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Abstract: The purpose of this timely review is to critically appraise and to assess the potential significance of best-published microbial inactivation kinetic data generated by pulsed light (PL). The importance of selecting different inactivation models to describe the PL inactivation kinetics ishighlighted. Current methods for the detection of viable-but-nonculturable (VBNC) organisms post PL-treatments are outlined along with the limitations of these methods within food microbiology. Finally, the importance of further molecular and combinational research to tackle the potential threat posed by VBNC organisms with regard to public health and food safety is presented.

***Cover Letter**

Dear Dr Finglas

As correspondent author, I am submitting a review article on: Efficacy of conventional growth dependent methods to determine pulsed-light lethality kinetic data; which revise and explain the kinetics of microbial inactivation by pulsed light (PL) technology and brings on the table the problems associated with conventional enumeration methods in the light of the identification of viable but non culturable (VBNC) microorganisms post-PL treatments. Since I believe that excellent authors make excellent articles, I have written this review together with Prof. Rowan, a pioneer of this technology and who has authored more than 75% of the articles published on PL and VBNC microorganisms; and with Prof. Valdramidis, who is expert on kinetics and co-authored the most successful software for microbial kinetic analysis. I hope that this article be accepted and becomes a key reference for the subject, after the good acceptation of my last review on pulsed light published by TFST, which has become the most cited review article in the field, with more than 100 citations so far.

Sincerely yours.

Dr. Vicente M. Gómez-López

Efficacy of conventional growth dependent methods to determine pulsed-light lethalitykinetic data: a review

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ABSTRACT

The purpose of this timely review is to critically appraise and to assess the potential significance of best-published microbial inactivation kinetic data generated by pulsed light (PL). The importance of selecting different inactivation models to describe the PL inactivation kinetics ishighlighted. Current methods for the detection of viable-but-nonculturable (VBNC) organisms post PL-treatments are outlined along with the limitations of these methods within food microbiology. Finally, the importance of further molecular and combinational research to tackle the potential threat posed by VBNC organisms with regard to public health and food safety is presented.

Keywords: pulsed light, inactivation kinetics, viable but not culturable microorganisms, Weibull.

HIGHLIGHTS

- Pulsed light inactivation kinetics is reviewed
- Microbial growth dependent culture methods overestimate pulsed light lethality
- Pulsed light kinetics usually follow non-log-linear patterns
- Pulsed light inactivation occurs through multi-target process
- Alternative enumeration methods to conventional agar plates are needed.

Introduction

Recent developments among consumers regarding the demand for fresh, minimally processed foods with a preferably long shelf life has resulted in emerging research into new non-thermal technologies to ensure appropriate preservation and safety of treated foodstuffs. However, this growing consumer preference for minimally processed foodstuffs is accompanied by public health concerns surrounding efficacy of such approaches to adequately deal with food-borne diseases (Rowan, 2004; Kramer & Muranyi, 2014). The trend towards fresh-cut produce usually cannot be decontaminated by conventional thermal methods, and washing or sanitizing approaches do not provide a sufficient reduction in microbial numbers to afford safety consumers (Sapers, 2001). Therefore, there is a pressing requirement for the development of nonthermal decontamination approaches to meet these demands and to address the requirement for producing safe fresh produce.

Pulsed light (PL) is a non-thermal method for microbial inactivation based in the application of one or several high power ultra-short duration pulses of broad spectrum light between 200 and 1100 nm (Gómez-López, Ragaert, Devebere, & Devlieghere, 2007). Typical processing times are in the order of few seconds and besides its advantages of rapid and cost-effective treatments, PL does not leave any unwanted residual compounds on foodstuffs. As many other microbial inactivation technologies, the appropriate characterization of the kinetics of microbial inactivation is fundamental for process optimization. PL is a fast and cost effective process where considerable research already proving efficient for killing various microbial pathogens and spoilage species in or on various matrices (Rowan, Kirk, & Tomkins, 1999; Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005; Woodling & Moraru, 2007; Farrell, Garvey, Cormican, Laffey, & Rowan, 2009a; Farrell, Garvey, & Rowan, 2009b; Hayes, Laffey, McNeil, & Rowan, 2012; Hayes, Kirk, Garvey, & Rowan, 2013; Levy, Aubert, Lacour, & Carlin, 2012). The decline of a microbial population during treatment can be monitored in time units or fluence (J/cm^2), which is a measure of the amount of energy incident on foods. Fluence is the parameter that allows inter-laboratory comparisons of PL efficacy and scaling up for commercial food treatment processes. In order to achieve a safe food, foodborne pathogens must be killed by applying suitable fluence. However, loss of culturability is typically taken as the single criteria for determining cell death where no deeper investigations into associated molecular or physiological contributing

factors that underpin PL-mediated killing of treated microbial cells are examined. Despite the fact that inactivation curves by PL technology are framed exclusively on culture-based methods, no published study to date has reported on the significance or impact (if any) of variations observed in different inactivation kinetic plots in terms of PL treatment efficacy.

Inactivation mechanism by pulsed light, in brief

Since the kinetics of microbial inactivation is related to the inactivation mechanism, a brief overview of PL inactivation mechanism is provided here. It is generally assumed that the UV component is the most important wavelength region for the bactericidal effects of PL (Gomez-Lopez et al., 2007) as UV illumination causes photochemical modification of microbial genomic material mainly by the photocatalytic formation of cyclobutane thymine dimmers and by causing a variety of mutagenic and cytotoxic DNA lesions (Bohrerova, Shemer, Lantis, Impellitteri, & Linden, 2008). Wang, MacGregor, Anderson, & Woolsey (2005) showed that the maximum inactivation of *Escherichia coli* by PL is obtained at 270 nm, a wavelength that is highly absorbed by DNA. Conversely, studies have also reported on the irreversible disruption of microbial cells by PL implying that destruction is caused by a multi-target process comprising inter-related photochemical, photothermal or photophysical effects (Wekhof, 2000, Takeshita et al., 2003; Krishnamurthy, Tewari, Irudayaraj, & Demirci, 2010; Farrell, Hayes, Laffey, & Rowan, 2011; Cheigh, Park, Chung, Shin, & Park, 2012; Kramer & Muranyi, 2014). Photophysical effects relate to structural damages occasioned by the constant disturbance caused by the high-energy pulses. While photothermal effects relates to the localized heating of microbial cells due to light pulses that can lead to cell explosion (Krishnamurthy et al., 2010). In such instances, exploded microbial cells are incapable of entering the VBNC state.

Models describing the microbial inactivation by pulsed light

Quality and safety kinetics can be described by mathematical models using theoretical analysis and experimental results. Depending on the mechanistic knowledge upon which these models are built, they can be subdivided into deductive or inductive (Hills, 2001), also described as mechanistic or empirical (McDonald & Sun, 1999). Deductive

kinetic models are based on the general laws, that is, (bio) chemical/physical, and use them to build realistic mathematical expressions, while inductive models have as a starting point the available data. The exact mechanism of PL induced lethality has not been fully characterized and, most importantly, has not been translated to quantitative measures that could be used for developing equations. For this reasons, most of the published models used to describe inactivation curves by PL treatments have been built on inductive approaches, as they are not based on *a priori* knowledge of the underlying biological mechanisms. Nevertheless, the existing modelling approaches can be further exploited to quantitatively describe the influence of processing conditions on the properties of the studied substrates, e. g., to assess the food safety of a product treated by PL. This review revises the modelling structures published in PL literature so far. These structures are described based on the previous re-parametrisation or normalisation e. g., log transformation of the microbial populations, and transformations advised by the authors of this chapter for permitting easy parameter identification. It is noteworthy that the use of the independent variable changes depending on how the experiments are built and data are collected, in some cases is fluence (in units of J/cm^2) and in others time (in units of second). Hereafter, the models are given in the original version that have been reported in the literature; with appropriate transformation fluence and time could be interchanged. An overview of these mathematical structures and features can be seen in figure 1.

