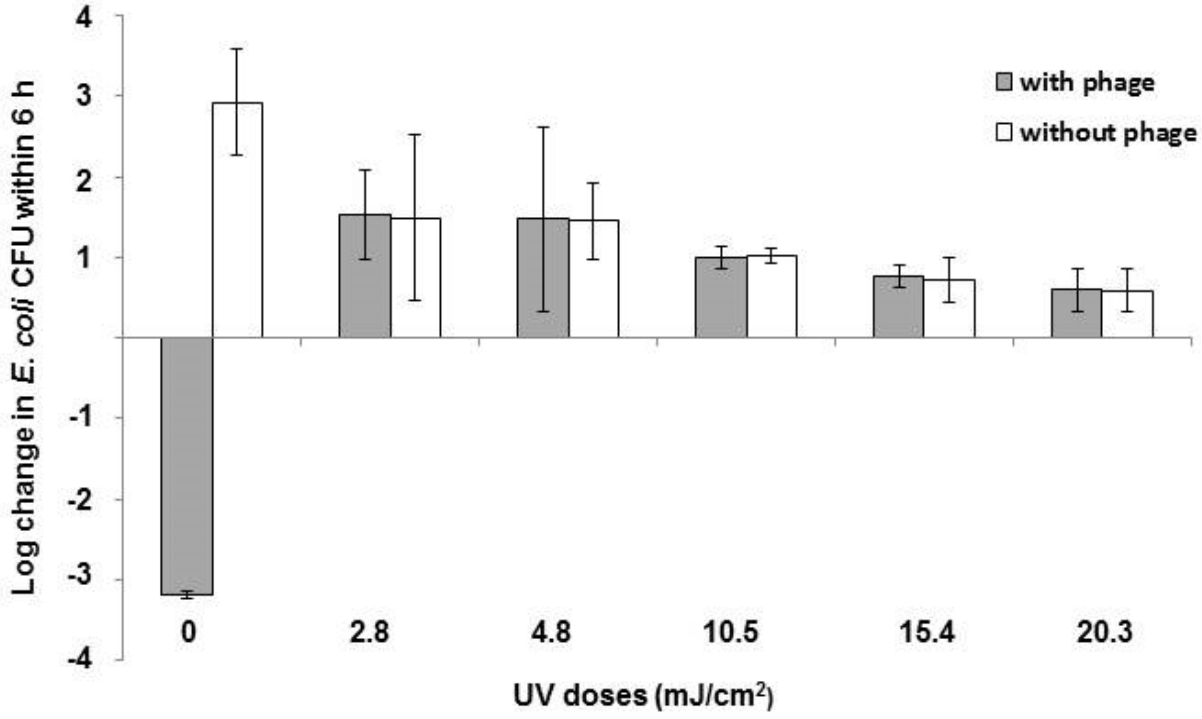
