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Comparison of *Legionella pneumophila* and *Pseudomonas fluorescens* Quantification Methods for Assessing UV LED Disinfection

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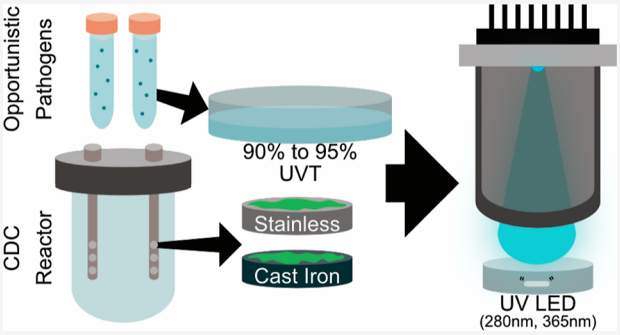
ABSTRACT: This study assesses the efficacy of ultraviolet light-emitting diodes (UV LEDs) for deactivating *Legionella pneumophila* (pure culture) and *Pseudomonas fluorescens* (pure culture and biofilms) on relevant drinking water distribution system surfaces (cast iron and stainless steel). UV LED treatment at 280 nm demonstrated superior performance compared to that at 365 nm, achieving a 4.8 log reduction value (LRV) for *P. fluorescens* pure cultures and, for biofilms, 4.02 LRV for stainless steel and 2.96 LRV for cast iron at 280 nm. Conversely, the results were less effective at 365 nm, with suspected photolytic reactions on cast iron. Quantification of *L. pneumophila* yielded varying results: 4 LRV using standard plate counts, 1.8 LRV with Legiolert, and 1 LRV with quantitative polymerase chain reaction at 280 nm, while the results were less than 1.5 LRV at 365 nm. This study provides insights into managing opportunistic pathogens and biofilms, emphasizing the need for improved quantification tools to better assess treatment efficacy.



KEYWORDS: *Legionella pneumophila, Pseudomonas fluorescens, water disinfection, biofilms, opportunistic pathogens, waterborne pathogens, UV LEDs*

1. INTRODUCTION

Biofilms in drinking water distribution systems (DWDS) pose significant concerns because of their capacity to host and create an optimal environment for the growth and survival of diverse pathogenic microorganisms, making it hard to develop efficient techniques for their control.[1,2] Opportunistic pathogens (OPs) such as *Aeromonas* spp., *Acinetobacter* spp., *Helicobacter* spp., *Methylobacterium* spp., *Mycobacteria* spp., *Stenotrophomonas* spp., *Pseudomonas* spp., and *Legionella* spp. are most commonly found in distribution systems.[3] One of the current pathogens of concern is *Legionella pneumophila*, a chlorine-resistant waterborne pathogen, which causes a severe pulmonary infection (pneumonia) known as Legionnaire's disease,[4,5] and it is transmissible by inhalation of aerosols generated in settings where hot water and steam is used.[6] Under specific temperature conditions and low disinfectant levels,[7] *L. pneumophila* can persist within biofilms by producing bacterial adhesins, which helps them to get attached to biofilms and survive within. The specific strain of *Pseudomonas fluorescens* has been reported as a typical host for *L. pneumophila*.[5,8]

The standard practice for drinking water disinfection in North America is the chemical addition of chlorine gas (Cl_2), sodium hypochlorite (NaOCl), or monochloramine (NH_2Cl). These substances produce a residual disinfection effect in the water, helping to mitigate microbial regrowth and contamination during distribution.[9,10] Although chemical disinfection has been used for centuries, it has limited efficiency because of poor penetration within biofilms.[11,12] Chemical disinfectants also react with natural organic matter (NOM) and can produce disinfection byproducts (DBPs), which are toxic and suspected carcinogens.[13,14]

Ultraviolet (UV) light inactivates protozoa, viruses, and bacteria by disrupting the genetic material.[15] Conventional UV treatments include the use of mercury-based low-pressure (LP) or medium-pressure (MP) lamps.[16,17] The primary challenges for conventional UV devices are that they have high energy consumption, contain mercury, are prone to fouling, and lack wavelength selectivity.[18,19] Emerging technologies like UV light-emitting diodes (UV LEDs) could be a suitable solution for the inactivation of biofilms and OPs in distribution systems

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because of their small footprint, design versatility, wavelength selectivity, and instantaneous powering.[20,21]

UV light is divided into four primary bands: VUV (vacuum UV) from 100 to 200 nm, UVC from 200 to 280 nm, UVB from 280 to 315 nm, and UVA from 315 to 400 nm.[22] The current standard wavelength for UV treatment is 254 nm. However, new information indicates that wavelengths closer to 280 nm (UVC) hold promise as a reliable and energy-efficient option for water disinfection.[23,24] Additionally, debates persist regarding wavelengths >315 nm (UVA), which have the potential to inflict irreparable damage to DNA, proteins, and repair enzymes, thereby impeding photoreactivation mechanisms.[25,26] These emerging findings underscore the necessity of exploring alternative wavelengths beyond the conventional 254 nm.[20,27,28]

There is limited literature examining the use of UV LEDs for inactivating planktonic and biofilm-associated pathogens.[21] Consequently, it is crucial to enhance our understanding of biofilm formation in water distribution systems when using UV LEDs as a treatment method. This involves conducting studies to investigate the behavior of OPs, such as *L. pneumophila*, within biofilms, examine biofilm formation on various representative surfaces relevant to DWDS, assess the effectiveness of UV LEDs with wavelengths >254 nm, and analyze biofilms in different water compositions.