The description of each model is given below together with examples of their use to describe PL inactivation kinetics of several microorganisms in different substrates. In order to assess the relation between a certain microorganism-matrix pair and a specific model, one must be aware of the way that such relationship is established because of the variety of analysis approach by the different authors. Some authors report the fitting capacity of a single model, while others test several ones and choose the best fit. Even this approach differs in the use of a variety of statistical indexes. Therefore, for a given dataset, it cannot be excluded that another non-tested model may have had a better fitting capacity.

Log-linear model

The model of Bigelow (1921) to describe log-linear kinetics has been applied for PL studies (as reported by Izquier & Gomez-Lopez, 2011).

$$\log_{10}(N_f) = \log_{10}(N_o) - k_{\max} \cdot \frac{F}{\ln(10)} \qquad (1)$$

This version replaces the original use of treatment time as independent variable by F (fluence, J/cm^2). N_f (CFU/g) is the number of survivors, N_0 (CFU/g) is the initial number of microorganisms, and k_{max} is the inactivation rate (cm^2/J).

Table 1 shows the literature where the log-linear model has been used. It is noteworthy that the log-linear pattern has not been identified when foods are the substrate.

Biphasic model

The model of Bigelow (1921) can be extended for describing two subpopulations with different microbial resistances. The biphasic model described originally by Cerf (1977) is a classic example. Ferrario, Alzamora, & Guerrero (2013) used a version that reads as follows:

$$\log_{10}(N_f) = \log_{10}(N_o) + \log_{10}\left(f \cdot \exp(-k_{\max 1} \cdot t) + (1-f) \cdot \exp(-k_{\max 2} \cdot t)\right) \qquad (2)$$

Where*f* is the fraction of the initial population corresponding to the subpopulation more sensitive to the treatment, (1- *f*) is the fraction of the initial population corresponding to the subpopulation more resistant to the treatment and k_{max1} and k_{max2} are the specific inactivation rates of the two populations, respectively.

Table 2 compiles the literature where biphasic model has been used. It is obvious that the model has only been used to describe the inactivation by PL in fruit juices and by only one research group. These inactivation curves were characterized by a higher sensitive subpopulation (f >0.77).

Sigmoidal model

The microbial responses could be more complicated and follow a more sigmoidal like behaviour, composed by three distinctive phases: a shoulder, a log-linear inactivation phase and a tail. Geeraerd, Herremans, & Van Impe (2000) developed a mathematical structure that can describe this behaviour, and it is presented in the following equation:

$$\log_{10}(N_f) = \log_{10}\left(10^{\log_{10}(N_o)} - 10^{\log_{10}(N_{res})}\right) + \log_{10}\left(\exp(-k_{\max} \cdot t) \cdot \frac{\exp(k_{\max} \cdot S_l)}{1 + (\exp(k_{\max} \cdot S_l) - 1) \cdot \exp(k_{\max} \cdot t)} + 10^{\log_{10}(N_{res})}\right) \qquad (3)$$

This structure (appearing here with the most recent modifications reported by Valdramidis et al. (2004)) was considered by Marquenie et al. (2003) using t (in seconds) as independent variable. S_l [min] is a parameter that stands for the length of the shoulder. Similarly to the previous models k_{max} is the specific inactivation rate [1/min], and N_{res} is the residual population density [cfu/ml].

This equation can be reduced to the following structure if tailing is not present in the collected data:

$$\log_{10}(N_f) = \log_{10}(N_o) + \log_{10}\left(\exp(-k_{\max}\cdot t)\cdot\frac{\exp(k_{\max}\cdot S_l)}{1+(\exp(k_{\max}\cdot S_l)-1)\cdot\exp(k_{\max}\cdot t)}\right) \quad (4)$$

If tailing is present but not shoulder, the equation takes the following form:

$$\log_{10}(N_f) = \log_{10}(10^{\log_{10}(N_o)} - 10^{\log_{10}(N_{res})}) + \log_{10}\left(\exp(-k_{\max}\cdot F) + 10^{\log_{10}(N_{res})}\right) \quad (5)$$

The latter structure has been considered by Izquier & Gómez-López (2011) having F as independent variable in the place of t.

The microorganism-matrix combinations following the sigmoidal model reported in the literature are shown in table 3. The microorganisms also include conidiafor which inactivation kinetic has been described. Nevertheless, this does not imply that all fungi follow this inactivation pattern. For example, Aron-Maftei, Ramos-Villarroel, Nicolau, Martín-Belloso, & Soliva-Fortuny (2014) reported no shoulder in the inactivation of naturally occurring moulds on wheat grain.

Weibull model

The Weibull model is a structure that is commonly used for describing non-linear kinetics. Different notations have been used for describing this model. One of these structures reads as follows:

$$\log_{10}(N_f) = \log_{10}(N_o) - (F/\delta)^p \quad (6)$$

where δ (J/cm^2) is the fluence for the first decimal reduction, and p (dimensionless) is a parameter describing concavity or convexity of the curve. The same type of equation has been considered from several researchers by using in some cases different notations, for example, a constant multiplied factors (e. g., multiplied by 1/2.303), α instead of δ

and p instead of β or sometimes by considering the use of time instead of the fluence as the studied independent variable, e. g. (Bialka, Demirci, & Puri, 2008; Sauer & Moraru, 2009; Keklik, Demirci, Puri, & Heinemann, 2012). The Weibull model is also used (refer to Ferrario et al., 2013, Uesugi, Woodling, & Moraru, 2007) in a re-parameterized form, which reads as follows:

$$log_{10}(N) = log_{10}(N_o) - b \cdot t^n \qquad (7)$$

In a similar way, the b value in the Weibull distribution function represents the rate of inactivation of the cells, while$n$ indicates the concavity of the survival curve (n > 1 refers to downward concavity and n < 1 to upward concavity). In all cases reported for microbial inactivation by PL n < 1, that means that the inactivation gets slower with the progress of the treatment.

It has to be highlighted that the direct comparison between the different estimated parameters is hampered by the variety of parameterizations and independent variables, which can be overcome by the standardisation of the Weibull model structure used by the different research groups. Previous researchers (refer to Mafart, Couvert, Gaillard, & Leguerinel, 2002) have shown interest on the use of Equation 6 mainly because parameter δ describes the time for the first log reduction and can permit direct comparison between numerous case studies.

The Weibull model is the most frequently used in the literature describing the inactivation of microorganisms by PL (table 4). It has been applied for the inactivation kinetics of Gram positive and Gram negative bacteria *in vitro* and on food contact surfaces, milk, meat products and fruit and vegetables.

Weibull with tail

Albert & Mafart (2005) extended the Weibull modelling structure for incorporating a tailing effect. When F is the independent variable, the reparameterisation results in the following model:

$$\log_{10}(N_f) = \log_{10} N_o[(10^{\log_{10}(N_o)} - 10^{\log_{10}(N_{res})}) \cdot 10^{\left(-\left(\frac{F}{F_1}\right)^p\right)} + 10^{\log_{10}(N_{res})}] \qquad (8)$$

Where N_f is the number of cfu after treatment at a fluence F, N_o is the initial number of the tested microorganism (in cfu), N_{res} is the number of surviving cells, F is the fluence

applied (J/cm^2), F_1 is the fluence allowing the first $\log_{10}$ reduction and p is a parameter which determines the curve convexity or concavity. This equation was studied by Esbelin, Mallea, Ram, & Carlin (2013), while it was also used by Ferrario et al. (2013) but working with treatment time, t, as the independent variable. The use of the Weibull with tail model has only been used in curves obtained with solid foods, as it can see in table 5.

