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Trends in Food Science & Technology 44 (2015) 79–92

TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

A review of quantitative methods to describe efficacy of pulsed light generated inactivation data that embraces the occurrence of viable but non culturable state microorganisms

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http://dx.doi.org/10.1016/j.tifs.2015.03.006

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 is highlighted. 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. Greater emphasis should be placed on elucidating appropriate inactivation kinetic model(s) to cater for the occurrence of these VBNC organisms that are underestimated in number using traditional culture-based enumeration methods. Finally, the importance of further molecular and combinational research to tackle the potential threat posed by VBNC organisms with regard to kinetic inactivation modelling and nexus to public health and food safety is presented.

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 (Kramer & Muranyi, 2014; Rowan, 2004). 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,

Debevere, & 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. PL is a fast and cost effective process where considerable research has already proved its efficiency for killing various microbial pathogens and spoilage species in or on various matrices (Farrell, Garvey, Cormican, Laffey, & Rowan, 2009; Farrell, Garvey, & Rowan, 2009; Gómez-López, Devlieghere, Bonduelle, & Debevere, 2005; Hayes, Kirf, Garvey, & Rowan, 2013; Hayes, Laffey, McNeil, & Rowan, 2012; Levy, Aubert, Lacour, & Carlin, 2012; Rowan, Kirf, & Tomkins, 2009; Woodling & Moraru, 2007). As many other microbial inactivation technologies, the appropriate characterization of the kinetics of microbial inactivation is fundamental for process optimization.

Data generated for PL inactivation kinetics is represented as function of several parameters, such as fluence, treatment time and number of pulses. As a photochemical process, fluence is the best parameter up to now to characterize PL effects since it provides information on the amount of energy impinging the object, and can allow results intercomparison and scaling up. The latest update of the glossary of terms used in photochemistry of the IUPAC (Braslavsky, 2007) that defines fluence as the radiant energy "incident on a small sphere from all directions divided by the cross-sectional area of that sphere", with SI units in J/m^2, although J/cm^2 is more commonly used in PL. Units such as J/g or J/l refer to dose (absorbed energy) (Braslavsky, 2007), but PL dosimetry usually measures impinging energy and most of the UV light is not absorbed by microorganisms (Bolton & Linden, 2003). The small number of authors using those units should be encouraged to elaborate on the rationale and justification underpinning its use. Nonetheless, the shape of inactivation curves is the same regardless of the use of fluence, time or number of pulses as independent variable or the units using to characterize population changes (CFU/ml or CFU/g) since the dynamics of microbial inactivation depends on the impinging energy.

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. A performance criteria must be achieved by the treatment, such as the minimum 5-log reduction in pertinent microorganism required by the FDA to cider and juice processors (FDA, 2001). 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, and 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 (Cheigh, Park, Chung, Shin, & Park, 2012; Farrell, Hayes, Laffey, & Rowan, 2011; Kramer & Muranyi, 2014; Krishnamurthy, Tewari, Irudayaraj, & Demirci, 2010; Takeshita *et al.*, 2003; Wekhof, 2000). 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 Fig. 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. It is important to consider that not all data is considered relevant for performing regression analysis studies. For example, survival data should exhibit at least a reduction of 1 log unit, which is considered to be within the range of the experimental error in plate count data (Mossel, Corry, Struijk, & Baird, 1995). Additionally, for a particular combination of conditions a minimum of 10 data points should be collected and, the majority of them, should be positioned at areas of inflection where the rate of change of the microbial kinetics is maximal (Walker & Jones, 1993).

