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Searching for Activity Markers that Approximate (VBNC) *Legionella pneumophila* Infectivity in Amoeba after Ultraviolet (UV) Irradiation

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Abstract: *Legionella pneumophila* is an increasingly recognized threat to public health via aerosol exposures; with a variety of control measures including: water temperature/flow management and free chlorine used to reduce the risk of infection within healthcare centers. Despite these efforts, *L. pneumophila* often recolonizes plumbing systems after specific treatments, which prompted us to examine ultraviolet (UV) irradiation for a point-of-use, secondary control measure. Currently, there is no data on the efficacy of high (>254 nm) wavelength UV-C (100–280 nm) light inactivation of *L. pneumophila* with resuscitation of viable but non-culturable (VBNC) cells. We report for the first time *L. pneumophila* dose-responses for 268.6 nm and 288.6 nm UV-C, as compared to 256 nm, and demonstrate UV induced VBNC *L. pneumophila* remaining infectious to *Acanthamoeba polyphaga* during co-culture experiments. Findings were correlated to molecular-based activity assays to identify additional measures of *L. pneumophila* viability following UV disinfection compared to culture. A collection of viability markers may provide a more representative measure of risk compared to current culture-based detection, since UV-C irradiated *L. pneumophila* lose culturability, yet retain activity, increased ATP production, and the ability to be resuscitated by amoeba co-culture. This finding is significant as it identifies potential concern from VBNC cells following UV-C disinfection and the need for further research into the efficacy of UV inactivation as a point-of-use application for *L. pneumophila* control and management.

Keywords: *Legionella*; UV inactivation; viable but non-culturable (VBNC); amoeba; resuscitation; activity assay; flow cytometry

1. Introduction

Legionella pneumophila is a Gram-negative, water-based, opportunistic pathogen that can cause two distinct diseases, a mild one known as Pontiac fever and potentially fatal Legionnaires' disease (LD). Incidence rates of LD have jumped in recent years with improved surveillance and an increase in high risk groups [1]. Major exposure sources include premise plumbing, cooling towers, and hot tubs, where warm water can stagnate, allowing for *L. pneumophila* to amplify within biofilms and free-living protozoa [2,3]. Due to these concerns, risk managers have resorted to numerous chemical disinfectants, thermal treatments, and point-of-use (POU) measures to reduce viable *L. pneumophila* levels within premise plumbing, particularly in healthcare centers [4].

Chlorination and water temperature management are the most common control measures for *L. pneumophila* within premise plumbing. However, several studies have demonstrated the re-colonization of buildings after thermal shock or superchlorination treatments, as *L. pneumophila* residing within biofilms and protozoan (including cyst) hosts remain protected [5,6]. Furthermore,

these treatments have been shown to induce a viable but non-culturable (VBNC) state in *L. pneumophila*, a biophysiological response to environmental stress, resulting in loss of culturability on conventional artificial media, yet cells can be resuscitated [7–9]. As a result, point-of-use applications, including ultraviolet (UV) light and filters have been promoted as secondary control measures.

UV disinfection has been described as an attractive approach to pathogen inactivation in water systems, as it does not add any chemicals [10]. It is most effective at POU, when considering the lack of residual disinfectant in circulating systems that may allow for recolonization, with a recommended dose of 16 mJ·cm^{-2} for supplemental germicidal treatment of disinfected water systems (Class-B POU) [4,11]. Until recently, most UV disinfection studies have focused on monochromatic 254 nm light, being the most commonly applied wavelength for UV water treatment processes via mercury vapor lamps [12]. Previous studies on the susceptibility of *L. pneumophila* to 254 nm UV-C (100–280 nm) reported a 4 $\log_{10}$ reduction in culturable cells at a dose of 5.7 mJ·cm^{-2} [13]. However, the presence of a light-dependent repair mechanism in *L. pneumophila* has reportedly turned a 3 $\log_{10}$ reduction into a 0.5 $\log_{10}$ reduction following incubation in the presence of visible light [14].

