Response to Comment on "UV Disinfection Induces a VBNC State in Escherichia coli and Pseudomonas aeruginosa "

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UV Disinfection Induces a Vbnc State in *Escherichia coli* and *Pseudomonas aeruginosa*

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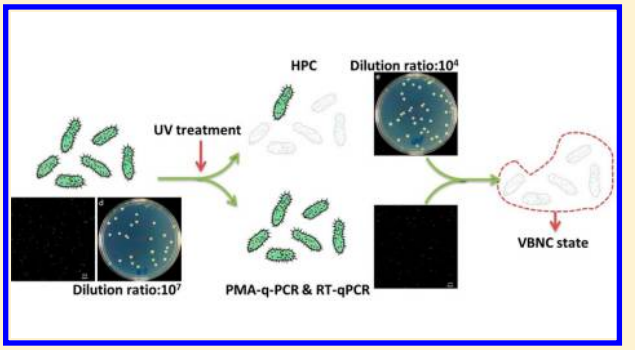
Supporting Information

ABSTRACT: The occurrence of a viable but nonculturable (VBNC) state in bacteria may dramatically underestimate the health risks associated with drinking water. Therefore, the potential for UV treatment to induce a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa* was investigated. UV disinfection effectively reduced the culturability of *E. coli* and *P. aeruginosa*, with the destruction of nucleic acids demonstrated using *gad*A long gene fragment qPCR amplification. Following UV radiation, copy numbers for the high transcriptional levels of the 16S rRNA gene varied insignificantly in both strains, confirming results from plate counting assays indicating that VBNC states were induced in both strains. Furthermore, the virulence genes *gadA* and *oprL* remained highly expressed, suggesting that the VBNC bacteria still displayed pathogenicity. Propidium monoazide qPCR indicated that cell membranes remained intact even at a UV dose of 300 mJ/cm^2. The RT-qPCR results after UV and chlorine treatments in *E. coli* were significantly different (8.41 and 5.59 log units, respectively), further confirming the induction of VBNC bacteria induced by UV radiation. Finally, resuscitation was achieved, with *E. coli* showing greater resuscitation ability than *P. aeruginosa*. These results systematically revealed the potential health risks of UV disinfection and strongly suggest a combined disinfection strategy.



INTRODUCTION

Effective disinfection technologies are essential for safe drinking water. Thus, strict criteria on total bacteria and/or waterborne pathogens have been issued by various countries and international organizations.[1,2] All of these criteria apply plate counting methods based on culturing the bacteria in the water samples for quantification. The rationale behind the use of these methods is that the viable bacteria are culturable, and the culturability of the bacteria represents their viability. However, Xu et al.[3] was the first to report that under various stresses many, even most, environmental bacteria can enter a viable but nonculturable (VBNC) state. Bacteria in the VBNC state fail to form colonies under suitable environmental stresses.[4] However, these cells remain alive and are capable of renascent metabolic activity under the appropriate stimulation.[5] Many emerging waterborne pathogens in drinking water reportedly enter a VBNC state, including *Helicobacter pylori*,[6] *Legionella pneumophila*,[7] *Pseudomonas aeruginosa*,[8] *Listeria monocytogenes*,[9] *Vibrio* spp.,[10,11] and *Yersinia enterocolitica*.[12] A public health concern regarding bacteria in the VBNC state in the drinking water system has been raised because the occurrence of these VBNC bacteria would obviously underestimate total bacterial cell counts.

Many factors induce bacteria into a VBNC state, such as adverse nutrition levels,[13] temperatures,[3] and osmotic pressures.[14] Disinfectants can become chemical inducers of the VBNC state. For example, chlorine disinfection in secondary-treated wastewater has been shown to induce *Escherichia coli* and *Salmonella typhimurium* into a VBNC state.[14] Turetgen reported that an environmental isolate of *L. pneumophila* more easily enter into a VBNC state than did a subculture of this strain at 2 ppm of monochloramine after 24 h.[15] In addition, ultraviolet (UV) disinfection[16] and ozone[17] also reportedly induce bacteria into a VBNC state. The physiological and biochemical properties of bacteria can change when they are in a VBNC state. For example, Signoretto et al.[18] reported that a 3-fold increase in particular DAP-DAP cross-linking and an increase in mucopeptides bearing a covalently bound lipoprotein were discovered while studying the cell wall peptidoglycan of *E. coli* induced by UV−C to enter the VBNC state.