Current culture methods for the quantification of *L. pneumophila* can take several days to complete. For instance, standard plate count (SPC) with buffered charcoal yeast extract (BCYE) agar is the traditional and most used method for most regulations; results are typically reported in 7−14 days.[29] According to studies, the Legiolert test could replace SPC as it is as accurate.[30,31] Unlike standardized SPC, Legiolert does not require serial dilutions.[31,32] The Centre for Disease Control (CDC) suggests using quantitative polymerase chain reaction (qPCR) as a monitoring tool, which normally takes around 2 h per sampling event. However, this assay may not differentiate between live, dead, or viable but not culturable (VBNC) bacteria, thus requiring culture methods.[29,33]

The objective of this study was to investigate the growth and disinfection with UV LEDs of the OP *L. pneumophila* and the biofilm-producing bacteria *P. fluorescens*. Specifically, monocultures of both bacteria were cultivated in aqueous solutions using culture broth, and *P. fluorescens* was grown in CDC biofilm reactors with two pipe materials, stainless steel (SS) and cast iron (CI). Dose−response curves were generated to assess the effectiveness of UV LEDs at 280 and 365 nm for inactivating these organisms. Also, quantification tools for *L. pneumophila* (SPC, Legiolert, and qPCR) were assessed to compare the effectiveness in measuring treatment outcomes using UV LEDs.

2. MATERIALS AND METHODS

2.1. Opportunistic Pathogens Monocultures. *P. fluorescens* (ATCC 17569) and *L. pneumophila* (ATCC 33152) were chosen to test the inactivation of OPs and were selected for being waterborne pathogens, their ability to form biofilms (*P. fluorescens*), and their persistence in DWDS. Frozen stocks preserved with 25% glycerol solution were used to prepare overnight cultures (1:10 dilution). *P. fluorescens* was inoculated in tryptic soy broth (TSB) (Becton Dickinson and Co., MD, USA) and incubated at 27 °C at 175 rpm for 24 h. *L. pneumophila* was inoculated in Legionella enrichment broth (Sigma-Aldrich, Darmstadt, Germany) and incubated at 37 °C and 175 rpm for 24 h. Cell washing was performed to obtain a UV transmittance between 90 and 95% (UVT in %). Cultures were cleaned by forming a pellet by centrifugation, and then the supernatant was replaced with sterile dechlorinated tap water (GAC-filtered and autoclaved at 15 psi and 121 °C for 30 min); this process was repeated three times. Sterile dechlorinated tap water was chosen to ensure that overall metals, NOM, and salts were still present within the water matrix to mimic environmental conditions. Although autoclaving likely changed the NOM composition, especially the organic matters that are heat sensitive, we still wanted to avoid using deionized water (DI) to have a much more representative matrix from a water distribution system. This approach, as demonstrated in prior research but involving wastewater,[34] offers an alternative to exclusively using synthetic matrices in disinfection experiments. The working solution was prepared with a 1:100 dilution of the washed cells of target microorganisms with an initial concentration of $\sim 10^7$ (colony forming unit) cfu/mL. A sample of the working solution was taken for UV-254 analysis with a spectrophotometer (DR-6000, Hach Company, Loveland, CO, USA) to obtain the UVT %. Samples were poured into sterile crystallizing dishes with a micro stir bar to maintain mixing. For *P. fluorescens* samples, serial 10-fold dilutions were prepared and then plated in tryptic soy agar (TSA) plates by using the spreading technique for SPC. The plates were incubated at 27 °C for 24 h until colony counting; the results were reported in cfu/mL.

L. pneumophila samples were quantified using three different methods. SPC was performed using BCYE agar (Becton Dickinson and Co., MD, USA) supplemented with L-cysteine and iron pyrophosphate $[Fe_4(P_2O_7)_3]$. Using serial dilutions, samples were plated on BCYE agar plates and incubated at 37 °C for 72 h, according to a standardized approach for *L. pneumophila* identification and quantification.[35] The Legiolert test is based on an enzymatic reaction that color-indicates (brown color) the presence of *L. pneumophila* through the utilization of a substrate, which accelerates its reproduction; the results are reported after 7 days of culture.[36] Legiolert samples were prepared following the manufacturer's instructions, incubated at 37 °C for 7 days, and then the results were reported to have the most probable number (MPN)/100 mL.[36,37] The samples of this study were also analyzed using a commercial *L. pneumophila* qPCR test kit (LuminUltra Technologies, NB, Canada). The test first required a DNA extraction, in which the sample is lysed, and then the DNA is purified. Then, qPCR preparation required adding the assay mix (primers and enzymes) and preparing a negative and positive control. Samples were then placed into a Q-16 qPCR device.[38]

2.2. Experimental Setup for Biofilm Growth in CDC Reactors. A modified version of the standard operating procedure ASTM E2562-22 was used to grow OP biofilms in CDC reactors.[39] Specifically, a water bath was used to maintain the temperature at 27 °C. Approximately 10^7 cfu/mL of *P. fluorescens* cells (1 mL) was inoculated into a CDC biofilm reactor (CBR 90-1, BioSurface Technologies Corp., Bozeman, MT) containing 495 mL of sterile dechlorinated tap water and 5 mL of sterile TSB. The reactor was operated in batch mode for 24 h at low rpm (~50 rpm). Then, the reactor was set to continuous flow reactor mode with 3.33% of TSB solution and a flow rate of 10 mL/min for another 24 h.