A major limitation of culture-based detection of *L. pneumophila* is the potential underestimation of risk resulting from the inability to detect VBNC cells. Despite restricted metabolic rates, VBNC cells have been shown to retain pathogenic properties [15]. The ability of amoeba co-cultures to resuscitate VBNC *L. pneumophila* was first reported after non-culturable bacterial cells that were subjected to nutrient scarce sterile tap water for 180 d quickly regained culturability following co-culture with *Acanthamoeba castellanii* [16]. Since this initial study, researchers have reported the resuscitation of VBNC *L. pneumophila* following inactivation in synthetic drinking water by free chlorine, monochloramine, and heat [17–20]. However, we are not aware of published data on the infectivity of UV-induced VBNC *L. pneumophila*; yet, given the previously reported photoreactive potential of this bacteria, we hypothesize its potential.

Despite this, amoeba co-culture is time consuming and can be vulnerable to contamination. Due to these concerns, researchers have turned to other markers of activity/viability that can be coupled to molecular-based detection platforms. A variety of markers might be useful for the detection of viable *L. pneumophila*, including membrane permeable and impermeable dyes (live-dead staining), metabolic activity indicators (electron transport activity, esterase enzyme function), and measures of growth potential (ATP concentration), Figure 1. The coupling of immunomagnetic separation purification with flow cytometry (IMS-FCM) and an activity marker represent a practical platform for the rapid detection of total legionellae and viable *L. pneumophila* within environmental water samples [21].

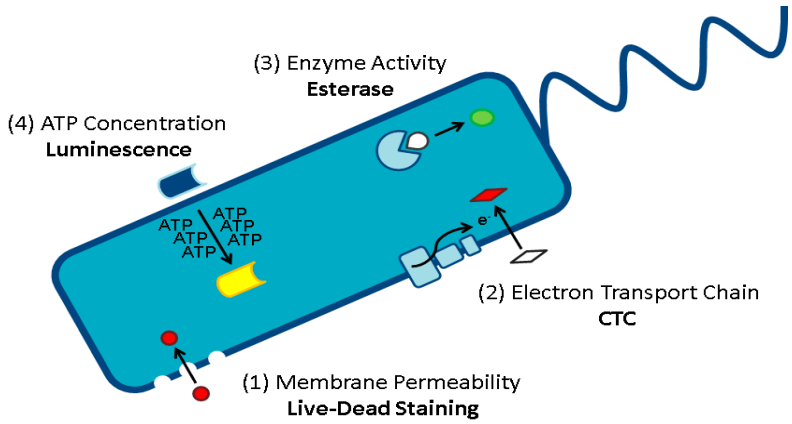

Figure 1. Fluorescent and luminescent viability assays examined with experimental amoeba co-culture data.

Here, we present an evaluation of UV-C light-emitting diodes (LED) disinfection efficacy to cell suspensions of *L. pneumophila* and compared measures of ATP, esterase activity, electron transport

function reduction and membrane permeability, alongside amoeba co-culture to investigate VBNC cell formation and light/dark repair phenomena following three different UV-C light treatments.

2. Materials and Methods

2.1. Cultivation of Legionella Pneumophila

Primary stock *L. pneumophila* Philadelphia-1 strain (ATCC 33152) was prepared by plating onto buffered charcoal yeast extract (BCYE) agar (obtained from Alberta Provincial Laboratory, Edmonton, AB, Canada) and incubating at 37 °C for 96 h. A single colony was then inoculated into AYE medium (10 g *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 10 g yeast extract, 0.4 g L-cysteine, and 0.25 g iron pyrophosphate per litre), and incubated in a shaker at 37 °C for 18 h, when the cells reached late log phase growth according to a predetermined bacterial growth curve calibrated to absorbance at 600 nm and colony forming units (CFU) [22].

2.2. Cultivation of Free-Living Amoebae

Acanthamoeba polyphaga (ATCC 30461) was grown in PYG 712 medium (20 g proteose peptone, 1 g yeast extract, 0.985 g magnesium sulphate heptahydrate, 0.0588 g calcium chloride, 1.0 g sodium citrate, 0.0196 g ammonium iron(II) sulphate hexahydrate, 0.67 g sodium phosphate dibasic heptahydrate, 0.34 g potassium phosphate monobasic, and 18 g of glucose per litre) [23]. The amoeba cultures were grown for 4 d at 25 °C before re-culturing by pipetting 200 µL of the culture into 5 mL of fresh PYG medium.

