# Influence of food intrinsic factors on the inactivation efficacy of cold atmospheric plasma: impact of osmotic stress, suboptimal pH and food structure

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# ABSTRACT

Cold atmospheric plasma (CAP) has proved to have great potential as a mild food decontamination technology. Different process parameters, including food intrinsic factors, are known to influence the resistance of the cells towards the treatment. The importance of osmotic stress (0, 2, 6% (w/v) NaCl) and suboptimal pH (5.5, 6.5, 7.4) on the CAP efficacy to inactivate *Salmonella* Typhimurium and *Listeria monocytogenes* is studied for various food structures. The helium-oxygen plasma was generated by a dielectric barrier discharge reactor, treating samples up to ten minutes. If grown under osmotic stress or at suboptimal pH, microbial cells adapt and become more resistant during CAP treatment (stress hardening). Additionally, the microorganisms and the food structures also influence the inactivation results. This study illustrates the importance of increasing knowledge on food intrinsic factors, to be able to predict the final CAP inactivation result.

**Keywords:** cold atmospheric gas plasma; osmotic stress; suboptimal pH; food structure; growth morphology.

# 1. INTRODUCTION

Despite all efforts, more than 300,000 food poisonings are still reported every year in the EU (EFSA and ECDC, 2015). Combined with an increasing consumer demand for minimally processed foods, this encourages the development of innovative mild decontamination techniques, like Cold Atmospheric Plasma (CAP). By applying a voltage to a gas stream, the gas atoms or molecules become ionized once the breakdown voltage is exceeded, creating plasma. The plasma phase of matter consists of a mixture of electrons, ions, atomic species, free radicals and UV photons, all able to inactivate microorganisms (Deng, Shi, & Kong, 2006; Perni et al., 2007). The CAP mode of action for inactivation of microbial cells may be explained at different levels (Fernandez & Thompson, 2012; Laroussi & Leipold, 2004; Moisan et al., 2002; Niemira, 2012). Reactive oxygen and nitrogen species interact with macromolecules, like lipids, amino acids and nucleic acids, and cause changes that lead to microbial death or injury. Next to this, charged particles accumulate at the surface of the cell membrane and induce its rupture. In addition, UV photons modify the DNA of the microorganisms. However, Deng et al. (2006) demonstrated that reactive plasma species play a dominant role as compared to the effect of charged particles and UV. When studying the influence of the reactive gas species on the inactivation of *Bacillus subtilis* spores using a helium-(oxygen) plasma, oxygen atoms, metastable oxygen molecules, OH, nitrogen containing species and ozone are responsible for spore inactivation. The low temperature during the CAP treatment, short treatment times, together with the fact that no residues remain on the product after the treatment (Moisan et al., 2001), sum up some of the most important advantages of using CAP for food treatment. However, as plasma is able to adapt bacteria at a cellular level, cells can become sublethally injured as a result from the treatment. This possible disadvantage may pose public health concerns since sublethal injured cells are susceptible to recovery (Noriega, Velliou, Van Derlinden, Mertens, & Van Impe, 2014).

Different process parameters influence the efficacy of the plasma treatment. First of all, all plasma characteristics play an important role, like the treatment time, the plasma power, the voltage and frequency applied, together with the gas flow rate and gas composition (Deng et al., 2007; Han, Patil, Keener, Cullen, & Bourke, 2014; Lerouge, Wertheimer, & Yahia, 2001). The CAP set-up itself also has an impact on the decontamination efficacy, as plasma can be produced using, e.g., a plasma jet or a dielectric barrier discharge (DBD) electrode, and a sample can be treated directly or indirectly (Ehlbeck et al., 2011; Fernandez & Thompson, 2012; Fridman et al., 2007). Finally, both the microorganism treated and the sample itself, influence the efficacy of the treatment (Afshari & Hosseini, 2012; Fernandez & Thompson, 2012). The species of the microorganism, together with its microbial load and the growth phase of the treated cells, play an important role during the CAP inactivation, as reported in literature (Fernandez & Thompson, 2012; Fernandez, Shearer, Wilson, & Thompson, 2012; Lerouge et al., 2001). Preservation of the sample, prior to CAP treatment, also affects the inactivation efficacy. For example, prolonged storage times may trigger the formation of biofilms, resulting in the production of polysaccharide matrices (Giaouris, Chorianopoulos, & Nychas, 2005). These matrices shield the cells against the CAP produced, promoting resistance towards the inactivation treatment (Laroussi, 2009; Vleugels et al., 2005). Regarding the treated sample itself, studies often focus on the CAP inactivation of cells on a specific food product (Fernandez, Noriega, & Thompson, 2013; Gurol, Ekinci, Aslan, & Korachi, 2012; Kim et al., 2011; Selcuk, Oksuz, & Basaran, 2008). General studies taking into account the influence of the (food) sample, or its preservation, on the CAP efficacy are limited.

However, food intrinsic factors affect the resistance of the microorganisms towards CAP treatment as well. For example, regarding the influence of the intrinsic food structure of the sample on the CAP efficacy, two different factors can play an important role. First, as a

consequence of the food structure, the growth morphology of the cells affects the efficacy of the treatment. Colony immobilization results in cells that are more resistant towards CAP as compared to treatment of cells grown planktonically (Smet et al., 2016). Secondly, the carrier on which cells are deposited during the treatment is important, influencing the CAP efficacy (Lerouge et al., 2001). The interference of plasma species with the cells depends on the type of carrier. In Smet et al. (2016), the CAP inactivation for *S.* Typhimurium and *L. monocytogenes* cells on a liquid carrier, a solid(like) surface and a filter were compared. Cells dispersed inside a liquid carrier are the most difficult to inactivate as most highly reactive plasma species are not able to penetrate the liquid. Additionally, food surfaces can have complex topographies, protecting the cells against plasma-generated species (Fernandez et al., 2013). Next, the acidity of the sample influences the CAP treatment. *S.* Enteritidis cells on agar containing microscope slides were more susceptible towards CAP treatment at pH 5 as compared to pH 7 (Kayes et al., 2007). For the same microorganism inactivated in a liquid carrier, an increasing acidity results in a decreasing CAP resistance (Rowan et al., 2007a).

In the present work, the role of food intrinsic factors on the efficacy of CAP inactivation is further investigated by focusing on the influence of osmotic stress, in combination with suboptimal pH values. These stresses are respectively represented by growth of *Salmonella* Typhimurium and *Listeria monocytogenes* cells in model systems at different salt concentrations (0, 2, 6% (w/v) NaCl) and pH values (5.5, 6.5, 7.4). Additionally, sublethal injury resulting from CAP treatment is assessed for all experimental conditions.

# 2. MATERIALS AND METHODS

## 2.1 Experimental plan

The influence of osmotic stress and suboptimal pH on the CAP efficacy to inactivate *Salmonella* Typhimurium and *Listeria monocytogenes* was studied. Cells were grown under

different salt concentrations (0, 2, 6% (w/v) NaCl) and pH values (5.5, 6.5, 7.4). The suboptimal pH range was selected to mimic pH values representing most food products. A broad range of salt levels were examined, also taking into account very salty food products. Additionally, a third intrinsic factor, the influence of the food structure, was studied. Two growth morphologies, planktonic cells or surface colonies, are a consequence of the intrinsic food structure. Surface colonies were promoted by the addition of 5% (w/v) gelatin to the growth medium, an amount often found in food products (e.g., meat). During inactivation, cells were CAP treated in a liquid carrier or on a solid(like) surface, mimicking treatment of solid and liquid food products. Figure 1 summarizes this experimental plan. As indicated on the figure, different combinations regarding the food structure, thus growth morphology and inactivation support system, were investigated. The logical combinations are (1) cells grown planktonically, followed by inactivation in a liquid carrier and (2) cells grown as surface colonies which are also CAP inactivated on a solid(like) surface. This situation arises when cells have grown in a certain food product, which is treated by CAP. However, two less evident combinations were also studied. First, cells grown planktonically that are inactivated on a solid(like) surface. For example, this situation can appear when microorganisms grow in water used to rinse of dirt from fresh produce, and attach to the food product which is plasma treated. Secondly, cells grown as surface colonies that are inactivated in a liquid carrier. This last scenario holds for cells grown on the surface of the food product, e.g., fresh produce, which is further on in the process blended into a juice and treated with plasma.

### 2.2 Microorganisms and pre-culture conditions

*Salmonella enterica* serovar Typhimurium SL1344 was kindly provided by the Institute of Food Research (IFR, Norwich, UK). The culture was stored at -80°C in Tryptone Soya Broth (TSB (Oxoid LTd., Basingstoke, UK)) supplemented with 25% (v/v) glycerol (Acros

Organics, NJ, USA). For every experiment, a fresh purity plate was prepared from the frozen stock culture by spreading a loopful onto a Tryptone Soya Agar plate (TSA (Oxoid Ltd., Basingstoke, UK)) incubated at 37°C for 24 h. One colony from this plate was transferred into 20 mL TSB and incubated under static conditions at 37°C for 8 h (Binder KB series incubator; Binder Inc., NY, USA). Next, 200 µL from this stationary phase culture was added to 20 mL of fresh TSB and incubated under the same conditions for 16 h.

*Listeria monocytogenes* LMG 13305 was obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM, Ghent, Belgium). The culture was stored at -80°C in TSB supplemented with 0.6% (w/v) yeast extract (Merck, Darmstadt, Germany) (TSBYE). For each experiment, a new purity plate was prepared on Brain Heart Infusion (BHI (Oxoid Ltd., Basingstoke, UK)) supplemented with 1.2% (w/v) agar (Agar technical n°3, Oxoid Ltd., Basingstoke, UK) and incubated for 24 h at 37°C. One colony from the purity plate was transferred into 20 mL BHI, incubated at 37°C for 8 h under static conditions, refreshed in BHI and incubated again for 16 h.

Cell cultivation under the above defined conditions yielded early-stationary phase populations for both *S.* Typhimurium and *L. monocytogenes*, at about $10^9$ CFU/mL. These cultures were used to inoculate the corresponding media at the appropriate concentration.

### 2.3 Growth stage prior to CAP inactivation

During cell growth, the effect of the food structure results in different growth morphologies. Planktonic cells or surface colonies were grown under different experimental conditions of salt concentrations and pH until the early stationary phase was reached. Results from preliminary growth experiments were used to verify this point on the growth curve (Smet, Noriega, Van Mierlo, Valdramidis, & Van Impe, 2015). The preparation and the growth

conditions of both the liquid system (planktonic cells) and solid(like) system (surface colonies) are discussed below.

### 2.3.1 Liquid systems: preparation and planktonic growth conditions

For *S.* Typhimurium, the appropriate amount of salt (0, 2, 6% (w/v) NaCl, Sigma Aldrich, MO, USA) was added to TSB without dextrose (Becton, NJ, USA) and the pH (DocuMeter, Sartorius, Goettingen, Germany) was adapted by the addition of 5 M HCl (Acros Organics, NJ, USA). BHI was used for *L. monocytogenes*. Cells were grown in petri dishes (diameter 5.5 cm) filled with 7 mL of the medium inoculated at $10^3$ CFU/mL. This cell density was obtained by serial decimal dilutions of stationary phase cells, using dilution medium with the same pH and amount of salt as the final growth conditions. After shaking, the inoculated growth medium was dispensed into petri dishes and placed, under static conditions, in a temperature controlled incubator (KB 8182, Termaks, Bergen, Norway) at 20°C, mimicking the temperature during the CAP treatment. Cells were grown until the early stationary phase was reached.

