International Journal of Food Microbiology

Volume 126, Issues 1-2, 15 August 2008, Pages 140-152

An explanation for the effect of inoculum size on MIC and the Growth/No-Growth Interface

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Abstract

The inoculum effect (IE) is the phenomenon observed where changes in the inoculum size used in an experiment alters the outcome with respect to, for example, the minimum inhibitory concentration of an antimicrobial or the growth/no growth boundary for a given set of environmental conditions. Various hypotheses exist as to the cause of the IE such as population heterogeneity and quorum sensing, as well as the null hypothesis – that it is artefactual. Time to detection experiments (TTD) were carried out on different initial inoculum sizes of several bacterial species (*Aeromonas hydrophila, Enterobacter sakazakii, Salmonella* Poona*, Escherichia coli and Listeria innocua*) when challenged with different pH and with combined pH and sodium acetate. Data were modelled using a modification to a Gamma model (Lambert and Bidlas 2007, Int. J. Food Microbiology 115, 204 – 213), taking into account the inoculum size dependency on the TTD obtained under ideal conditions. The model suggests that changes in minimum inhibitory concentration (MIC) or in the Growth-No growth boundary with respect to inoculum size are due to using a smaller or larger inoculum (i.e. is directly related to microbial number) and is not due to other, suggested, phenomena. The model used further suggests that the effect of a changing inoculum size can be modelled independently of any other factor, which implies that a simple 1 to 2 day experiment measuring the TTD of various initial inocula can be used as an adjunct to currently available models.

Keywords: Predictive modelling, inoculum effect, preservation, MIC, Growth No growth boundary

Introduction

Predictive microbiology (PM), or the "quantitative microbial ecology of foods" (McMeekin et al., 1997; McMeekin and Ross 2002) attempts to provide a mathematical rationale for microbial growth under a variety of environmental conditions – e.g. temperature, pH, a_w and the effect of preservatives. PM is the quantification of the hurdle concept developed by Leistner (Leistner, 1995; Leistner and Gorris, 1995; Leistner, 2000). Within the multi-factor modelling generally performed, the effect of the initial inoculum size on microbial growth is not, however, commonly investigated; the amount of resource required to produce such a multi-factorial model using traditional methodology (i.e. plates and agar) is often a barrier to such an investigation. Furthermore the assumption that inoculum size has no effect on microbial growth once growth is initiated would suggest that such experiments would be irrelevant and some studies have confirmed this. Buchanan, Smith, McColgan, Marmer, Golden and Dell (1993) examined the growth of *Staphylococcus aureus* using inoculum levels between 10^1 and 10^6 cfu ml^{-1} over 4 temperatures; the inoculum size had "little if any effect on the growth kinetics". Bhaduri, Turner-Jones, Buchanan and Phillips (1994) stated that in studies with *Yersinia enterocolitica* inoculum levels between 10^3 and 10^5 cfu ml^{-1} had little effect on the LPD or GT. A most convincing result with *Escherichia coli* O157:H7 was reported by Buchanan, Bagi, Goins and Phillips (1993); the effect of inoculum size on the growth kinetics was evaluated using two aerobic variable combinations: (1) 28°C, pH 7.2 0.5% NaCl; and (2) 19°C, pH 7.0, 5% NaCl. An inoculum range of between approx. $10^{1.9}$ to $10^{5.9}$ cfu ml^{-1} was examined. Regression analysis indicated that there was no significant effect on LPD, GT or MPD related to inoculum size for a given set of environmental conditions.