Increasingly more attention has been focused on UV radiation as an emerging disinfection technology because it is more effective against oocysts and cysts of *Cryptosporidium* and *Giardia* than either chlorination or ozonation and without disinfection byproducts produced.[19] In Europe, UV radiation has been applied since the 1980s for the disinfection of drinking and reclaimed water to reduce heterotrophic plate counts.[20,21] The germicidal mechanism of UV radiation differs from that of

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Table 1. Target Genes and Primers Used in This Study

species gene	target primers	primers	primer sequence	product size	reference
E.coli	16S	341F/534R	CCTACGGGAGGCAGCAG/ATTACCGCGGCTGCTGG	194	24
E.coli	16S	27F/1492R	AGAGTTTGATCCTGGCTCAG/TACGGCTACCTTGTTACGACTT	1465	25
E.coli	*gadA*	GAD1/GAD2	GGTGATGCGCATTATGTGTC/CGGGTGATCGCTGAGATATT	100	26
E.coli	*gadA*	GADL1/GADL2	GGTTCTTCCGAGGCCTGTAT/CATAATGCGCATCACCACGA	900	26
P.aeruginosa	*oprL*	OPR1/OPR2	GACGTACACGCGAAAGACCT/GCCCAGAGCCATGTTGTACT	99	27

[a]Annealing temperature all were 60 °C.

conventional disinfection chemicals. The latter, including chlorine, chloramine, chlorine dioxide, and ozone, generally oxidize the bacterial cell and break the cellular membranes of a large portion of the killed cells. By contrast, UV disinfection generally maintains cellular integrity because it targets nucleic acids inside the cells. However, to date, only a few publications have examined whether UV disinfection induces bacteria into the VBNC state, and the answers to some important questions remain unclear, including the effect of various UV dosages, the characterization of the VBNC state induced, and the activity of bacteria, especially pathogenic bacteria, in this state.

Several studies have demonstrated that bacteria in a VBNC state may resuscitate under suitable environments. For example, *Vibro vulnificus* was reactivated from a low-temperature induced VBNC state through a temperature upshift[22] and *L. pneumophila* demonstrated resuscitation properties by the addition of *Acanthamoeba castellanii* to the microcosm.[23] However, it is currently unknown whether bacteria induced into a VBNC state by UV disinfection resuscitate and maintain their physiological characteristics. This question warrants study because of the importance of culturable bacteria to safe drinking water.

Thus, we employed several methods, including heterotrophic plate counts (HPC), reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), and propidium monoazide quantitative polymerase chain reaction (PMA-qPCR), to examine how UV disinfection affects the induction and recovery of bacteria from the VBNC state using *E. coli* and *P. aeruginosa* as test strains. The transcription levels of virulence genes and the integrity of cells induced into a VBNC state were investigated. Here, we are the first to report that the resuscitation of UV radiation-induced VBNC bacteria was observed under moderate nutrient and temperature conditions. Therefore, our findings strongly indicate reconsideration of "the role of" UV disinfection for microbial safety in drinking water treatment systems.

MATERIALS AND METHODS

Strains. *Pseudomonas aeruginosa* (ATCC 15692) and *E. coli* (CMCC 44103) were purchased from the Guangdong Microbiology Culture Center (Guangdong, China).

UV Disinfection. The tested strains were grown in LB broth at 37 °C for 16 h so that the cell density could reach 10^9 CFU/mL as determined using a standard plate count method (nutritional agar, at 37 °C for 24 h). The tubes containing 20 mL of cultured broth were then centrifuged at 9000*g* for 10 min. After discarding the supernatant, the remaining pellet was washed twice and resuspended in 20 mL of sterile saline in a sterile Petri plate placed on a magnetic stirrer to ensure homogeneity of the cultures. A bench-scale collimated beam apparatus (Supporting Information (SI) Figure S1) was used to irradiate the samples. This apparatus contained a low-pressure (15 W) mercury UV−C lamp (254 nm, 280 μW/cm^2, Philips, USA). The selected UV lamp was housed above a collimating tube (33 cm) affixed to internal aluminum foils that helped to focus the UV beam onto the sample. The duration of UV radiation was 0, 3, 6, 9, 12, 15, or 18 min. All of the experiments were conducted in the dark. The inactivation efficiency was determined as log (N_0/N_t), and where N_0, N_t are the plate count levels (CFU/mL) of the specific bacteria before and immediately after UV disinfection, respectively.

DNA Extraction and qPCR. All DNA extractions were performed on 1 mL subsamples using a FastDNA Spin Kit (TIANGEN, #DP302−02) following the manufacturer's protocol. The target genes and primers used in this study are shown in Table 1. The qPCR was performed using the Roche 480 Real-Time PCR system (Roche, Basel, Switzerland) according to the manufacturer's instructions: one cycle at 95 °C for predenaturation for 5 min; 40 cycles at 95 °C for 15s, 60 °C for 45s; denaturation was one cycle based on previous experiments. Conditions for the 1465 bp and 900 bp primers were similar but used the annealing/extension conditions of 60 °C for 1 min and EvaGreen was used instead of SybrGreen to produce the fluorescent signal.