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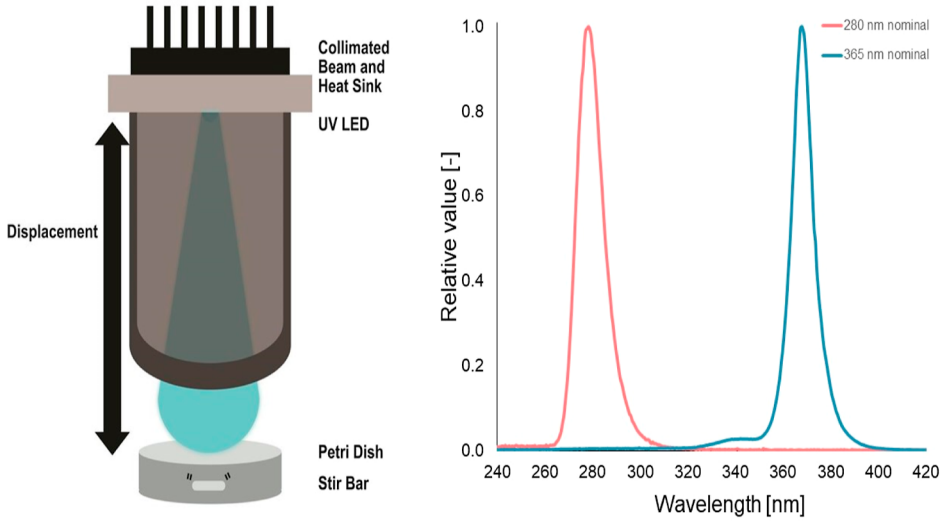

Figure 1. (Left) Schematic of the UV LED system used in this study. Displacement represents the distance from the LEDs to the sample. Sample plane diameter was the internal diameter of the Petri dish. UV LED beam light is represented by the blue shape. (Right) UV LED emission spectra of the device used in this study.

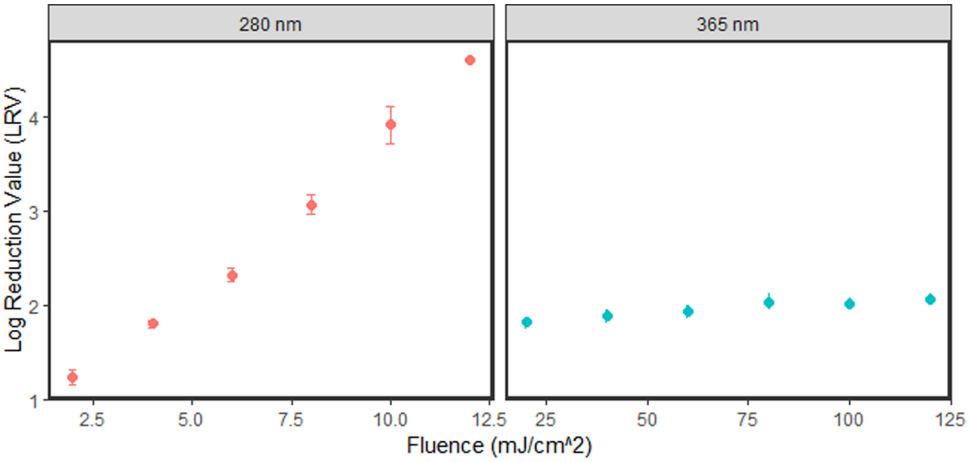

Figure 2. Inactivation of *P. fluorescens* monocultures (in aqueous solution) after UV LEDs, $n = 4$. The cells were washed to obtain a UVT = ∼ 95% and treated at 280 and 365 nm. Cells were quantified using SPC on TSA plates. Error bars represent the standard deviation from the mean.

Two different coupons were used as a growth surface: SS, to simulate general industrial equipment surface,[40] and CI, as it is one of the most frequent materials used for DWDS in North America.[41] All coupons were 1.27 cm in diameter. The biofilm recovery method was adopted from Gora et al.[42] (2019). In summary, coupons were extracted aseptically, and then biofilms were recovered by swabbing the coupon's surface and resuspending it by vortex into 10 mL of sterile phosphate-buffered saline to homogenize. Serial dilutions were prepared, then plated on TSA plates, and incubated at 27 °C for 24 h.