Observations, however, that inoculum size could have an effect on the duration of the lag phase have been reported. These studies have examined low inoculum size effects (typically <<10 cfu ml^{-1}) when populations are exposed to harsh conditions. Augustin, Brouillaud-Delattre, Rosso, and Carlier (2000) showed that the lag time of *Listeria monocytogenes* was extended when the cells were

77 severely stressed by starvation. This was observed at very low cell densities and explained by an
78 increase in the variation of individual cells' lag time. Indeed these low inoculum size effects are quite
79 general and reflect the distribution of injury in a microbial population, which becomes apparent when
80 such low inoculum studies are performed (Pin and Baranyi 2006). Guillier, Pardon and Augustin
81 (2005) described the effect of various stresses on the distribution of individual lag times of *L.*
82 *monocytogenes*, and work by Métris, George and Baranyi (2006) has shown the evolution of the injury
83 distribution of small populations of *L. innocua* as the concentration of acetic acid in the medium is
84 increased. The initial application of these 'single-cell kinetic' studies in foods has also been reported
85 (D'Arrigo, García de Fernando, Velasco de Diego, Ordóñez, George and Baranyi 2006). The use of
86 automated turbidometry in these studies has proven very useful and one point is consistently made – at
87 higher inocula, the time to detection is the time taken for the 'fittest' organism to complete repair and
88 divide. Hence the time to detection of higher inocula (using turbidometry) are those organisms found
89 on one-side of the distribution tail. The comparison and the evolution of the distribution injury
90 between the population and the fittest organisms following an inimical procedure has been reported by
91 Lambert and Ouderaa (1999). Although D'Arrigo et al (2006) state that " the lag times of populations
92 initiated with small inocula cannot be measured accurately with traditional microbiology techniques
93 such as bacterial counts", the method of Lambert and Ouderaa (1999) allows the distributions to be
94 obtained using this traditional technique.
95
96 At the other extreme of inoculum size – at high cell densities an inoculum effect has been
97 observed with many organisms, but the phenomenon has been attributed to several mechanisms.
98 Prominent amongst these is quorum sensing – the ability of microbial cells to communicate amongst
99 themselves (Surette, Miller and Bassler 1999; Miller and Bassler 2001; Smith, Fratamico and Novak
100 2004; Zhao, Montville and Schaffner 2006). The inoculum effect (IE) has also been defined as the
101 increase in the minimum inhibitory concentration (MIC) of an inhibitor as the initial microbial
102 inoculum is increased (Steels, James, Roberts and Stratford 2000). Essentially, it is argued, more
103 inhibitor is needed to inhibit a larger microbial load, and this would appear to be a common-sense
104 view. Interestingly, in medical microbiology (where Leistner (2000) has suggested that food

105 microbiologists look for complementary approaches to similar phenomena) inoculum effects on MIC
106 are clinically important where, for example, high densities of β-lactamase pathogens are found such as
107 in endocarditis and meningitis (Thomson and Moland 2001) and in invasive fungal infections (Gehrt,
108 Peter, Pizzo and Walsh 1995), although the IE has also been considered to be artefactual - "an in-vitro
109 laboratory phenomenon" (Thomson and Moland 2001).
110
111 But there are also published studies, which, in contradiction to the assumption given above,
112 suggest that the inoculum size has a direct influence on the prediction of growth. These studies are
113 generally concerned with the growth/no growth interface (G/NG) for a given set of environmental
114 conditions. Masana and Baranyi (2000) showed that for identical combinations of NaCl/pH,
115 differences between low and medium levels of inocula were observed, with the medium inoculum
116 more able to grow at the more extreme conditions. They also reported the increased variability as
117 conditions became harsher, also shown by Ratkowsky, Ross, McMeekin, and Olley (1991).
118 Koutsoumanis and Sofos (2005) described the effect of inoculum size on the growth boundary of *L.*
119 *monocytogenes* for combinations of temperature, pH and aw. Minimum growth values for pH and aw
120 were found to vary with inoculum size. The effects of inoculum size on microbial growth initiation
121 observed in their study suggested to them that growth limits for individual cells in microbial
122 populations were heterogeneous. More recently, Skandamis et al. (2007) have examined the effect of
123 inoculum size on the G/NG interface of *E.coli* O157:H7 and again have shown that the lower the
124 initial inoculum the more its G/NG boundaries are influenced by stringent conditions. One other
125 obvious explanation of these observations would be the argument used for the IE on MIC.
126
127 Robinson, Aboaba, Kaloti, Ocio, Baranyi, and Mackey (2001) and also in an complimentary
128 study Pascual, Robinson, Ocio, Aboaba and Mackey (2001) showed that the mean lag time of *L.*
129 *monocytogenes* increased with decreasing inoculum size as growth conditions became harsher.
130 Furthermore, they noted that the variance between replicate inocula also increased as the conditions
131 grew harsher. Above a certain threshold of NaCl concentration (1.2 mol l^{-1}) no well inoculated with a
132 single cell showed growth, and a much higher inoculum was required as the conditions became