RNA Isolation. Suspended cells (1 mL) were concentrated using centrifugation (12 000*g*, 10 min, 4 °C). Trizol reagent (Invitrogen, Cat. No. 15596−026) was used to extract and purify total RNA from *E. coli* and *P. aeruginosa* according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically at an optical density of 260/280 (OD260/280) (AS-Nano100−1072; AOSHENG, Shanghai, China). In the subsequent reverse transcription process, The GoScrip Reverse Transcription System (A5001, Promega) was used for first strand cDNA synthesis using 1 μg of RNA. To determine if the amplification product was exclusively from the RNA, a control without reverse transcriptase (RT) was included. Before proceeding with qPCR, reverse transcription was inactivated using a heat block set at 70 °C for 15 min.

PMA Treatment. PMA is a dye that penetrates membrane-damaged cells and binds with their DNA. The PMA-bound DNA cannot be amplified in the ensuing PCR. This characteristic is often applied to determine cellular integrity using the PMA−PCR method; thus, this method was employed in the present study.[28] The PMA (Biotium, Inc., Hayward, CA) was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mM and stored at −20 °C in the dark. The PMA (10 μL) was added to 990 μL of culture aliquots in microcentrifuge tubes. After incubating for 5 min in the dark with occasional mixing, the samples were light-exposed for 15 min using a 650 W halogen light source (220 V, 3400K, OSRAM, Munich, Germany). The sample tubes were laid horizontally on ice to avoid excessive heating and placed approximately 15 cm from the light source. After photoinduced cross-linking, the cells were pelleted at 12 000*g* for 10 min prior to DNA isolation.

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Live/Dead Bacteria Staining and Confocal Laser Scanning Microscopy (CLSM). Cultures of *E. coli* (120 mL) were shaken at 37 °C and 180 rpm for 17 h in LB medium until they reached the plateau phase. Twenty mL of cultures were transferred to a Petri dish and 100 mL of which were transferred to a flask. The aliquots were centrifuged, washed, and resuspended as described in the "UV Disinfection". The cultures in the plateau phase (20 mL in Petri dishes) were radiated using 100 mJ/cm^2 of UV, whereas the cultures in the 100 mL flasks were treated with NaClO solutions having a final chlorine concentration of 1.1, 2.2, 3.3, 4.4, or 5.5 mg/L. After the flasks were shaken to mix completely, the cultures were disinfected by the chlorine for 30 min.

The fluorescent probes SYTO 9 and PI were used with CLSM to distinguish intact from membrane-permeabilized cells. For these assays, 1 mL sample from each aliquot was obtained and centrifuged at 12 000*g* for 3 min. The supernatant was removed and the pellet was resuspended in 1.5 mL of 0.9% NaCl buffer. The cell suspensions (200 μL) were incubated with 1.5 μL of a dye mixture containing SYTO 9 and PI (LIVE/DEAD *Bac*Light Bacterial Viability Kit, Invitrogen, Carlsbad, CA), with a final concentration of 3.5 μM SYTO 9 and 20 μM PI, for 15 min at room temperature in the dark. The suspensions were then centrifuged twice at 3000*g* for 10 min and washed in sterile PBS, and 10 μL of each suspension incubated with the dye mixture was trapped between a slide and an 18 mm square coverslip. Samples were examined with an LSM710 CLSM (Carl Zeiss Jena, Germany) equipped with a Nikon Coolpix E 950 digital camera (Nikon Corporation, Tokyo, Japan).

Resuscitation Experiments. The ability of any bacterium to resuscitate is essential if the VBNC state is truly a survival strategy. Whether the culturable cells which appear following removal of the inducing environmental stress are a result of true resuscitation or of regrowth of a few residual culturable cells has been debated. Some researchers have argued that resumption of *V. vulnificus* culturability following a temperature upshift originated from the regrowth of residual culturable cells remaining among the nonculturable population.[29] Thus, some studies employed diluted populations of VBNC cells in which it was unlikely for any culturable cells present to subsequently regrow.[30] In the present study, a 10-fold serial dilution method was applied to determine the resuscitation potential of *E. coli* and *P. aeruginosa* induced by UV radiation into a VBNC state. The resuscitation from VBNC state was distinguished from regrowth by a series dilution method. First, the tested strain after the UV radiation was cultured. The culture was then diluted into a series of levels much lower than 1 CFU/mL, that is, 10°, 10^{-1}, 10^{-}2 and 10^{-3} CFU/mL based on plate counting results. The 100 μL of each diluted culture and 900 μL LB broth were mixed and transferred into a 24-well microtiter plate at 37 °C for 24 h. Ten parallel wells were prepared for one dilution. These series of mixtures meant the CFU levels of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} CFU/mL, respectively. If any one of these wells got turbid, the resuscitation could be demonstrated.

Statistical Analysis. Analysis of Variance (ANOVA) was conducted using the SPSS 11.5 statistical software package. A single factor ANOVA was used to determine significant differences in the results between replicate trials. The results are expressed as mean ± SE and were deemed statistically significant when P-values were less than 0.05.