Mixed Weibull model

Ferrario et al. (2013) (table 6) used the two mixed Weibulian distributions of Coroller, Leguerinel, Mettler, Savy, & Mafart (2006) which could describe the kinetics of sub-populations having different resistance.

$$\log_{10} N_f = \log_{10} N_o + \log_{10}\left(\frac{1}{1+10^{a}}\right) + \log_{10}\left[10^{\left(-\left(\frac{t}{\delta_1}\right)^{p+\alpha}\right)} + 10^{\left(-\left(\frac{t}{\delta_2}\right)^{p}\right)}\right] \quad (9)$$

where t (seconds) is used instead of F, p is a shape parameter, a is the $\log_{10}$ proportion between the sensitive fraction (f) and the resistant one (1 - f), δ_1 and δ_2 are the time for the first decimal reduction of the subpopulation 1 and subpopulation 2, respectively.

Interpreting the models

It is not clear why a specific microorganism differs in the pattern of inactivation (applied kinetic model) as function of the substrate. While tailing is more likely to occur in irregular solid opaque substrates than in stirred liquids due to shadow effects, other factors regulating how lethality curves deviate from linearity remain obscure. Subtle differences in data acquisition could lead to different kinetic models, for example, between biphasic and double Weibull, since even though a relatively high number of points could be used to build the inactivation curve, the portions of the curve determining which model yields the best fit could consist of relatively few points. As discussed earlier, the specific models tested in data analysis will not necessarily exclude the appropriateness of the rest. It is known that food matrix affect PL efficacy (Gómez-López et al., 2005) due to competition with bacteria for light absorption, but other extrinsic factors may play a role, such as pH, which can in turn have synergistic or opposite influences in each one of the multi-target lethal inactivation process. Some

254 possible explanations for the occurrence of some features of the PL inactivation curve
255 are given below.

256 The shoulder phase of PL-generated inactivation kinetic data

257 While there are different models that include a shoulder, such as the log-linear with
258 shoulder and the biphasic and shoulder (Geeraerd, Valdramidis, & Van Impe, 2005),
259 only the sigmoidal model has been used to describe the PL inactivation kinetics. This
260 fact should not be strange since cases of complete inactivation are very scarce, and the
261 occurrence of tailing is common, and shoulders and tails give place to a sigmoidal
262 pattern. The Weibull model can also fit shoulders although not explicitly (Geeraerd et
263 al., 2005) and could mask the existence of shoulders, however the kinetic curves
264 analyzed in this revision and described by the Weibull model show a sudden drop of
265 survivor population after the first pulse. Besides the few microorganism-matrix
266 combinations listed in table 3, there are other few examples in the literature where
267 shoulders appear evident such as the classical paper of MacGregor et al. (1998) on the
268 inactivation of *E. coli, E. coli* O157:H7 and *Listeria monocytogenes*, and those by
269 Farrell et al. (2009ab) on 13 bacteria and the yeast *Candida* respectively, and all of them
270 on agar surfaces.

271 The biological meaning of the shoulder could be related to the multi-target nature of the
272 microbial inactivation by PL; the damage initially occurring in microbial cells is not
273 enough to make them become unculturable, until a threshold is reached where cells loss
274 the capability to divide. This interpretation is in line with the so-called vitalistic
275 approach (refer also to Geeraerd et al., 2000). Besides its biological meaning, it can be
276 considered more important to assess its relevance in PL microbial inactivation. Taking
277 into account the microbial inactivation curves characterized as per fluence basis,
278 Luksiene, Gudelis, Buchovec, & Raudeliuiene (2007) reported a shoulder length of just
279 0.08 mJ/cm^2, while Lasagabaster & Martinez (2014) reported 0.045-0.073 J/cm^2, which
280 looks relatively irrelevant compared to the value of 12 J/cm^2, which is the maximum
281 allowed by the FDA (1996). Moreover, a possible relationship between the existence of
282 shoulder and the type of bacteria arises from the work of Farrell et al. (2009a) where 13
283 bacteria were tested under similar conditions, the eight Gram positive bacteria exhibited
284 shoulder but the five Gram negative not, with the exception of *Pseudomonas*

aeruginosa, which showed a shoulder but only at the lowest lamp discharge, as it has been also reported for several species of *Candida* (Farrell et al., 2009b).

It is possible that shoulders are missing from several inactivation curves reported in the literature because researchers applied already too high fluences for the first pulse, therefore specific tests using very low fluences could resolve shoulders. However, even though more basic research is needed based on fluence-characterized treatments to elucidate the possible presence of shoulder as a typical feature of PL inactivation curves, those results will be meaningful only from the point of view of fundamental research; from the point of view of practical implementation, very small shoulders could be disregarded for process design. The evidence accumulated so far indicates that shoulders are infrequently observed, and when so, too short to be relevant in practice.

The inactivation phase in PL-mediated inactivation kinetic data

Since all reported inactivation curves have been obtained by using culture methods, the inactivation can be primarily ascribed to the formation of cyclobutane pyrimidine dimmers, which give place to chlonogenic death: the loss of ability of cells to duplicate. Regarding the deviations of linearity, the mechanistic and the vitalistic concepts (developed quite some years ago by Cerf (1977) are the main concepts explaining these phenomena in predictive microbiology. According to the vitalistic concept, on one hand, individual cells are not identical (e. g., due to phenotypic variation between cells (Humpheson, Adams, Anderson, & Cole, 1998)) which can be assigned to a mechanism at the molecular level (Van Boekel, 2002), which may vary between individuals. Consequently, the non-identical behaviour resulting from exposure to stresses, which results to deviations from loglinear inactivation kinetics at population level. This variation has been described by some authors in terms of the statistical properties of different underlying distributions (e. g., Weibull) of resistances or sensitivities (Mafart et al., 2002; Van Boekel, 2002; Peleg & Cole, 1998). Possible approaches to validate the vitalistic theory could be to assess the resistance of microorganisms surviving more drastic treatments and compare it with the or assess the resistance of decreasingly smaller fractions of the population in order to determine whether the continuously decreasing death rate curves become progressively exponential as cell counts decrease.

On the other hand, considering the mechanistic theory as it was discussed and reviewed by Geeraerd et al. (2000) and Cerf (1977) deviations could be related to the fact that

some micro-organisms are inaccessible by the main processing parameter (in the current case light), to acquired microbial resistance during the treatment, or to experimental artefacts, such as, clumping of micro-organisms, the presence of genetically different microbial populations or other experimental protocol issues.

The comparison of results should be performed carefully, especially with data analysed by the Weibull model where diverse reparameterizations have been used. Taken this into account, a limited insight on the effects of different variables on the kinetic parameters can be performed in spite of the relatively high amount of data derived from the Weibull model for PL inactivation. The effect of substrate on PL inactivation kinetics can be observed when *Salmonella enterica* is inactivated upon inoculation on different fruit surfaces. The PL inactivation of *S. enterica* on raspberry surface gives anα is 4.16 min and β 0.71, and 0.05 min and 0.32 respectively when inoculated on strawberry (Bialka et al., 2008). Another comparison shows also differences in the PL inactivation of *E. coli* in liquid substrates, with α 5.70 for buffer and 1.60 for apple juice (Hsu & Moraru, 2011), showing that the inactivation is faster in the most translucid liquid.

The tail phase in PL-mediated inactivation kinetic data

There are some cases where a residual survival population persists at constant or nearly constant levels no matter how long the treatment is prolonged, which is known as tailing. Tailing seems to be common in the microbial inactivation by PL. From the practical point of view, it implies that once reached the tail, prolonging the treatment will not yield further microbial inactivation but it can deteriorate the food where the microorganism is. Having also in mind this practical implication, the null or nearly null microbial inactivation is not only present in those inactivation models in which the tail is explicitly present (sigmoidal, Weibull plus tail), but also in the inactivation curves where a second inactivation phase can have a very low inactivation rate. Furthermore, it is possible that tailing can emerge in inactivation curves where it has not being identified when higher fluences are applied, since complete inactivation has been rarely reported, Krishnamurthy, Demirci, & Irudayaraj (2007) is an exception.