harsher for the observation of growth. Robinson et al. (2001) argued that that since the maximum specific growth rate (μ_{max}) was independent of cell history and was uniquely determined by its environment (a given assumption), for any set of conditions, a plot of the logarithm of inoculum size versus detection time should yield a straight line whose slope was proportional to the specific growth rate, provided lag time was constant and unaffected by inoculum size (a similar point had been made previously by Cuppers and Smelt 1993). The deviation from linearity observed by Robinson et al.(2001) implied that population lag was not independent of inoculum size and therefore hypothesised a cooperative population effect, "the ability to initiate growth under severe salt stress depends on the presence of a resistant sub-fraction of the population, but that high cell densities appear to assist the adaptation of those cells to the unfavourable growth conditions". As conditions became more stressful the scatter observed increased to about 10 doubling times implying that the variability in detection time was attributed to greatly extended lags. The hypotheses made by the authors seemed to be suggesting both the phenomena attributed to high inoculum density studies and to the low density studies were in operation for the inoculum sizes often used in PM - namely $10^3 - 10^5$ cfu ml^{-1}.

Recently, we have reported on the predictive modelling of some pathogenic bacteria using the Gamma concept (Zwietering, Wijtzes, De Wit and Riet, 1992) as an axiomatic base. This concept hypothesises that combined environmental factors (temperature, pH, a_w, etc) independently affect the growth of microorganisms. The growth of *A. hydrophila* in combinations of temperature, pH, salt, weak acids and $NaNO_2$ has been reported using the rapid technique of time to detection (TTD), with the same approach used in studies of *Enterobacter sakazakii* and *Salmonella* Poona (Lambert and Bidlas 2007a; 2007b; 2007c; 2007d). The method we have developed along with the predictive models used to analyse the data were considered ideal to investigate the effect that the initial inoculum size had on parameters such as the minimum pH for growth and the MIC of some common preservatives.

Herein we describe these investigations and give a possible explanation of the changes that occur in MIC as inoculum size changes and the impact this has on defining a growth/no growth boundary.

2. Materials and methods

2.1. Culture Preparation

Aeromonas hydrophila (ATCC 7966), *Salmonella enterica* ssp. *enterica* serovar Poona (NCTC 4840), *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 25922) and *Enterobacter sakazakii* (factory isolate, FSM263) were grown overnight in flasks containing 80 ml Tryptone Soya Broth, TSB (Oxoid CM 129) shaking at 30^{o}C. The cells were harvested, centrifuged to a pellet (512g, 10 mins, 15^{o}C), washed and re-suspended in peptone solution (0.1%). The optical density (OD) of the inoculum was standardised to OD= 0.5 at 600nm (approximately 7x10^{8} cfu ml^{-1}, Table 1). This standardized culture was either subject to decimal dilutions (in TSB) or further diluted in TSB to achieve an initial inoculum (Io) of approximately 1x10^{5} cfu ml^{-1} in the microtitre plate (see Table 1).

2.2 Analysis

All analyses were performed in a Bioscreen Microbiological Analyser (Labsystems Helsinki, Finland). In general the methods of Lambert & Pearson (2000) was used, whereas for combined inhibitors, a chequerboard (grid) arrangement using the method of Lambert and Lambert (2003) was used.

2.2.1. Inoculum size dependency of the time to detection

An initial culture with OD=0.5 was consecutively decimally diluted 9 times in TSB. These cultures (250µl) were placed in the columns of a 10x10 Bioscreen microtitre plate, giving 10 replicates per inoculum size. The plates were incubated at 30^{0}C for two days. The optical density (OD) of the wells was recorded at 600nm every 10 minutes.