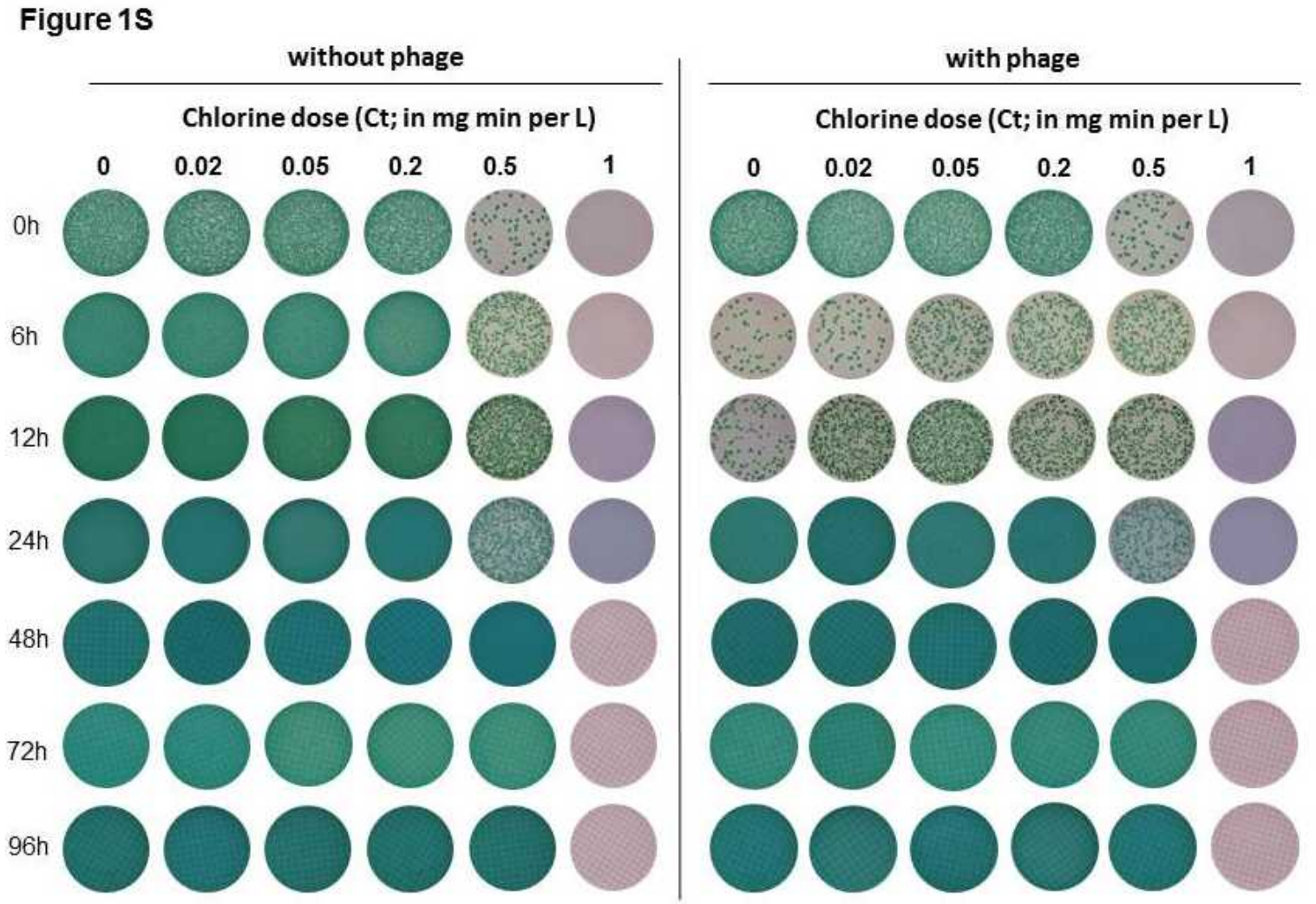