### 2.3.2 Solid(like) systems: preparation and (surface) colonial growth conditions

Together with the appropriate amount of NaCl, gelatin at 5% (w/v) (gelatin from bovine skin, type B, Sigma-Aldrich, MO, USA) was added to TSB or BHI. After heating for 20 min at 60°C in a thermostatic water bath (GR150-S12, Grant Instruments Ltd, Shepreth, UK), the gelatin melted and the medium was adapted to the appropriate pH. The gelled medium was then filter-sterilized using a 0.2 mm filter (Filtertop, 150 mL filter volume, 0.22 µm, TPP, Switzerland), kept liquid at 60°C, and 7 mL was pipetted into sterile petri dishes (diameter 5.5 cm), which was left to solidify. Next, the solid(like) plates were surface inoculated at approximately 3.0 x $10^2$ CFU/$cm^2$ (surface area 23.8 $cm^2$, corresponding to $10^3$ CFU/mL), by

using serial decimal dilution of stationary phase cells with the appropriate dilution medium. Following this step, 20 µL of the corresponding dilution was spread onto each petri dish. After being sealed, plates were placed in a temperature controlled incubator at 20°C under static conditions and grown until the early stationary phase was reached.

**2.4 Sample inoculation for CAP inactivation**

Early stationary phase cells were CAP treated as these cells have the highest resistance and are predominantly encountered in a natural environment (Hurst, 1977; Rees, Dodd, Gibson, Booth, & Stewart, 1995).

When the planktonic cells or surface colonies reached the early stationary phase, samples were again diluted, using dilution medium with the same NaCl concentration and pH value, and were again inoculated in/on the selected support system. When inactivated in a liquid carrier, the sample was properly diluted (or re-melted in case of surface colonies) to obtain a cell density of 5.5 log(CFU/mL), and 100 µL was pipetted on empty 5 cm petri dishes, which were closed until CAP treatment. Regarding inactivation on a solid(like) surface, the gelled surface was prepared in a 5 cm petri dish (surface area 19.6 $cm^2$), at similar experimental conditions regarding salt level and pH as the initial growth medium. Following this, 50 µL of the appropriately diluted sample of either planktonic or surface colony cells was pipetted and spread on the gelled surface, which was allowed to dry for 40 min in the laminar flow cabinet (Telstar Laboratory Equipment, Woerden, the Netherlands). This results in a final cell density of 5.5 log($CFU/cm^2$) before inactivation. Cell inoculation on a (membrane) filter (cyclopore PC circles, 0.2 µm, diameter 2.5 cm, Whatman, Maidstone, UK) was identical to the procedure for solid(like) surfaces, except that only 12.5 µL was pipetted and spread on the filter area (4.9 $cm^2$), resulting again in a final inoculum density of 5.5 log($CFU/cm^2$).

**2.5 CAP: equipment and inactivation procedure**

The dielectric barrier discharge reactor used to study microbial inactivation is illustrated in Figure 2. The discharge was generated between two electrodes (diameter 5.5 cm), covered by a dielectric layer (diameter 7.5 cm). In this set-up, the electrode gap can be varied from 0 to 1 cm (fixed at 1 cm in these experiments). An enclosure (22.5 cm x 13.5 cm x 10 cm) around the electrode increases the residence time of the plasma species around the sample while also providing a more controlled environment. The enclosure was not airtight, and so oxygen and nitrogen from the environment were present. The plasma power supply transforms a low voltage DC input (0-60V) into a high voltage AC signal (0-20kV), at a frequency up to 30 kHz.

The plasma was generated in a gas mixture of helium (purity 99.996%, at a flow rate of 4 L/min) and oxygen (purity ≥ 99.995%, at a flow rate of 40 mL/min). The two flows were mixed before entering the plasma chamber (total flow rate 4.04 L/min). Thus, a 1% (v/v) admixture of oxygen was added to the helium. For this flow rate, the residence time in the enclosure was approximately 45 s. Due to the use of this helium/oxygen mixture, the key reactive gas species generated are: helium metastables (He*, $He_2$*), atomic oxygen (O), excited atomic oxygen: O(1D), O(1S), exited oxygen: $O_2$(1D), $O_2$(1S), vibrationally excited oxygen: $O_2$(v=1-4), ozone ($O_3$), hydrogen species (H, OH, $HO_2$, $H_2O_2$), nitrogen oxides (NO, $NO_2$, $NO_3$, $N_2O$) and oxygen ions ($O_2^+$, $O_4^+$, $O^-$,$O_2^-$,$O_3^-$, $O_4^-$) (Murakami, Niemi, Gans, O'Connell & Graham, 2014).

Samples were placed between the 0.8 cm gap of the DBD electrodes, and after flushing the reactor with the helium-oxygen gas mixture for 4 min, the high-voltage power source was energized and the plasma was generated. Both electrical and optical methods of characterization were employed to ensure a stable plasma discharge. Samples were treated up to 10 minutes (3 min for the filters) at a peak-to-peak voltage around 7 kV, frequency of

15 kHz and dissipated plasma power of 9.6 W. For these experimental conditions, the temperature increase of the sample, measured directly after treatment, was about 2°C. Additionally, as was reported in Smet et al. (2016), at longer treatment times (≥ 5 min) the liquid carrier significantly evaporated due to the CAP generated, at a rate of 0.0087 ± 0.0006 g/sec. Control tests confirmed the evaporation was not due to the gas flow, as for tests executed without any current the sample volume remained intact.

**2.6 Cell recovery and microbiological analysis**

To detect the cell density following CAP inactivation, the viable plate counting technique was used. Following the CAP treatment, some cells might be sublethally injured. Sublethal injury (SI) was defined as a consequence of exposure to a chemical or physical process that damages but does not kill a microorganism (Hurst, 1977). In order to calculate the percentage of sublethal injury (% SI) resulting from CAP treatment, viable plate counting on both general and selective media was performed to determine the cell density after CAP treatment. For cells inactivated in a liquid carrier, 900 µL of saline solution (0.85% (w/v) NaCl) was added to the sample. Afterwards, the diluted sample (1 mL) was collected from the petri dish and transferred to a sterile Eppendorf, in order to prepare serial decimal dilutions. For cells inactivated on the solid(like) surface, the content of the petri dish was transferred to a stomacher bag, liquefied in a thermostatic water bath at 37°C and homogenized in the stomacher for 30 seconds. 1 mL was taken from this bag, and serial decimal dilutions were prepared with saline solution. Regarding inactivation on a filter, the filter was transferred to a stomacher bag containing 5 mL of the saline solution and homogenized in the stomacher for 30 seconds. Similarly, 1 mL was pipetted from this bag, to prepare serial decimal dilutions. For each sample, 2-4 dilutions were plated (49.2 µL) onto TSA or BHI-Agar plates (general media) and XLD-Agar (*S.* Typhimurium, Xylose Lysine Deoxycholate Agar, Merck & Co,

New Jersey, USA) or PALCAM-Agar (*L. monocytogenes*, VWR Chemicals, Leuven, Belgium) plates (selective media) using a spiral plater (Eddy-Jet, IUL Instruments). Plates with general media were placed at 37°C for 24 h before counting, while selective plates were stored up to 48 h at 30°C. Cell counts shown in the figures are the mean of all countable dilutions for each sample.

**2.7 Modelling, parameter estimation and estimation of sublethal injury**

The model of Geeraerd, Herremans and Van Impe (2000), was used to fit experimental data. This model describes a microbial inactivation curve consisting of a shoulder, a loglinear inactivation phase and a tail:

$$N(t) = (N_0 - N_{res}) \cdot \exp(-k_{max} \cdot t) \cdot \left(\frac{\exp(k_{max}.t_l)}{1+(\exp(k_{max}.t_l)-1)\cdot\exp(-k_{max}\cdot t)}\right) + N_{res} \quad (1)$$

with $N(t)$ [CFU/mL] the cell density at time $t$ [s], $N_0$ [CFU/mL] the initial cell density, $N_{res}$ [CFU/mL] a more resistant subpopulation, $k_{max}$ [1/s] the maximum specific inactivation rate and $t_l$ [s] the length of the shoulder. The regression analysis was performed using the log transformation of Equation 1. The final *log reduction* is calculated from the difference between log $N_0$ *and* log $N_{res}$, using log *N(t=600 sec)(or* log *N(t=180 sec)*, for inactivation on a filter) if log $N_{res}$ was not yet reached.

Parameters of the Geeraerd et al. (2000) model were estimated via the minimization of the sum of square errors (SSE), using the *lsqnonlin* routine of the Optimization Toolbox of Matlab (The Mathworks Inc.). Simultaneous with parameter estimation, the parameter estimation errors were determined based on the Jacobian matrix. The Root Mean Squared Error (RMSE) was added as an absolute measure of the goodness of the model fit to the actual observed data.

In order to calculate the percentage of sublethal injury (% SI), theoretical concentrations obtained from the model were used. The percentage of injured survivors after exposure to

CAP treatment was determined using the following equation (Busch & Donnelly, 1992), providing the extent of the injured population at each exposure time, and the obtained values were used to simulate the percentage of sublethal injury with respect to treatment time:

$$\%\ \text{Sublethal Injury} = \frac{\text{counts on non selective medium} - \text{counts on selective medium}}{\text{counts on non selective medium}} . 100 \qquad (2)$$

### 2.8 Statistical analysis

Analysis of variance (ANOVA) test was performed to determine whether there were significant differences amongst means of logarithmically transformed viable counts, at a 95.0% confidence level ($\alpha = 0.05$). The Fisher´s Least Significant Difference (LSD) test was used to distinguish which means were significantly different from which others. Standardized skewness and standardized kurtosis were used to assess if data sets came from normal distributions. These analyses were performed using Statgraphics Centurion XVI.I Package (Statistical Graphics, Washington, USA). Test statistics were regarded as significant when P was $\leq 0.05$.

## 3. RESULTS AND DISCUSSION

Figure 3 and 4 represent the inactivation curves of stationary phase *S.* Typhimurium and *L. monocytogenes* cells exposed to CAP treatment. For each microorganism, cells were inactivated in a liquid carrier (a, b, c), on a solid(like) surface (d, e, f) or on a filter (g, h, i). Prior to the CAP treatment, cells were grown at three different experimental conditions, in order to assess the influence of osmotic stress in combination with a suboptimal pH: pH 7.4, 0% (w/v) NaCl (a, d, g), pH 6.5, 2% (w/v) NaCl (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i). Additionally, different food structures of the model system result in cells grown planktonically or as surface colonies. As a control, for the most optimal experimental

condition at pH 7.4 and 0% (w/v) NaCl (a, d, g), cells from the pre-culture were also directly treated. The experimental data were fitted with the Geeraerd et al. (2000) model. Table 1 and 2 summarize the estimated main inactivation parameters from the Geeraerd model, i.e., the length of the shoulder phase $t_l$, the inactivation rate $k_{max}$, the cell density in the tail log $N_{res}$, the overall *log reduction* and the corresponding statistical analysis.

Regarding the response of both microorganisms to CAP exposure, under optimal experimental conditions inactivation kinetics of the Gram-negative *S.* Typhimurium and Gram-positive *L. monocytogenes* have similar shapes. However, *L. monocytogenes* is more resistant to the CAP treatment, resulting in the observation of lower inactivation efficacies. As previously discussed by Smet et al. (2016), the diversity in the CAP efficacy can be explained by the different cell wall structures of the two microorganisms. Gram-positive bacteria are often found to be more resistant towards CAP treatment than Gram-negative bacteria (Ermolaeva et al., 2011; Lee, Paek, Ju, & Lee., 2006). The addition of osmotic stress or suboptimal pH to the cell environment does not change this observation, as also at pH 6.5, 2% (w/v) NaCl (b, e, h) and pH 5.5, 6% (w/v) NaCl (c, f, i) *L. monocytogenes* has a lower inactivation efficacy as compared to *S.* Typhimurium (Figure 3 and 4).

