Effect of sublethal temperatures on Pulsed Light inactivation of bacteria

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ABSTRACT

This study investigated the effect of sublethal temperatures on the efficacy of Pulsed Light (PL) treatment for the inactivation of *Listeria innocua*, *Escherichia coli* ATCC 25922, and *Pseudomonas fluorescens*. A thin layer of clear, liquid phosphate buffer inoculated with one of the challenge organisms, at a concentration of about 10^8 CFU/mL, was equilibrated to a temperature ranging from 5 °C to 50 °C and then treated with PL, at doses between 1.02 and 12.29 J/cm². All treatments were performed in triplicate. In the temperature range of 5 °C to 40 °C, the average maximum reductions for *L. innocua*, *E. coli*, *P. fluorescens* were 6.27 ± 0.23 log CFU, 6.66 ± 0.36 log CFU, and 6.15 ± 0.19 log CFU, respectively. Temperature did not affect PL inactivation of *E. coli* or *P. fluorescens*, but a modest synergistic effect between PL and temperature was observed for *L. innocua* treated above 40 °C.

Industrial relevance: This study suggests that PL inactivation of bacteria is independent of temperature in the sublethal range 5 °C to 40 °C. The practical benefit of this finding is that PL treatments can be conducted in a wide range of environmental temperatures, without any change in the outcome of the treatment.

Keywords: Pulsed light; Temperature; *E. coli*; *L. innocua*; *P. fluorescens*

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1. Introduction

Pulsed light (PL) technology is a non-thermal microbial inactivation method that can improve food safety and shelf life, with minimal effects on the food's original nutritional and sensory attributes. PL is approved by the U.S. Food and Drug Administration for the decontamination of food and food contact surfaces, at doses not exceeding 12 J/cm² (FDA, 1996). PL has been shown to be effective in the inactivation of foodborne pathogens and their surrogates on foods and food contact surfaces (Rajkovic, Tomasevic, Smigic, Uyttendaele, Radovanovic, & Devlieghere, 2010; Proulx, Hsu, Miller, Sullivan, Paradis, & Moraru, 2015).

The treatment consists of short duration pulses of high intensity broad-spectrum light ranging in wavelength from 200 to 1100 nm. It is generally accepted that PL treatment primarily inactivates microorganisms through the absorption of UV light by DNA (Takeshita, Shibato, Sameshima et al., 2003; Wang, MacGregor, Anderson, & Woolsey, 2005; Woodling & Moraru, 2007; Uesugi, Hsu, Worobo & Moraru, 2016). There are also reports that attribute some of the lethal effects of PL to photothermal effects, which lead to cytoplasmic membrane damage, cell content leakage, and ultimately cell death (Takeshita et al., 2003; Krishnamurthy, Tewari, Irudayaraj, & Demirci, 2010; Cheigh, Hwang, & Chung, 2012).

The efficiency of PL treatments is dependent on several factors, including fluence and spectral distribution of the light, the challenge microorganism, and the interaction of light with the substrate. Hsu & Moraru (2011) determined that fluence varies significantly within a PL chamber, making sample positioning relative to the lamp and lamp geometry crucial factors to consider when designing an efficient and uniform PL treatment. Surface properties of the substrate also affect the outcome of PL treatment. If the substrate contains UV absorbing

1 components such as proteins, pigments, or phenolic compounds, PL effectiveness can be
2 reduced, as less UV light is available to reach target microorganisms. For solid substrates,
3 microorganisms may lodge in surface irregularities or penetrate into the solid beyond PL's
4 penetration depth, limiting the microorganisms' exposure to PL (Woodling & Moraru, 2005).
5 For instance, high UV reflectivity was found to reduce inactivation on Al packaging materials
6 (Ringus & Moraru, 2013).

7 No data currently exists in the literature on the effect of environmental temperatures on
8 PL efficacy. This is very relevant for practical applications of PL, since a temperature effect may
9 on one hand lead to differences in inactivation if treatments are conducted at different
10 temperatures, and on the other hand any potential synergistic effects may allow the development
11 of mild temperature – PL combination treatments. For instance, synergistic effects between mild
12 heat and continuous UV were observed for *Escherichia coli* (Gayán, Monfort, Álvarez, &
13 Condón, 2011), *Salmonella* (Gayán, Serrano, Raso, Álvarez, & Condón, 2012), *Staphylococcus*
14 *aureus* (Gayán, García-Gonzalo, Álvarez, & Condón, 2014), and *Listeria monocytogenes*
15 (Gayán, Serrano, Pagán, Álvarez, & Condón, 2015), when performing the UV treatments in the
16 50 °C – 60 °C temperature range.