2.2.2. pH and inoculum size dependency

2.2.2.1 *Listeria innocua and Salmonella* Poona

For *L. innocua* four plates of TSB (200 µl) at pH 7, 5.5, 5.2 and 4.8 (adjusted with HCl) were prepared. To each well of the first column of the microtitre plate was placed 200 µl of pH adjusted

diluted standard inoculum (giving 7.31 log cfu ml^{-1} in the first column), this was then half-fold diluted across the plate, giving an initial inoculum range of 7.31 to 4.60 log cfu ml^{-1} ; this gave 10 replicates per inoculum size per pH. For *S*. Poona two identical grids of pH (4.1, 4.2, 4.5, 4.7, 5.2, 5.5, 5.8, 6.2, 6.5, 7.1, adjusted with HCl and NaOH as appropriate) and diluted standard inoculum (pH adjusted to column pH, giving ten initial inocula with a range of 4.8 to 2.1 log cfu ml^{-1}) were prepared. Both sets of experiments were incubated at 30^{o}C for 3 days, with the OD recorded every 10 minutes at 600nm.

2.2.2.2. A. hydrophila, Ent. sakazakii, E.coli, S. poona & L. innocua

The first row of the microtitre plates with grids of pH and Na acetate (see section 2.2.3 below) were devoid of Na acetate (pH controls) and were used to examine the effect of pH (range from pH 4 to 7) and inoculum size on the TTD.

2.2.3 Weak acid analysis

In general up to 8 identical microtitre plates were prepared as follows; sodium acetate (1 g) was dissolved in TSB and the volume made up to 100ml. The solution was split into 10 equal portions and the pH adjusted to give a pH range from 7 to 4 (typical target pH were 3.5, 4.0, 4.2, 4.5, 4.8, 5.2, 5.5, 5.8, 6.2, 6.5). A Bioscreen (Labsystems Helsinki, Finland) 10x10 micro-array plate was prepared in which each of the columns (except the wells of the first row) had 200 µl of TSB added at a pH equivalent to one of the bottles. To the first row was added the appropriate solution of sodium acetate, 400 µl (1%, pH = column pH), and half-fold diluted down the plate, discarding the final 200 µl of solution.

To every well of each identical microtitre plate was added a known dilution of the standard culture (pH adjusted to column pH, 50µl). The plate was then incubated for 3 days at 30^{o}C.The Bioscreen was set to take an optical density (OD) reading at 600nm every 10 minutes.

2.2.4. Time to Detection

The criterion used for the time to detection (TTD) was the time taken for the OD at 600nm to reach a defined value (in this work OD = 0.2 was used). In the presence of inhibitors it was generally assumed that the time taken to reach a particular OD was equivalent to microbial numbers reaching a specific value. Under certain conditions (normally close to a G/NG boundary and often with reduced temperatures) and with specific microorganisms, changes in morphology occur (e.g. with *Listeria monocytogenes* see Bereksi, Gavini, Bénézech and Faille 2002). No gross morphology changes were observed microscopically under the most inimical conditions used in this work.

To obtain a precise time for OD = 0.2, linear interpolation of the OD/time values which straddled the target OD was used. This was achieved using an Excel macro which scanned the OD/time data for the times at which the OD crossed the defined TTD criterion.

2.3. Model Fitting

2.3.1 Inoculum size dependency of the time to detection

The inoculum size dependency was modelled using a simple linear model (Eq.(1), Cuppers and Smelt 1993).

$$TTD = C - m \log_{10} I \qquad (1)$$

Where C = time taken for 1 cell to multiply to the detection value of 0.2 OD and m is the time taken for a 10 fold increase (1 $\log_{10}$) in microbial numbers for a given, constant temperature (30^{o}C in these experiments), I is the inoculum size (cfu ml^{-1}). No variance stabilising transform was used in the fitting of this equation to the inoculum size–only data. The increased 'scatter' at the lowest inoculum size used was ignored in the fitting of the regression line (Figure 2). For a given inoculum size grown under ideal conditions at a specified temperature the TTD recorded is the shortest possible (in the given media) and can be considered as the reference time to detection, TTD_{ref}.