2.3. Disinfection by Ultraviolet Radiation

The efficacy of ultraviolet (UV) light disinfection on *L. pneumophila* (ATCC 33152) was examined by irradiating at multiple UV-C light wavelengths and fluences, measuring the $\log_{10}$ reduction of culturable cells. The experiment followed the National Science Foundation (NSF) International standards for evaluating microbiological UV susceptibility, where UV dose is calculated from measured intensity, absorbance of suspended medium, and exposure time [11]. The UV intensity was measured with an UVX-25 radiometer (UVP, Upland, CA, USA) set to the 0–200 $\mu W \cdot cm^{-2}$ range, with calibration factors for the respective wavelengths tested. The UV irradiance was measured at the same distance from the LEDs as the irradiated samples, with the collimating beam attached, with absorbance being recorded at each respective wavelength being tested.

Bacterial cells were grown for 18 h to late log phase and serial diluted to a working concentration of 10^6 $CFU \cdot mL^{-1}$ in 0.85% saline. An aliquot (28.3 mL) of this suspension was placed in a 55 mm × 20 mm Petri dish to a final water depth of 1 cm and mixed with a magnetic stir bar. Prior to UV exposure, a 100 µL sample of the bacterial cell suspension was serial diluted and plated onto BCYE agar to determine initial *L. pneumophila* concentration above the detection limit of 10 $CFU \cdot mL^{-1}$. The suspensions were irradiated with 256, 268.6, or 288.6 nm UV light while using a PearlBeam™ LED collimating beam (AquiSense Technologies, Erlanger, KY, USA), with each diode emitting within a narrow band of polychromatic UV-C light (some 10 nm $\frac{1}{2}$ max bandwidth, Supplementary Information, Figures S1–S3). Plates were incubated at 37 °C for 96 h before counting revealed the $\log_{10}$ reduction in CFU resulting from UV exposure.

2.4. Photoreactivation of Non-Culturable L. Pneumophila after UV Inactivation

In order to study light-activated repair, the stock suspension of *L. pneumophila* (ATCC 33152) was exposed to UV light doses of 0, 10, 20, 30, and 40 $mJ \cdot cm^{-2}$. From the UV irradiated cell suspensions, 1 mL samples were divided into dark (control) or light conditions at room temperature, with the samples being plated at 0 and 24 h, post-irradiation. Secondary controls with non-UV irradiated *L. pneumophila* in 0.85% saline were run to determine whether the cells could replicate within the osmotically neutral, minimal medium, given the presence of residual growth medium stemming from

dilutions of late log-phase cells (Supplementary Information, Table S1). Statistical significance of observed differences in dose-response and photoreactivity was quantified while using an unpaired, two-tailed Student's *t*-test (Microsoft Excel 2010).

2.5. Resuscitation of UV Irradiated L. pneumophila with A. polyphaga

An axenic subculture of *A. polyphaga* (ATCC 30461) was diluted to 10^5 amoebae·mL^{-1} in PYG 712 medium in order to assess the resuscitation capabilities of 256 nm UV irradiated, non-culturable *L. pneumophila* within a free-living amoeba host. A 5 mL aliquot was then transferred to a 15 mL centrifuge tube, where approximately 5×10^6 CFU·mL^{-1} of non-stressed (control) or UV stressed (non-culturable) *L. pneumophila* (ATCC 33152) was added to create a 50:1 multiplicity of infection (MOI). The bacteria-amoebae mixture was centrifuged at 233 rcf for 5 min to stimulate contact between cells. The co-culture was then vortexed briefly to re-suspend cells before 1 mL suspensions were added into 24 well plates that were incubated for 4–5 days at 37 °C. A set of control samples containing control or non-culturable, UV irradiated *L. pneumophila* without *A. polyphaga* were run to examine the effects of free-living amoebae and the amoeba growth medium on the resuscitation potential. The concentration of culturable *L. pneumophila* was determined by plating samples from the 24 well plates on BCYE agar at 0, 24, 48, 72, and 96 h post infection. A 3 mL syringe with 20 G needle was used to vigorously agitate the mixtures to disperse *A. polyphaga* allowing access to internalized *L. pneumophila,* following optimized procedures [24].