## 3.1 Effect of osmotic stress and suboptimal pH on CAP inactivation efficacy

### *3.1.1 Effect of osmotic stress and suboptimal pH for cells inactivated in a liquid carrier*

For all experimental conditions, the inactivation curves of *S.* Typhimurium cells treated in a liquid carrier (a, b, c) present a long shoulder phase followed by a log linear inactivation phase, regardless their growth morphology. This long shoulder phase reflects a resistant population and implies that the microorganisms need to have a certain CAP treatment level before the cells are lethally damaged. As the stress level rises due to an increase of the salt

level in combination with a decrease of the pH value, the reduction in cell density due to CAP treatment reduces. This trend is very significant when comparing kinetics for cells grown under optimal conditions (pH 7.4, 0% (w/v) NaCl (a)) with the inactivation dynamics of cells grown under high environmental stress (pH 5.5, 6% (w/v) NaCl (c)). Regarding the inactivation parameters for *S.* Typhimurium, no significant differences are observed for $t_l$ values between the three experimental conditions when the cells are grown planktonically. For cells grown as surface colonies, the length of the shoulder phase increases under high osmotic and acidic stresses. The inactivation rate, $k_{max}$, tends to decrease when the environmental stress level increases, irrespective of the growth morphology. No tailing phase is present for cells inactivated in a liquid carrier, thus log $N_{res}$ is undefined. Finally, *log reductions* for cells inactivated in a liquid carrier tend to be the lowest at pH 5.5, 6% (w/v) NaCl (c), which is valid for both planktonic cells and surface colonies.

In case of *L. monocytogenes*, most survival curves exhibit again a long shoulder phase followed by the log linear inactivation. In all cases, the final reduction is limited, especially in comparison to results for *S.* Typhimurium. Especially at the most stressing condition, pH 5.5 and 6% (w/v) NaCl, almost no reduction in cell concentration is observed. No shoulder is present in the kinetics at pH 5.5, 6% (w/v) NaCl (c) for both growth morphologies, or at pH 7.4, 0% (a) NaCl for planktonic cells. Therefore, no conclusion can be made regarding the influence of osmotic stress and suboptimal pH on the inactivation parameter $t_l$ for *L. monocytogenes* cells inactivated in a liquid carrier (a, b, c). Similar to the results for *S.* Typhimurium and regardless the growth morphology of the *L. monocytogenes* cells, $k_{max}$ and the *log reduction* are the lowest for more stressing experimental conditions, while $N_{res}$ is undefined.

338 *3.1.2 Effect of osmotic stress and suboptimal pH for cells inactivated on a solid(like) surface*

339 For *S.* Typhimurium cells inactivated on a solid(like) surface (d, e, f), a shoulder phase is
340 detected for low stress levels (pH 7.4, 0% (w/v) NaCl (d) and pH 6.5, 2% (w/v) NaCl (e)).
341 Following the log linear inactivation phase, sometimes also a tailing phase is present,
342 indicating the presence of a CAP resistant population. This tail was again observed for all
343 growth morphologies at the optimal experimental condition (d), and for planktonic cells at the
344 most severe environmental stresses (e, f). Although no significant differences are present, the
345 inactivation rate decreases slightly when the stress level increases, which is observed for all
346 growth morphologies. A tailing phase is often observed for cells inactivated on a solid(like)
347 surface, but no general trend for $N_{res}$ concerning the influence of the pH value or salt
348 concentration is found. Concerning the influences of osmotic stress and a suboptimal pH on
349 the *log reductions* for *S.* Typhimurium cells inactivated on a solid(like) surface, the reduction
350 tends to be the highest at optimal conditions (d). As the shoulder was not detected for all
351 experimental cases, no conclusion regarding its length can be drawn.
352 Inactivation kinetics for *L. monocytogenes* cells inactivated on a solid(like) surface follow a
353 similar trend (d, e, f). While a shoulder is never observed, and thus $t_l$ is undefined, the linear
354 inactivation phase is always followed by a long tail. As for *S.* Typhimurium, $k_{max}$ values of
355 *L. monocytogenes* decrease when environmental stresses increase, while $N_{res}$ values tend to
356 increase at high osmotic and acidic stresses. Also at pH 5.5, 6% (w/v) NaCl (f), *log reductions*
357 are lower.
358
359 *3.1.3 Effect of osmotic stress and suboptimal pH for cells inactivated on a filter*
360 For *S.* Typhimurium cells inactivated on a filter (g, h, i), the inactivation kinetics do not
361 follow any specific trend. A shoulder phase is only observed for low (pH 7.4, 0% (w/v) NaCl
362 (g)) and medium stress levels (pH 6.5, 2% (w/v) NaCl (h)), so no general conclusion

363 regarding the length of this shoulder can be drawn. Again, $k_{max}$ values for both growth
364 morphologies decrease slightly when the stress level increases. $N_{res}$ is undefined as most
365 conditions do not have a tail. Again, *log reductions* tend to be the lowest at pH 5.5, 6% (w/v)
366 NaCl (i).
367 When *L. monocytogenes* cells are inactivated on a filter (g, h, i), a tail is always observed
368 while the shoulder phase is mainly present at high stress levels. Regarding the inactivation
369 parameters, $t_l$ is often undefined. The decrease in *log reduction* and $k_{max}$ with increasing
370 environmental stress is very limited, and regarding the inactivation rate no significant
371 differences are observed. The tailing phase is always present, and $N_{res}$ slightly increases with
372 an increase of the environmental stress.
373
374 Growth conditions or intrinsic factors do not only influence microbial growth, but are also
375 able to affect the stress response of microorganisms towards CAP treatment. Regardless the
376 inactivation support or the growth morphology, the more stressing the growth conditions
377 concerning pH value and NaCl concentration, the more resistant the microorganisms are
378 towards CAP treatment, resulting in lower inactivation efficacies. For example, if a
379 microorganism would be able to grow in a salty food product (e.g., cheese), CAP treatment
380 might not be sufficient to ensure the food safety. Many bacteria interpret osmotic stress as a
381 signal to prepare for more stringent conditions in the future by inducing a general system of
382 stress protection (O'Byrne & Booth, 2002). Similarly, acid-adapted cells were found to have
383 increased tolerance towards various stresses, including thermal and osmotic stress (Leyer &
384 Johnson, 1993). As a non-thermal technology, CAP is the optimal choice to treat (acid) fruits.
385 However, acid adaption of cells raises problems when the CAP treatment is not able to
386 inactivate them. These stress related phenomena can be explained by cross protection or stress
387 hardening, which refers to an increased resistance to lethal factors, e.g. CAP, after adaptation

388 to environmental stresses (Lou & Yousef, 1997). More specifically, the adaptation to different
389 environmental stresses (acid, ethanol, $H_2O_2$, heat, NaCl) was reported to increase the
390 resistance of *L. monocytogenes* to hydrogen peroxide. This can be explained by the induction
391 of a sigma factor, accounting for the general resistance to environmental stresses in microbial
392 cells (Wesche, Gurtler, Marks, & Reyser, 2009).
393 Limited research is available on the influence of intrinsic or extrinsic factors on the CAP
394 efficacy. Fernandez et al. (2013) studied the effect of the growth temperature on the CAP
395 inactivation of *S.* Typhimurium. In the observed range of temperatures from 20°C to 45°C,
396 the growth temperature did not significantly affect the resistance of the microorganism
397 towards the CAP treatment. However, an increased resistance to CAP treatment, after
398 adaptation to environmental stress has been previously reported. For example, in Smet et al.
399 (2016), the influence of the intrinsic food structure on the CAP efficacy to inactivate
400 *S.* Typhimurium and *L. monocytogenes* was studied. During bacterial growth, different growth
401 morphologies arise as a direct consequence of the intrinsic food structure. As reported, the
402 type of growth morphology influences the CAP efficacy. CAP inactivation experiments with
403 cells grown as surface colonies result in lower log reductions as compared to experiments
404 with planktonic cells, indicating an increased resistance of the surface colonies towards CAP.
405 Starvation stress, resulting from nutrient limitations which surface colonies endure, can create
406 cells resistant to the subsequent CAP inactivation treatment (Li, Sakai, Watanabe, Hotta, &
407 Wachi, 2013). Similar to stress due to the intrinsic food structure or growth temperature, cross
408 protection plays an important role on the CAP inactivation efficacy if cells are grown under
409 osmotic stress or at suboptimal pH values. All environmental stresses, due to the pH, salt
410 level, food structure or growth temperature can result in an increased resistance towards a
411 subsequent CAP treatment.
412

## 3.2 Effect of food structure on CAP inactivation efficacy for environmental conditions under osmotic stress at suboptimal pH

The food structure can affect the CAP inactivation on two different levels. The growth morphology of the cells, a direct consequence of the intrinsic food structure, influences the CAP inactivation efficacy. Secondly, the food structure plays an important role during the treatment itself, by means of the inactivation support system in/on which the cells are deposited.

In most cases, these two levels regarding food structure are related. A liquid food product (e.g., a juice) where cells have grown planktonically, can be treated directly and thus the cells are also treated in a liquid carrier. This holds as well if the inactivation support is a solid(like) surface (e.g., meat, fruits, vegetables) infected with surface colonies. However, due to food processing, exceptions can arise. For example, planktonic cells can grow in the washing water used during processing. This could result in cross-contamination onto the food products, which are afterwards treated with CAP. Similarly, all surfaces that come into contact with a (liquid or solid(like)) food product, form a potential risk for contamination if infected with colonies. Finally, if colonies have grown on a the surface of a fruit or vegetable, and this product is blended into a juice, the resulting fruit or vegetable juice itself can be treated with CAP (liquid carrier).

This section discusses, on both levels, the effect of the food structure on the CAP inactivation efficacy for the different (stressing) experimental conditions.

### *3.2.1 Effect of the growth morphology on the CAP inactivation efficacy*

In Smet et al. (2016), the influence of the food structure on the CAP efficacy was examined under optimal experimental conditions regarding osmotic and acidic stress (pH 7.4, 0% (w/v) NaCl (a, d, g)). The influence of food structure during growth was studied by investigating the

role of the growth morphology in the CAP inactivation efficacy, indicating cells were grown planktonically in a liquid environment or as surface colonies on a solid(like) surface. Lower inactivation efficacies for cells grown as surface colonies at pH 7.4, 0% (w/v) NaCl (a, d, g), regardless of the inactivation support system, indicate an increased resistance of these immobilized cells towards CAP inactivation of both *S.* Typhimurium and *L. monocytogenes*. This conclusion still holds when the environmental stress is more severe (Figure 3 and 4). If cells of both microorganisms are inactivated in a liquid carrier and grown at pH 6.5, 2% (w/v) NaCl (b) or pH 5.5, 6% (w/v) NaCl (c), the CAP inactivation is again the highest for planktonic cells, although in some cases the inactivation parameters do not indicate statistically significant differences. In a stressing environment, cells inactivated on the solid(like) surface exhibit lower *log reductions* when grown as surface colonies as compared to cells grown planktonically. Next to this, the inactivation rate follows the order $k_{max,\ planktonic\ cells} \geq k_{max,\ surface\ colonies}$ while log $N_{res}$ is always lower for cells grown planktonically. A similar behavior was detected for cells grown under stressing conditions and CAP treated on a filter. In a solid(like) environment growth takes place as (surface) colonies and the transport is based on diffusion, limiting among others the nutrient delivery (Antwi et al., 2006; Malakar et al., 2000; Wimpenny & Coombs, 1983). This nutrient limitation results in starvation stress, indicating the survival of bacteria in oligotrophic conditions (Wesche et al., 2009), which can promote the resistance of the cells against the subsequent CAP treatment (Li et al., 2013).