2.3.2. Gamma composite model

The general form of the model used in these studies has been described previously (Lambert and Bidlas 2007 a-d). In the studies discussed herein, the reciprocal transformation of the time to detection

data (TTD) consistently gave superior fits to the observed data than the logarithmic transformation, hence equation (2) was used.

$$RTD_{obs} = RTD_{ref} \exp\left\{-1\left[\sum_{i=1}^{n}\left(\frac{inhibitor_i}{P_{2i-1}}\right)^{P_{2i}}\right]\right\} \quad (2)$$

Where RTD_{obs} and RTD_{ref} are the observed reciprocal time to detection (or rate to detection) and the reference rate to detection (normally the reciprocal of the shortest time to detection, $1/TTD_{ref}$), The summation term gives the function for the inhibitory effect of n inhibitors, each of which is defined by two parameters, $P_{2i-1}$and P_{2i}: the parameter P_{2i-1} is the concentration (normally mg l^{-1} is used, but percent has also been used) of the inhibitor which gives an inhibition of growth relative to the optimal RTD of 1/e (approx. 0.368), the exponents (P_{2i}) are slope parameters and can be considered a measure of the dose response. We also define here the summation term of Eq. (2) as the effective concentration (EffC).

The inoculum size dependency of Eq.(2) was modelled by replacing the RTD_{ref} by the inoculum size dependent function, Eq. (1), i.e. $RTD_{ref} = 1/(C\text{-}m\log_{10}I)$; in the text this model is referred to as the 'composite model'.

The concentration of the weak acid and anion produced from the total added salt of the weak acid, at a specific pH, was calculated using the Henderson-Hasselbalch equation (3).

$$[HA] = \left(\frac{[Total \quad salt]}{1+10^{pH-pKa}}\right) \quad (3)$$

Where [HA] is the concentration in solution at a given pH for a given total concentration of salt with a defined equilibrium constant given by the pKa. MIC were calculated from the intercept of the maximum slope of plot of RTD against log concentration (Lambert and Pearson 2000). This is given by

$$MIC_{calc} = P_{2i-1} \exp\left(\frac{1}{P_{2i}}\right) \quad (4)$$

The minimum pH was calculated using hydrogen ion concentration and then transformed back to pH.

Analyses were done using the JMP Statistical Software (SAS Institute Cary NC USA), using non-linear regression with the minimised sum of squares as the search criterion.

3. RESULTS

3.1. Time to detection under optimal conditions

Under optimum environmental conditions the time to detection of a given culture will depend only on the size of the initial inoculum itself, assuming that optimal conditions are inoculum size independent. Using decimal dilutions of approximately $7x10^8$ cfu ml^{-1} cultures, OD/incubation time profiles (30^oC, pH 6.5) were obtained for *A. hydrophila, Ent. sakazakii, E. coli, S.* Poona and *L. innocua*. Figure 1 shows the results for *A. hydrophila*. From the initial standard inoculum (OD = 0.5) it can be seen that successive decimal dilutions display the same OD/incubation time curve except that it is displaced further down the time axis with increasing decimal dilutions. With the 8^{th} decimal dilution, some wells (2/10) failed to grow within the 2-day incubation time; hence the maximum average OD is approximately 20% lower than the higher inoculants. With the 9^{th} decimal dilution no wells (0/10) showed growth within the 2-day incubation period.

From each of the OD/time profiles, the time to reach an OD = 0.2 was obtained (ten replicates per initial inoculum size). A plot of the time to 0.2 OD against the log of the inoculum size gives the well-known linear relationship (Eq.(1), Cuppers and Smelt 1993); Figure 2 gives an example of such a plot for *Ent. sakazakii*. As the initial inoculum size is decreased the variance in the replicate data increased. This was observed in every case studied. Table 1 gives the parameters obtained for all the organisms discussed. The inoculum size at OD = 0.2 at 600nm can be obtained from the parameters of Table 1 by solving the equation for TTD = 0. The maximum specific growth rate can also be obtained from the gradient values given in Table 1, through calculation of the doubling time.