2.6. Activity Assays

Three fluorescent assays measuring membrane permeability, electron transport chain function, and enzyme activity were examined for potential correlation with amoeba resuscitation data analyzed by flow cytometry (Gallios™ flow cytometer, Beckman Coulter, Brea, CA, USA). Each assay was standardized according to the manufacturer protocols or best-practices from available literature. Upon calibration, each assay was used to determine the percent change in viable cell population following disinfection at increasing doses up to 2.5 times dose the required to achieve 6 $\log_{10}$ reductions in culturable cells. For each activity assay, 10^6 CFU·mL^{-1} *L. pneumophila* were exposed to 10, 20, 30, or 40 mJ·cm^{-2} 256 nm UV-C light. Prior to stress events, aliquots of *L. pneumophila* were taken to serve as the non-stressed (control) proportions, as well as a 70 °C heat exposed and 95% ethanol treated dead controls, to which each disinfected sample was compared against. Fluorescent dyes and colourless reagents were added prior to flow cytometric analysis and were incubated in accordance with recommended protocols. All fluorescent data was recorded on the Gallios™ flow cytometer, analyzed using Kaluza™ software (Beckman Coulter, Brea, CA, USA). The same protocol was used for the luminescence-based activity assay with measurements while using a FLUOstar Omega filter-based multi-mode microplate reader (BMG Labtech, Ortenburg, Germany).

The Live/Dead® BacLight™ bacterial viability kit (Molecular Probes, Eugene, OR, USA) was used to determine the proportion of bacteria with damaged cell membranes. The assay followed the manufacturer's instructions, with 3 µL of 20 mM PI and 3 µL of 3.34 mM Syto-9, being added to a 2 mL sample of bacteria, followed by 15 min incubation at room temperature. 5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC) (Sigma Aldrich, St. Louis, MO, USA), was used to determine the proportion of bacteria with active electron transport chains, using a previously published assay; in short, with 100 µL of a 50 mM CTC solution being added to 1 mL samples, followed by 1 h incubation at 37 °C [25]. The colourless molecule, 6-carboxyfluorescein diacetate (CFDA) (Sigma Aldrich, St. Louis, MO, USA), was used to determine the proportion of bacteria with esterase enzyme activity. Method standardization followed established literature, with 2.5 µL of 10 mM stock 6-CFDA being added to 500 µL samples with 50 µL of 10 mM Ethylenediaminetetraacetic acid (EDTA), followed by a 30 min incubation at 35 °C [26]. The luminescent BacTiter-Glo™ Microbial Cell Viability Assay kit (Promega, Madison, WI, USA) was used to quantify the intracellular ATP concentrations. The protocol followed the manufacturer's instructions, with 100 µL of the luminescence reagent being

added to 100 µL cell suspensions in an opaque 96 well plate. The relative ATP concentration was determined by comparing the cellular luminescence values to a standard curve with known ATP concentrations. Top luminescence of each well was recorded while using a multi-mode microplate reader in contrast to the fluorescent assay detection by flow cytometry.

3. Results and Discussion

Many bacterial species exhibit light and dark-specific DNA damage repair mechanisms, with the former being referred to as photoreactivation. The effectiveness of UV-C light on *L. pneumophila* has been shown to be dependent on the absence of visible light post irradiation [14]. Hence, the ability of *L. pneumophila* (ATCC 33152) to repair DNA damage following exposure to UV light was examined, as seen in Figure 2.