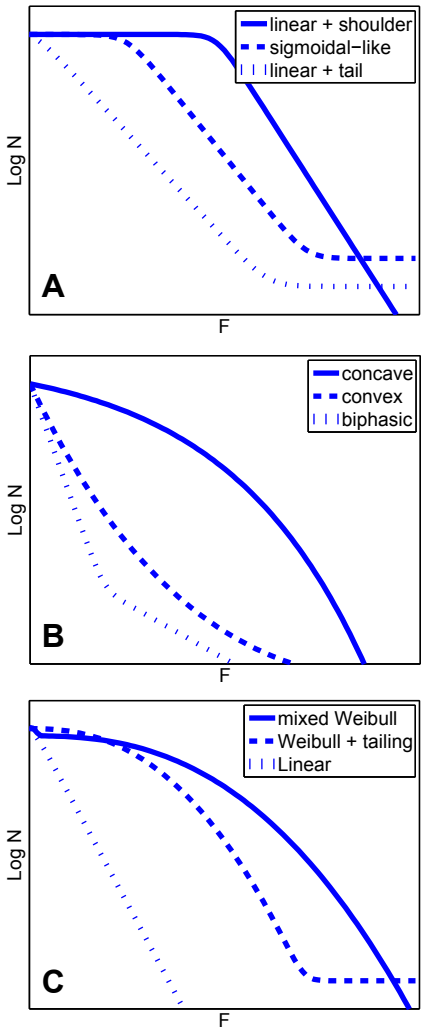

Fig. 1. Commonly observed types of inactivation curves during PL processing expressed as $\log_{10}$ *N* versus *F*. Plot A: sigmoidal-like, linear with a preceding shoulder, log-linear with a tailing. Plot B: biphasic, concave upwards or downwards. Plot C: Linear, Weibull incorporating a tailing effect,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

All current published inactivation curves have been generated in batch systems, in the case of liquids, for example, pouring it in a test cell, which could be a conventional Petri dish. Few cases use an orbital shaker to promote turbulence and generate a homogeneous exposure of the liquid (Miller, Sauer, & Moraru, 2012; Sauer & Moraru, 2009).

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) for decontamination of vegetable surfaces.

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

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, and Guerrero (2013) used a version that reads as follows:

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

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}

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

Microorganism	Matrix	Reference
E. coli	Water	Otaki *et al.* (2003)
E. coli	Agar	Farrell, Garvey, Cormican, *et al.* (2009)
Listeria monocytogenes	Agar	Bradley, McNeil, Laffey, and Rowan (2012)
Pseudomonas aeruginosa	Buffer	Ben Said and 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)

and k_{max2} are the specific inactivation rates of the two populations, respectively.

This model has been used by only one research group (Ferrario *et al.*, 2013), which described the kinetics of PL microbial inactivation for *E. coli* in commercial and natural apple juice and commercial orange juice, and *Listeria innocua* and *Saccharomyces cerevisiae* in natural apple juice. 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:

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

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:

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

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

The sigmoidal model has been applied for the description of the PL inactivation kinetics on agar of *L. innocua* (Lasagabaster & Martínez, 2014) and *Salmonella* Typhimurium (Luksiene, Gudelis, Buchovec, & Raudeliuniene, 2007), and the fungi *Botrytis cinerea* and *Monilia fructigena* on agar (Marquenie *et al.*, 2003). Nevertheless, this does not imply that all fungi follow this inactivation pattern. For example, Aron-Maftei, Ramos-Villarroel, Nicolau, Martín-Belloso, and 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

downward or upward concavity 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; Keklik, Demirci, Puri, & Heinemann, 2012; Sauer & Moraru, 2009). The Weibull model is also used (refer to Ferrario *et al*., 2013; Uesugi, Woodling, & Moraru, 2007) in a re-parameterized form (Peleg & Cole, 1998), which reads as follows:

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

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 2). 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 and 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:

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

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/mL or CFU/gr depending on how people report their experimental data), 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 direction of curve concavity exactly as for equation (7). This equation was studied by Esbelin, Mallea, Ram, and Carlin (2013) to describe the inactivation of *Aspergillus niger* spores, and Cheigh, Hwang, and Chung (2013) for the kinetics of

Table 2. Microorganism-matrix combinations following the Weibull model grouped by its different reparameterizations.

Microorganism	Matrix	Reference
E. coli O157:H7	Strawberry	Bialka *et al.* (2008)
	Cider	Sauer and Moraru (2009)
	Apple juice	
E. coli	Buffer	Hsu and 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 and Moraru (2011)
	Plastics	Ringus and 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 and Gómez-López (2011)
	Cabbage	
	Carrots	

Listeria monocytogenes inactivation on fillets of salmon, flatfish and shrimp.

Mixed Weibull model

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

Ferrario *et al.* (2013) used the two mixed Weibulian distributions of Coroller, Leguerinel, Mettler, Savy, and Mafart (2006) which could describe the kinetics of subpopulations having different resistance,where t (seconds) is used instead of F, p is a shape parameter, α 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. The authors found that this equation was the best for describing the PL inactivation of *Salmonella* Enteritidis in commercial apple juice and *S. cerevisiae* in that matrix and in natural melon juice.