*3.2.2 Effect of the inactivation support system on the CAP inactivation efficacy*

The effect of the food structure during the CAP treatments is studied in Smet et al. (2016) by assessing the kinetics of cells, grown under optimal experimental conditions (pH 7.4, 0% (w/v) NaCl (a, d, g)). In the current work cells are inactivated on three different inactivation support systems: a liquid carrier, a solid(like) surface or a filter. Regardless if *S.* Typhimurium

or *L. monocytogenes* cells are grown under optimal environmental conditions (a, d, g), or at more stressing pH values and salt levels (b, c, e, f, h, i), the effect of the intrinsic food structure during the treatment on the CAP efficacy remains the same (Figure 3, 4). At optimal environmental conditions, the lack of a shoulder phase for cells inactivated on a solid(like) surface or on a filter leads to a very rapid inactivation as compared to cells inactivated inside a liquid carrier. These different shapes in survival curves are always observed for the different inactivation support systems, independent of the environmental growth condition. Regarding the inactivation parameters, as expected $k_{max}$ values are either similar or slightly lower for cells inactivated in a liquid carrier, grown at pH 6.5, 2% (w/v) NaCl (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i). Therefore, also under more severe environmental stress, cells in a liquid carrier prove to be more difficult to inactivate. As many highly reactive plasma species already react at the plasma-liquid interface and do not penetrate very deep into the liquid medium, cells in a liquid carrier are more challenging to inactivate. Cells treated on a solid(like) surface or on a filter are easily attained by the plasma species during the treatment, resulting in a higher inactivation efficacy (Oehmigen et al., 2010). As commented in Section 2.5, the liquid carrier partly evaporates at longer treatment times ($\geq$ 5 min). This could possibly result in a shift of treatment of cells on the plastic petri dish instead of in the liquid carrier which can contribute to the fact that inactivation of cells in the liquid carrier only starts at longer treatment times.

As previously mentioned, food products containing high salt concentrations or products with lower pH values might not be well suited to be CAP treated as cells could be able to adapt to these environmental stresses and gain resistance towards subsequent CAP treatment. The above findings regarding the influence of the food structure indicate this effect might be magnified if cells are either grown as surface colonies or inactivated in a liquid carrier.

### 3.3 CAP and sublethal injury of cells: effect of osmotic and acidic stress

As sublethal injured cells are able to recover or even gain resistance, they pose major public health concerns. Thus it is important to investigate the relation between novel inactivation technologies and SI of cells (Noriega et al., 2014). Sublethal injury of cells treated with CAP was studied by plating the treated cells on both general and selective plating media. The percentage of sublethal injury (SI) as a function of treatment time is illustrated in Figure 5 and 6.

Most studies focus on enumerating the microbial survivors on general media while limited research has been performed focusing on the sublethal injury of cells following CAP treatment. By using respiratory staining (RS), Rowan et al. (2007b) proved the existence and rapidly quantified the extent of sublethal injury for CAP treated pathogens. As discussed in Smet et al. (2016), under optimal conditions (pH 7.4, 0% (w/v) NaCl) the SI of *S.* Typhimurium cells is higher than for *L. monocytogenes*. This trend can be extended to the more stressing experimental conditions at pH 6.5, 2% (w/v) NaCl (b, e, h) and pH 5.5, 6% (w/v) NaCl (c, f, i), as the SI detected for *L. monocytogenes* will always be the lowest. Regardless the environmental growth condition, a maximum for the SI evolution in time is often detected. This maximum illustrates the phenomenon of injury accumulation finally culminating into cell death (Noriega, Velliou, Van Derlinden, Mertens, & Van Impe., 2013), and coincides to the start of a new phase in the inactivation kinetics. As for most *L. monocytogenes* experiments the kinetics show a tailing phase, the maximum in the SI evolution corresponds to the transition into this last phase. In case of *S.* Typhimurium, a maximum is detected at optimal conditions (a, d, g) or moderately stressing environmental conditions (pH 6.5, 2% (w/v) NaCl (b, e, h)). Also for this microorganism, the maximum coincides with the transition to either the linear inactivation phase or the tailing phase, if

present. However, at more stressing environmental conditions, different trends are sometimes observed, and the evolution of SI with treatment time is still increasing. This holds for *S.* Typhimurium at the most stressing condition (c, f, i), as for these experiments no tail is (yet) reached. Finally, for experiments with a limited overall reduction, the SI evolution with treatment time remains constant

As reported in Smet et al. (2016), no trend regarding the influence of the food structure on the SI evolution after CAP treatment is present, which is again valid in a stressed environment. However, there is a direct influence of the osmotic stress and suboptimal pH on the SI as a function of the CAP treatment time. In general, if the stress level increases due to prior growth at high salt concentrations and low pH values, also the level of SI during CAP inactivation increases. For instance, for *S.* Typhimurium at pH 5.5, 6% (w/v) NaCl (c, f, i), some cells are even sublethally injured prior to the CAP treatment, which can be explained due to the high salt concentrations present in the media during growth.

# 4. CONCLUSION

The role of food intrinsic factors on the efficacy of CAP inactivation is further investigated by focusing on the influence of osmotic stress and suboptimal pH on the inactivation kinetics of *S.* Typhimurium and *L. monocytogenes*. The presence of high salt concentrations in the growth medium or suboptimal pH values, induces stress hardening, creating cells resistant towards the subsequent CAP treatment. Additionally, regardless the osmotic stress level or the pH value in the system, both the type of microorganism and the food structure remain to influence the inactivation results. The maximum in the SI evolution as a function of the treatment time, indicates an injury accumulation of the treated cells that finally culminates into cell death. This research again confirms that food intrinsic factors, influence the CAP inactivation efficacy. This indicates the importance of knowledge on the different food

intrinsic factors or thus the food properties, e.g., regarding salt concentration, pH value or intrinsic food structure, to be able to predict the final CAP inactivation result. This knowledge makes it possible to assess whether or not CAP can be an efficient mild technology to treat a specific food product.

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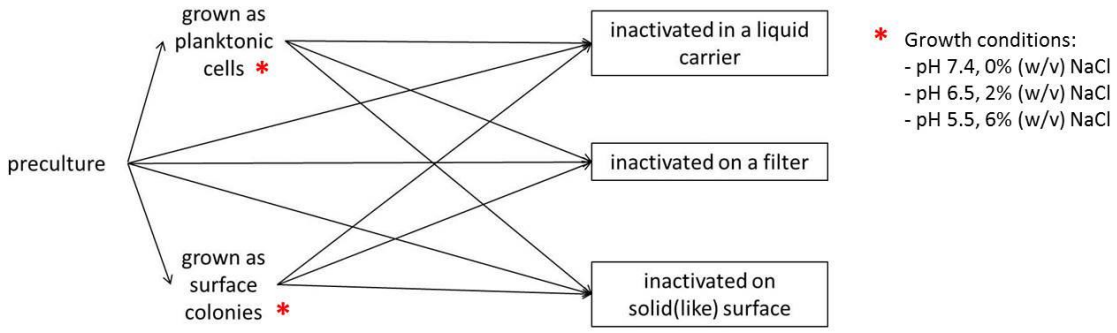
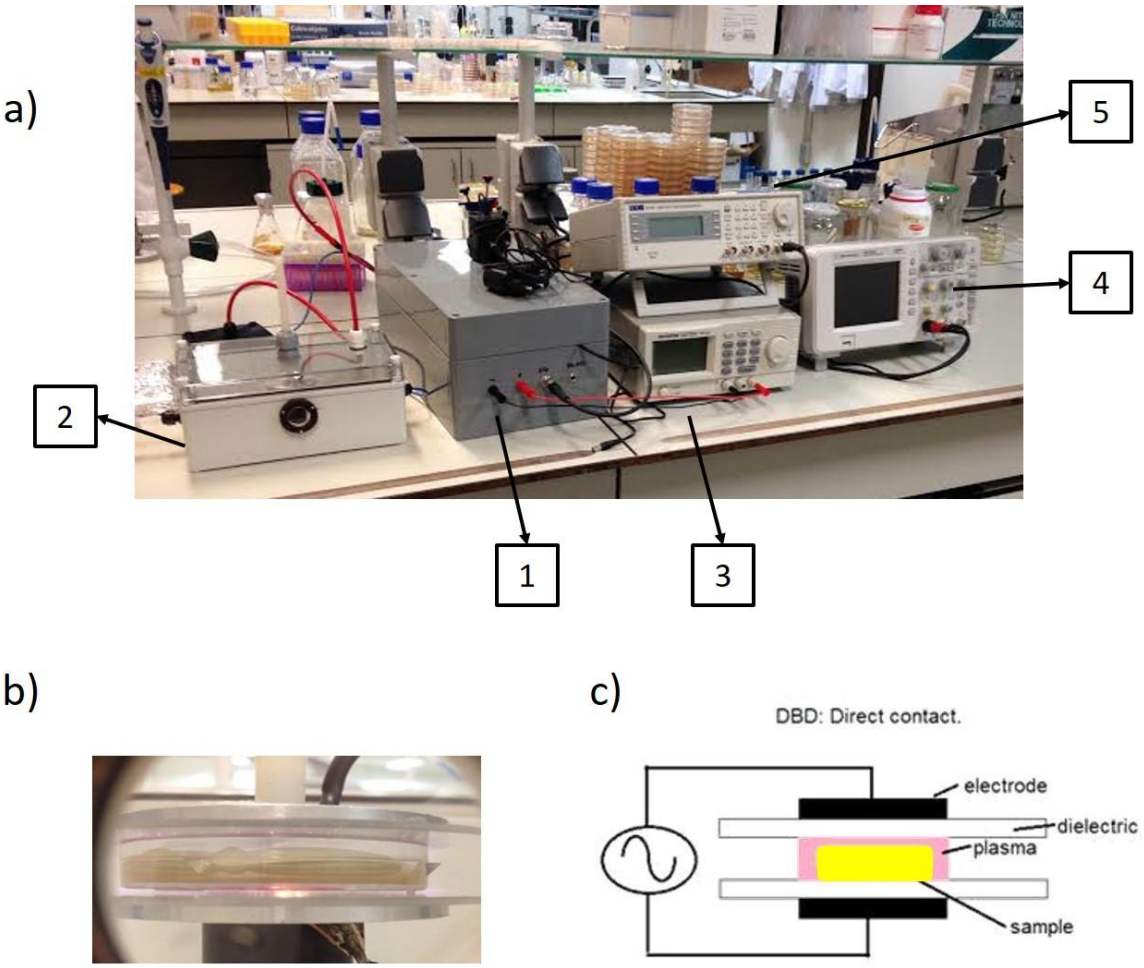



689

690 Figure 1: Different combinations tested at each experimental condition. Cells were inactivated
691 in a liquid carrier (a, b, c), on a solid(like) surface (d, e, f) and on a filter (g, h, i). Prior to
692 CAP treatment, cells were grown at pH 7.4, 0% (w/v) NaCl (a, d, g), pH 6.5, 2% (w/v) NaCl
693 (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i), and either planktonically or as surface colonies.
694 For pH 7.4, 0% (w/v) NaCl (a, d, g), as a control, cells from the preculture were directly
695 treated. (b) Examples for the different combinations.




696

697 Figure 2: (a) the CAP set up: (1) plasma power source, (2) Dielectric Barrier Discharge
698 reactor (22.5 cm x 13.5 cm x 10 cm), (3) DC power supply, (4) oscilloscope and (5) function
699 generator. (b) DBD electrode inside reactor (electrode: diameter 5.5 cm, dielectric: 7.5 cm)
700 petri dish containing sample: petri dish with diameter 5 cm). (c) Schematic representation
701 DBD electrode with sample.