Figure 4. Change in colony forming units (CFUs) of *E.coli* on MLGA agar within 6 h after exposure to different UV doses and absence of presence of a bacteriophage. Samples without UV irradiation served as controls. Error bars show standard deviations from three independent repeats.

Supplementary Figures



Supplementary Fig. S1 Effect of increasing chlorine disinfection strengths on the culturability of *E.coli* in the absence or presence of a lytic bacteriophage over a time course of 96 h. Phage (if applicable) was added directly after disinfection and dechlorination at an MOI = 1. Samples were taken after indicated times and bacteria were collected by membrane filtration. Filters were placed on MLGA agar, where *E. coli* forms green colonies. Plates were incubated at 35 °C for 24 h. Samples without chlorine addition served as controls. Representative results from three independent repeats are shown.

Figure 2S

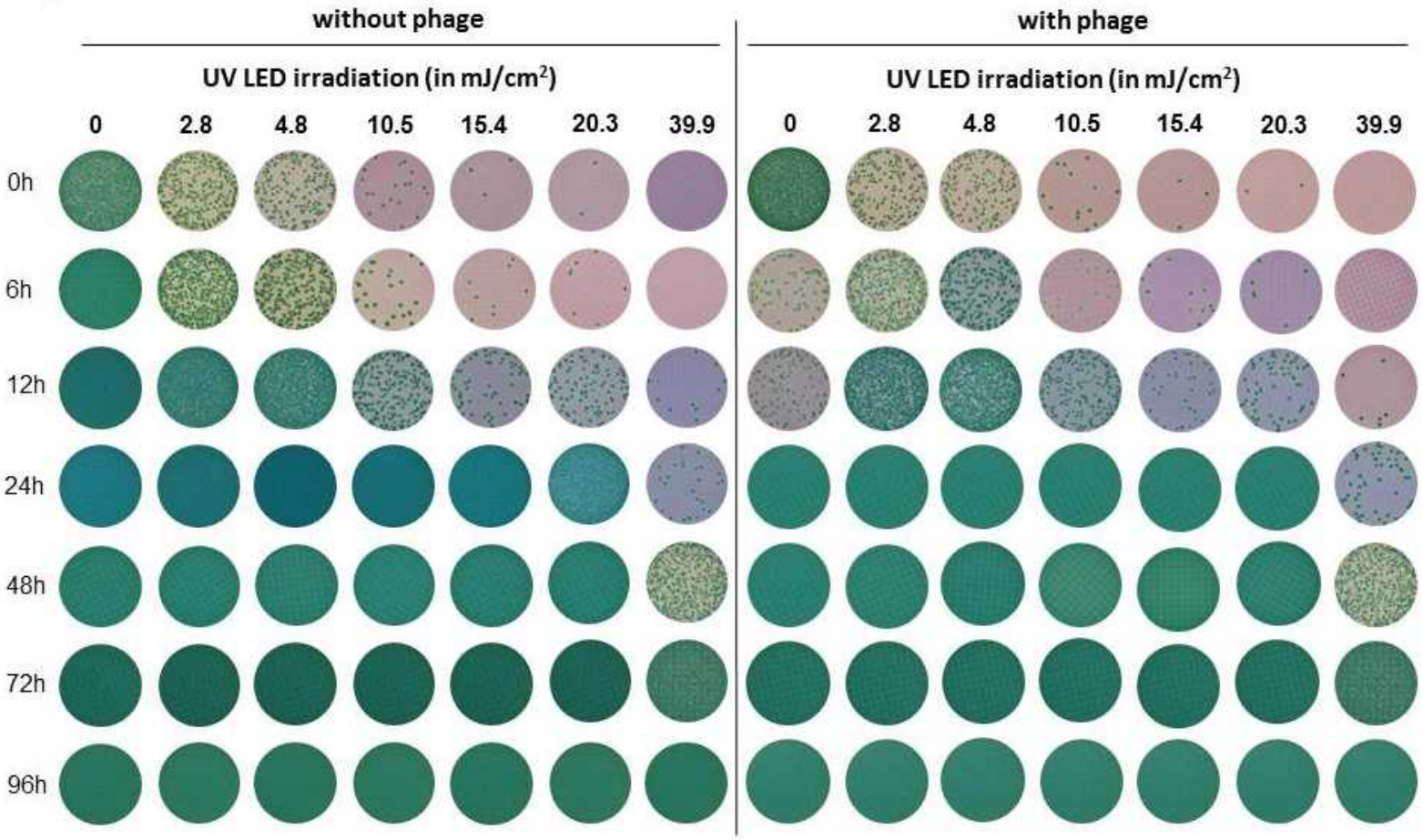


Figure 2S. Effect of increasing UV irradiation doses on the culturability of *E.coli* in the absence or presence of a lytic bacteriophage over a time course of 96 h. Phage (if applicable) was added directly after disinfection at an MOI = 1. Samples were taken after indicated times and bacteria were collected by membrane filtration. Filters were placed on MLGA agar, where *E. coli* forms green colonies. Plates were incubated at 35°C for 24 h. Samples without UV irradiation served as controls. Representative results from three independent repeats are shown.