17 The objective of this study was to evaluate the effect of temperature on the PL
18 inactivation of *Listeria innocua*, *E. coli* ATCC 25922, and *Pseudomonas fluorescens* in a liquid
19 substrate. *L. innocua* and *E. coli* ATCC 25922 were determined to be non-pathogenic surrogates
20 of *L. monocytogenes* and *E. coli* O157:H7 for PL treatments (Sauer & Moraru, 2009; Uesugi &
21 Moraru, 2009). *Pseudomonas spp.* are commonly found spoilage bacteria in foods, particularly in
22 dairy products (Ternström, Lindberg, & Molin, 1993).

2. Materials and Methods

2.1. Challenge organisms

L. innocua FSL C2-008 and *P. fluorescens* D3-266 were obtained from frozen stocks maintained by the Food Microbiology and Safety Laboratory at Cornell University (Ithaca, NY). *E. coli* ATCC 25922 was obtained from frozen stocks maintained by the Worobo Laboratory at Cornell University. The cultures were streaked onto tryptic soy agar (TSA; Difco, Becto Dickinson, Franklin Lakes, NJ) plates and incubated for 24 ± 2 h at 37 ± 2 °C for *L. innocua* and *E. coli* and at 30 ± 2 °C for *P. fluorescens*.

2.2. Substrate preparation

A single colony of the challenge microorganism was isolated from TSA plates and transferred into tryptic soy broth (TSB; Difco, Becto Dickinson, Sparks, MD). The broth was incubated with shaking at 210 rpm for 24 ± 2 h at 37 ± 2 °C for *L. innocua* and *E. coli* and at 30 ± 2 °C for *P. fluorescens*. Incubation times were selected to yield cells in stationary growth state. The culture was diluted 10 fold in Butterfield's Phosphate Buffer (BPB; pH 7.2 ± 0.2 at 25 °C; Weber Scientific, Hamilton, NJ). The initial inoculum level was 10^8 CFU/mL for *L. innocua* and *E. coli* and 10^6 CFU/mL for *P. fluorescens*. One milliliter of the inoculated BPB was transferred into Nunc Lab-Tek II 1 well Chamber Slides (Thermo Fisher Scientific, Waltham, MA). The chamber slide and substrate were brought to the temperature of interest prior to PL exposure by placing the sample in an incubator for at least 15 min to ensure that the target temperature (± 0.5 °C) had been reached. The temperatures studied were 5 °C, 20 °C, 30 °C, 40 °C, and 50 °C, which were in the sublethal range for the three challenge organisms were chosen. Some thermal inactivation of *E. coli* and *P. fluorescens* occurred at 50 °C, which will be discussed later. The

temperature of each sample was measured immediately prior to PL treatment using a Type K temperature probe (ThermoWorks, Salt Lake City, UT).

2.3. PL treatments

PL treatments were performed using a Xenon RS-3000C SteriPulse System (Xenon Corp., Wilmington, MA). A xenon flashlamp within the unit emits light in the wavelength range 200 to 1100 nm, with a pulse width of 360 μ s and a pulse frequency of 3 pulses per second. Chamber slides were centrally placed on an adjustable stainless steel shelf located 5.8 cm below the flashlamp housing. Samples were exposed to 1, 3, 6, 9, or 12 pulses of light, corresponding to fluence values of 1.02, 3.07, 6.14, 9.22, and 12.29 J/cm², respectively. A waiting period of at least 60 s between treatments was allowed, to prevent overheating of the flashlamp.

2.4. Fluence measurements

A pyroelectric detector head (PE25BB-DIF) connected to a Nova II energy meter (Ophir Optonics, North Andover, MA) was used to quantify the fluence (PL dose) received by the samples. The detector was placed inside the PL unit treatment chamber with the diffusor opening of the detector centrally located 5.8 cm below the flashlamp housing. The detector cable and base were covered in aluminum foil, leaving only the diffuser opening exposed. Fluence measurements were conducted for 1, 3, 6, 9, and 12 pulses. Measurements were performed at least 60 s apart to prevent overheating of the flashlamp and pyroelectric detector. All measurements were performed in triplicate.