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. A clear effect of experimental conditions can be seen in some liquids, where the inactivation curve differs between tests performed under static or turbulent conditions. In the case of *E. coli* inoculated in apple juice, sample shaking causes that tailing occurs at higher inactivation levels in turbulent tests in comparison to static tests leading to a difference higher than 4 log in residual population (Sauer & Moraru, 2009). The turbulence effect is more drastic in milk inoculated with *E. coli*, where tailing appears at about 2 J/cm^2 under static conditions while the inactivation curve is linear even at 14.85 J/cm^2 under turbulence, which is also evidenced by the shape parameter, which is 0.13 for skin milk under static conditions and 0.98 under turbulence (Miller *et al.*, 2012). It is possible that under static conditions only the bacterial cells at the upper levels of the liquid column are reached by light, which gets attenuated when penetrates through the liquid sample. While shaking increase the homogeneity of the exposure of the bacterial population. These results are important from the practical point of view, since PL application to liquids will be likely performed in flow-through systems where turbulence can be promoted.

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

The shoulder phase of PL-generated inactivation kinetic data

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

The biological meaning of the shoulder could be related to the multi-target nature of the microbial inactivation by PL; the damage initially occurring in microbial cells is not enough to make them become unculturable, until a threshold is reached where cells loss the capability to divide. This interpretation is in line with the so-called vitalistic approach (refer also to Geeraerd *et al.*, 2000). This

threshold would not be reached until enough photons of adequate energy reach the target points of the microorganisms. The recovery methods can also influence the shoulder of inactivation curves, since there is a possible contributory role of variation in media formulation used for dilution, enumeration and resuscitation of treated cells such as oxygen scavengers. This is relevant as variation in absence or presence of shoulder affects could be attributed to damage of house keeping genes responsible for production of catalase and superoxide dismutase, which affect the ability of sub-populations of PL-treated cells to grow on highly nutritious media. Lewis (2000) described localised metabolic suicide as the ability for stress or damaged cells to tolerate oxygenated environs was decoupled. This, greater consideration must be placed of media design by way of resuscitation to address possible variability seen with shoulder plots.

Besides its biological meaning, it can be considered more important to assess its relevance in PL microbial inactivation. Taking into account the microbial inactivation curves characterized as per fluence basis, (Luksiene *et al.*, 2007) reported a shoulder length of just 0.08 mJ/cm^2, while Lasagabaster and Martínez (2014) reported 0.045−0.073 J/cm^2, which looks relatively irrelevant compared to the value of 12 J/cm^2, which is the maximum allowed by the FDA (1996). Moreover, a possible relationship between the existence of shoulder and the type of bacteria arises from the work of Farrell, Garvey, Cormican *et al.*(2009) where 13 bacteria were tested under similar conditions, the eight Gram positive bacteria exhibited 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, Garvey & Rowan, 2009).

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 log linear 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; Peleg & Cole, 1998; Van Boekel, 2002). 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. For example, α is 4.16 min on raspberry surface but much lower on strawberry surface (0.05 min), while β is 0.71 and 0.32 for raspberry and strawberry surfaces respectively (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).

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, and 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 solids area 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 (Cudemos, Izquier, Medina-Martínez, & Gómez-López, 2013; Farrell, Garvey, Cormican *et al*., 2009), 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 and 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 that rely exclusively on such agar plate count or growth-dependent enumeration (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 (Hayes *et al*., 2013; Kramer & Muranyi, 2014; Rowan, 1999, 2004). Consequently, there is a pressing need to establish whether or not existing culture-based kinetic models can be applied to VBNC organisms so that reliable and repeatable determination of their destruction post PL-treatment occurs. 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 microbial state 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 (Fakruddin, Bin Mannan, & Stewart, 2013; Sardessai, 2005). The acknowledgement 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 (Cheigh *et al*., 2012; Farrell *et al*., 2011; Kramer & Muranyi, 2014; Takeshita *et al*., 2003), 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; Rowan, 2011; Sawaya, Kaneko, Fukushi, & Yaguchi, 2008). 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, VBNC 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; Rowan *et al.*, 2008; Yaqub, Anderson, MacGregor, & Rowan, 2004), 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) reported that VBNC cells pose a distinct threat to public health and food safety dispelling opinion that such 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). Cappelier *et al.* (2007) also reported that avirulent VBNC 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 (Fakruddin *et al.*, 2013; Rowan, 2011). It is now appreciated that VBNC cells represents a distinct survival strategy enabling problematical microorganisms to adapt to adverse environmental conditions (Rowan, 2004). Harsh environmental triggers that have been reported to 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 (Kramer & Muranyi, 2014; Rowan, 2011).