3.2. Effect of pH

3.2.1. *Listeria innocua*. The effect of pH (4.8, 5.2, 5.5 and 7) on the time to detection of a range of initial inoculum sizes ($\log_{10} I$ = 7.3 to 4.6) gave the observations shown in Figure 3. The parameters of the best fit regression line at pH 7 (C =1163.4 SE = 2.55 and m = 143.5 SE = 0.424) are similar to

298 those given in Table 1 for this organism although at a different pH (t-test: P = 0.105 and 0.014 for *m*
299 and *C* respectively). From the figure as the growth pH is decreased to 4.8 the variance of the replicates
300 increases especially with the smallest inocula used (at pH 7 the average standard deviation ($SDev_{av}$) of
301 all the replicates at this pH was found to be $SDev_{av}$ = 3.29 +/- 1.14; pH 5.5, $SDev_{av}$ = 3.26 +/- 0.6; pH
302 5.2, $SDev_{av}$ = 3.59 +/- 0.69; pH 4.8, $SDev_{av}$ = 12.4 +/- 5.99). Fitting the composite model (Eq. 2 with
303 RTD_{ref} given by Eq.1) to the 400 data points gave the parameters described in Table 2, Figure 3
304 compares the observations with the modelled data. The minimum pH was calculated (Eq. 4) as 4.42
305 (+/-0.013).Extrapolation to TTD = 0 of the best fit regression lines gives an intercept on the $\log_{10} I$
306 axis = 8.18 +/- 0.06 ($\log_{10}$ cfu ml^{-1}) ; from the modelled parameters TTD = 0 occurred at $\log_{10} I$ = 8.09
307 ($\log_{10}$ cfu ml^{-1}).
308
309 3.2.2. *Salmonella* Poona. The TTD from the incubation at 30°C of two identical (10x10) grids of pH
310 and initial inoculum size were obtained and Figure 4 gives a plot of the average RTD with respect to
311 the $\log_{10}$ inoculum size. Out of 200 wells 190 showed growth (the ten wells that failed to show growth
312 during the 3 day incubation period were all at pH = 4.08, with $\log_{10} I < 3.8$); the figure suggests that
313 some of these wells may have shown visible growth if incubated longer. The combined data (190
314 values) were modelled using the composite model. Table 2 gives the regression parameters obtained
315 and Figure 5 shows a plot of the modelled RTD with respect to $\log_{10}$ inoculum size. The minimum pH
316 was calculated (Eq. 4) as 3.89 (+/-0.03). As the optimum pH is approached, the function describing the
317 effect of the pH tends to a value of 1, hence the curve in Figure 5 (pH 7.1) is given by the inoculum
318 size dependency only, i.e. RTD = 1/(C-m $\log_{10}$ I). This can be considered as the optimal-curve for the
319 given media and incubation temperature. The model suggests that as the pH is decreased this 'optimal
320 curve' is multiplied by a constant (for a given pH) which is < 1.
321
322 3.2.3. *Aeromonas hydrophila.* The TTD of an inoculum dilution sequence of *A. hydrophila* ($\log_{10}$5
323 CFU ml^{-1} to $\log_{10}$1 CFU ml^{-1}) was studied over a range of pH (3.78-6.49). No visible growth was
324 recorded in any well with pH< 4.56 during the five-day incubation period. A plot of $\log_{10} I$ against
325 TTD for the various pH used showed that a linear relationship between $\log_{10} I$ and TTD exists for a

given pH. No substantial deviation from linearity was observed (best fit regression lines: pH 6.49, TTD = 673 – 80.5 $\log_{10} I$, r^2 = 0.999 ; pH 6.22, TTD = 747.1 – 95.2 $\log_{10} I$, r^2 = 0.978; pH 5.82, TTD = 771 – 92.8 $\log_{10} I$, r^2 = 0.992; pH 5.51, TTD = 884.7 – 105.9 $\log_{10} I$, r^2 = 0.988; pH 5.20, TTD = 1273–151.7 $\log_{10} I$, r^2= 0.999; pH 4.84, TTD = 2359 – 269.2 $\log_{10} I$, r^2 = 0.996).