2.3. Bench-Scale UV LED Disinfection Treatment. UV disinfection efficiency of OP monocultures and biofilms was performed using a UV LED collimated beam (PearlLab Beam 280/310/365, AquiSense Technologies). A modified version of the method developed for UV bench-scale experiments by Bolton and Linden[43] (2003) was used to calculate the correction factors and exposure times for the specific characteristics of the collimated beam used in this study. The center point irradiance (E_0) was calculated using a UV radiometer (Ocean Optics USB 4000), by placing it in the center of the sample plane, and using the SpectraSuite software to calculate the irradiance (E_0) in $\mu W/cm^2$. The following experimental conditions were input: sample plane diameter, sample plane displacement, measurement spacing (set at 5 mm), sample volume (for liquid samples), and UVT % (UV-254 previously measured). A schematic representation of the UV LED setup and the UV spectra of the LEDs is provided in Figure 1.

The log reduction value (LRV) was calculated to quantify the inactivation efficiency of treatments presented in this study using all of the quantification methods previously described. LRV was calculated using eq 1

$$\mathrm{LRV} = \log_{10}\left(\frac{N_0}{N}\right) \tag{1}$$

where N_0 is the concentration of microorganisms before treatment (control, no UV exposure), and N is the treated concentration (after UV treatment). The LRV for each quantification method was calculated using each experiment's control.

2.4. Statistical Methods, Linear Regression Model, and *k*-Values. The k value is the first-order inactivation rate

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constant, representing the rate of microbiological decrease per unit. This value enables more perceptible and straightforward comparisons among various inactivation scenarios. The k values were calculated using a log−linear regression to analyze the disinfection performance using eq 2

$$\frac{N}{N_0} = 10^{-kF} \tag{2}$$

where F is the UV fluence at the exposure time.

All calculations of linear regressions for k values were done using the free statistical tool GInaFit v1.6, following the approach developed by Geeraerd et al.[44] (2005). The figures were made using *R Studio*.[45]

3. RESULTS AND DISCUSSION

3.1. Inactivation of *P. fluorescens* Monocultures with UV LEDs. The inactivation of *P. fluorescens* monocultures after disinfection with UV LEDs was higher at 280 nm than at 365 nm, achieving a 4.8 ± 0.042 LRV at 12 mJ/cm^2. The 365 nm wavelength achieved a LRV of only 1.96 ± 0.009 (Figure 2). Saha et al.[46] (2014) reported a much lower LRV when using a 254 nm LP UV lamp to inactivate *P. fluorescens*, achieving a 1.1 LRV at 22.5 mJ/cm^2. However, the UVT was low compared to that in the present study (0.003% vs 95%). A higher UVT represents a better passage of light thought the sample, thus reducing the chance of light being absorbed by other constituents (such as dissolved solids, proteins, sugars, etc.) instead of the organisms.[15] This process is called shielding, where UV-absorbing molecules protect organisms from UV photodamage.[47] Therefore, it is better to have a higher UVT to prevent this mechanism.

Another study that treated *P. fluorescens* with 254 nm[48] demonstrated 1.1 LRV at 22.5 mJ/cm^2 on a metalworking fluid. While the comparison between these previous studies is not ideal,[46,48] we have identified a research gap, which this study is trying to address. These studies presented their results in terms of reduction percentages or survival concentration instead of LRV, and the exposure time was reported instead of fluences. Consequently, these variations in methodology make it challenging to compare directly the findings across these studies. The data presented in Figure 2 showed that a LRV greater than 3 is achieved at fluences of at least 8 mJ/cm^2 at a wavelength of 280 nm. Our findings demonstrated high efficacy at 280 nm while achieving lower LRV at 365 nm. Specifically, the hierarchy of LRV values was as follows: 280 > 365 > 254 nm. This paper contributes to the database of studies demonstrating that 280 ± 2 nm has improved performance over 254 nm as a wavelength for disinfection for challenge microorganisms.[24,49]

The k values obtained for *P. fluorescens* monocultures (Table 2) show that in the case of 280 nm, the k value is 0.36 ± 0.033, which is similar to the values obtained in a study by Rattanakul and Oguma[50] (2018) for another strain of *Pseudomonas* (i.e., *Pseudomonas aeruginosa*; k = 0.42), in which it was also treated at 280 nm. Lower k values were observed at 365 nm (0.002 ± 0.0003), likely because the action spectra of UVA are less effective per physical unit (mJ/cm^2) compared to UVC and UVB.[51] No previous studies have treated *P. fluorescens* at 365 nm.

3.2. Inactivation of *L. pneumophila* Monocultures with UV LEDs and Comparison between the Quantification Methods. We employed a relative standard deviation (RSD) calculation to evaluate the initial quantification of the three methods using samples from individual control samples (without UV treatment). It is important to note that these methods employ different units of measurement, making any direct comparison challenging, resulting in noticeable variations within the data. The analysis showed that within their respective replicates, qPCR demonstrated the lowest variability at 0.38%, followed by Legiolert with relatively low variability at 1.48%. In contrast, SPC exhibited the highest variability among the tree quantification methods, at 10.11%. The results can be found in Table 1.