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 (Hayes *et al.*, 2013; Rowan, 1999, 2004). Evidence suggests that these harsh environment cues may trigger a switch to the adaptive survival VBNC state in PL treatments (Kramer & Muranyi, 2014; Rowan, 2011). Otaki *et al.* (2003) along with Gómez-López *et al.* (2005) reported the occurrence of photoreactivation after PL treatments. Photoreactivation is the recovery of culturability of PL-treated microorganisms after exposure to visible light. It is limited to small recoveries (<1 log) and to the application of low fluencies. Photoreactivation studies constituted one of the earliest approaches to provide evidence that not all PL-treated microorganisms which are transferred to agar plates retain vital molecular and cellular functions but are incapable of forming colonies.

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

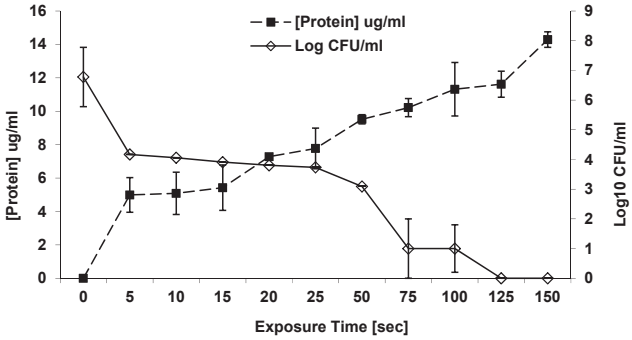

Fig. 2. Reduction in total fungal protein levels (μg/ml) in *C. albicans* D7100 as a consequence of increased pulsing or amount of pulses applied. (Farrell *et al.*, 2011, with permission from Elsevier™, *Journal of Microbiological Methods, 84,* 317–326).

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, and Alzamora (2014) studied the inactivation of *S. 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 and Muranyi (2014) studied the influence of PL treatment on structural and physiological properties of *L. 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 and 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, and Egil (2006) used flow cytometric studies to report statistical different levels of metabolic activity of *L. 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, and Abdennaceur (2010) 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. Kramer & Muranyi (2014) 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 and 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 and 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 and 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, Kirf, & 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 nonviable 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 a likely 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.

How best then to determine the efficacy of PL-treatments to destroy VBNC organisms with the knowledge that traditional culture-based microbial kinetic modelling informs process operational parameters yet are not appropriate for VBNC. Identification of an appropriate model to facilitate enumeration of VBNC post PL treatments using our knowledge acquired from culture-based kinetics would advance the field of food processing and would have far reaching tangential implications for other related domains that are deploying similar PL disinfection approaches including inter alia drinking water. In the very limited number of PL-treatment studies published thus far that reported to occurrence of VBNC, the various vitality markers described plot in a linear fashion and possibly aligned with log-linear kinetic inactivation method (Farrell *et al.*, 2011; Ferrario *et al.*, 2014; Kramer & Muranyi, 2014). One logical approach to connecting VBNC with culture-based kinetic inactivation methods would be to focus of modelling the occurrence of vital markers (such as inter alia onset of apoptosis) and to factor this into operation of PL treatments.

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 evidence recently 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 VBNC 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 foods is 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. Intensive research is warranted to investigate the most suitable vitality marker(s) for PL-treated cells and to then construct appropriate kinetic curves based on these along with comparing and contrasting kinetic curves produced using traditional culture based techniques under similar experimental conditions to inform process efficacy.

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 to elucidate the 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.

Acknowledgements

This research was partly supported by a Marie Curie FP7-Reintegration-Grant within the 7th European Community Framework Programme under the project Development of novel Disinfection Technologies for Fresh Produce (DiTec), while it is also partly funded by the COST ACTION FA1202 BacFoodNet and by the Erasmus + Programme Q-Safe of the European Union, Project No: 2014-1-MT01-KA200-000327. The support of project PMAFI/29/14 sponsored by UCAM is also acknowledged.

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