RESULTS AND DISCUSSION

Impact of UV Treatment on the Culturability of *E. coli* and *P. aeruginosa*. The *E. coli* and *P. aeruginosa* cultures were treated with various UV doses to evaluate UV disinfection efficiency. The results showed that UV radiation effectively reduced the culturability of *E. coli* (all $P < 0.05$) and *P. aeruginosa* (all $P < 0.05$) (Figure 1). At a dose of 50 mJ/cm^2,

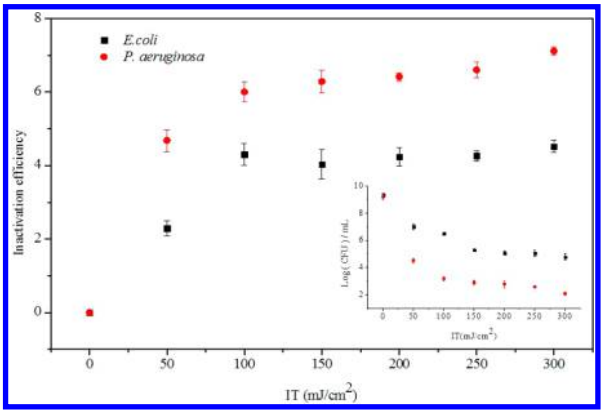

Figure 1. Inactivation efficiency of *E. coli* and *P.aeruginosa* after UV treatment of different doses based on plate counting. The embedded diagram shows the HPC results of *E. coli* and *P.aeruginosa* after treatment under different UV doses based on plate count. The product of I (UV radiation intensity, μW·cm^{-2}) and T (UV radiation time, S) is the UV dose. The error bars represent ± SE.

the HPC of *E. coli* decreased from 2 × 10^9 CFU/mL to 1.02 × 10^7 CFU/mL and the HPC of *P. aeruginosa* decreased from 1.53 × 10^9 CFU/mL to 3.2 × 10^4 CFU/mL. The corresponding inactivation efficiencies for *E. coli* and *P. aeruginosa* were 2.29 and 4.68 logs, respectively, and the efficiencies reached 4.3 and 6.0 logs at a dose of 100 mJ/cm^2, respectively. The inactivation efficiencies of *E. coli* and *P. aeruginosa* both remaining relatively stable at the higher doses, which just as a "tailing zone" from 100−300 mJ/cm^2 of UV dose.

Many species of bacteria exhibit an initial lag in the slope or tailing zone when inactivated by UV exposure. Such a lag or tailing zone has been attributed to multiple-hit kinetics or other related phenomena.[31] However, an initial lag was not observed in the present study because the lowest UV dose was 50 mJ/cm^2, which far exceeds a typical dose, such as 10 mJ/cm^2, that results in a lag phase for *Bacillus subtilis* spores.[32]

Evidence of Nucleic Acid Damage in *E. coli*. The mechanisms for UV light inactivation of microorganisms vary with the wavelength used. The disinfection effect of UV−C light is mainly due to the formation of cis-syn cyclobutane pyrimidine dimers in the genome DNA of the organisms rather than the result of membranolysis, which is induced by other disinfectants, such as chlorine and ozone.[33,34] UV-induced lesions inhibit the normal replication of the genome and result in inactivation of the microorganisms. To confirm nucleic acid damage after UV treatment in the present study, in other words, the formation of cis-syn cyclobutane pyrimidine dimers in the genome DNA, qPCR amplifying long and short DNA fragments was conducted in *E. coli*.

During the qPCR process, the prolongation is interrupted at the nucleotide acid site in a dimer structure. More dimers are generated in the longer gene fragments than in the shorter fragments during UV radiation. The amplification of long gene

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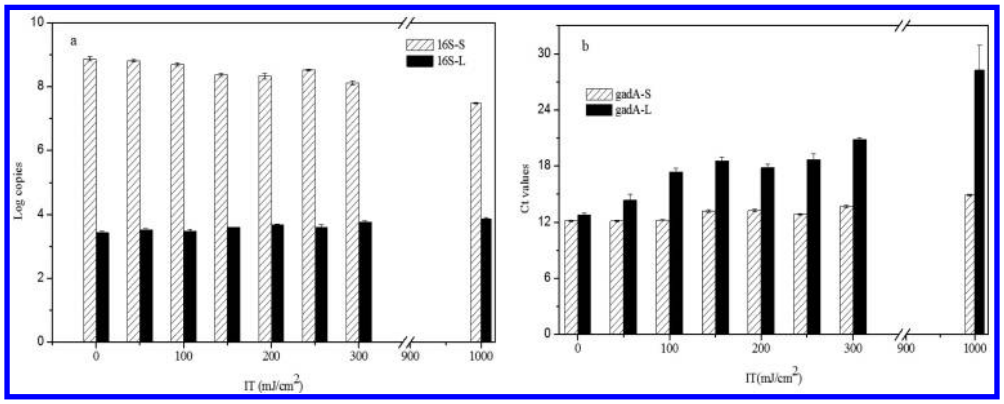

Figure 2. a: Log copies of long/short 16S gene fragments of *E. coli* under different UV dose based on qPCR; b: Ct values of long/short *gadA* gene fragments of *E. coli* under different UV dose based on qPCR. The product of I (UV radiation intensity, $\mu W \cdot cm^{-2}$) and T (UV radiation time, S) is the UV dose. The error bars represent ± SE.