There are several theories on the possible explanation of tailing, some general and others specific of the PL process. The vitalistic approach supports that the existence of different sub-populations can cause tailing when one sub-population is very resistant to

the treatment (Marquenie et al., 2003). In the frame of a mechanistic theory, since UV light penetration is poor, any opaque body between the light source and the microorganism can shield it from inactivation, which is known as shadow effect. The shadow effect will then generate a tail in the inactivation curve because part of the microbial population will never be reached by light. In solids, microorganisms can be shielded by surface features such as the achnes of strawberries or the druplets of raspberries (Bialka et al., 2008) or by surface irregularities of food contact surfaces (Ringus & Moraru, 2013). In liquids, turbidity and suspended solidsarea main obstacles for microbial inactivation although appropriate mixing can maximize the exposure to light of all microorganisms present in the liquid mass (Gómez-López, Koutchma, & Linden, 2012). It has also demonstrated that high population densities can produce tailing when microorganisms overlap each other, those at the top get inactivated but simultaneously protect those at the bottom (Farrell et al., 2009a; Cudemos, Izquier, Medina-Martínez, & Gómez-López, 2013), the same occurs in liquids when there is cumpling of cells (Uesugi et al., 2007). Another approach states that the probability of different targets being reached by photons is reduced when the survivor population is low (McDonald et al., 2000).

It is worth mentioning that the tailing could be just an experimental artefact, such asnon-homogeneity in illumination (Unluturk, Atilgan, Handan Baysal, & Tari, 2008). Special care must be taken in non-confounding tailing with reaching the maximum detectable level of inactivation (Lasagabaster & Martínez, 2013). The limit of detection defines the levels in which classical cultural microbiological methods can be performed. Some researchers tried to exclude this artifact by performing additional experiments based on Most Probably Numbers (Sauer & Moraru, 2009) and reporting the same deceleration. It is critical that new microbiological methods are developed to eliminate these experimental artifacts.

Zero or values below statistical significance in an enumeration test based on classical microbiological techniques may consist of artificial below the limit results. These results have been described as censored results that are not quantified but are assumed to be less than a threshold value (Duarte, 2013). Current trends in predictive microbiology are suggesting the use of these data by the applications of imputation, e. g. Lorimer & Kiermeier (2007) or maximum likelihood estimation methods, e. g. (Busschaert, Geeraerd, Uyttendaele, & Van Impe, 2011). These statistical approaches

could stand as alternatives to novel microbiological techniques that can contribute to decreasing the levels of detection or enumeration of microbial bacteria.

Relevance of agar plate count culture data

While the foregoing sections have revealed significant differences in kinetic data attributed to PL-treatments, there is also a growing body of evidence to support the viewpoint that food technologies who rely exclusively on such agar plate count or growth-dependentenumeration (kinetic) data may very well be significantly underestimating the proportion of microbial survivors post PL treatments. Recent studies have shown that a still unknown proportion of microorganisms supposedly killed by PL enter what is commonly termed as a viable but not culturable (VBNC) state (Rowan, 1999; Rowan, 2004; Hayes et al., 2013; Kramer & Muranyi, 2014). According to the early work of Oliver (1993), a bacterium in the VBNC state is defined as "a cell which is metabolically active, which being incapable of undergoing the cellular division required for growth in or on a medium normally supporting grown of that cell". While the relevance and significance of a VBNC microbialstate post PL-processes have yet to be fully appreciated, molecular and combinational research suggests that a significant sub-population of non-culturable microorganisms retain pathogenicity that may pose a threat to public health and food safety (Sardessai, 2005; Fakruddin, Bin Mannan, & Stewart, 2013). The acknowledgment of the relevance of this phenomenon in PL treatment also raises questions as to the efficacy of using culture-based data alone for food safety determinations. While only a limited number of studies to date have investigated the impact of PL on microbial viability at the molecular and cellular level (Takeshita et al., 2003; Farrell et al., 2011; Cheigh et al., 2012; Kramer & Muranyi, 2014), they all have revealed alarming discrepancy between conventional plate counts and different viability staining parameters whereby PL-treatment does not cause immediate shutdown of vitality functions even when the number of colony forming units decreased by more than 6 $\log_{10}$ per sample.

Culture dependent vs culture independent methods for assessing pulsed light efficacy

Viable but non-culturable state

The evidence for the existence of VBNC cells has increased since the introduction of this concept by Byrd and Colwell in the 1980's (Byrd, Xu, & Colwell, 1991),

particularly in food and drink that elicits a myriad of inter-related sub-lethal microbial stresses such osmotic stress (Dunaev, Alanya, & Duran, 2008; Sawaya et al., 2008; Rowan, 2011). Microbial pathogens in VBNC state may still retain their capacity to cause infections (Cappelier, Besnard, Roche, Velge, & Federighi, 2007; Rowan, 2011). VBNC state microorganisms cannot be cultured on routine microbiological media, yet maintain their viability and pathogenicity. Unlike semi-starved bacteria, viable but nonculturable cells will not resume growth when nutrients and culture-friendly conditions are provided. Fakruddin et al. (2013) report that VBNC cells exhibit active metabolism in the form of respiration or fermentation (Besnard, Federighi, & Cappelier, 2000; Yaqub et al., 2004; Rowan et al., 2008), incorporate radioactive substances (Rollins & Colwell, 1986), and have active protein synthesis (Farrell et al., 2011) but cannot be cultured or grown on conventional laboratory media. Albeit currently unknown in terms of its' severity or scope, recent observations reveal that environmentally-stressed pathogenic organisms that exist in the VBNC state may potentially present as yet an undefined risk to consumers. Rowan (2004, 2011) reported previously that VBNC organisms may potentially be more virulent that those grown on artificial laboratory-based culture media due to exposure to adverse environmental stressors that are commonly associated with food processing such as such as high salt or acidity causing enhanced virulence factor expression. Fakruddin et al. (2013) report that VBNC cells pose a distinct threat to public health and food safety dispelling opinion thatsuch pathogens are unable to induce infection/disease despite retaining their virulent properties. Researchers have revealed that when VBNC pathogens pass through an animal host (Baffone et al., 2003), resuscitation and resumption of metabolic activity have led to infections and diseases (Baffone et al., 2003; Sardessai, 2005). The first evidence of pathogenicity of nonculturable cells was demonstrated of fluid accumulation in the rabbit ileal loop assay by VBNC *Vibrio cholera* O1, followed by human volunteer experiments (Amel, Amine & Amina, 2008). Capperlier et al. (2007) also reported that avirulent viable but nonculturable cells so *L. monocytogenes* needs to presence of an embryo to be recovered in egg yolk and regain virulence after recovery.

Though historically there has been disputes surrounding the existence of VBNC cells, extensive molecular studies has resolved this debate (Rowan, 2011; Fakruddin et al., 2013). It is now appreciated that VBNC cells represents a distinct survival strategy enabling problematical microorganismsto adapt to adverse environmental conditions (Rowan, 2004). Harsh environmental triggers that have been reported to be cause the

occurrence of VBNC cells include nutrient starvation, sharp changes in pH or salinity, osmotic stress, oxygen availability, extreme temperatures, exposure to food preservatives and heavy metals, chlorination of wastewater and decontamination processes such as pasteurization of milk (Fakruddin et al., 2013). Recently there has been a growing awareness about the potential for minimal processing technologies such as PL to produce VBNC cells (Rowan, 2011; Kramer & Muranyi, 2014).

Culture dependent vs culture independent methods

Since the landmark work of Rowan et al. (1999), most of the published studies to date have used conventional agar-based culture methods for the enumeration of survivors to PL treatments. The purpose of subsequent studies has been to demonstrate efficacy of PL application for microbial destruction at an appropriate technology readiness level (TRL) suitable for market update and deployment. However, measuring of microbial lethality associated with PL treatments has been far from straight forward as inactivation varies depending on operational parameters (such as applied voltage, number of pulses, distance from light source that are collectively captured under the term UV dose or fluence), biological factors (such as type, nature and number of microbial species present, nature of the suspension menstrum, presence of antibiotics or dyes, shading effects), presence of an enrichment/resuscitation phase post treatments to name but a few (Rowan, 1999, 2004; Hayes et al, 2013). Evidence suggests that these harsh environment cues may trigger a switch to the adaptive survival VBNC state in PL treatments (Rowan, 2011; Kramer & Muranyi, 2014).