The data were modelled using the composite model; Table 2 gives the parameters found from the fitting of the model. The minimum pH was calculated (Eq. 4) as 4.52 (+/-0.05). The values for the parameters describing the inoculum size effect were similar to those in Table 1 (t-test: P = 0.55 and 0.043 for m and C respectively). From the regression lines the intercept on the $\log_{10} I$ axis occurred at 8.34+/- 0.29.

3.2.4. *E. coli* (ATCC 25922). A smaller range of initial inoculum (7 initial inocula; $\log_{10} I$: 5.5 – 3.4) was used with ten initial pH (range 6.51 to 3.50). No growth was observed in any well with pH < 4.50. Plots of the TTD against the $\log_{10} I$ gave linear relationships for a given pH (best fit linear regression lines for the observables: pH 6.51, TTD = 824.6 – 96.9 $\log_{10} I$, r^2 = 0.990; pH 6.2, TTD = 858.9 – 100.7 $\log_{10} I$, r^2 = 0.993; pH 5.83, TTD = 933.9 – 108.3 $\log_{10} I$, r^2 = 0.987; pH 5.54, TTD = 1128.4 – 137.0 $\log_{10} I$, r^2 = 0.996; pH 5.22, TTD = 1419.2 – 173.8 $\log_{10} I$, r^2 = 0.999; pH 4.83, TTD = 2305.6 – 289.6 $\log_{10} I$, r^2 = 0.992 ; pH 4.50, TTD = 3977.4 – 511.3 $\log_{10} I$, r^2 = 0.987). The data were modelled using the composite model and the regression parameters are given in Table 2. According to the composite model the TTD at a given pH is simply given by the multiplication of Eq.1. by a constant factor (calculated using Eq.2). The magnitude of this factor is dependent on the harshness of the environmental conditions. Table 3 shows a comparison of the ratios between the observed and modelled TTD at different pH values for different initial inocula relative to pH 6.505 with $I = 10^{5.4}$. For example a shift from pH 6.50 to pH 4.83 will result in an increase in the TTD recorded at pH 6.50 by a factor of 2.5. The observed ratios and the modelled ratios are in general agreement. The minimum pH was calculated (Eq. 4) as 4.11 (+/-0.05).

3.2.5. *Ent. sakazakii* (FSM 263).

Plots of log inoculum, using a half-folding dilution from an initial inoculum of $10^{5.4}$ (7 initial inocula, range 5.4 to 3.6) against the observed TTD gave linear relationships for a given pH. During the 3-day incubation growth was observed only in wells with pH 4.24 or greater (best fit linear regression lines: pH 6.54, TTD = 742.3 – 91.6 $\log_{10} I$, r^2 = 0.995; pH 6.18, TTD = 734.9 – 90.1 $\log_{10} I$, r^2 = 0.996; pH 5.79, TTD = 768.4 – 93.7 $\log_{10} I$, r^2 =0.996 ; pH 5.51, TTD = 854.9 – 102.4 $\log_{10} I$, r^2 = 0.994; pH 5.19, TTD = 1084.1 – 131.1 $\log_{10} I$, 0.998; pH 4.79, TTD = 1663.3 – 200.7 $\log_{10} I$, r^2 = 0.997; pH 4.55, TTD = 2185.7 – 266.5 $\log_{10} I$, r^2 = 0.992; pH 4.24, TTD = 7870.3 – 1074.1 $\log_{10} I$, r^2= 0.976). The data were modelled using the composite model and the regression parameters are given in Table 2. The minimum pH was calculated (Eq. 4) as 4.10 (+/-0.06).