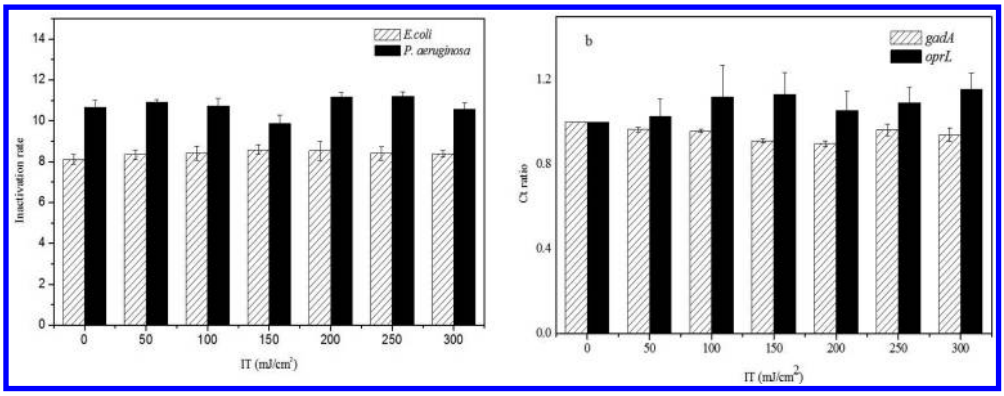

Figure 3. a: Log copies of *E. coli* and *P.aeruginosa* after treatment under different UV dose based on RT-qPCR. b: Ct ratio of *gad*A and *opr*L after treatment under different UV dose based on RT-qPCR. and the value of 1 was set as control group. The product of I (UV radiation intensity, $\mu W \cdot cm^{-2}$) and T (UV radiation time, S) is the UV dose. The error bars represent ± SE.

fragments have a much higher probability of being terminated, so these qPCR results better reflect the formation of dimers, that is, the DNA damage induced by UV radiation.

Figure 2a showed the results of 16S rRNA long and short DNA fragment qPCR of *E. coli*. The results of 16S rRNA qPCR with a 194 bp product showed that the number of copies was maintained at log 8.11 to 8.87, whereas the results of 1465 bp product maintained the copy number at log 3.42 to 3.76. Thus, the UV inactivation ratios of the longer and shorter fragments were nearly unchanged, indicating the difficulty in forming dimers in the 16S rRNA gene, because this gene is an ordinary reference one and can usually be stably expressed.[35] Figure 2b showed the results of *gadA* long/short DNA fragment qPCR in *E. coli* after UV radiation. The glutamic acid decarboxylase (GAD) system provides useful probes for detecting the ability of pathogenic bacteria to tolerate an acidic environment[36] and allows pathogenic bacteria to become active in an acidic environment, such as the stomach, when they infect a host. GAD is coded by *gadA*, which was used to indicate the virulence of *E. coli* (CMCC 44103) in this study. The Ct values for *gadA* qPCR were maintained at 12.12 to 13.67 using 100 bp and 12.8 to 20.83 using 900 bp product following UV exposure of 0 to 300 mJ/cm^2, and at 14.91 (100 bp) and 28.26 (900 bp) following 1000 mJ/cm^2 of UV radiation, respectively. The results showed that more severe DNA damage occurred with the increased UV radiation dose. The UV disinfection mechanism discussed above could well explain the results. There is a higher possibility that the omissions of dimmers occur when PCR products are shorter, which may lead to the underestimating of DNA damage. By contrast, longer qPCR amplified fragments (900 bp) may detect more dimmers. In other published studies, the longer DNA fragments were also used to magnify the signals of DNA damage.[37,38] For example, an equivalent DNA segment (approximately 1000 bp) was normalized to enhance capture of DNA damage events under UV radiation.[37]

From the results of the culturability of *E. coli* and *P. aeruginosa*, increasing the UV dose could result in reducing culturability of *E. coli* and *P. aeruginosa*, which hinted the significant DNA damage induced by UV radiation. However, the qPCR results of 16S rRNA short/long and *gad*A short gene fragments did not reflect this phenomenon. By contrast, the nucleic acid damage could be clearly demonstrated if *gad*A long gene fragments were used as qPCR target. Therefore, when the qPCR method was chosen to identify if nucleic acid was damage induced by UV radiation, the type and length of the gene fragments were important as these might result in different conclusions.