To complicate the prediction process further, recent evidence clearly shows that PL treatment kills yeast through a multi-hit or mechanistic process that affects cell membrane permeability along with DNA and macromolecule stability and functionality depending on the UV dose applied. Specifically, Farrell et al. (2011) reported on the various mechanisms of cellular response in clinical strains of *Candida albicans* to PL treatments. Significant increase in the permeability of the cell membrane as function of the amount of UV pulsing applied was demonstrated by both, propidium iodide uptake and protein leakage (Fig. 2). The latter finding correlated well with increased levels of lipid hydroperoxidation in the cell membrane of PL-treated yeast. PL-treated yeast cells displayed a specific pattern of reactive oxygen species (ROS) production during treatments, where ROS bursts observed during the initial phases of PL treatment was

consistent with the occurrence of apoptotic cells. Increased amount of PL treatment also resulted in the occurrence of late apoptotic and necrotic cells with commensurate transition from nuclear to cytoplasmic accumulation of ROS and cell membrane leakage. Enhanced nuclear damage was observed in PL-treated cells as determined by the Comet assay. Cellular repair was observed in all yeast during sub-lethal exposure to PL-treatments. These complex structural and physiological studies revealed that microorganisms may survive PL depending on the regime of treatments and in order to comprehensively achieve complete lethality it is important to understand and appreciate all operating conditions including target organism(s) under investigation and to mitigate for VBNC. This will have follow-on implications for effective microbial modelling of survivors post PL treatments and interpreting associated death rate kinetic data.

Ferrario, Guerrero, & Alzamora (2014) studied the inactivation of *Saccharomyces cerevisiae* using flow cytometry in combination with different fluorescent stains and compared PL-mediated disinfection with conventional plate count enumeration. They found that the loss of culturability was much higher than the correspondent increase in permeabilized cells. Using a similar approach, Kramer & Muranyi (2014) studied the influence of PL treatment on structural and physiological properties of *Listeria innocua* and *E. coli*. Findings were consistent with the observations of Farrell et al. (2011) where a significant discrepancy between conventional plate counts and different viability staining parameters was reported, showing that PL treatment does not cause immediate shutdown of vitality functions even when the number of colony forming units decreased by more than 6 $\log_{10}$ per sample. Kramer & Muranyi (2014) also showed that loss of culturability occurred at considerably lower fluences than shutdown of cellular functions like depolarization of cell membranes, the loss of metabolic, esterase and pump activities or the occurrence of membrane damage. The authors concluded that a considerable proportion of PL-treated bacteria appeared to have entered the VBNC state. While oxidative stress with concomitant damage to DNA molecule were showed to be directly responsible for loss of microbial culturability as opposed to direct rupture of cell membranes or inactivation of intracellular enzymes, it would appear that the microbial lethality occurs due to accumulation of multiple insults inflicted on the treated cells where the rate of onset is influence in part by the amount of fluence applied. This complex cellular response to PL-treatment is reflected in different death rate kinetic data exhibited by microbial food spoilage and pathogens.

Flow cytometric investigations in combination with different fluorescent probes provide valuable insight into the physiological states and are suitable approach to gain further appreciation of the impact of microbial disinfection processes (Kennedy, Cronin, & Wilkinson, 2011; Nocker et al., 2011). Berney, Weilenmann, & Egil (2006) used flow cytometric studies to report statistical different levels of metabolic activity of *Listeria innocua* and *E. coli* levels detectable after PL treatment despite colony count enumeration data dropping to below the detection limit. However, application of higher energy levels of PL caused a gradual shutdown of cellular functions. Indeed, immediately after applying a fluence of 0.76 J/cm^2, high fractions of both bacterial populations were still able to maintain polarized cell membranes even though colony counts reduced to more than 99.99% in each case. These studies revealed that PL-treated bacteria entering this VBNC state may still show several vital functions, although they are incapable of growth in or on laboratory nutrient media.

Ben Said, Otaki, Shinobu, & Abdennaceur (2012) also reported the occurrence of VBNC bacteria after PL treatments by investigating phage susceptibilities of *Streptococcus typhi*. Infectivity of the host bacteria was still detectable intimating viability although culturability was lost. Otaki et al. (2003) along with Gómez-López et al. (2005) reported the occurrence of photoreactivation after PL treatments. Kramer & Muranyi (2013) observed that due to highly variable results obtained in different reported studies concerning potential rupture of treated microorganisms by PL, it appears likely that the occurrence of photothermal or photophysical inactivation mechanisms is to some extent likely to be attributed to their size, cellular structure and UV light absorption properties. Besides obvious damages to DNA (Kramer & Muranyi, 2014), microbial inactivation by PL could be linked to alterations of proteins and lipids where researchers reported on the occurrence of lipid peroxides and carbonylated proteins and lipid hydroperoxidation in the cell membrane of treated yeasts (Farrell et al., 2011).

Kramer & Muranyi (2014) reported that measurement of intracellular esterase activity proved to be a weak parameter to investigate cell viability post PL-treatments because high levels of CF-stained bacteria could be detected even when cells were already nonculturable and de-energised. The detection of enzyme activity does therefore not necessarily suggest cell viability. Kramer & Muranyi (2014) also showed that exclusion of the dye PI that is often used as a criterion for live bacteria could not be seen as a

suitable marker for viability as high levels of cells with intact membranes were detected after treatment with lethal energy doses. Also, Kramer & Muranyi (2014) reported detection of significant levels of ROS at 0.50 J/cm^2, which corresponds to a fluence where increasing loss of culturability occurred with PL-treatments. This corroborated earlier work of Farrell et al. (2011) which demonstrated that augmented levels of ROS were evident in nonculturable cells. The latter authors uniquely reported that the onset of apoptosis is possibly a suitable candidate marker to intimate microbial destruction as this state in PL-treated yeast occurs after lethal doses of PL are delivered.

Recently, PL has also been used for the destruction of the waterborne enteroparasite *Cryptosporidium parvum* that requires either use of complex mammalian *in vitro* cell culture techniques or use of *in vivo* rodent infection models to confirm efficacy of destruction (Garvey, Farrell, Cormican, & Rowan, 2010; Garvey, Hayes, Clifford, Kirk, & Rowan, 2013). An alternative method for assessing viability post PL treatments is the measurement of cellular adenosine triphosphate (ATP), which is the basic unit of energy currency in viable cells. ATP is not present in non-viable cells, as it is degraded after death. ATP has been used as an indicator of viability of microorganisms including *C. parvum* (King, Keegan, Monis, & Saint, 2005). ATP measurement is alikely candidate method for rapidly determining the viability or activity of this parasite pre and post PL disinfection particularly as oocyst excystation requires the generation and use of ATP. Garvey et al. (2013) reported on disinfection levels as determined via ATP measurement pre and post UV exposure were also compared with the combined *in vitro* HCT-8 cell culture-qPCR assay which was shown previously to correlate with the gold standard mouse infectivity model (Garvey et al., 2010**).** Quantitative PCR is growing in popularity as a culture-independent means of assessing microbial lethality post treatments (Garvey et al., 2010, 2013). Their studies showed that PL effectively killed *C. parvum* with a 5.4 $\log_{10}$ loss in oocyst viability after exposure to a UV fluence of 8.5 μJ/cm^2 as determined by the *in vitro* cell culture - *q*PCR assay. The ATP assay was shown to be significantly less effective in measuring loss of oocyst viability in similarly PL-treated samples for all combination of treatment regimes studied. Overestimation of survivors by the ATP assay may suggest that a sub-population of *C. parvum* oocysts may exist in a VBNC state.