2.5. Recovery and enumeration of survivors

Following PL treatment, the 1 mL sample was transferred from the chamber slide to a test tube containing 7 mL of TSB. The chamber slide was rinsed twice with 1 mL of TSB to yield a final volume of 10 mL. Serial dilutions in BPB were performed and the appropriate dilutions

were plated in duplicate on TSA plates. Plates were incubated for 24 ± 2 h at 37 ± 2 °C for *L. innocua* and *E. coli* and at 30 ± 2 °C for *P. fluorescens*. Survivors were enumerated using standard plate counting. When counts below 25 CFU/plate were expected the three-tube most probable number technique was used (Downes, Ito, & APHA, 2001). Microbial reductions were calculated as $\log[N/N_0]$, where N is the number of survivors after PL treatment and N_0 is the initial inoculum level as determined by positive controls. As positive controls, samples prepared in the same manner as the test samples and exposed to temperature conditions, but not PL treatment, were used.

2.6. Modeling of inactivation kinetics

The PL inactivation kinetics for *L. innocua*, *E. coli*, and *P. fluorescens* across the temperature range of 5 °C to 50 °C was described using the Weibull model. The Weibull model (Eq. 1) has been shown before to accurately predict PL inactivation of *L. innocua* in clear liquid substrates at room temperature (Uesugi, Woodling, & Moraru, 2007):

$$\text{Log}(N/N_0) = -b \times F^n \quad (1)$$

where N is the number of survivors after the PL treatment, N_0 is the initial inoculum level, b is the scale parameter, n is the shape parameter, and F is the fluence (J/cm^2). The shape parameter indicates the shape of the survivor curve, with $n > 1$ indicating concave down survival curves, $n < 1$ concave up survival curves, and $n = 1$ indicates linear survival curves.

2.7. Replication and statistical analysis

Both biological and technical triplicates were conducted for all experimental conditions. ANOVA and Tukey's HSD test were used to determine if differences in inactivation between treatments at different temperatures were significant ($p < 0.05$), using the statistical program RStudio (R Core Team, 2014).

3. Results and discussion

3.1. Effect of temperature on PL inactivation of the challenge organisms

PL inactivation curves for *L. innocua*, *E. coli* ATCC 25922 and *P. fluorescens* at different sample temperatures are shown in Figs. 1-3. It should be noted that in all cases inactivation levels ($\log N/N_0$) were calculated using as control a sample that was handled and equilibrated at the same temperature as the experimental samples, but was not treated with PL. All inactivation curves were non-linear, with inactivation showing a rapid increase with fluence up to about 6 J/cm², after which only a slight increase in inactivation with fluence was observed for *L. innocua*, *E. coli*, and a very clear plateau for *P. fluorescens*.

For *L. innocua*, the maximum reductions achieved were 6.06 ± 0.24 log CFU, 6.41 ± 0.57 log CFU, and 7.58 ± 0.53 log CFU at 5 °C (refrigeration temperature), 20 °C (room temperature), and 50 °C, respectively. No inactivation effect due to temperature alone was observed for any of the temperature conditions used. Differences between inactivation levels at 5 °C, 20 °C, 30 °C, and 40 °C were not statistically significant for most of the fluence levels tested. However, PL reductions achieved at 50 °C were significantly greater ($p < 0.05$) than those achieved at temperatures below 40 °C, for all fluence levels, with the exception of an outlier observed at 6.14 J/cm² for the 5 °C treatment (Fig. 1).

The increased PL inactivation at 50 °C can be due to a synergism between temperature and PL treatment under those temperature conditions. Gayán, Serrano, Pagán, Álvarez, & Condón (2015) attributed the synergistic effect between continuous UV and temperature for the inactivation of *L. monocytogenes* at temperatures between 50 °C and 60 °C to an increase in the fluidity of the cell membrane of the target microorganism. Since UV is a major contributor to inactivation in the fluence range studied here (Woodling, Uesugi & Moraru, 2007), increased cell

membrane fluidity may also explain the modest synergism between temperature and PL in the present study.

As for *L. innocua*, temperature did not have a significant effect in the PL inactivation of *E. coli* ATCC 25922 in the temperature range of 5 °C to 40 °C (Fig. 2). Thermal inactivation of *E. coli* ATCC 25922 occurred at 50 °C, and therefore the maximum temperature included in this analysis is 40 °C. The maximum PL reductions for this organism were 7.13 ± 0.87 log CFU, 6.66 ± 0.38 log CFU, and 6.25 ± 0.38 log CFU at 5 °C, 20 °C, and 40 °C, respectively.