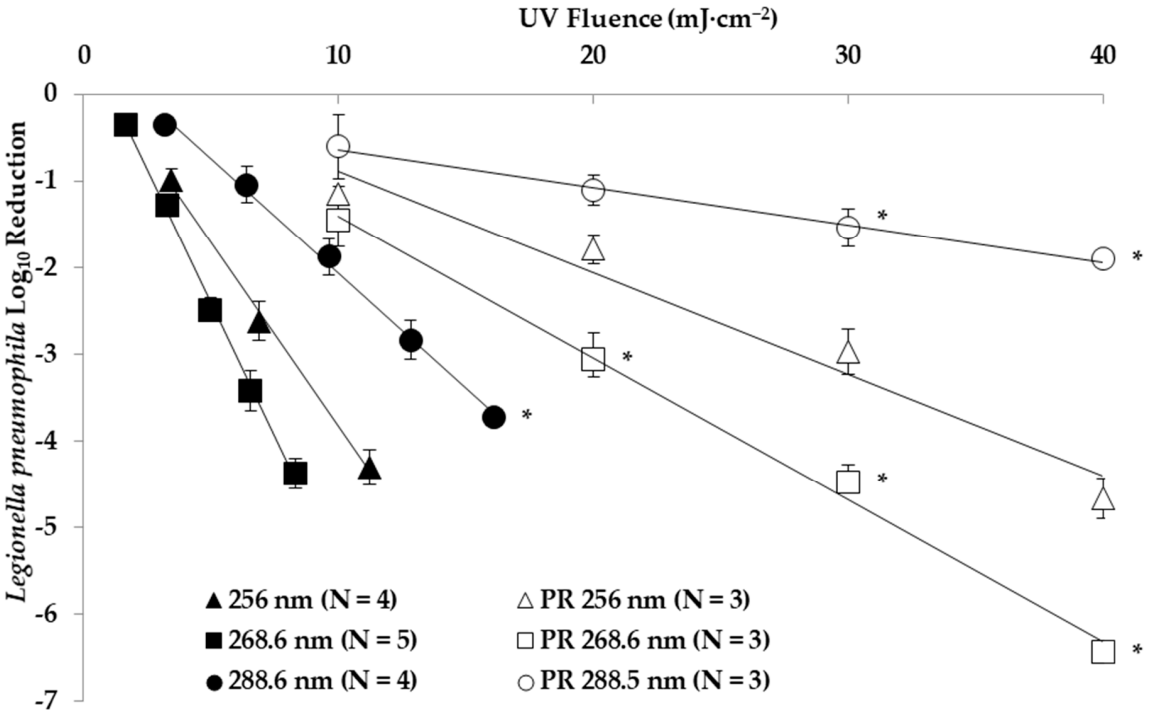

Figure 2. Ultraviolet (UV) dose-response curve for *L. pneumophila* (ATCC 33152) exposed to LEDs emitting at peaks of 256, 268.6, or 288.6 nm UV-C light (solid ■) as well as photoreactivated cells (PR □), with response reported as the reduction in culturable cells plated on BCYE agar, with a detection limit of 10 CFU·mL^{-1}. The average starting concentration of culturable *L. pneumophila* was 2.3 × 10^6 CFU·mL^{-1}, with error bars equivalent to ± SEM (standard error of the mean) for N replicate, independent trials. Statistically significant differences compared to standard 256 nm UV-C are denoted by (*).

Figure 2 shows that 268.6 nm UV-C was the most effective wavelength for achieving up to 6 log$_{10}$ reduction in culturable *L. pneumophila* after accounting for DNA repair mechanisms. The difference in efficacy was observable at higher UV doses, where a 40 mJ·cm^{-2} exposure resulted in an apparent complete loss of culturable cells at 268.6 nm, with approximately 10^2 and 10^5 *L. pneumophila* remaining culturable at 256 and 288.6 nm, respectively. Statistical analysis revealed significant differences in the mean photoreactivation values for 266.8 nm when compared to 256 nm at 20 mJ·cm^{-2} (P = 0.034), 30 mJ·cm^{-2} (P > 0.01), and 40 mJ·cm^{-2} (P = 0.017) and 288.6 nm as compared to 256 nm at 30 mJ·cm^{-2} (P = 0.01) and 40 mJ·cm^{-2} (P > 0.001), respectively. A plausible explanation for the photoreactivation phenomenon of legionellae must account for their ecological preference to inhabit environments where exposure to UV and visible light are more common than in engineered water systems [27]. *Legionella*

spp. have been shown to express a melanin-like pigment when grown in certain conditions, although there appears to be no relationship with UV light sensitivity [28]. The ecological significance of *L. pneumophila* photoreactivation remains unclear, however VBNC cells that can be re-cultured by visible light suggests that the organism might remain infectious to preferred hosts.

Without accounting for photoreactivation potential, estimated UV doses of 14.7, 10.2, and 21.8 mJ·cm^{-2} would result in 6 $\log_{10}$ reductions in culturable cells at 256, 268.6, and 288.6 nm, respectively. Figure 2 demonstrates that these doses are insufficient to yield a true 6 $\log_{10}$ reduction after accounting for DNA damage repair mechanisms, when considering culturable cells were observed at each wavelength following exposure to 30 mJ·cm^{-2}. In addition, the differences in culturable cell counts directly following exposure and after 24 h incubation in ambient light clearly indicate that UV-C exposure induces a VBNC state in *L. pneumophila*. While *L. pneumophilia* cells were non-culturable directly after exposure, light-activated DNA damage repair mechanisms promote re-culturability, demonstrating that the apparent VBNC *L. pneumophila cells* remain viable during this time. Thus, UV-C light exposure induces a VBNC state in *L. pneumophila*, which can regain culturability through incubation in ambient light conditions.