Table 1. Results of Initial Quantification Methods for *L. pneumophila* (Log-Transformed)[a]

quantification method	Legiolert	SPC	qPCR
units	MPN/100 mL	cfu/mL	GU/mL
results	6.47	10.91	7.84
	6.31	10.88	7.86
	6.27	8.94	7.78
	6.22	8.85	7.84
total average	6.32	9.89	7.83
standard deviation (SD) ±	0.108	1.153	0.034
relative SD (%)	1.48	10.11	0.38

[a]The results were obtained from individual control samples (without treatment), and each sample was divided into three quantification methods. The presented results include units, total average for all measurements, standard deviation, and RSD per method. SPC: standard plate counts; qPCR: quantitative polymer chain reaction; MPN: most probable number; cfu: colony-forming unit; GU: gene units.

Results show that all three quantification methods for *L. pneumophila* achieved different LRVs (Figure 3). Quantification by SPC shows a progressive reduction for UV exposure at 280 nm, reaching 4 ± 0.665 LRV at 20 mJ/cm^2. This result is similar to other studies of UV disinfection at 280 nm with *L. pneumophila*.[50,52] The maximum observed values for Legiolert and qPCR samples were 1.8 ± 0.685 LRV and 1 ± 0.005 LRV at 20 mJ/cm^2, respectively. According to the data, it is more perceptible the difference in outcomes after 6 mJ/cm^2, where each LRV per quantification method starts to spread out as the fluence increases.

UV treatments may cause *L. pneumophila* to enter a VBNC state, where the cells are still alive but damaged after specific treatments and in stressful environments.[53−55] VBNC cells are not detected by culture methods (such as SPC), but colorimetric and fluorescence methods can still count them. The Legiolert test uses an enzyme to color-indicate the presence of *L. pneumophila*;[36] therefore, this could lead to the quantification of VBNC and viable cells, thus generating a low LRV and a difference of around 2 LRV between SPC and Legiolert results.

Most qPCR tests only detect short genome sequences, which can lead to an overestimation of viable organisms.[56] This means that qPCR analysis of UV-treated samples detects the presence of the bacterium only by amplifying the part of the genome that indicates the genus and species of *L. pneumophila*. Quantifying the presence of that specific amplicon does not indicate if a cell is damaged, dead, alive, or viable. Hence, the qPCR test has the potential to detect VBNC, as well as both dead and alive cells, leading to results with low LRV. The mechanisms of genome damage are more complex than the short amplicon qPCR could fully detect.

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Table 2. Summary of Previous Biofilm-Pathogen Inactivation Studies Using UV LEDs[a]

organism	strain	wavelength (nm)	matrix	material	LRV	fluence (mJ/cm^2)	k values	reference
Ec	IFO 3301	280	monoculture	*NA*	4.2	9	0.561	50
Ec	CGMCC 1.3373	365*	monoculture	*NA*	4	19.6	0.7	23
Ec	ATCC 15597	278	monoculture	*NA*	5	6.5	0.342	49
Ec	O157: H7	268	monoculture	*NA*	4.88	7	0.428	66
Ec	K-12 (ATCC R 29425)	285	monoculture	*NA*	5	16	0.403	67
Pa	*NR*	265	biofilm	silicon	2.4	8.3	*NR*	62
Pa	*NR*	265	biofilm	FEP teflon	4.3	166	*NR*	62
Pa	PAO1	295	biofilm	membrane filter	3.9	20	*NR*	63
Pa	ATCC 10145	280	monoculture	*NA*	4	9	0.511	50
Pa	ATCC 15442	285	monoculture	*NA*	4.5	16	0.483	67
Pa	PAO1	265	biofilm	polycarbonate	1.3	8	*NR*	42
Pa[1]	*NR*	282	biofilms	polycarbonate	2.08	40	0.26	21
Pa[3]	*NR*	282	biofilms	polycarbonate	2.5	40	0.17	21
Pa	*NR*	282	biofilm	resuspended	6.5	10	0.26	21
Lp	ATCC 33152	365	monoculture	*NA*	3	1300	*NR*	26
Lp	ATCC 33152	280	monoculture	*NA*	4	9	0.453	50
Lp	Sg1	280	monoculture	*NA*	4	16	*NR*	55
Lp	Sg4	280	monoculture	*NA*	4	16	*NR*	55
Lp	Sg6	280	monoculture	*NA*	5.5	16	*NR*	55
Lp	ATCC 33152	280	monoculture	*NA*	4	20	0.27	
Lp	ATCC 33152	365	monoculture	*NA*	1.5	200	0.009	
Pf	ATCC 17569	280	monoculture	*NA*	4.5	12	0.36	
Pf	ATCC 17569	365	monoculture	*NA*	2	120	0.003	
Pf	ATCC 17569	280	biofilm	stainless steel	4	100	0.181	
Pf	ATCC 17569	365	biofilm	stainless steel	0.2	1000	0.000	
Pf	ATCC 17569	280	biofilm	cast iron	2.8	100	0.006	
Pf	ATCC 17569	365	biofilm	cast iron	1.3	1000	0.0003	

[a]Below the dotted line are the results of this study. Organisms: *Ec*, *E. coli*; *Pa*, *P. aeruginosa*; *Lp*, *L. pneumophila*; *Pf*, *P. fluorescens*. Monocultures: pure cultures in an aqueous solution. *NA*: not applicable; *NR*: not reported; LRV: log reduction value. *Treatment made with TiO_2. [1]One-day-old biofilm. [3]Three-day-old biofilm. Sg: Serogroup.