Conclusions

The inactivation kinetic of microbial cells due to PL treatment has been described using different models, frequently non-log-linear. Even though harmonisation between the modelling structures and the right choice of parameters is necessary to compare the effectiveness of the technologies between laboratories worldwide, it appear that the diversity of models is a product of a mechanism of inactivation that is not simple but occurs through a complex multi-targeted molecular and cellular process where the rate of microbial destruction is critically influenced by the level of fluence applied combined with nature of the methods used to enumerate cell survivors. A number of mechanisms have been described associated to photochemical, photophysical and photothermal effects. Therefore, numerous modelling structures have been proposed that can also capture non-linear kinetics.

Increasing evidencerecently recognises that significant numbers of microorganisms cannot be cultured successfully with conventional growth dependent techniques such as agar plates, membrane filtration and broth enrichment post PL-treatments. A wide range for nonsporulating Gram positive and negative bacteria can exist in the Viable but Non Culturable state, which is a survival strategy that enables the PL-treated microorganism to employ enhanced resistance to combat adverse conditions that are commonly associated with stresses imposed during food processing. Pathogenicity is maintained by some species during VBNC state inferring that such survivors may still pose a potential threat to consumers is beginning to be considered. The real risk of low numbers of VBNC survivors in minimally processed foodsis limited and there is a pressing need to gain a greater appreciation of the true levels of viable organisms in raw materials and the manufacturing environment. However, the full impact of VBNC microorganisms on industrial food processes has not been given consideration due in part to the widespread conventional use of culture dependent growth techniques that are incapable of detecting such organisms.

A deeper study of PL lethality is therefore needed in order to identify new methods of enumeration and identification with the potential for detecting VBNC organisms post treatments in such foods may bring about a radical reappraisal of processing parameters and detection limits. New research is required to ascertain the ability of VBNC survivors tolerating and replicating within established in vivo infection models post PL-treatments. Greater information is also required toelucidatethe existence of commonly shared cellular mechanisms (and associated gene expression regulators and gene

markers) that govern cellular conversion to this VBNC state. Moreover, there is a dearth of knowledge regarding specific underlying molecular and associated cellular mechanisms governing transition and persistence of food and waterborne microorganisms in this VBNC state, in addition to obviously establishing what specific environmental conditions or triggers cause these changes in culturable state. Further research is, however, also urgently needed to identify a suitable cellular marker to tag microbial cell death and to investigate the relationship (if any) between detection of this 'cell death marker' and corresponding culture dependent plate count data that is currently used in the food industry.

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901 246-251.

902 **Table 1. Microorganism-matrix combinations following the log-linear model.**

Microorganism	Matrix	Reference
E. coli	Water	Otaki et al., 2003
E. coli	Agar	Farrell et al., 2009a
Listeria monocytogenes	Agar	Bradley et al., 2012
Pseudomonas aeruginosa	Buffer	Ben Said & Otaki, 2013
Zygosaccharomyces bailii	Glucose solutions	Hayes et al., 2012
Zygosaccharomyces rouxii	Glucose solutions	Hayes et al., 2012
Saccharomyces cerevisiae	Glucose solutions	Hayes et al., 2012
Coliphage T4	Water	Otaki et al., 2003

903

904 **Table 2. Microorganism-matrix combinations following the biphasic model.**

Microorganism	Matrix	Reference
E. coli	Commercial apple juice	Ferrario et al., 2013
E. coli	Commercial orange juice	Ferrario et al., 2013
E. coli	Natural apple juice	Ferrario et al., 2013
Listeria innocua	Natural apple juice	Ferrario et al., 2013
Saccharomyces cerevisiae	Natural apple juice	Ferrario et al., 2013

905

906 **Table 3. Microorganism-matrix combinations following the sigmoidal model.**

Microorganism	Matrix	Reference
Listeria innocua	Agar	Lasagabaster & Martinez, 2014
Salmonella Typhimurium	Agar	Luksiene et al., 2007
Botrytis cinerea	Buffer	Marquenie et al., 2003
Monilia fructigena	Buffer	Marquenie et al., 2003

907

908 **Table 4. Microorganism-matrix combinations following the Weibull model**
909 **grouped by its different reparameterizations.**

Microorganism	Matrix	Reference
E. coli O157:H7	Strawberry	Bialka et al., 2008
	Cider	Sauer & Moraru, 2009
	Apple juice	
E. coli	Buffer	Hsu & Moraru, 2011
	Skim milk	Miller et al., 2012
	Whole milk	
Listeria monocytogenes	Chicken frankfurters	Keklik et al., 2012
Listeria innocua	Clear liquid	Uesugi et al., 2007
	Buffer	Hsu & Moraru, 2011
	Plastics	Ringus & Moraru, 2013
	Commercial orange juice	Ferrario et al., 2013
	Natural melon juice	
Salmonella Typhimurium	Chicken breast	Keklik et al., 2012
Salmonella Enteritidis	Shell eggs	Keklik et al., 2012
	Natural apple juice	Ferrario et al., 2013
Natural microflora	Lettuce	Izquier & Gómez-López, 2011
	Cabbage	
	Carrots	

910

911 **Table 5. Microorganism-matrix combinations following the Weibull with tail**
912 **model.**

Microorganism	Matrix	Reference
Listeria monocytogenes	Salmon fillet	Cheigh et al., 2013
Listeria monocytogenes	Flatfish fillet	Cheigh et al., 2013
Listeria monocytogenes	Shrimp fillet	Cheigh et al., 2013
Aspergillus niger spores	Agar	Esbelin et al., 2013

913

914 **Table 6. Microorganism-matrix combinations following the mixed Weibull model.**

Microorganism	Matrix	Reference
Salmonella Enteritidis	Commercial apple juice	Ferrario et al., 2013
Saccharomyces cerevisiae	Commercial apple juice	Ferrario et al., 2013
Saccharomyces cerevisiae	Natural melon juice	Ferrario et al., 2013

915

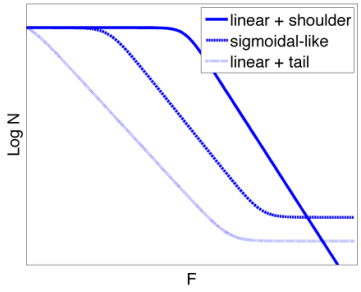
916 FIGURE CAPTIONS

917 Fig. 1. Commonly observed types of inactivation curves during PL processing
918 expressed as $\log_{10} N$ versus F. Plot A: sigmoidal-like, linear with a preceding shoulder,
919 log-linear with a tailing. Plot B: biphasic, concave and convex. Plot C: Linear, Weibull
920 incorporating a tailing effect, two mixed Weibullian distributions.

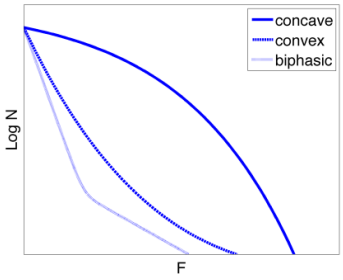
921 Fig. 2. Reduction in total fungal protein levels (µg/ml) in *C. albicans* D7100 as a
922 consequence of increased pulsing or amount of pulses applied. (Farrell et al., 2011, with
923 permission from Elsevier™, *Journal of Microbiological Methods, 84*, 317-326).

Figure 1.