Here we also report the infectivity of non-culturable UV-C exposed *L. pneumophila* when co-cultured in the dark with *A. polyphaga* (Table 1).

Table 1. *Legionella pneumophila* (ATCC 33152) resuscitation in *Acanthamoeba polyphaga* (ATCC 30461) following exposure to 256 nm UV-C light over 4 d incubation at 37 °C, with culturable cell counts reported in $\log_{10}$ CFU·mL^{-1} ± SEM for N = 3 replicate, independent trials, with a detection limit of 10 CFU·mL^{-1}. Absence of growth denoted as ND (Non-Detectable).

UV Fluence (mJ·cm^{-2})	*Legionella* Concentration in Amoeba Co-Culture ($\log_{10}$ CFU·mL^{-1})				
	Day 0	Day 1	Day 2	Day 3	Day 4
0	6.2 ± 0.5	7.4 ± 0.6	7.6 ± 0.6	8.0 ± 0.6	8.6 ± 0.7
16	ND	3.2 ± 0.3	5.0 ± 0.5	6.0 ± 0.6	6.1 ± 0.5
40	ND	ND	ND	ND	ND

Table 1 demonstrates an amplification of 16 mJ·cm^{-2} UV stressed, non-culturable (immediately following UV treatment) *L. pneumophila* following co-culture in a free-living amoeba host over the course of four days. Within four days of co-culture, the bacteria reached estimated microbial risk assessment infection levels of concern, <10^6 CFU·mL^{-1}, through amplification in free-living amoeba. This finding is significant in that it demonstrates that the NSF International standards for Class-B POU secondary disinfection systems could be inadequate for removal of pathogenic *L. pneumophila*. Furthermore, culture-based detection would indicate no risk, as the bacteria are non-culturable directly following UV treatment, yet remain infectious and may regain culturability over time (potentially also with lung macrophages) [29].

While UV disinfection was insufficient at a dose of 16 mJ·cm^{-2}, resuscitation was not observed for *L. pneumophila* exposed to 40 mJ·cm^{-2} 256 nm UV-C light. A possible explanation for this observation involves Figure 2, which shows the corrected UV-C dose-response curves accounting for light-activated DNA damage repair. At a UV dose of 16 mJ·cm^{-2}, the corrected dose-response curve suggests >10^5 CFU·mL^{-1} *L. pneumophila* remain viable, despite being non-culturable. In contrast, at 40 mJ·cm^{-2} there was >10^2 CFU·mL^{-1} *L. pneumophila* that may remain viable that could be re-cultured via photoreactivation pathways. Therefore, the observed resuscitation of VBNC UV stressed *L. pneumophila* following 16 mJ·cm^{-2} exposure, but not at 40 mJ·cm^{-2} exposure may be attributed to the number of viable bacteria present in the samples. While, in theory, only one viable bacterium is necessary to infect and amplify within free-living amoeba, the high detection limit (10 CFU·mL^{-1}) make it unlikely to observe resuscitation with low viable cell counts. Therefore, to provide greater evidence of 40 mJ·cm^{-2} efficacy against *L. pneumophila*, it is recommended that co-culture lasts upwards of 7–10 days and that 1 mL samples (or cell concentrates) are plated to lower the detection limit to 1 CFU·mL^{-1}. Table 1

highlights the first reported instance of UV-C induced VBNC *L. pneumophila* infecting a free-living amoeba host, hence further research is recommended to evaluate the true efficacy of UV disinfection on other water-based pathogens.

The ability of *L. pneumophila* to move from a non-culturable state following exposure to UV to an infectious dose within a few days highlights the challenges associated with legionellae control and management in plumbing fixtures, such as shower heads. While amoeba co-culture is considered the truest measure for assessing public health risk that is associated with VBNC *L. pneumophila*, the method is time-consuming, prone to contamination, and relies on culture-based detection, which has a series of limitations that have prompted the search for novel molecular-based approaches, including flow cytometry. Therefore, a variety of fluorescent or luminescence-based activity assays were examined to explore the correlations with the experimental resuscitation data (Figure 3).