The Transcriptional Levels of 16S rRNA and Virulence Genes Following UV Treatment in *E. coli* and *P. aeruginosa*. The transcriptional levels of 16S rRNA and the virulence genes in *E. coli* and *P. aeruginosa* after UV treatment were determined using RT-qPCR. The results of the RT-qPCR amplified 16S rRNA showed that UV doses ranging from 0 to 300 mJ/cm^2 had little effect on the copy number, which varied insignificantly from 8.11 to 8.58 for *E. coli* (P = 0.07−0.297)

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and from 9.87 to 11.17 for *P. aeruginosa* (P = 0.06−0.837) (Figure 3a). The half-life of bacterial mRNA is generally only 3 to 5 min. Thus, only live bacteria can persistently transcribe mRNA, and, hence, the continued activity of mRNA is an excellent indicator for bacterial viability, especially for studies determining the VBNC state.[39] Potential genes used as indicators for viability include *rfbE*, *fliC*, *stx1*, *stx2*, *mobA*, *eaeA*, *hly*, and 16S rRNA to determine suitable target genes for detection of the pathogen *E. coli* O157:H7 using RT-qPCR.[40] The cellular levels of 16S rRNA and mRNA for the *tuf*, *rpoS*, and *relA* genes have also been analyzed to assess metabolic activity of the *Vibrio cholerae* strain EI Tor 3083 and determine if they entered the VBNC state.[41] During a disinfection event, oxidative stress may render bacteria nonculturable but metabolically active and viable,[42] severely underestimating the number of viable cells as determined using HPC. The results from the RT-qPCR and plate counting assays in the present study demonstrated that UV radiation induced a VBNC state in *E. coli* and *P. aeruginosa*. Specifically, UV radiation at doses ranging from 0 to 300 mJ/cm^2 generated viable but nonculturable *E. coli* and *P. aeruginosa*.

In addition to establishing viability, RT-qPCR also determines the transcriptional level of pathogenic genes indicating the virulence of pathogens in a VBNC state. The *oprL* gene encoding the peptidoglycan-associated lipoprotein (PAL) in *P. aeruginosa* is highly conserved. PAL is the last major outer-membrane protein to significantly contribute to the thermal death of bacteria and can be utilized as a target gene for RT-qPCR detection.[43] Therefore, in the present study, RT-qPCR was applied to determine the transcriptional levels of *gadA* and *oprL* to indicate the virulence of *E. coli* and *P. aeruginosa* in the VBNC state. The transcriptional levels of *gadA* and *oprL* following UV exposures at doses from 0 to 300 mJ/cm^2 were not significantly downregulated (Figure 3b). The values of the Ct ratios varied from 0.89 to 1 for *gadA* (all P < 0.05) and from 1 to 1.21 for *oprL* (P = 0.087−0.551, except P = 0.017 for UV 300 mJ/cm^2), demonstrating that even under high UV radiation doses *gadA* and *oprL* were expressed at levels similar to those in control cells that received no disinfection treatment.

For cells in a VBNC state, the distinction between viability and culturability is especially significant for pathogens because loss of culturability may not guarantee loss of pathogenicity. If pathogenicity is sustained, pathogens in the VBNC state may pose an unrecognized public health threat. Unfortunately, extensive data sets are unavailable for this aspect of VBNC microorganisms. The highly sensitive RT-qPCR assay has been previously applied to examine the activity of pathogenic genes that indicate the toxicity of pathogens in a VBNC state.[44,45] In the present study, the observed high transcriptional levels of the *gadA* and *oprL* genes verified the continued pathogenicity and tolerance of the bacteria to environmental stresses even after treatment with very high UV doses.

Integrity of UV-Treated *E. coli*. Characteristic of VBNC bacteria, the bacterial cells examined in the present study failed to form colonies for plate counting, while displaying cellular integrity and activity in gene expression assays (discussed below). In many circumstances, bacteria can be inactivated by treatments (e.g., UV light) that do not directly damage the membrane but injure the genetic material instead. As a universal method, PMA treatment was unsuccessful at distinguishing between live and UV-killed *E. coli* O157:H7.[46] However, the use of longer PCR amplicon sizes may improve the efficacy of PMA−PCR, as was previously demonstrated when applied to heat-killed *E. coli* O157:H7 and *Enterobacter aerogenes*.[47] In the present study, PMA was cross-linked to DNA in cells following UV radiation. PCR amplification of specific gene targets (16S rRNA and *gadA*) were assessed using short and long amplicon sizes to discern the reduction in the cell signal after the UV treatment, and qPCR was used to measure the effect. SI Figure S2 shows that the results of the PMA treatment were similar to those results shown in Figure 2. That is, the results of PMA-qPCR revealed no decrease in copy number for short or long gene fragments of 16S rRNA and no increase in Ct values for short gene fragment of *gadA* gene.