PL inactivation of *P. fluorescens* had a similar behavior as for *E. coli*: temperature did not affect inactivation in the temperature range of 5 °C to 40 °C, but thermal inactivation of *P. fluorescens* occurred at 50 °C. The maximum PL reductions achieved for *P. fluorescens* at 5 °C, 20 °C, and 40 °C were 6.41 ± 0.18 log CFU, 6.16 ± 0.21 log CFU, and 6.07 ± 0.16 log CFU, respectively (Fig. 3). Some outliers were observed for this microorganism for the 3.07 J/cm² and 6.14 J/cm² treatments at 5 °C and 30 °C, respectively, but the reason for these outliers is unclear. *Pseudomonas spp.* are known to produce extracellular polymeric substances (EPS), which can vary with temperature (Sandford, 1979). This could have promoted cell clustering and consequently light scattering and/or shading of the bacterial cells, which may account for the variability of inactivation data obtained for this particular microorganism.

Comparing the three bacterial strains, it can be concluded that *L. innocua* is the most PL resistant and *P. fluorescens* is the least resistant to PL at low temperatures and low fluence levels, although the difference in PL sensitivity of the three microorganisms decreases as temperature and PL dose increase. The lower PL sensitivity of *L. innocua* compared to the other bacterial species is consistent with the results of previous studies. Proulx et al. (2015) found that *L. innocua* was more resistant to PL than *E. coli* and *P. fluorescens* when treated on the surface

of cheese slices, while Jay et al. (2005) reported that gram-positive bacteria were less sensitive to UV treatments than gram-negative bacteria.

Except for a synergistic effect between temperature and PL treatment observed for *L. innocua* at 50 °C, no synergism between PL and sublethal temperatures was observed for the challenge organisms and treatment conditions studied here. While this may seem to be contradictory to the synergistic effects between UV and temperature observed before, the explanation may be due to the much shorter treatment time of PL (a few seconds) as compared to continuous UV (tens of seconds).

From a practical perspective, there is a benefit to the fact that temperature does not affect PL effectiveness, since the same level of microbial inactivation by PL can be expected when PL treatments are conducted in a wide range of temperatures, from refrigeration conditions (5°C) to warm processing environments (40°C).

3.2. Inactivation kinetics

The Weibull kinetic parameters for PL inactivation of the three organisms, at all temperatures, were also determined, and are shown in Table 1.

For *L. innocua*, the only challenge organism that showed a synergism between temperature and PL inactivation, a significant temperature dependence of the shape and scale Weibull parameters was observed (Fig. 4), which can be expressed as:

$$n = 0.584 - 0.005t \quad (2)$$

$$b = 1.577e^{0.013t} \quad (3)$$

$$\text{Log}(N/N_0)_{L. innocua} = -1.577e^{0.013t} \times F^{(0.584 - 0.005t)} \quad (4)$$

where t = temperature of the sample (°C), and F = fluence (J/cm²).

Eq. 4 can be used to calculate PL inactivation of *L. innocua* in a clear liquid at any temperature within the range 5 °C to 50 °C.

PL inactivation of *E. coli* and *P. fluorescence* was independent of temperature, which is confirmed by the fact that the shape (*n*) and the scale (*b*) parameters determined for different temperatures were not statistically different from each other (*p*>0.05) (Table 1). Therefore, average rather than temperature dependent Weibull parameters were determined for these two microorganisms.

PL inactivation of *E. coli* in a clear liquid substrate in the temperature range of 5 °C to 40 °C can be characterized by a shape parameter $n = 0.30 \pm 0.05$ and a scale parameter $b = 3.21 \pm 0.20$ were obtained for the temperature range 5 °C to 40 °C. The PL inactivation kinetic model can be written as:

$$\text{Log}(N/N_0)_{E. coli} = -3.21 \times F^{0.30} \quad (5)$$

For *P. fluorescence*, a shape parameter $n = 0.20 \pm 0.03$ and a scale parameter $b = 3.95 \pm 0.34$ were obtained for the temperature range 5 °C to 40 °C, and the form of the kinetic model is:

$$\text{Log}(N/N_0)_{P. fluorescence} = -3.95 \times F^{0.20} \quad (6)$$

3.3. Validation of kinetic models

A validation of the kinetic models shown in Eq. 4-6 was carried out by comparing predicted and experimental PL inactivation values obtained for a treatment temperature and fluence level different than those used to determine the Weibull parameters. Validation was conducted at 45 °C for *L. innocua*, and at 35 °C for both *E. coli* and *P. fluorescens*.

Fig. 5 shows the predicted PL inactivation curves and a validation point for each of the three microorganisms, under the conditions specified above.