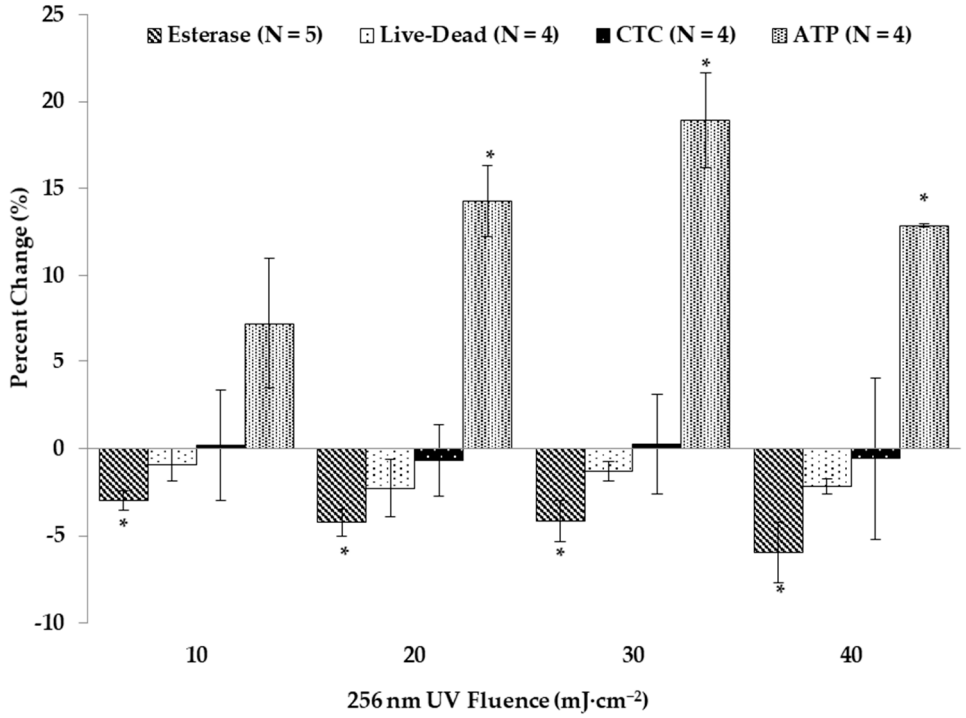

Figure 3. Esterase, CTC, live-dead, and ATP-luminescence activity assays for $<10^6$ *L. pneumophila* (ATCC 33152) irradiated with increasing doses of 10, 20, 30, and 40 mJ·cm^{-2} UV-C at 256 nm. Data is reported as percent change in activity when compared to non-stressed (0 mJ·cm^{-2}) *L. pneumophila*, with error bars equivalent to ± SEM for N replicate, independent trials. Statistically significant differences compared to null hypothesis = 0, are denoted by (*).

Figure 3 demonstrates significant changes in esterase activity ($P < 0.03$) and ATP concentration ($P < 0.01$), while membrane permeability and electron transport function remain unchanged following 256 nm UV exposure (Supplementary Information, Table S4). The decrease in esterase activity suggests that UV-C may be causing photochemical reactions that reduce enzyme function through protein folding [30]. The increase in ATP production following UV stress may arise from increased exonuclease activity and substrate level phosphorylation, as cellular enzymes repair damaged DNA [31]. The ATP concentration appears to spike at 30 mJ·cm^{-2} before declining at a fluence of 40 mJ·cm^{-2}, suggesting that the higher fluence may be effective in reducing viable cell counts through induction of DNA damage. Thus, the activity trends presented here confirm that UV-C inactivation affects proteins as well as DNA, and that the increased ATP concentration following stress may provide insight into the true risk associated with these VBNC *L. pneumophila*.

While the ATP concentration was found to increase, there was minimal change identified by CTC or live-dead stains, which indicates that cells still possess all the markers of being active. However, despite the knockout of one 'viability' marker, cells may remain a risk if they can repair damage over time, or purposefully reduce metabolic activity upon induction of a VBNC state in response to stress. Some have recommended that dead cells be reclassified as those that have irreversibly lost all ability to reproduce, elongate, and synthesize proteins [32]. Thus, it is apparent that a true marker of resuscitation potential account for different components possessed by viable cells, including enzyme function, membrane permeability, electron transport function, and ATP production.