Based on the principles of PMA staining, it can be concluded that PMA staining coupled with quantitative PCR is a powerful tool for determining cellular integrity. For example, a linear relationship existed between the results obtained using ultrasonic detection by PMA-qPCR and those obtained using the plate counting method for the inactivation efficiencies of *E. coli* and *B. subtilis*.[28] In many studies, the integrity of the cellular structure based on PMA-qPCR analysis was used to represent cellular activity. For example, PMA treatment was directly used on a membrane filter for the quantification of VBNC *Legionella* using qPCR,[48] and *Enterococcus* was detected with both the standard culture-based and PMA-qPCR assays to quantitate VBNC cells.[49] The results of the present study demonstrated that the cellular structure was intact following UV treatment, even at doses as high as 300 mJ/cm^2.

Confirmation of the VBNC State Induced by UV Disinfection. To verify the reliability of the remarkable results from RT-qPCR that a wide range of UV radiation doses induced a VBNC state in *E. coli* and *P. aeruginosa*, a second disinfectant, chlorine, utilizing a different mechanism of action was employed to inactivate *E. coli*. When the effects were examined using HPC, we found no significant difference in the results following a UV dose of 100 mJ/cm^2 and those following 2.2 mg/L of chlorine (6.49 and 6.69 log units, respectively; P = 0.331). By contrast, as determined using RT-qPCR, the results after the UV and chlorine treatments in *E. coli* were significantly different (8.41 and 5.59 log units, respectively; P = 0.005). Thus, although the two inactivation methods appeared to have similar efficiencies based on the HPC results, the RT-qPCR results suggested that the different inactivation techniques did not display the same effects. (Figure 4).

Previous researchers have encountered equivalent issues. McKillip et al.[50] demonstrated that the rRNA content in *Staphylococcus aureus* and *E. coli* O157:H7 was suitable to monitor bacterial viability under extreme heat conditions (121 °C for 15 min), but that it could not indicate viability following moderate heat inactivation (80 °C for 20 min) or UV irradiation (254 nm light source and 1200 mW/cm^2). Therefore, these authors suggested that rRNA may not be a suitable target molecule for monitoring bacterial viability. However, McKillip et al. may not have realized that *S. aureus* and *E. coli* O157:H7 enter into a VBNC state following moderate heat inactivation or UV irradiation and that such treatments may not regulate the expression of the 16S gene. By contrast, chlorine treatments downregulated the transcriptional level of 16S gene in our study, indicating that the high 16S rRNA transcriptional levels assessed using RT-qPCR to determine the viability of UV-treated *E. coli* and *P. aeruginosa* originated from their VBNC state.

As mentioned above, cellular integrity is necessary for VBNC bacteria. The SYTO 9 and PI staining followed by CLSM

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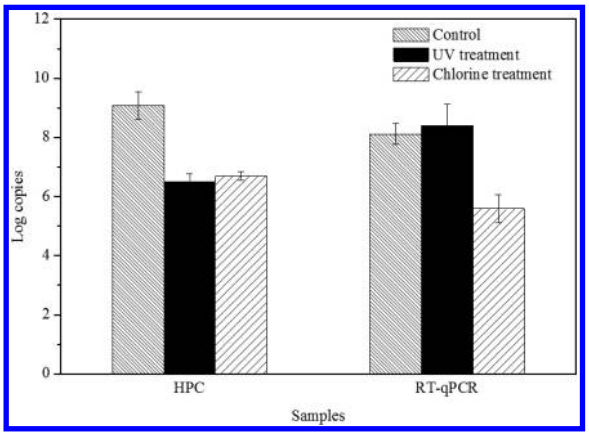

Figure 4. Results of *E. coli* after treatment under UV (100 mJ/cm^2) and chlorine (2.2 mg/L) based on HPC and RT-qPCR. The reason to exhibit disinfection effect of 2.2 mg/L chlorine was that the HPC result of chlorine (2.2 mg/L) disinfection was nearest with that of UV (100 mJ/cm^2). The error bars represent ± SE.

imaging was used here to show how UV radiation induced the bacteria into this VBNC state. As shown in Figure 5a, green fluorescence represented viable bacteria that maintained their structural integrity, and Figure 5d shows that the colony size was uniform with smooth surfaces. Figures 5-b and Figures 5e show that after UV radiation cell membrane integrity was maintained and the cell shape was just as smooth as that in control cells. By contrast, marked changes occurred after chlorine treatment. In Figure 5c only red dots are observed, demonstrating that chlorine destroyed the integrity of the cell membrane. SI Figure S3-f shows a lack of uniformity in the size of and the rough edges of the cultured colonies, indicating that bacteria were unhealthy after chlorine treatment.