The Weibull model slightly overestimated the inactivation of *L. innocua* under these conditions: the reductions were 6.52 log CFU and 5.78 ± 0.61 log CFU for the predicted values (with temperature dependent parameters) and experimental values, respectively. The overestimation of the inactivation at 45 °C may be due to a slight model error caused by the synergism between PL and temperature, particularly at 50 °C. Nonetheless, the difference between the predicted and experimental values are of less than 1 log, and as such can be considered within the accepted range of biological variability. For comparison purposes, in Fig. 5 both the inactivation curve for *L. innocua* predicted using average values for all temperatures for n (0.44 ± 0.09) and b (2.38 ± 0.64), represented by the dotted line, and the inactivation curve using the temperature dependent values (Eq. 2 and 3), represented by the solid line, were plotted. Although slight differences between the two curves exist, they are rather small, of less than 0.5 log for any fluence level considered here. This suggests that using these average n and b values may lead to an acceptable prediction of PL inactivation for *L. innocua*.

For the two gram negative microorganisms, the experimental and predicted reductions were much closer than for *L. innocua*. For *E. coli* treated at 35 °C with 10.2 J/cm^2 these values were 6.67 ± 0.71 log CFU and 6.44 log CFU, respectively. For *P. fluorescens* treated at 35 °C and 10.2 J/cm^2 the inactivation values were 6.06 ± 0.13 log CFU (experimental) and 6.24 log CFU (predicted). This demonstrates that Eq. 5 and 6 can be used to accurately predict PL inactivation of the two organisms in a clear liquid, in the sublethal temperature range of 5 °C to 40 °C.

4. Conclusions

The results of this study indicate that PL treatment effectiveness is independent of temperature for *E. coli* and *P. fluorescens* in clear liquid substrates within the temperature range

of 5 °C to 40 °C, but a modest synergistic effect of the two treatments was observed for *L. innocua* at 50 °C. The Weibull model was able to closely predict the inactivation kinetics of these three challenge microorganisms in clear liquids, at sublethal temperatures and fluence levels below the FDA limit of 12 J/cm². The lack of a temperature effect has a practical benefit for food processing, since the outcomes of the treatment will not be affected by the temperature conditions typically found in processing environments. Moreover, the temperature of the treated product or substrate need not be considered when predicting PL inactivation within processing temperatures of 5 °C to 40 °C.

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TABLES

Table 1. Weibull parameters for the PL inactivation of the three challenge microorganisms at different temperatures

Temperature during PL treatment, °C	R ² of model fit	Shape parameter (n)	Scale parameter (b)
<i>L. innocua</i>			
5	0.94	0.54 ± 0.08 ^a	1.81 ± 0.32 ^a
20	0.96	0.50 ± 0.01 ^a	2.05 ± 0.05 ^a
30	0.97	0.47 ± 0.03 ^{ab}	2.02 ± 0.13 ^a
40	0.98	0.37 ± 0.00 ^{bc}	2.65 ± 0.15 ^b
50	1.00	0.33 ± 0.02 ^c	3.37 ± 0.30 ^c
<i>E. coli</i>			
5	1.00	0.34 ± 0.03 ^a	3.11 ± 0.10 ^a
20	0.99	0.33 ± 0.03 ^a	3.05 ± 0.13 ^a
30	1.00	0.28 ± 0.03 ^a	3.26 ± 0.25 ^a
40	0.99	0.26 ± 0.04 ^a	3.41 ± 0.14 ^a
<i>P. fluorescence</i>			
5	1.00	0.21 ± 0.02 ^a	4.15 ± 0.06 ^a
20	0.99	0.19 ± 0.02 ^a	4.11 ± 0.20 ^a
30	1.00	0.18 ± 0.03 ^a	3.82 ± 0.36 ^a
40	1.00	0.22 ± 0.04 ^a	3.73 ± 0.50 ^a

* Different letters within the same column, for the same microorganism, are significantly different from each other (p<0.05)

FIGURES

Fig. 1. PL inactivation curves for *L. innocua* at different treatment temperatures

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Fig. 2. PL inactivation curves for *E. coli* at different treatment temperatures

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Fig. 3. PL inactivation curves for *P. fluorescens* at different treatment temperatures

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3 **Fig. 4.** Weibull shape (n) and scale (b) parameters as a function of temperature (t), for the PL
 4 inactivation of *L. innocua* in the temperature range 5 °C-50 °C

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3 **Fig. 5.** Comparison between Weibull predicted PL inactivation curves and experimental points
 4 obtained at 10.2 J/cm^2 for *E. coli* ATCC 25922 (at 35°C), *P. fluorescens* (at 35°C) and *L.*
 5 *innocua* (at 45°C)