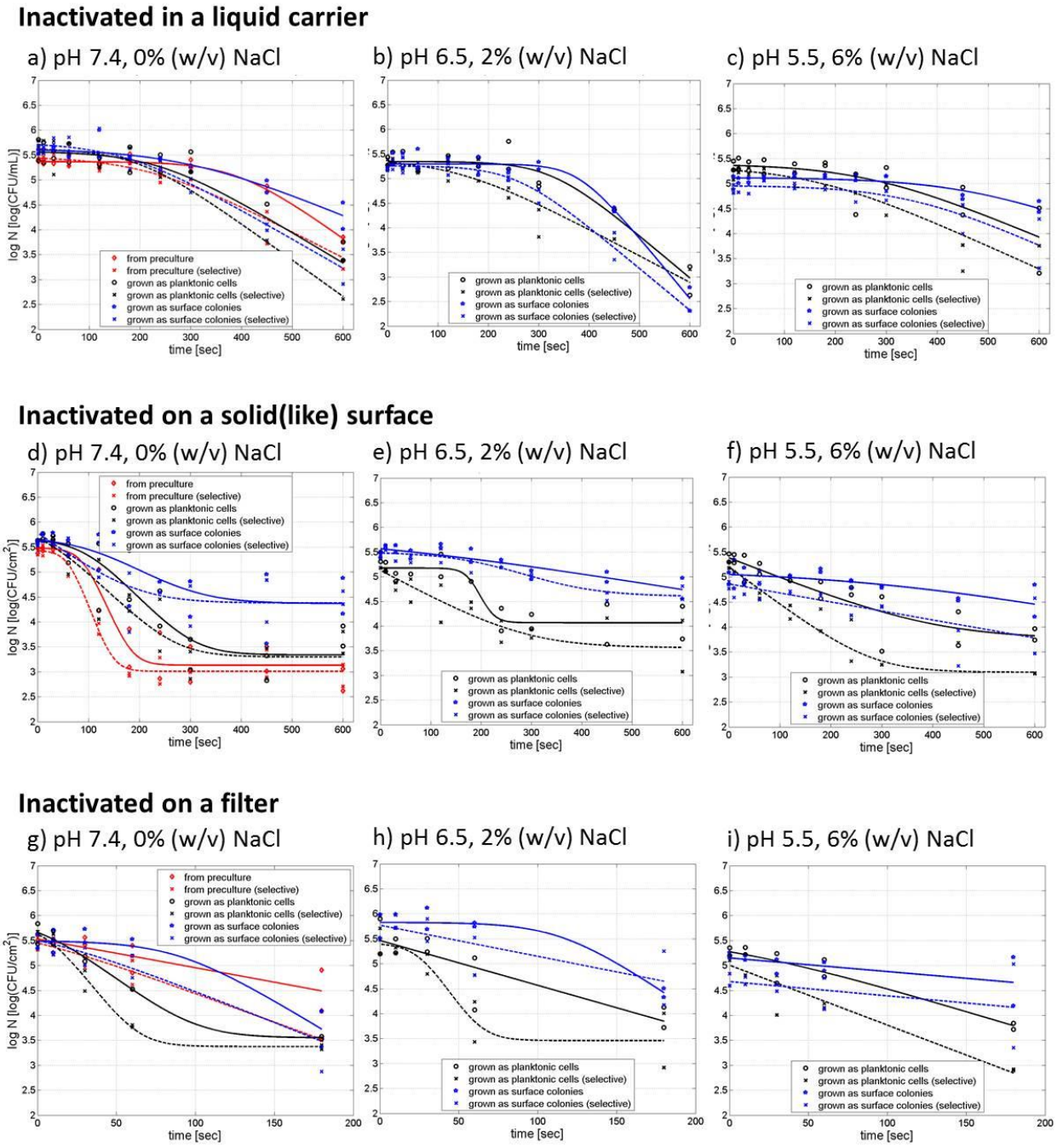
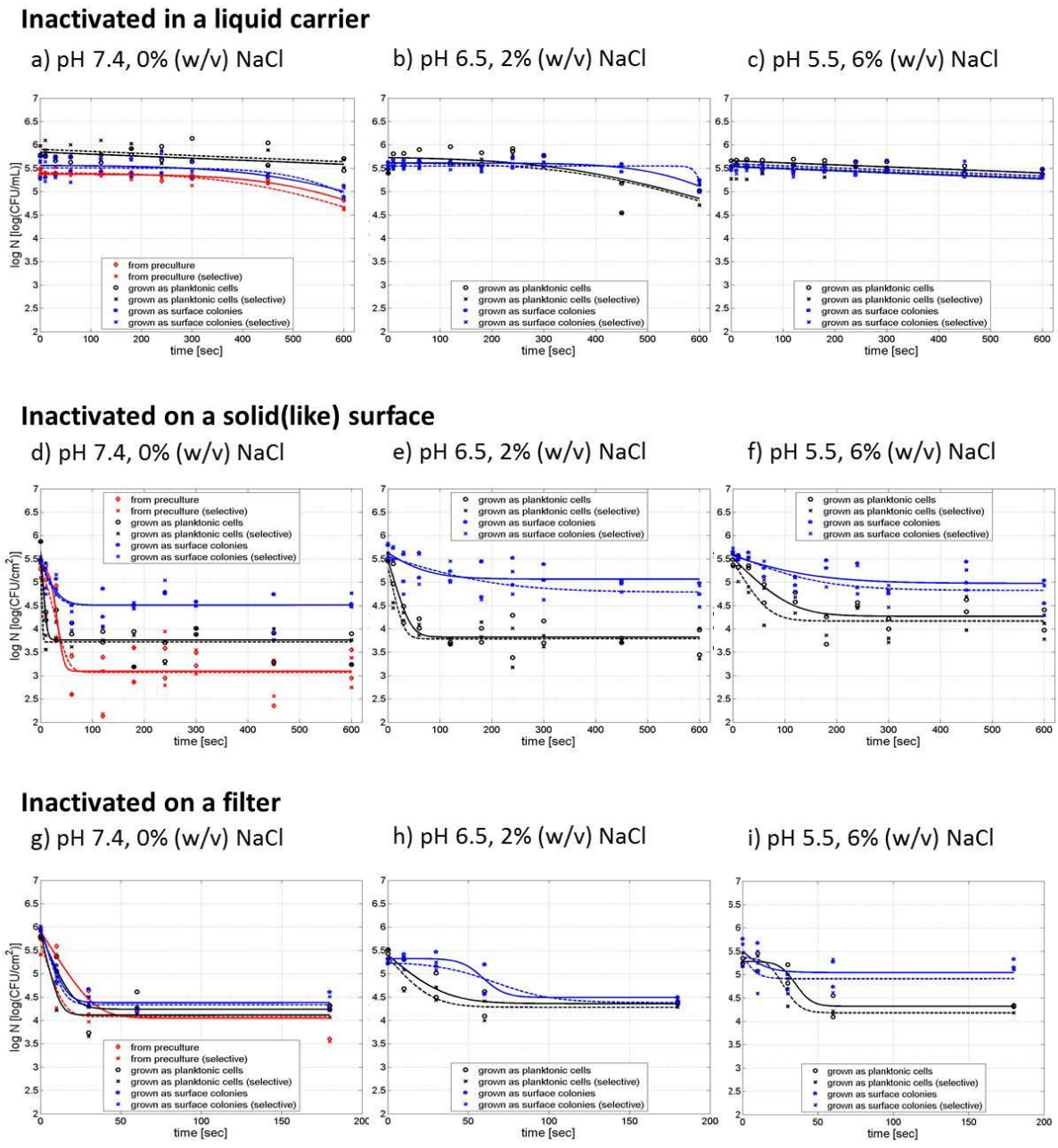



Figure 3: Survival curves of stationary phase *S.* Typhimurium after exposure to CAP. Cells were inactivated in a liquid carrier (a, b, c), on a solid(like) surface (d, e, f) and on a filter (g, h, i). Prior to CAP treatment, cells were grown at pH 7.4, 0% (w/v) NaCl (a, d, g), pH 6.5, 2% (w/v) NaCl (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i), and either planktonically or as surface colonies. For pH 7.4, 0% (w/v) NaCl (a, d, g), as a control, cells from the preculture were

708 directly treated. Experimental data (symbols) and global fit (line) of the Geeraerd et al. (2000)

709 model: total viable population (o, solid line) and uninjured viable population (x, dashed line).

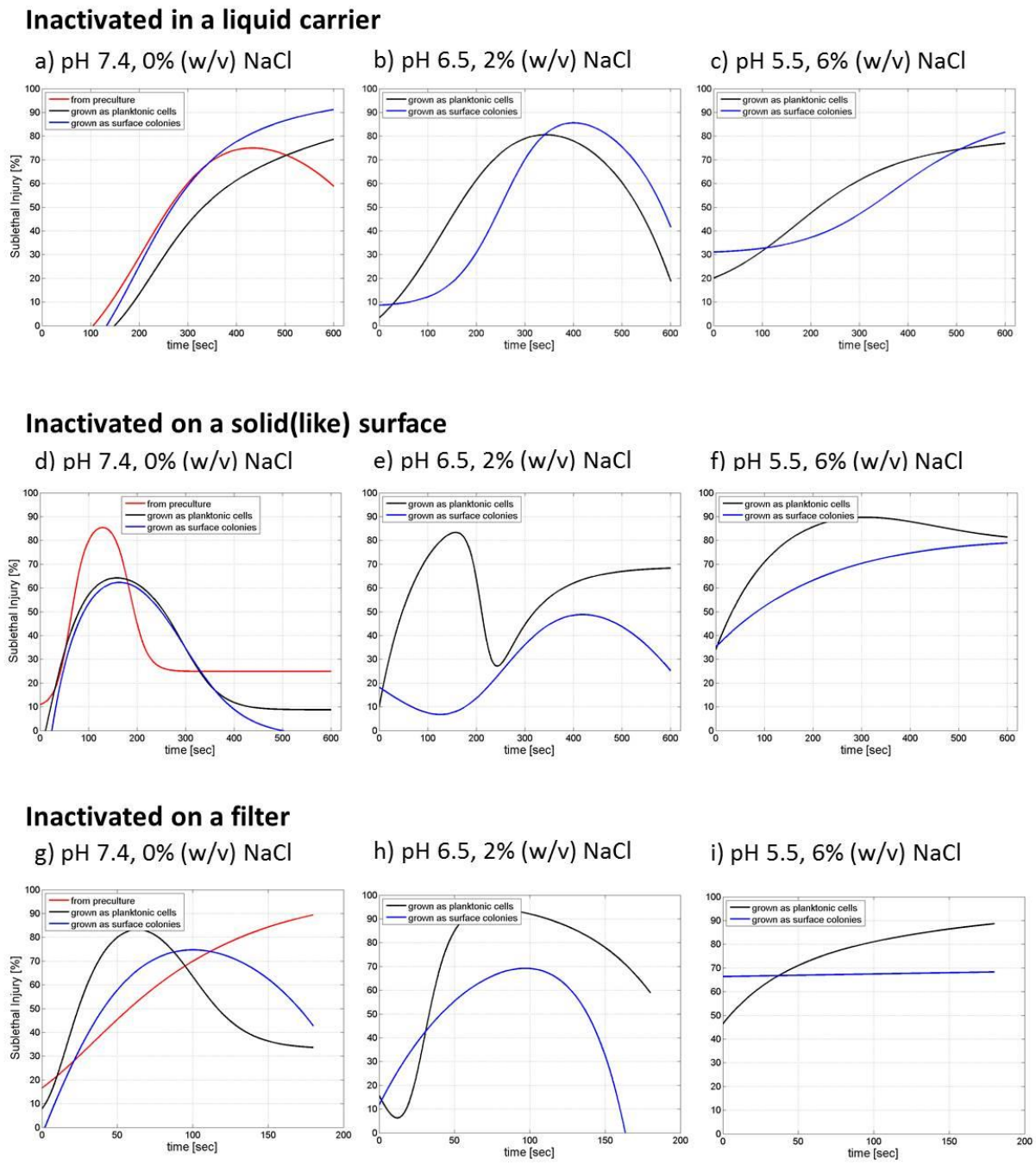



710

711 Figure 4: Survival curves of stationary phase *L. monocytogenes* after exposure to CAP. Cells
712 were inactivated in a liquid carrier (a, b, c), on a solid(like) surface (d, e, f) and on a filter (g,
713 h, i). Prior to CAP treatment, cells were grown at pH 7.4, 0% (w/v) NaCl (a, d, g), pH 6.5,
714 2% (w/v) NaCl (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i), and either planktonically or as
715 surface colonies. For pH 7.4, 0% (w/v) NaCl (a, d, g), as a control, cells from the preculture
716 were directly treated. Experimental data (symbols) and global fit (line) of the Geeraerd et al.