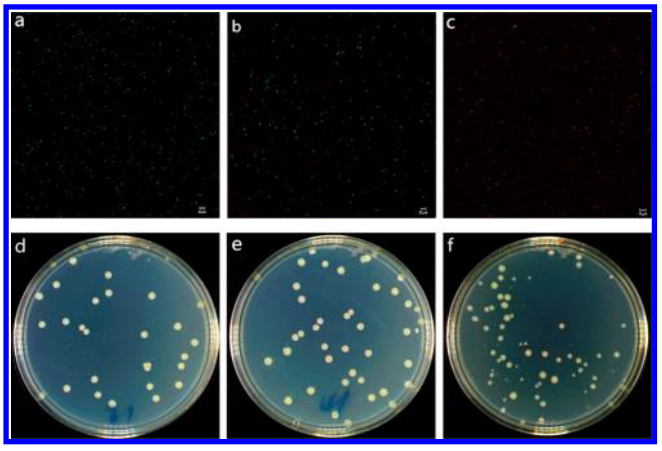

Figure 5. CLSM and plate culture images of *E. coli* after treatment under UV (100 mJ/cm^2) and chlorine (2.2 mg/L) based on HPC and RT-qPCR. a, b, and c are CLSM images of no-treatment control, after UV dose of 100 mJ/cm^2 radiation and after chlorine addition of 2.2 mg/L respectively. d, e, and f are plate culture images of no-treatment control (cell density was $10\hat{9}.08$ CFU/mL), after UV dose of 100 mJ/cm^2 radiation (cell density was $10\hat{6}.49$ CFU/mL) and after chlorine addition of 2.2 mg/L separately (cell density was $10\hat{6}.69$ CFU/mL). (The *bar* = 20 μm).

Resuscitation cCharacteristics. A dilution study was conducted in triplicate to examine the resuscitation potential and characteristics, and different resuscitation results were detected (Table 2). For *E. coli*, all of VBNC cells resuscitated when culturable cells were diluted to 10^{-1} CFU/mL, whereas only those VBNC cells induced by 50 mJ/cm^2 of UV radiation resuscitated at a dilution of 10^{-2} CFU/mL. For *P. aeruginosa*, those VBNC cells induced by 25 mJ/cm^2 of UV disinfection resuscitated when culturable cells were diluted to 10^{-1} CFU/mL. These results showed that when induced into a VBNC state after UV radiation both *E. coli* and *P. aeruginosa* could be resuscitated under the appropriate temperature (37 °C) and nutrients (LB broth), with *E. coli* resuscitating more easily than *P. aeruginosa*. The concentrations of *E. coli* and *P. aeruginosa* both reached 10^9 CFU/mL in all cases where resuscitation was achieved, similar to cultured control cells (data not shown).

Resuscitation is a very important characteristic for VBNC bacteria. Thus, VBNC cells responding to appropriate environmental stimuli, such as a temperature upshift or the addition of nutrients, become metabolically active and culturable. Many pathogens in the VBNC state are unable to initiate disease, but they can retain virulence potential and initiate infections following resuscitation to an actively metabolizing state. For example, VBNC *Vibrio harveyi* inoculated into zebra fish did not express the hemolysin gene or cause death, but the resuscitated cells were lethal.[51] Similarly, VBNC *V. vulnificus* injected into mice lost virulence in proportion to the length of time injected, but resuscitation occurring within the mice could initiate virulence and fatal infections.[52]

The results of our resuscitation study are important for the microbial safety of drinking water. *E. coli* and *P. aeruginosa* are often used as indicators of pathogenic bacteria, and their resuscitation in our study revealed the potential for the resuscitation of other pathogenic microorganisms following UV treatment. Our results are important to consider in developing strategies to reduce pathogens in drinking water treatment

Table 2. Resuscitation of *E. coli* and *P. aeruginosa* from the VBNC State As Observed in 10 Individual Wells Which Contained Bacteria Samples (100 μL) of the Same Dilution and 900 μL of LB Broth[a]

	E.coli (wells)				*P.aeruginosa* (wells)			
IT(mJ/cm^2)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
25	0/1/1	0/0/0	0/0/0	0/0/0	1/1/0	0/0/0	0/0/0	0/0/0
50	3/7/1	2/1/1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
100	4/2/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
150	1/2/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
200	2/0/1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
250	1/1/1	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
300	4/1/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0

[a]The study was conducted triplicate, and results of three times were all exhibited. The units of $10^{-1} \sim 10^{-4}$ all were CFU/mL.

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systems as they indicate that potential resuscitation should be evaluated to ensure the biological safety of UV disinfection.

Environmental Implications. This study demonstrated the potential of UV disinfection to induce VBNC bacteria. Although the cell counts based on the results of the HPC assay decreased and nucleic acid damage was more severe with increasing doses of UV radiation, the integrity of the cell membrane was maintained and the expression levels of 16S rRNA and two other functionally important genes were equivalent to those of control cells. The resuscitation potential of bacteria needs to be taken seriously. A public health risk may exist when UV radiation is utilized as a drinking water treatment system.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The information regarding the bench scale collimated beam apparatus (Figure S1), PMA-qPCR results (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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