Figure 3 may present the first comprehensive analysis of *L. pneumophila* activity following UV stress, with most literature being focused on *Escherichia coli* to date. However, the available publications on *E. coli* did not analyze ATP activity, yet found minimal change in esterase or CTC for 50 mJ·cm^{-2} UV [32]. The increase in ATP production following UV irradiation may explain why UV was the only inactivation regime yielding *L. pneumophila* that could be resuscitated by amoeba co-culture. Thus, luminescent viability assays of intracellular ATP concentration in *L. pneumophila* might provide a useful measure of infectivity risk following inactivation by UV-C light exposure.

4. Conclusions

Here, we have reported for the first time, higher UV-wavelength dose-response curves, comparative photoreactivity plots, and UV-C induced VBNC *L. pneumophila* remaining infectious to an amoeba host. These findings were used to examine potential activity markers that correlate with amoeba infectivity following UV-C exposure.

The corrected *L. pneumophila* dose-response curve for UV-C exposure (Figure 2) suggests that the NSF International guidelines for effective POU UV treatment should be revisited. Recall that the NSF International/ANSI standard document mandates a UV dose of 16 mJ·cm^{-2} for Class-B POU systems, which are used for supplemental germicidal treatment of disinfected water supplies; while a dose of 40 mJ·cm^{-2} is deemed sufficient for pathogen removal when operated as the primary inactivation treatment (Class-A POU) [11]. Neither of these treatments would be sufficient to achieve up to a 6 $\log_{10}$ inactivation of *L. pneumophila* at 256 or 288.6 nm, with only the 268.6 nm exposure apparently causing complete loss of culturable cells at 40 mJ·cm^{-2}. Operating a POU applicator at these low efficacy wavelengths might lead to poor inactivation performance and increased risk of legionellosis cases, particularly in biofilm rich environments, such as shower heads where light may reach, and amoeba may also grow. Accordingly, further research is required to determine the true population of VBNC and potentially infectious *L. pneumophila* following UV inactivation. Once this is achieved, quantitative microbial risk assessment (QMRA) modeling could provide an improved estimate of risk, allow for better monitoring of hazards, and ideally identify where to mitigate cases.

The non-culturable state induced in response to 256 nm UV-C irradiation could have major implications for public health risk. The current culture-based approach for detecting *L. pneumophila* could lead one to conclude no risk when exposed to tap water that had been irradiated with UV-C light at a point-of-use filter. While it is unclear what fraction of VBNC *L. pneumophila* may remain after Class-A POU treatment at 40 mJ·cm^{-2}, should biofilm develop post UV treatment in a POU device, such as in a shower head/aerator, it is possible that within 5 days of initial UV exposure, VBNC *L. pneumophila* could amplify within biofilm associated amoebae to levels approaching the critical concentration estimate by QMRA to likely cause infection via aerosol exposures [33]. Overall, it is extremely important that we continue to examine other means for detecting *L. pneumophila* within premise plumbing, as current culture methods will not account for VBNC cells that remain infectious and a potential risk to public health.

The analysis of activity assays used in the current study indicates that plate-culture does not provide an accurate measure of viability (and potential infectivity), since cells loose ability to be cultured prior to major changes in the activity assays. Given that current detection relies on traditional plate culture, the VBNC state represents a major concern for evaluating true public health risk and the

efficacy of legionellae control and management. Thus, a collection of viability markers may provide a more representative measure of risk when compared to current culture-based detection, since UV-C irradiated *L. pneumophila* lost culturability, yet showed no major loss of activity, increased ATP production, and the ability to be resuscitated by amoeba co-culture.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/10/9/1219/s1. Table S1: Photoreactivation control experimental data, Table S2: Dose response statistics, Table S3: Photoreactivation statistics, Table S4: Statistical analysis, Figure S1: LED UV emission spectra 256 nm, Figure S2: LED UV emission spectra 268.6 nm, Figure S3: LED UV emission spectra 288.6 nm, Figure S4: *Legionella* growth in amoeba, Figure S5: Amoeba co-culture controls, Figure S6: Live-Dead flow cytometry plot, Figure S7: CTC flow cytometry plot, Figure S8: Esterase flow cytometry plot, Figure S9: Methodology flow chart.

Author Contributions: M.R.G. and N.J.A. conceived and designed the experiments; R.D. aided in amoeba co-culture training and oversight of their culture; M.R.G. performed the experiments, analyzed the data and wrote the paper as part of his MSc. study under the supervision of N.J.A.

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Conflicts of Interest: The authors declare no conflict of interest.

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