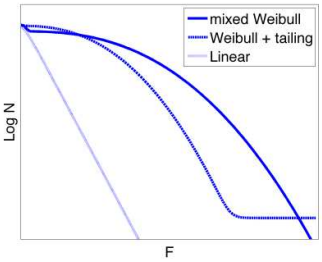
A.


linear + shoulder
sigmoidal-like
linear + tail
Log N
F

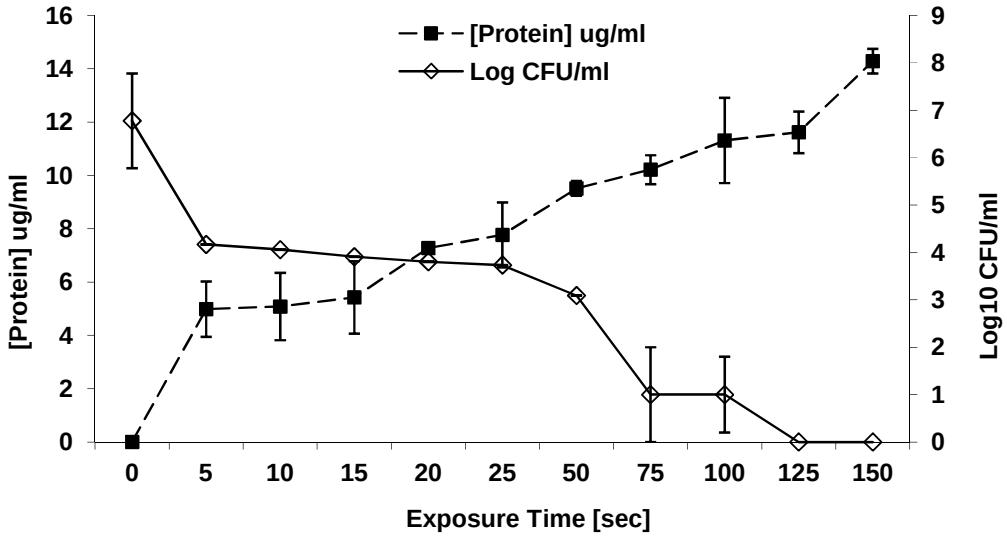
B.


concave
convex
biphasic
Log N
F

C.


mixed Weibull
Weibull + tailing
Linear
Log N
F

933 **Figure 2.**



934

*Submission Checklist

Efficacy of conventional growth dependent methods to determine pulsed-light lethality kinetic data: a review

Cover letter

Manuscript

Checklist

Highlights

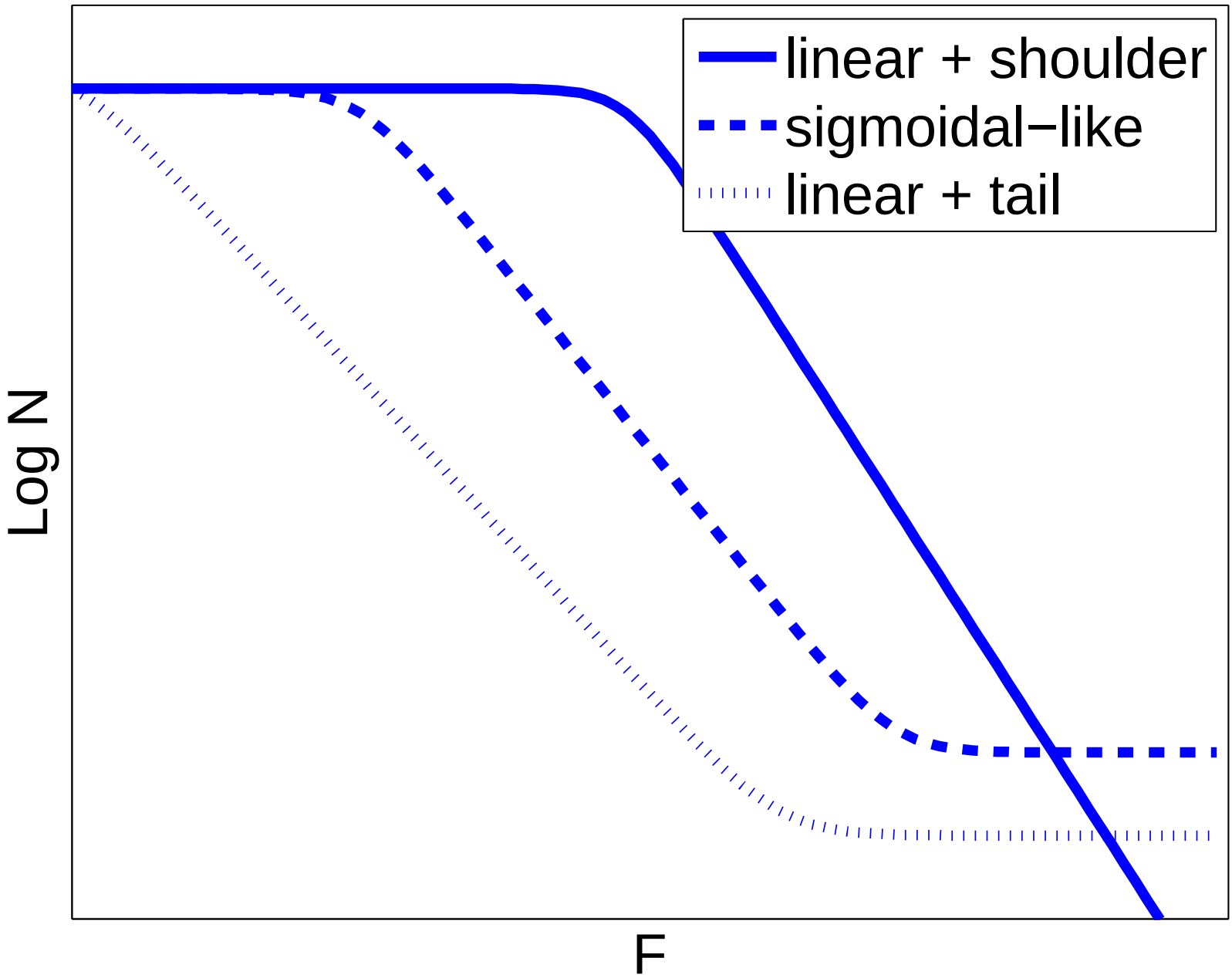
Figure 1A

Figure 1B

Figure 1C

Figure 2

Figure(s)



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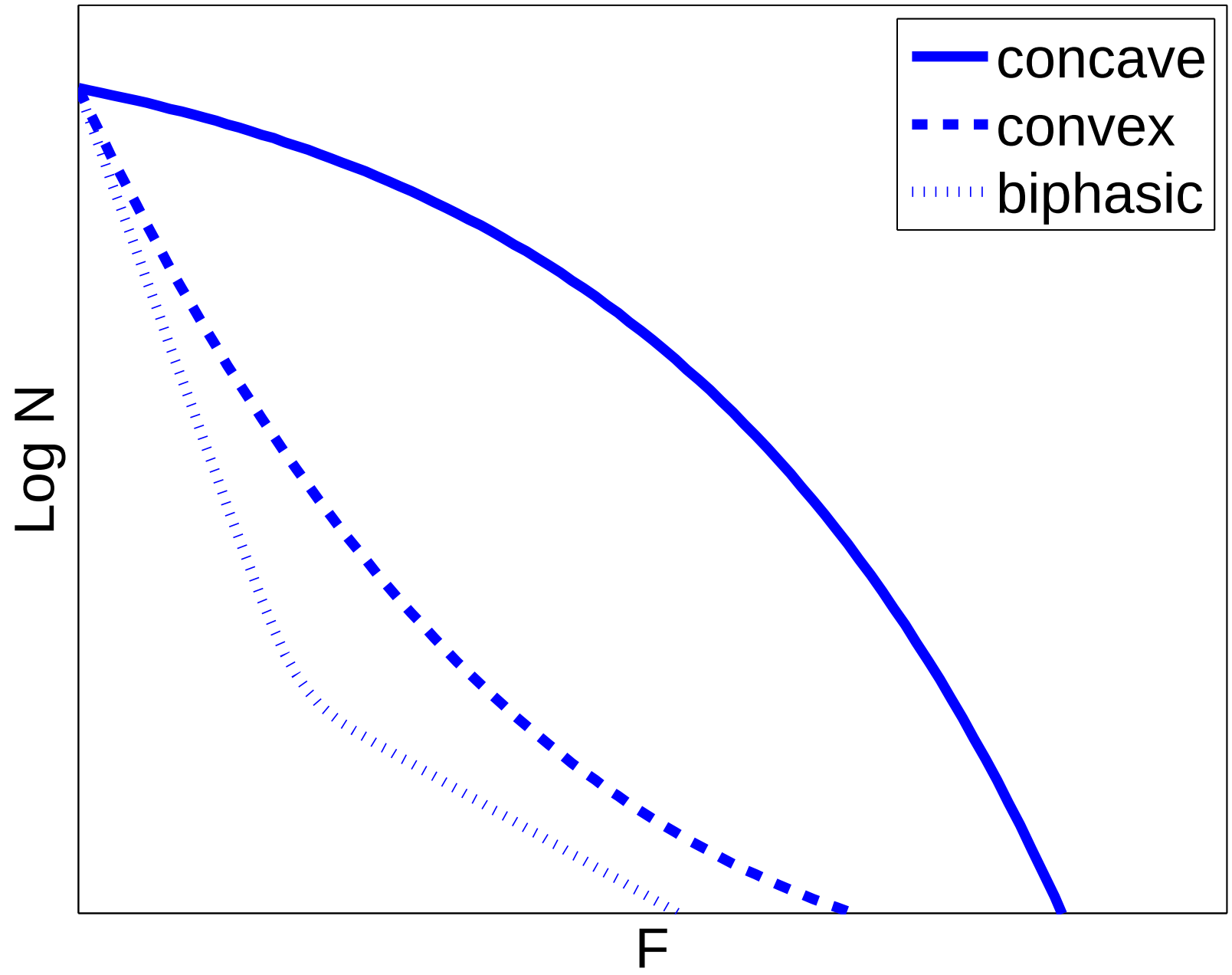