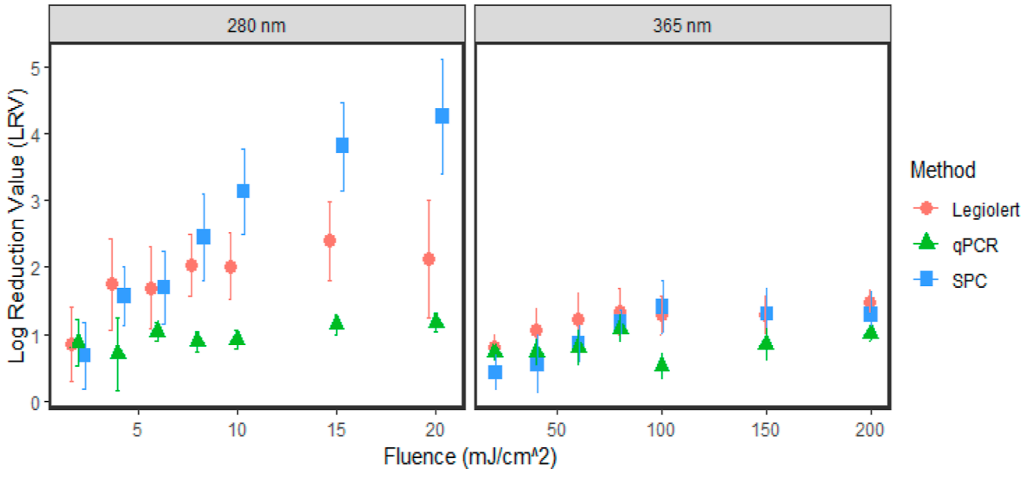

Figure 3. Inactivation of *L. pneumophila* (in aqueous solution) after UV LEDs. The cells were washed to obtain a UVT = ∼ 95% and treated with 280 and 365 nm. Cells were quantified using SPC with BCYE agar, Legiolert test, and quantitative polymerase chain reaction (qPCR) assay. Error bars represent the standard deviation from the mean; n = 4.

According to our results, the qPCR amplicon effectively captures 1 ± 0.005 LRV, as indicated by the significantly reduced error bars beyond 4 mJ/cm^2. Fluences below this threshold appear to cause more random damage to the targeted section of the amplicon, and by the time it reaches approximately 4 mJ/cm^2, nearly all of the section is damaged. Therefore, qPCR can still capture some of the genome damage, but not enough to determine if this damage would result in an inactivated cell or a VBNC cell, therefore the gap of nearly 3 LRV between SPC and qPCR outcomes.

The choice of quantification tools can obscure the effect of treatment and show different information regarding the state of *L. pneumophila* cells. Additionally, the results of *L. pneumophila* testing alone do not measure health risks and potential disease.[29]

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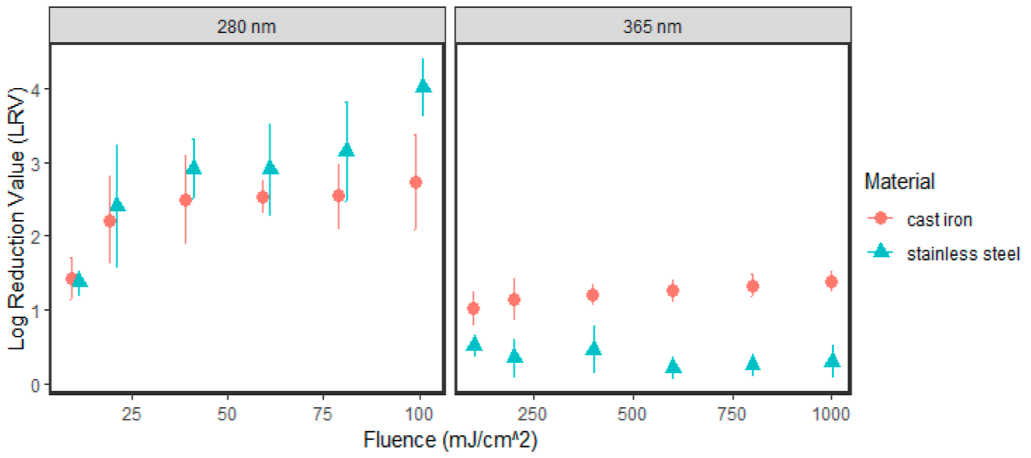

Figure 4. Inactivation of *P. fluorescens* biofilms after the use of UV LEDs. The organism was inoculated into CDC reactors for 24 h in batch mode and another 24 h in flow-through mode. Stainless steel (SS) and cast iron (CI) coupons were used as a growth surface. The feeding solution was prepared with a 3.33% TSB solution. Biofilms were quantified using SPC with TSA plates. Error bars represent the standard deviation from the mean, $n = 4$.

