

PHOTOINACTIVATION OF *LEGIONELLA RUBRILUCENS* BY VISIBLE LIGHT

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In this study, the photoinactivation of *Legionella* by visible light is investigated. The success of this approach would offer new prospects for technical water disinfection and maybe even for therapeutic measures in cases of *Legionella* infections. Therefore, *Legionella rubrilucens* was dispensed on buffered charcoal yeast extract medium agar plates and illuminated with different doses of violet light generated by 405 nm light-emitting diodes (LEDs). A strong photoinactivation effect was observed. A dose of 125 J/cm² reduced the bacterial concentration by more than 5 orders of magnitude compared to *Legionella* on unirradiated agar plates. The necessary dose for a one log-level reduction was about 24 J/cm². These results were obtained for extracellular *L. rubrilucens*, but other *Legionella* species may exhibit a similar behavior.

Keywords: *Legionella rubrilucens*, 405 nm irradiation, photoinactivation, disinfection

Introduction

Legionellae are ubiquitous in aquatic environments and can therefore be found in natural reservoirs as well as in human water distribution systems. In humans, they can trigger severe pulmonary infections (Legionnaires' disease) and flu-like disorders (Pontiac fever). The main pathogen of the severe and fatal courses of the disease is the species *Legionella pneumophila*, which was named after the first documented outbreak of legionellosis in 1976 in Philadelphia during a convention of the American Legion (US military veterans) [1].

There are different approaches to control *Legionella* in drinking water systems. Frequently, chlorination is employed but often does not have the desired effect. This is at least partially due to the fact that legionellae often live in biofilms or reside intracellularly in amoebae, which offer protection against many disinfection measures.

Ultraviolet C (UV-C) irradiation is a well-known alternative to chemical disinfection. However, even in this approach, it was observed that amoebae hosts offer some kind of cover to legionellae. In order to achieve the desired

disinfection effect on legionellae inside amoebae, almost twice the UV-C dose is required compared to planktonic legionellae. Usually, mercury vapor lamps are used as UV light sources for this kind of disinfection. Their 254 nm radiation destroys the DNA by formation of thymine dimers [2]. Unfortunately, UV-C radiation has some disadvantages in the control of legionellae. On the one hand, legionellae have the ability to repair this kind of DNA damage in the presence of light with the help of the enzyme photolyase [3], and on the other hand, the penetration depth of UV-C radiation in water is very low. Depending on the degree of contamination, it can be determined within the millimeter or centimeter range [4].

For these reasons, the photoinactivation of *Legionella* by violet light at 405 nm is investigated in this paper. The success of photoinactivation with visible radiation as a disinfectant was already observed on 40 different bacterial species [9], but to our knowledge, legionellae have not been tested so far. The penetration depth of 405 nm radiation in water – about 10 m [5] – is significantly higher compared to UV-C light. The potential mechanism, which was first discussed in Refs. [6–8] is based on the presumed

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existence of endogenous porphyrins such as coproporphyrin III, protoporphyrin IX, and uroporphyrin III. These internal photosensitizers absorb 405 nm light, which subsequently leads to the formation of reactive oxygen species that attack cell structures such as the cell membrane and destroy the bacteria from within.

Due to safety and regulatory issues, the experiments and results presented here are based on *Legionella rubrilucens*, which is generally regarded as nonpathogenic, even though a first case of a pathogenic coinfection with *L. rubrilucens* was previously reported [10].

Materials and methods

The bacterium *L. rubrilucens* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) as freeze-dried culture (DSM No. 11884) and cultivated in buffered yeast extract medium (BYE) [11]. For photoinactivation experiments, buffered charcoal yeast extract medium (BCYE) agar plates [11] were prepared in Petri dishes of 90 mm diameter. Bacterial suspensions were first diluted with phosphate-buffered saline (PBS) to about 2.4×10^8 CFU/ml by measuring the optical density (OD) at 600 nm in a photometer. The corresponding OD was about 0.24. A subsequent further dilution ensured that a 100- μ l sample contained less or much less than 1000 bacteria to increase interpretability of the results. Such 100 μ l volumes of diluted bacterial suspensions were thoroughly distributed on the agar surfaces prior to the irradiation.

For the 405 nm irradiation experiments, a setup with 5 light-emitting diodes (LEDs) type LZ1-10UB00-00U7

from LED Engin, Inc. (USA) was constructed, resulting in a high but homogenous irradiance of 9.4 ± 0.3 mW/cm² over an area of 20×20 cm² that allowed the simultaneous irradiation of 4 agar plates. The radiation experiments were performed with different exposure times to achieve different irradiation doses. The maximum applied dose of 125 J/cm² took an illumination time of 3 h and 40 min.

After irradiation, the agar plates were stored for 4 days at 37 °C and high humidity in an incubator before they were photographed for easier colony counting, and the observed number of colonies on irradiated agar plates were compared to controls not subjected to irradiation. All experiments were performed three times with at least three evaluated agar plates in each run.