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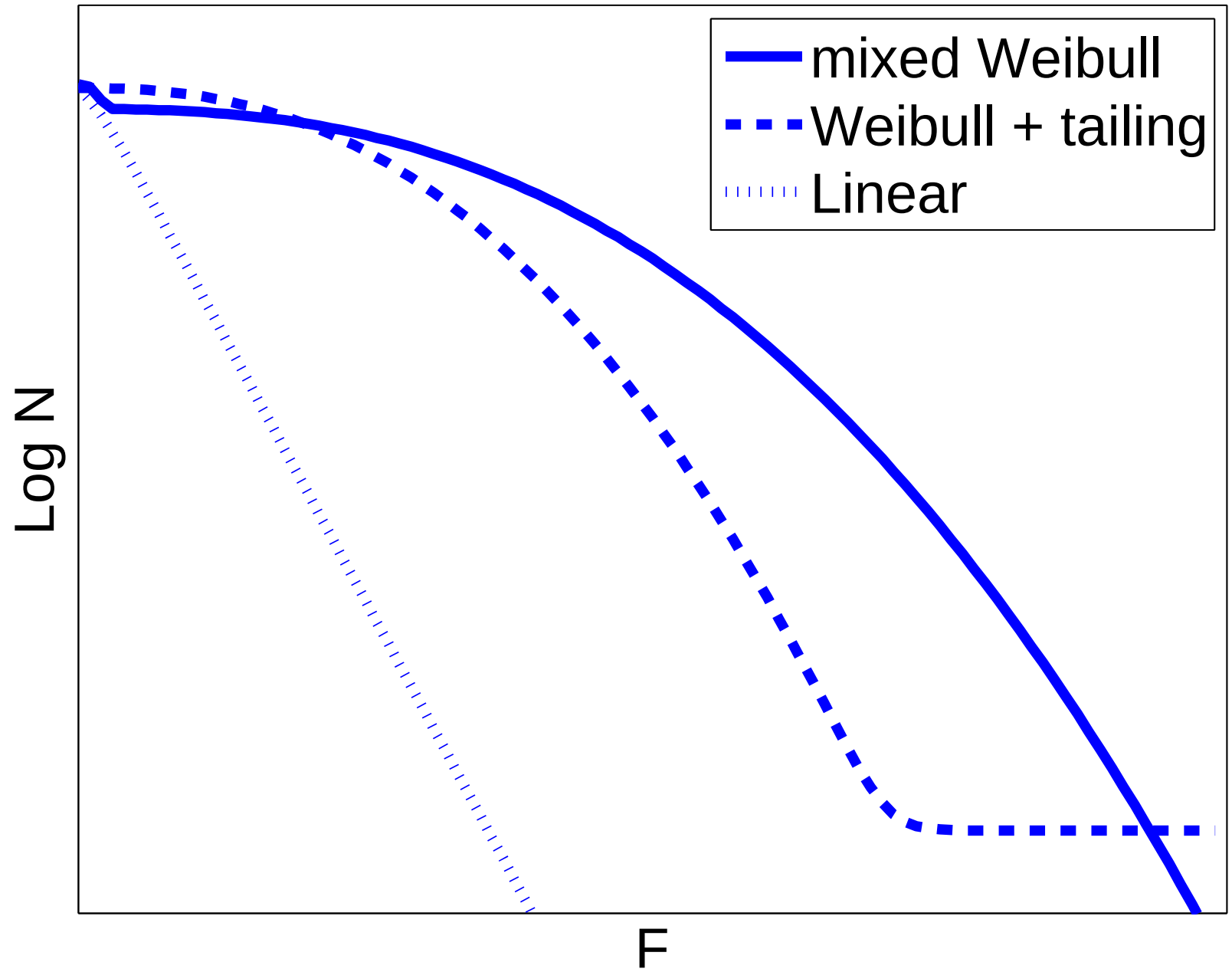

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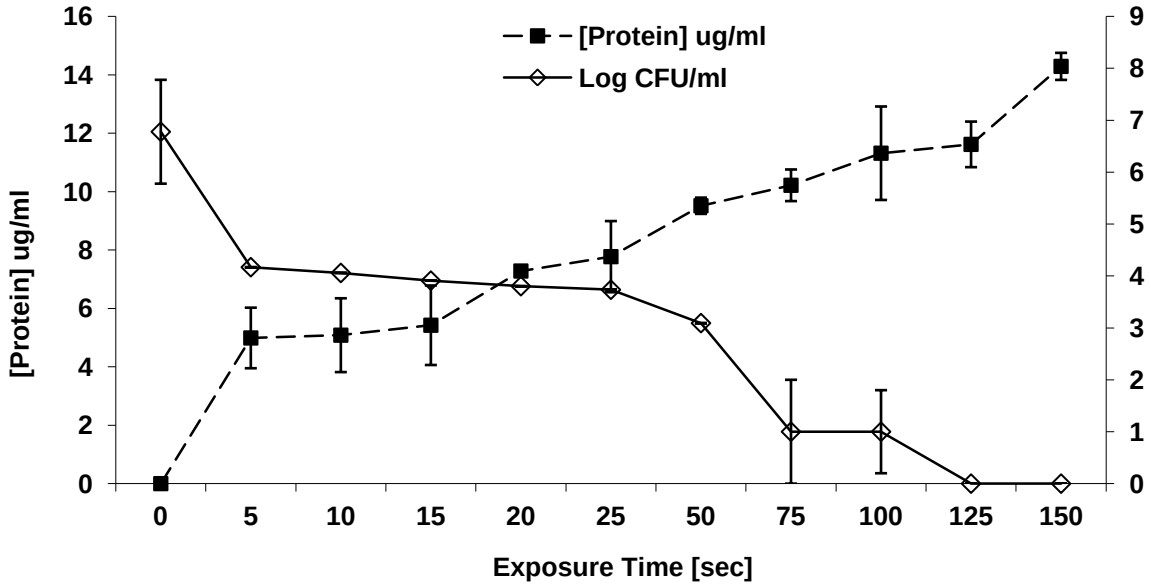
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Log CFU/ml
[Protein] ug/ml
Exposure Time [sec]
0
5
10
15
20
25
50
75
100
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14
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***Highlights (for review)**

- Pulsed light inactivation kinetics is reviewed
- Microbial growth dependent culture methods overestimate pulsed light lethality
- Pulsed light kinetics usually follow non-log-linear patterns
- Pulsed light inactivation occurs through multi-target process
- Alternative enumeration methods to conventional agar plates are needed.