According to studies, genome quantification for viruses is more accessible than for bacteria, as they target RNA instead of DNA sequencing.[56,57] As not many studies are addressing this issue, it leads to the importance of properly quantifying DNA damage, as it is the main mechanism of UV disinfection. Dusserre et al.[58] (2008) used qPCR, SPC, and flow cytometry to detect VBNC cells of *L. pneumophila* treated with chemical disinfection; results show that *L. pneumophila* PCR detects culturable bacteria, VBNC forms, and dead bacterial DNA at low chlorine concentrations without differentiating them. Chlorine can damage any part of the cell (including protein, membrane, and DNA, through oxidation), whereas UV primarily targets DNA; therefore, assessing the tools available for DNA damage quantification is crucial for evaluation of UV disinfection outcomes. Specifically, the qPCR test proved to be the fastest tool as it can produce results relatively rapidly (around 2 h per sampling event); however, it is essential to develop a qPCR-based technique to quantify cell damage for *L. pneumophila*. Current cultured-based methods can take up to 7 days to obtain results, which is a long time for a scenario where it is necessary to take immediate corrective actions (i.e., assessment of disinfection performance in drinking water).

In a study carried out by Buse et al.[55] (2022), three strains of *L. pneumophila* (serogroups 1, 4, and 6) were treated using a UV LED collimated beam. The strains were exposed to 255, 265, and 280 nm, with fluences ranging from 0.5 to 34 mJ/cm^2. The findings revealed that, in general, the lower fluences at 255 nm resulted in effective inactivation, although certain strains showed higher susceptibility to 280 nm; results show LRV between 4 and 5.5 at 16 mJ/cm^2. Despite the different strains of *L. pneumophila* used, the outcomes quantified by SPC in these studies align with the findings reported in the present study. The authors stated that the effectiveness of UV LEDs in deactivating *L. pneumophila* may vary, even among strains from the same species, leading to divergent inactivation results. It is important for researchers to consider strain choice with working with this species of challenge organism, as it will impact results.

UV disinfection at 365 nm did not exceed 1.5 LRV at fluences between 20 and 200 mJ/cm^2 in this study (Figure 3); specifically at 200 mJ/cm^2, SPC obtained 1.15 ± 0.269 LRV, and for Legiolert and qPCR, 1.42 ± 0.142 and 0.94 ± 0.006 LRV were obtained, respectively. In contrast, Allahyari et al. (2022) obtained ∼3 LRV of *L. pneumophila* with 1700 mJ/cm^2. Therefore, it is likely that very high fluences (i.e., >1000 mJ/cm^2) are required to inactivate *L. pneumophila* using only UVA wavelengths. As previously mentioned, it is still debated whether wavelengths higher than 315 nm induce photodamage to genomic DNA and repair proteins.[26] These mechanisms are not apparent in this study, as all the quantification methods showed similar results. It is hypothesized that the mechanism for 365 nm damage is captured within the range of fluences examined in this study (20−200 mJ/cm^2) but may not be captured if higher fluences were used.

Interestingly, the LRVs from Legiolert were higher than those from SPC for the 365 nm exposure. Therefore, it was hypothesized that 365 nm wavelength induces some cell or protein damage and relies less on genomic damage for disinfection. This suggests that proteins in 365 nm-exposed samples do not react with the enzyme present in the Legiolert (therefore no color change) but still foster some growth on culture media (BCYE).

The k value obtained for *L. pneumophila* (Table 2) at 280 nm is 0.27 ± 0.04, whereas the value reported by Rattanakul and Oguma[50] (2018) is ∼0.45; the differences are probably related to the conditions and setup used between studies, such as sample preparation and concentration, media preparation, type of collimation used, and photo radiometer measurements. The efficacy of UV LEDs in inactivating *L. pneumophila* can exhibit discrepancies even among strains belonging to the same species. Furthermore, variations in methodology can also contribute to divergent results.[55] The k value for 365 nm was 0.009 ± 0.01; a study using 365 nm and fluences of 1700 mJ/cm^2 utilizing UV LEDs for *L. pneumophila*[26] yielded a k value of 0.796 ± 0.178 × 10^{-3}.

The primary concern with UV disinfection is the photoreactivation of pathogens. Studies have shown that following 254 nm disinfection of *L. pneumophila*, photoreactivates faster than other pathogens, specifically *E. coli*.[59,60] This highlights the need for improved UV disinfection systems, specifically targeting *L. pneumophila*. These previous studies used 254 nm LP or MP UV lamps. Therefore, it is essential to investigate the effects of higher wavelengths, such as 280 and 365 nm, using UV LED devices to determine if they may or may not prevent

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or reduce photoreactivation by causing damage to nucleic acids and repair enzymes.[26] Photoreactivation studies were not in the scope of this study, but the authors agree that it is important to address them for future research.