717 (2000) model: total viable population (o, solid line) and uninjured viable population (x,

718 dashed line).




719

720 Figure 5: Evolution with time of the sublethal injury (%) of *S.* Typhimurium towards the
721 exposure time to CAP. Cells were inactivated as a liquid (a, b, c), on a solid(like) surface (d,
722 e, f) and on a filter (g, h, i). Prior to CAP treatment, cells were grown at pH 7.4, 0% (w/v)
723 NaCl (a, d, g), pH 6.5, 2% (w/v) NaCl (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i), and either
724 planktonically or as surface colonies. For pH 7.4, 0% (w/v) NaCl (a, d, g), as a control, cells
725 from the preculture were directly treated.

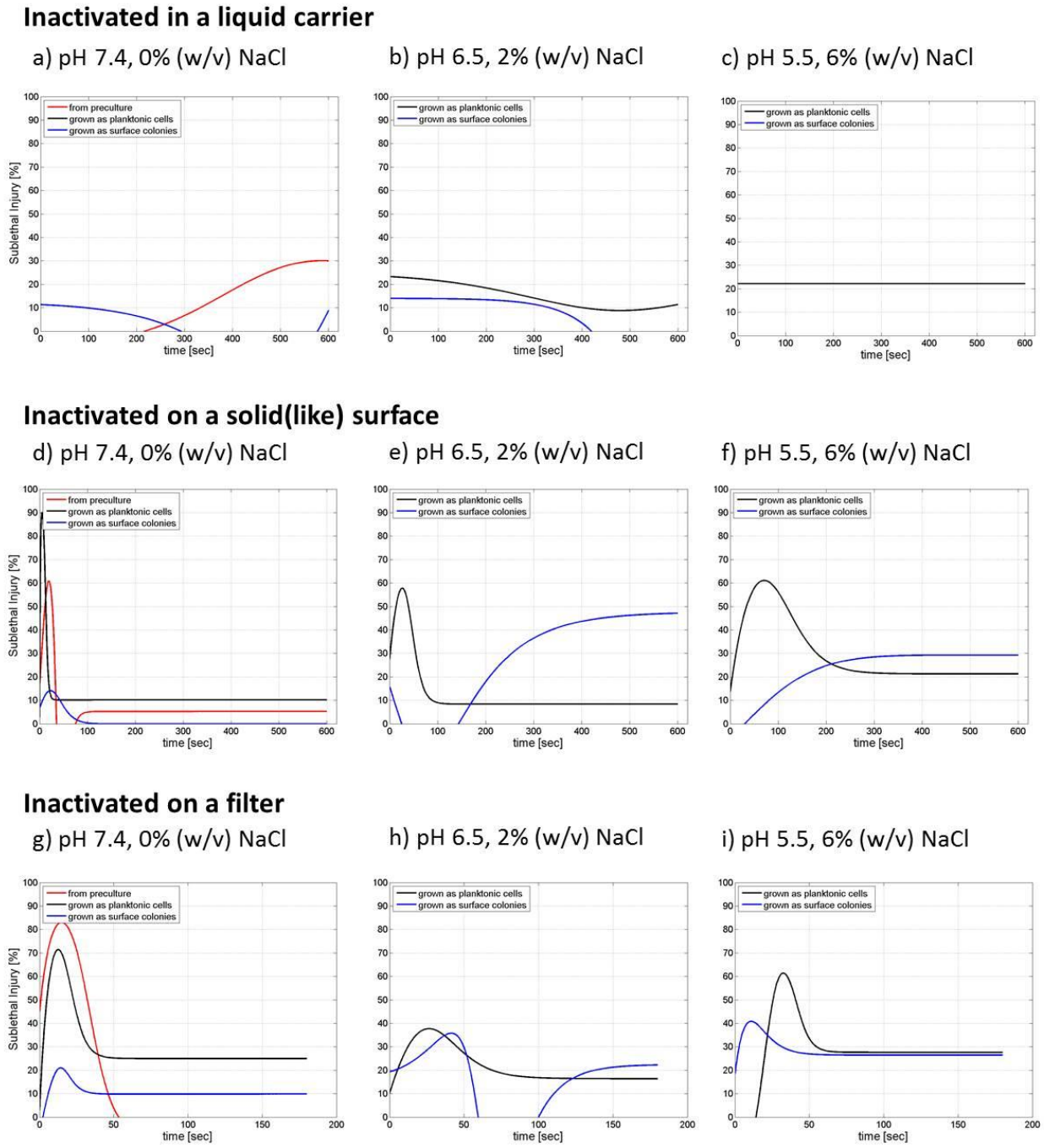



Figure 6: Evolution with time of the sublethal injury (%) of *L. monocytogenes* towards the exposure time to CAP. Cells were inactivated as a liquid (a, b, c), on a solid(like) surface (d, e, f) and on a filter (g, h, i). Prior to CAP treatment, cells were grown at pH 7.4, 0% (w/v) NaCl (a, d, g), pH 6.5, 2% (w/v) NaCl (b, e, h) or pH 5.5, 6% (w/v) NaCl (c, f, i), and either planktonically or as surface colonies. For pH 7.4, 0% (w/v) NaCl (a, d, g), as a control, cells from the preculture were directly treated.

733 Table 1. Inactivation parameters of the Geeraerd et al. (2000) model for *S.* Typhimurium after exposure to CAP. Cells were inactivated on a
734 liquid carrier, on a solid(like) surface and on a filter. Prior to CAP treatment, cells were grown at pH 7.4, 0% (w/v) NaCl, pH 6.5, 2% (w/v) NaCl
735 or pH 5.5, 6% (w/v) NaCl, and either planktonically, as surface colonies, or as the direct preculture (only for pH 7.4, 0% (w/v) NaCl).