Results and discussion

The mean values of counted *L. rubrilucens* colonies on irradiated or reference agar plates and their standard deviation as well as the achieved bacterial reduction are presented in *Table 1* and *Fig. 1*. A successful photoinactivation effect is clearly observable with a maximum bacterial reduction of more than five log levels for a dose of 125 J/cm². There might appear a small “shoulder like” behavior for low intensities, indicating a minimal photoinactivation sensitivity for low irradiation doses, but for increased doses, the ratio of surviving *L. rubrilucens* shows an exponential decrease, illustrated as straight line in the half-logarithmic diagram in *Fig. 1*. The average dose for a one log reduction with 405 nm light is about 24.7 J/cm², which lies within the so far observed range of bacterial photoinactivation sensitivities presented in Ref. [9].

Table 1. Applied 405 nm irradiation doses, bacterial concentrations after illumination, reference concentrations, and ratio of surviving bacteria

Illumination dose (J/cm ²)	Bacterial concentration after illumination (CFU/ml)	Bacterial reference concentration (CFU/ml)	Average ratio of surviving bacteria (standard deviation of average ratio)
25	5.85E+07	2.81E+08	
25	9.20E+07	2.92E+08	2.4E-01 (4.3E-02)
25	4.07E+07	2.41E+08	
50	1.39E+07	2.89E+08	
50	1.20E+07	1.66E+08	4.8E-02 (1.4E-02)
50	2.97E+06	1.28E+08	
75	5.43E+05	2.81E+08	
75	3.31E+06	2.92E+08	5.3E-03 (3.0E-03)
75	6.50E+05	2.41E+08	
100	3.08E+04	2.81E+08	
100	1.38E+04	2.92E+08	6.3E-05 (2.3E-05)
100	8.00E+03	2.41E+08	
125	7.37E+02	2.89E+08	
125	7.90E+02	1.66E+08	7.2E-06 (3.6E-06)
125	1.84E+03	1.28E+08	

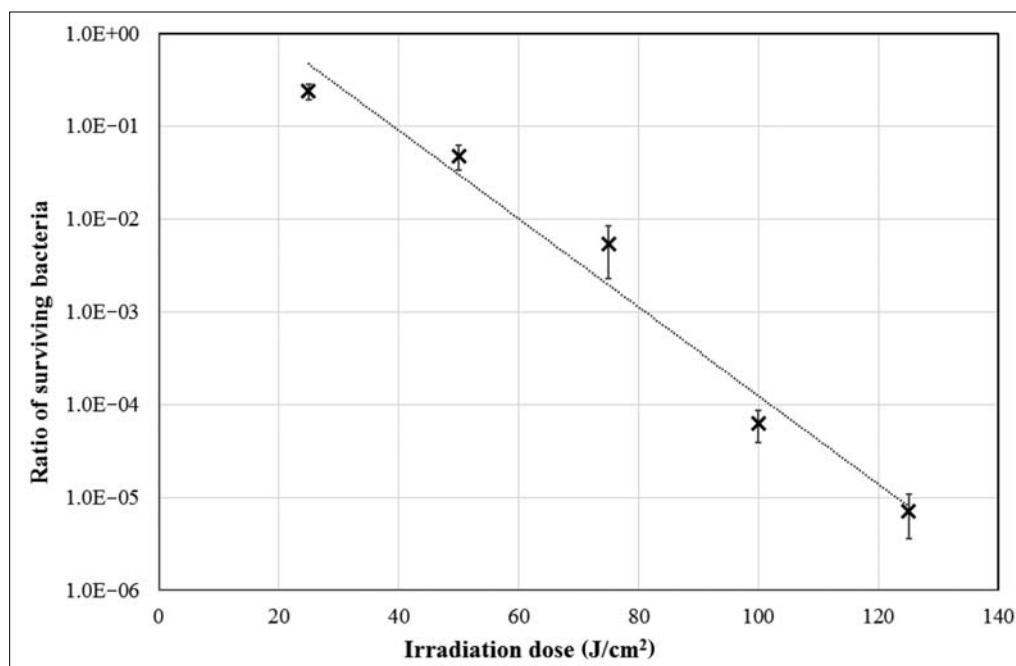


Fig. 1. Bacterial reduction for *L. rubrilucens* during 405 nm irradiation. Error bars depict the standard deviation of the average of three independent measurements

These results were obtained for extracellular *L. rubrilucens* and not for the species *L. pneumophila*, which is relevant for human infections. However, the results are probably transferable towards different legionellae based on the observation that there are only slight differences in the UV-C sensitivity of various *Legionella* species [12].

It is likely that higher doses are necessary for *Legionella* residing within amoebae, similar to the situation observed for UV-C irradiation [12]. This is most likely due to the known oxygen dependence of the photoinactivation effect [13] and the assumption that amoebae and biofilms offer an environment of reduced oxygen concentration compared to the situation of a bacterial suspension on agar plates with high oxygen diffusion rates.

Nevertheless, our results offer the prospect of new *Legionella* disinfection techniques especially by employing small high power 405 nm LEDs. They could be integrated in technical water systems like water dispensers, waterspout fountains, or in water tanks in domestic installations. Their advantages compared to UV-C generating mercury lamps are smaller size, lower costs, longer lifetimes, and no toxic materials. A further potential application might even be the therapy of infected patients, e.g., in cases of pneumonia. Human cells are much less sensitive to 405 nm [14] than *Legionella* or other bacterial species. Violet 405 nm light has already been tested to eliminate *Helicobacter pylori* in human patients [15, 16].

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Conflict of interest

The authors declare no conflicts of interest.

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