3.3. Growth and Inactivation of *P. fluorescens* Biofilms with UV LEDs. A better performance inactivation is shown for SS at 280 nm, reaching 4.02 ± 0.32 LRV at a fluence of 100 mJ/cm^2, in contrast with CI, which achieved disinfection of 2.96 ± 0.56 LRV at 100 mJ/cm^2. At 365 nm, even though the disinfection was below 1.4 LRV, the performance was significantly better for CI compared with that of SS, in which progressive disinfection reached 1.4 ± 0.13 LRV at 1000 mJ/cm^2 and 0.30 ± 0.20 LRV, respectively (Figure 4). This suggest that UV at 365 nm initiates another mechanism with CI compounds, which may induce photolysis that led to bonds rupture and photodegradation. Another study has observed 365 nm-induced biodegradation of compounds using *Pseudomonas* spp,[61] which suggests that this mechanism may occur at wavelengths closer to 365 nm. The differences between CI and SS 365 nm results are statistically significant (*p-value* of 4.04 × 10^{-11}).

To date, there is a lack of reported studies utilizing the precise strain of *P. fluorescens* within a biofilm matrix that has been exposed to UV LEDs, particularly at wavelengths >254 nm, thereby impeding specific comparisons. However, alternative strains of *Pseudomonas*, such as the more pathogenic *P. aeruginosa*, can be employed for reference. Studies involving *P. aeruginosa* biofilms on plastic-like materials have demonstrated that exposure to wavelengths ranging from 265 to 280 nm, with fluences <100 mJ/cm^2, yields LRV ranging from 1 to 5.[21,42,62,63]

A 10-fold increase in fluence was necessary to attain comparable outcomes in the inactivation of planktonic cells versus biofilm-bound cells of *P. fluorescens*. It is widely acknowledged that bacterial communities existing in biofilm structures exhibit heightened resistance to treatments, thereby posing a greater challenge in their management.[4,64]

The biofilm growth of *P. fluorescens* on SS was 7.96 log cfu/cm^2 ± 1.71, while on CI, it was 8.43 log cfu/cm^2 ± 1.64. The results demonstrate that the difference between biofilm growth on the materials was not significant (*p-value* of 0.403 with a 95% confidence interval [−2.57, 1.63]; log-transformed *t*-test).

Further investigations are necessary to confirm that the variation in inactivation outcomes is not attributed to the material itself but rather to the formation and characteristics of biofilms on the material, including factors like thickness, water channel formation, nutrient absorption, and extracellular polymeric substances composition, like carbohydrates and lipids.[65]

A closer look at the *k* values (Table 2) allows us to compare each treatment based on the inactivation constant of the same microorganism in different scenarios. At 280 nm, the inactivation rate constant of *P. fluorescens* monocultures (0.36 ± 0.033) was double the *k* value of SS biofilms (0.181 ± 0.254) and was 60 times higher than that of CI biofilms (0.006 ± 0.0004). Ma et al.[21] (2022) observed a *k* value of 0.17 ± 0.00 for a three-day-old *P. aeruginosa* biofilm exposed to a wavelength of 282 nm on a polycarbonate coupon. Despite variations in strains, conditions, and materials, the results show a similarity in the outcomes of UV LED inactivation when compared to the SS results of our study. At 365 nm, the rate constant of monocultures (0.002 ± 0.0003) was eight times higher than that of SS biofilms (0.00025 ± 0.0001) and six times higher than that of CI biofilms (0.00033 ± 0.0004). These results express that the best performance, regardless of the matrix, was at 280 nm for *P. fluorescens* UV LED inactivation.

Table 2 provides a consolidated overview of prior biofilm and monoculture inactivation studies utilizing UV LEDs, incorporating the findings from this study to facilitate direct comparisons. However, this table is not exhaustive as it includes only a limited range of wavelengths and organisms that are specifically relevant to the study being conducted.

This study suggests that *P. fluorescens* exhibits a higher level of sensitivity compared to *L. pneumophila* when exposed to identical wavelengths and fluences, which aligns with the findings reported in the existing literature.[50,68]

4. CONCLUSIONS

In this study, we examined the effectiveness of UV LED treatment (at wavelengths of 280 and 365 nm) on the OP *L. pneumophila* and biofilm-producing bacteria *P. fluorescens* and the comparison across quantification methods for assessing UV disinfection outcomes. Our findings indicate the following key points:

L. pneumophila was more effectively disinfected with UV LEDs at 280 nm than at 365 nm. Results at 365 nm did not exceed 1.5 LRV. However, quantification methods (SPC, Legiolert, and qPCR) yielded varying results at 280 nm, highlighting the need for improved quantification methods to assess UV LED disinfection accurately. Improved strategies, such as qPCR-based quantification, should be explored for more accurate evaluations.

P. fluorescens monocultures showed better disinfection at 280 nm compared to that at 365 nm. Biofilms of this pathogen on SS exhibited higher LRV at 280 nm, while CI samples showed higher LRV at 365 nm. A 10-fold increase in fluence was required to achieve comparable results between pure cultures (planktonic cells) vs biofilms. Further research is needed to understand the mechanisms at play in biofilm-bound cells.

UV LED treatments at wavelengths closer to 280 nm showed superior disinfection performance compared with conventional treatments at 254 nm and higher fluences.

In summary, UV LED-based disinfection processes offer effective biofilm and pathogen control but the development of better quantification methods is essential. Additionally, exploring the physical and biological mechanisms in biofilm-bound cells can provide valuable insights for future studies.

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Notes

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