| Inactivation support system | Growth conditions | | | Growth morphology | Population | Inactivation parameters | | | | RMSE | $_1$log reduction$_2^3$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH (-) | NaCl (% (w/v)) | Reference | | | $_1$log $N_0$ (log (CFU/mL))$_2^3$ / $_1$log $N_0$ (log (CFU/cm$^2$))$_2^3$ | $_1t_l$ (s)$_2^3$ | $_1k_{max}$ (1/s)$_2^3$ | $_1$log $N_{res}$ (log(CFU/mL))$_2^3$ / $_1$log $N_{res}$ (log (CFU/cm$^2$))$_2^3$ | | |
| *liquid* | 7.4 | 0 | a | Preculture | Total | $5.4 \pm 0.0_{A}$ | $_{(b)}392.2 \pm 21.1_{B}$ | $_{(a)}0.017 \pm 0.002_{B}$ | - | 0.1122 | $\approx {}_{(b)}1.6 \pm 0.0_{B}$ |
| | | | | | Uninjured | $5.4 \pm 0.1_{A}$ | $_{(b)}220.6 \pm 41.5_{A}$ | $_{(a)}0.012 \pm 0.002_{A}$ | - | 0.1996 | $\approx {}_{(a)}2.0 \pm 0.1_{A}$ |
| | | | | Planktonic cells | Total | $5.6 \pm 0.1_{B}^{b}$ | $_{(b)}238.3 \pm 74.3_{A}^{a}$ | $_{(a)}0.014 \pm 0.003_{AB}^{a}$ | - | 0.4204 | $\approx {}_{(a)}2.3 \pm 0.1_{C}^{b}$ |
| | | | | | Uninjured | $5.6 \pm 0.2_{AB}^{b}$ | $_{(b)}196.8 \pm 58.7_{A}^{a}$ | $_{(a)}0.017 \pm 0.003_{B}^{b}$ | - | 0.4567 | $\approx {}_{(b)}2.9 \pm 0.2_{C}^{c}$ |
| | | | | Surface colonies | Total | $5.6 \pm 0.1_{B}^{c}$ | $_{(b)}296.8 \pm 47.5_{AB}^{a}$ | $_{(a)}0.010 \pm 0.002_{A}^{a}$ | - | 0.1567 | $\approx {}_{(a)}1.3 \pm 0.1_{A}^{b}$ |
| | | | | | Uninjured | $5.7 \pm 0.1_{B}^{c}$ | $177.4 \pm 34.9_{A}^{a}$ | $_{(a)}0.014 \pm 0.001_{AB}^{a}$ | - | 0.2061 | $\approx {}_{(c)}2.5 \pm 0.1_{B}^{b}$ |
| | 6.5 | 2 | b | Planktonic cells | Total | $5.3 \pm 0.1_{A}^{a}$ | $325.4 \pm 31.6_{A}^{a}$ | $_{(a)}0.020 \pm 0.003_{A}^{b}$ | - | 0.2167 | $\approx {}_{(c)}2.3 \pm 0.1_{A}^{b}$ |
| | | | | | Uninjured | $5.3 \pm 0.1_{A}^{a}$ | $150.2 \pm 60.0_{A}^{a}$ | $_{(a)}0.013 \pm 0.002_{A}^{ab}$ | - | 0.3217 | $\approx {}_{(b)}2.4 \pm 0.1_{A}^{b}$ |
| | | | | Surface colonies | Total | $5.3 \pm 0.0_{A}^{b}$ | $_{(b)}378.4 \pm 18.7_{A}^{b}$ | $_{(a)}0.029 \pm 0.003_{B}^{b}$ | - | 0.1833 | $\approx {}_{(c)}2.7 \pm 0.0_{B}^{c}$ |
| | | | | | Uninjured | $5.3 \pm 0.0_{A}^{b}$ | $255.5 \pm 17.3_{B}^{a}$ | $_{(a)}0.020 \pm 0.001_{B}^{b}$ | - | 0.1443 | $\approx {}_{(c)}3.0 \pm 0.0_{B}^{c}$ |
| | 5.5 | 6 | c | Planktonic cells | Total | $5.4 \pm 0.1_{B}^{a}$ | $271.8 \pm 96.4_{A}^{a}$ | $_{(a)}0.010 \pm 0.003_{A}^{a}$ | - | 0.3326 | $\approx {}_{(a)}1.5 \pm 0.1_{B}^{a}$ |
| | | | | | Uninjured | $5.3 \pm 0.1_{B}^{a}$ | $174.0 \pm 64.0_{A}^{a}$ | $_{(a)}0.011 \pm 0.002_{A}^{a}$ | - | 0.2697 | $\approx {}_{(a)}2.0 \pm 0.1_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.0 \pm 0.0_{A}^{a}$ | $473.7 \pm 41.5_{B}^{c}$ | $_{(a)}0.009 \pm 0.003_{A}^{a}$ | - | 0.1079 | $\approx {}_{(a)}0.5 \pm 0.0_{A}^{a}$ |
| | | | | | Uninjured | $5.0 \pm 0.1_{A}^{a}$ | $351.1 \pm 70.1_{B}^{b}$ | $_{(b)}0.011 \pm 0.003_{A}^{a}$ | - | 0.2391 | $\approx {}_{(b)}1.2 \pm 0.1_{A}^{a}$ |
| *solid(like) surface* | 7.4 | 0 | d | Preculture | Total | $5.5 \pm 0.1_{A}$ | $_{(a)}88.9 \pm 21.4_{A}$ | $_{(b)}0.057 \pm 0.019_{B}$ | $3.1 \pm 0.1_{A}$ | 0.3386 | $_{(c)}2.4 \pm 0.1_{B}$ |
| | | | | | Uninjured | $5.4 \pm 0.1_{A}$ | $_{(a)}61.8 \pm 12.9_{A}$ | $_{(b)}0.065 \pm 0.014_{B}$ | $3.0 \pm 0.1_{A}$ | 0.1970 | $_{(b)}2.4 \pm 0.1_{B}$ |
| | | | | Planktonic cells | Total | $5.6 \pm 0.2_{A}^{b}$ | $_{(a)}95.8 \pm 54.3_{A}$ | $_{(a)}0.026 \pm 0.010_{A}^{a}$ | $3.3 \pm 0.2_{A}^{a}$ | 0.4455 | $_{(a)}2.3 \pm 0.3_{B}^{c}$ |
| | | | | | Uninjured | $5.7 \pm 0.2_{AB}^{b}$ | $_{(a)}33.0 \pm 57.6_{A}$ | $_{(a)}0.023 \pm 0.008_{A}^{a}$ | $3.3 \pm 0.2_{A}^{ab}$ | 0.3842 | $_{(a)}2.4 \pm 0.3_{B}^{b}$ |
| | | | | Surface colonies | Total | $5.6 \pm 0.2_{A}^{b}$ | $_{(a)}117.1 \pm 113.5_{A}^{a}$ | $_{(ab)}0.017 \pm 0.015_{A}^{a}$ | $4.4 \pm 0.3_{B}$ | 0.4549 | $_{(a)}1.2 \pm 0.4_{A}^{b}$ |
| | | | | | Uninjured | $5.8 \pm 0.2_{B}^{c}$ | - | $_{(a)}0.017 \pm 0.005_{A}^{b}$ | $4.4 \pm 0.2_{B}$ | 0.3666 | $_{(a)}1.4 \pm 0.3_{A}^{b}$ |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6.5 | 2 | e | Planktonic cells | Total | $5.2 \pm 0.1_{A}^{a}$ | $180.2 \pm 14.8_{A}$ | $_{(a)}0.073 \pm 0.063_{A}^{a}$ | $4.1 \pm 0.1^{b}$ | 0.2571 | $_{(a)}1.1 \pm 0.1_{A}^{a}$ |
| | | | | | Uninjured | $5.1 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.013 \pm 0.003_{A}^{a}$ | $3.6 \pm 0.2_{A}^{b}$ | 0.3446 | $_{(a)}1.5 \pm 0.2_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.6 \pm 0.1_{B}^{b}$ | $_{(ab)}140.7 \pm 199.4_{A}^{a}$ | $_{(a)}0.004 \pm 0.002_{A}^{a}$ | - | 0.1514 | $\approx {}_{(a)}0.9 \pm 0.1_{A}^{ab}$ |
| | | | | | Uninjured | $5.5 \pm 0.1_{B}^{b}$ | $214.0 \pm 40.4$ | $_{(a)}0.014 \pm 0.007_{A}^{b}$ | $4.6 \pm 0.1_{B}$ | 0.1377 | $_{(a)}0.9 \pm 0.1_{A}^{a}$ |
| | 5.5 | 6 | f | Planktonic cells | Total | $5.4 \pm 0.1_{B}^{ab}$ | - | $_{(a)}0.009 \pm 0.002_{A}^{a}$ | $3.8 \pm 0.2^{b}$ | 0.2830 | $_{(a)}1.6 \pm 0.2_{B}^{b}$ |
| | | | | | Uninjured | $5.2 \pm 0.1_{B}^{a}$ | - | $_{(b)}0.018 \pm 0.003_{B}^{a}$ | $3.1 \pm 0.2^{a}$ | 0.3246 | $_{(a)}2.1 \pm 0.2_{B}^{b}$ |
| | | | | Surface colonies | Total | $5.1 \pm 0.1_{A}^{a}$ | $350.3 \pm 189.6^{a}$ | $_{(a)}0.005 \pm 0.003_{A}^{a}$ | - | 0.1919 | $\approx {}_{(a)}0.6 \pm 0.1_{A}^{a}$ |
| | | | | | Uninjured | $4.9 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.004 \pm 0.000_{A}^{a}$ | - | 0.3223 | $\approx {}_{(b)}1.1 \pm 0.1_{A}^{ab}$ |
| | 7.4 | 0 | g | Preculture | Total | $5.5 \pm 0.2_{A}$ | $_{(a)}2.2 \pm 163.3_{A}$ | $_{(a)}0.014 \pm 0.011_{A}$ | - | 0.2917 | $\approx {}_{(a)}1.0 \pm 0.2_{A}$ |
| | | | | | Uninjured | $5.5 \pm 0.1_{A}$ | $_{(a)}13.6 \pm 32.6_{A}$ | $_{(a)}0.027 \pm 0.005_{A}$ | - | 0.2149 | $\approx {}_{(a)}2.0 \pm 0.1_{A}$ |
| | | | | Planktonic cells | Total | $5.7 \pm 0.1_{A}^{b}$ | $_{(a)}12.4 \pm 15.1_{A}$ | $_{(b)}0.057 \pm 0.017_{B}^{b}$ | $3.5 \pm 0.1$ | 0.1482 | $_{(a)}2.2 \pm 0.1_{C}^{b}$ |
| | | | | | Uninjured | $5.6 \pm 0.1_{A}^{b}$ | $_{(a)}10.1 \pm 7.6_{A}$ | $_{(b)}0.097 \pm 0.019_{B}^{ab}$ | $3.4 \pm 0.1$ | 0.1699 | $_{(a)}2.2 \pm 0.1_{A}^{a}$ |
| | | | | Surface colonies | Total | $5.5 \pm 0.1_{A}^{b}$ | $_{(a)}89.0 \pm 47.6_{A}$ | $_{(b)}0.045 \pm 0.022_{AB}^{a}$ | - | 0.2613 | $\approx {}_{(b)}1.8 \pm 0.1_{B}^{c}$ |
| | | | | | Uninjured | $5.5 \pm 0.2_{A}^{b}$ | $20.1 \pm 52.6_{A}$ | $_{(b)}0.029 \pm 0.009_{A}^{b}$ | - | 0.3880 | $\approx {}_{(b)}2.0 \pm 0.2_{A}^{c}$ |
| *filter* | 6.5 | 2 | h | Planktonic cells | Total | $5.5 \pm 0.2_{A}^{ab}$ | - | $_{(a)}0.021 \pm 0.004_{A}^{a}$ | - | 0.3967 | $\approx {}_{(b)}1.7 \pm 0.2_{A}^{a}$ |
| | | | | | Uninjured | $5.4 \pm 0.2_{A}^{b}$ | $25.4 \pm 15.5$ | $_{(b)}0.118 \pm 0.063_{B}^{b}$ | $3.5 \pm 0.3$ | 0.4374 | $_{(ab)}1.9 \pm 0.4_{A}^{a}$ |
| | | | | Surface colonies | Total | $5.8 \pm 0.0_{A}^{c}$ | $_{(a)}115.1 \pm 79.7$ | $_{(a)}0.049 \pm 0.059_{A}^{a}$ | - | 0.2019 | $\approx {}_{(b)}1.4 \pm 0.0_{A}^{b}$ |
| | | | | | Uninjured | $5.8 \pm 0.2_{A}^{b}$ | - | $_{(a)}0.014 \pm 0.005_{A}^{a}$ | - | 0.4058 | $\approx {}_{(b)}1.2 \pm 0.2_{A}^{b}$ |
| | 5.5 | 6 | i | Planktonic cells | Total | $5.3 \pm 0.1_{A}^{a}$ | $25.1 \pm 49.0$ | $_{(b)}0.022 \pm 0.007_{B}^{a}$ | - | 0.2178 | $\approx {}_{(a)}1.5 \pm 0.1_{B}^{a}$ |
| | | | | | Uninjured | $5.0 \pm 0.1_{A}^{a}$ | - | $_{(c)}0.028 \pm 0.003_{B}^{a}$ | - | 0.2775 | $\approx {}_{(a)}2.2 \pm 0.1_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.2 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.006 \pm 0.003_{A}^{a}$ | - | 0.2667 | $\approx {}_{(a)}0.5 \pm 0.1_{A}^{a}$ |
| | | | | | Uninjured | $4.7 \pm 0.2_{A}^{a}$ | - | $_{(ab)}0.007 \pm 0.005_{A}^{a}$ | - | 0.4573 | $\approx {}_{(a)}0.5 \pm 0.2_{A}^{a}$ |

[1] For each experimental condition, growth morphology and population type, parameters of the Geeraerd model bearing different subscripts (no lowercase letters in common) are significantly different ($P \leq 0.05$)

[2] For each inactivation support, experimental condition and population type, parameters of the Geeraerd model bearing different subscripts (no uppercase letters in common) are significantly different ($P \leq 0.05$)

[3] For each inactivation support, growth morphology and population type, parameters of the Geeraerd model bearing different superscripts (no lowercase letters in common) are significantly different ($P \leq 0.05$)

738 Table 2. Inactivation parameters of the Geeraerd et al. (2002) model for *L. monocytogenes* after exposure to CAP. Cells were inactivated on a
739 liquid carrier, on a solid(like) surface and on a filter. Prior to CAP treatment, cells were grown at pH 7.4, 0% (w/v) NaCl, pH 6.5, 2% (w/v) NaCl
740 or pH 5.5, 6% (w/v) NaCl, and either planktonically, as surface colonies, or as the direct preculture (only for pH 7.4, 0% (w/v) NaCl).

| Inactivation support system | Growth conditions | | | Growth morphology | Population | Kinetic parameters | | | | RMSE | $_1$log reduction$_2$[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH (-) | NaCl (% (w/v)) | Reference | | | $_1$log $N_0$ (log(CFU/mL))$_2$[3] / $_1$log $N_0$ (log (CFU/cm$^2$))$_2$[3] | $_1t_l$ (s)$_2$[3] | $_1k_{max}$ (1/s)$_2$[3] | $_1$log $N_{res}$ (log (CFU/mL))$_2$[3] / $_1$log $N_{res}$ (log (CFU/cm$^2$))$_2$[3] | | |
| *Liquid* | 7.4 | 0 | a | Preculture | Total | 5.4 ± 0.0$_A$ | 511.9 ± 18.0 | $_{(a)}$0.011 ± 0.002$_B$ | - | 0.0557 | ≈ $_{(a)}$0.6 ± 0.0$_B$ |
| | | | | | Uninjured | 5.4 ± 0.0$_A$ | 438.0 ± 30.5 | $_{(a)}$0.010 ± 0.002$_A$ | - | 0.0822 | ≈ $_{(a)}$0.8 ± 0.0$_C$ |
| | | | | Planktonic cells | Total | 5.8 ± 0.1$_C^a$ | - | $_{(a)}$0.001 ± 0.000$_A^a$ | - | 0.1818 | ≈ $_{(a)}$0.2 ± 0.1$_A^a$ |
| | | | | | Uninjured | 5.9 ± 0.1$_B^b$ | - | $_{(a)}$0.001 ± 0.000$_A^a$ | - | 0.1633 | ≈ $_{(a)}$0.3 ± 0.1$_A^a$ |
| | | | | Surface colonies | Total | 5.6 ± 0.1$_B^a$ | 493.0 ± 70.0 | $_{(a)}$0.009 ± 0.005$_B^{ab}$ | - | 0.1840 | ≈ $_{(a)}$0.6 ± 0.1$_B^b$ |
| | | | | | Uninjured | 5.5 ± 0.1$_A^a$ | 540.0 ± 53.5 | $_{(a)}$0.015 ± 0.012$_A^b$ | - | 0.2031 | ≈ $_{(a)}$0.5 ± 0.1$_B^b$ |
| | 6.5 | 2 | b | Planktonic cells | Total | 5.7 ± 0.1$_A^a$ | 369.3 ± 108.7$_A$ | $_{(a)}$0.008 ± 0.004$_A^b$ | - | 0.2638 | ≈ $_{(a)}$0.9 ± 0.1$_B^b$ |
| | | | | | Uninjured | 5.6 ± 0.1$_A^a$ | 407.6 ± 91.4$_A$ | $_{(a)}$0.009 ± 0.004$_A^b$ | - | 0.2403 | ≈ $_{(a)}$0.8 ± 0.1$_B^b$ |
| | | | | Surface colonies | Total | 5.6 ± 0.0$_A^a$ | 546.3 ± 24.4$_A$ | $_{(a)}$0.014 ± 0.005$_A^b$ | - | 0.0919 | ≈ $_{(a)}$0.5 ± 0.0$_A^b$ |
| | | | | | Uninjured | 5.5 ± 0.0$_A^a$ | 598.8 ± 2.2$_B$ | $_{(b)}$0.123 ± 0.001$_B^c$ | - | 0.0872 | ≈ $_{(a)}$0.4 ± 0.0$_A^{ab}$ |
| | 5.5 | 6 | c | Planktonic cells | Total | 5.7 ± 0.0$_B^a$ | - | $_{(a)}$0.001 ± 0.000$_A^a$ | - | 0.0815 | ≈ $_{(a)}$0.3 ± 0.0$_B^a$ |
| | | | | | Uninjured | 5.6 ± 0.1$_A^a$ | - | $_{(a)}$0.001 ± 0.000$_A^a$ | - | 0.1691 | ≈ $_{(a)}$0.3 ± 0.1$_A^a$ |
| | | | | Surface colonies | Total | 5.5 ± 0.0$_A^a$ | - | $_{(a)}$0.001 ± 0.000$_A^a$ | - | 0.0581 | ≈ $_{(a)}$0.2 ± 0.0$_A^a$ |
| | | | | | Uninjured | 5.6 ± 0.0$_A^a$ | - | $_{(a)}$0.001 ± 0.000$_A^a$ | - | 0.1382 | ≈ $_{(a)}$0.3 ± 0.0$_A^a$ |
| *solid(like) surface* | 7.4 | 0 | d | Preculture | Total | 5.3 ± 0.3$_A$ | 20.5 ± 27.6 | $_{(a)}$0.219 ± 0.576$_A$ | 3.1 ± 0.1$_A$ | 0.4751 | $_{(b)}$2.2 ± 0.3$_B$ |
| | | | | | Uninjured | 5.2 ± 0.3$_A$ | 9.8 ± 19.5 | $_{(ab)}$0.119 ± 0.088$_A$ | 3.1 ± 0.1$_A$ | 0.5108 | $_{(c)}$2.1 ± 0.3$_B$ |
| | | | | Planktonic cells | Total | 5.7 ± 0.2$_A^a$ | - | $_{(c)}$0.355 ± 0.096$_A^b$ | 3.8 ± 0.1$_B^a$ | 0.3335 | $_{(b)}$1.9 ± 0.2$_B^b$ |
| | | | | | Uninjured | 5.7 ± 0.2$_B^b$ | - | $_{(a)}$1.000 ± 13.908$_A^a$ | 3.7 ± 0.1$_B^a$ | 0.3103 | $_{(b)}$2.0 ± 0.2$_B^b$ |
| | | | | Surface colonies | Total | 5.5 ± 0.2$_A^a$ | - | $_{(a)}$0.070 ± 0.031$_A^b$ | 4.5 ± 0.1$_C^a$ | 0.2594 | $_{(b)}$1.0 ± 0.2$_A^b$ |
| | | | | | Uninjured | 5.4 ± 0.2$_{AB}^a$ | - | $_{(a)}$0.077 ± 0.048$_A^b$ | 4.5 ± 0.1$_C^a$ | 0.3503 | $_{(b)}$0.9 ± 0.2$_A^a$ |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6.5 | 2 | e | Planktonic cells | Total | $5.5 \pm 0.2_{A}^{a}$ | - | $_{(b)}0.088 \pm 0.021_{B}^{a}$ | $3.8 \pm 0.1_{A}^{a}$ | 0.2568 | $_{(b)}1.7 \pm 0.2_{B}^{b}$ |
| | | | | | Uninjured | $5.3 \pm 0.2_{A}^{a}$ | - | $_{(b)}0.120 \pm 0.035_{B}^{a}$ | $3.8 \pm 0.1_{A}^{a}$ | 0.2820 | $_{(c)}1.5 \pm 0.2_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.6 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.023 \pm 0.017_{A}^{a}$ | $5.1 \pm 0.1_{B}^{b}$ | 0.2629 | $_{(a)}0.5 \pm 0.1_{A}^{a}$ |
| | | | | | Uninjured | $5.6 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.010 \pm 0.006_{A}^{a}$ | $4.8 \pm 0.2_{B}^{b}$ | 0.3169 | $_{(b)}0.8 \pm 0.2_{A}^{a}$ |
| | 5.5 | 6 | f | Planktonic cells | Total | $5.5 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.027 \pm 0.008_{A}^{a}$ | $4.3 \pm 0.1_{A}^{b}$ | 0.2646 | $_{(c)}1.2 \pm 0.1_{B}^{a}$ |
| | | | | | Uninjured | $5.5 \pm 0.2_{A}^{ab}$ | - | $_{(a)}0.046 \pm 0.018_{B}^{a}$ | $4.2 \pm 0.1_{A}^{b}$ | 0.3330 | $_{(c)}1.3 \pm 0.2_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.6 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.012 \pm 0.007_{A}^{a}$ | $5.0 \pm 0.1_{B}^{b}$ | 0.2442 | $_{(b)}0.6 \pm 0.1_{A}^{a}$ |
| | | | | | Uninjured | $5.6 \pm 0.1_{A}^{a}$ | - | $_{(a)}0.013 \pm 0.007_{A}^{a}$ | $4.8 \pm 0.1_{B}^{b}$ | 0.2807 | $_{(b)}0.8 \pm 0.1_{A}^{a}$ |
| | 7.4 | 0 | g | Preculture | Total | $5.9 \pm 0.2_{A}$ | - | $_{(a)}0.127 \pm 0.030_{A}$ | $4.0 \pm 0.1_{A}$ | 0.2721 | $_{(b)}1.9 \pm 0.2_{B}$ |
| | | | | | Uninjured | $5.6 \pm 0.3_{A}$ | - | $_{(b)}0.252 \pm 0.100_{A}$ | $4.0 \pm 0.1_{A}$ | 0.3586 | $_{(b)}1.6 \pm 0.3_{A}$ |
| | | | | Planktonic cells | Total | $5.9 \pm 0.2_{A}^{b}$ | - | $_{(b)}0.190 \pm 0.067_{AB}^{a}$ | $4.2 \pm 0.1_{B}^{a}$ | 0.2804 | $_{(b)}1.7 \pm 0.2_{AB}^{b}$ |
| | | | | | Uninjured | $5.8 \pm 0.2_{A}^{b}$ | - | $_{(a)}0.333 \pm 0.085_{A}^{a}$ | $4.1 \pm 0.1_{A}^{a}$ | 0.2820 | $_{(b)}1.7 \pm 0.2_{A}^{b}$ |
| | | | | Surface colonies | Total | $5.9 \pm 0.1_{A}^{b}$ | - | $_{(b)}0.235 \pm 0.051_{B}^{a}$ | $4.4 \pm 0.1_{C}^{a}$ | 0.1876 | $_{(c)}1.5 \pm 0.1_{A}^{c}$ |
| | | | | | Uninjured | $6.0 \pm 0.1_{A}^{b}$ | - | $_{(b)}0.265 \pm 0.042_{A}^{a}$ | $4.3 \pm 0.1_{B}^{a}$ | 0.1516 | $_{(c)}1.7 \pm 0.1_{A}^{b}$ |
| *filter* | 6.5 | 2 | h | Planktonic cells | Total | $5.4 \pm 0.2_{A}^{a}$ | - | $_{(b)}0.075 \pm 0.035_{A}^{a}$ | $4.4 \pm 0.2_{A}^{a}$ | 0.2764 | $_{(a)}1.1 \pm 0.3_{A}^{a}$ |
| | | | | | Uninjured | $5.4 \pm 0.1_{A}^{a}$ | - | $_{(b)}0.100 \pm 0.037_{A}^{a}$ | $4.3 \pm 0.1_{A}^{b}$ | 0.2167 | $_{(b)}1.1 \pm 0.1_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.3 \pm 0.1_{A}^{a}$ | $53.0 \pm 31.3$ | $_{(a)}0.158 \pm 0.666_{A}^{a}$ | $4.5 \pm 0.2_{A}^{a}$ | 0.2123 | $_{(b)}0.8 \pm 0.2_{A}^{b}$ |
| | | | | | Uninjured | $5.2 \pm 0.1_{A}^{a}$ | $50.4 \pm 23.0$ | $_{(ab)}0.060 \pm 0.093_{A}^{a}$ | $4.4 \pm 0.1_{A}^{a}$ | 0.1915 | $_{(b)}0.8 \pm 0.1_{A}^{a}$ |
| | 5.5 | 6 | i | Planktonic cells | Total | $5.3 \pm 0.1_{A}^{a}$ | $29.7 \pm 5.6$ | $_{(a)}0.205 \pm 0.888_{A}^{a}$ | $4.3 \pm 0.1_{A}^{a}$ | 0.2097 | $_{(b)}1.0 \pm 0.1_{A}^{a}$ |
| | | | | | Uninjured | $5.4 \pm 0.1_{A}^{a}$ | $20.2 \pm 12.9$ | $_{(a)}0.203 \pm 0.240_{A}^{a}$ | $4.2 \pm 0.1_{A}^{ab}$ | 0.1866 | $_{(b)}1.2 \pm 0.1_{B}^{a}$ |
| | | | | Surface colonies | Total | $5.5 \pm 0.2_{A}^{a}$ | - | $_{(a)}0.113 \pm 0.164_{A}^{a}$ | $5.0 \pm 0.2_{B}^{b}$ | 0.3343 | $_{(ab)}0.5 \pm 0.3_{A}^{a}$ |
| | | | | | Uninjured | $5.4 \pm 0.3_{A}^{a}$ | - | $_{(a)}0.199 \pm 0.302_{A}^{a}$ | $4.9 \pm 0.2_{B}^{b}$ | 0.3817 | $_{(ab)}0.5 \pm 0.4_{A}^{a}$ |

[1] For each experimental condition, growth morphology and population type, parameters of the Geeraerd model bearing different subscripts (no lowercase letters in common) are significantly different ($P \leq 0.05$)

[2] For each inactivation support, experimental condition and population type, parameters of the Geeraerd model bearing different subscripts (no uppercase letters in common) are significantly different ($P \leq 0.05$)

[3] For each inactivation support, growth morphology and population type, parameters of the Geeraerd model bearing different superscripts (no lowercase letters in common) are significantly different ($P \leq 0.05$)