3.3. Effect of the initial inoculum size on the inhibition by Na acetate and pH

3.3.1. *E.coli*: TTD data from 6 identical 10 x10 grids of Na acetate and pH, each inoculated with a known amount of *E.coli*, were obtained. Figure 6 shows the observed data at pH 6.50 for the six initial inocula over the range of acetic acid applied. The pH controls (no Na acetate added) have observed TTD_{ref} = 294.6, 331.0, 362.5, 401.5, 443.3 and 474.2 mins for the half-folded dilution from an initial inoculum of $10^{5.52}$. As the concentration of acetic acid increases the TTD increases; from the simple linear regressions – as the initial inoculum decreases the gradient of the regression lines increases.

The data were modelled using the composite model with both Na acetate, (as acetic acid - calculated from Eq.3) and pH as the inhibitory effects. The non-linear regression parameters obtained are given in Table 4. Figure 7 shows plots of the observed and modelled data at pH 6.5 and 5.83; they show that there is a smooth reduction in the RTD as the acetic acid concentration increases or as the initial inoculum size decreases. Indeed the model states that the curve observed is obtained from the multiplication of the simple inoculum function (C- m $\log_{10} I$) with a constant dependent only on the pH and the acetic acid concentration. Figure 8 shows a plot of the calculated vs. the observed RTD and also a plot of the calculated RTD vs. the error (calculated RTD-observed RTD) for *E. coli*. There is an

excellent agreement between the modelled and observed data and the stochastic assumption (that the reciprocal transformation stabilises the variance) appears valid.

3.3.2. *L. innocua*. TTD data from 8 identical 10 x10 grids of Na acetate and pH each inoculated with a known amount of *L.innocua* (7 half-fold dilutions from an initial $1x10^5$ cfu ml^{-1} culture) were obtained; 410/800 wells showed growth within the 3-day incubation period. The growth data were modelled using the composite equation and the regression parameters found are given in Table 4. From plots of the calculated RTD against the observed RTD along with the error plot (calc. RTD – obs. RTD) the best fit regression lines were obtained: $RTD_{obs} = 1.002\ RTD_{calc} - 3x10^{-6}$, r^2=0.991; error $= -0.002\ RTD_{calc} + 3x10^{-6}$, $r^2 = 0.0004$ (data not shown).

Equation 2 was applied to each individual data set (with constant initial inoculum) and the parameters P_i for i = 1 to 4 obtained (Table 5). T-tests were performed on all combinations and in no case were there statistically significant differences ($P<0.05$) between any of the parameters for P_i with i = 1 to 4; for example between the highest and lowest inocula the t-test gave P =0.565, 0.234, 0.632 and 0.429 for P_1 to P_4 respectively. The minimum pH for growth (4.53 +/-0.015) and MIC of acetic acid (968 +/-48 mg l^{-1}) were obtained using Eq.3. From Table 5 the calculated pH_{min} for *L. innocua* is not statistically significantly different over the range of inocula investigated. Interestingly, the MIC of acetic acid shows a slight rise with *decreasing* inoculum size, however, the confidence intervals also increase with decreasing inoculum size and this is not statistically significant.

3.3.3. *Salmonella* Poona; TTD data from 5 identical 10 x10 grids of Na acetate and pH each inoculated with a known amount of *S.Poona* (4 half-fold dilutions from an initial $2x10^5$ cfu ml^{-1} culture) were obtained; 309/500 wells showed growth within the 3-day incubation period. The growth data were modelled using the composite equation and the regression parameters found are given in Table 4. The minimum pH for growth (3.80 +/-0.04) and MIC of acetic acid (917 +/-62 mg l^{-1}) were obtained using Eq.4. From plots of the calculated RTD against the observed RTD along with the error plot (calc.

RTD – obs. RTD) the best fit regression lines were obtained: $RTD_{obs} = 1.0023\ RTD_{calc} - 5x10^{-6}$, $r^2=0.993$; error = $-0.0023\ RTD_{calc} + 5x10^{-6}$, $r^2 = 0.0008$ (data not shown).

3.3.3. *Aeromonas hydrophila*; TTD data from 5 identical 10 x10 grids of Na acetate and pH each inoculated with a known amount of *A. hydrophila* (4 decimal dilutions from an initial $1x10^5$ cfu ml^{-1} culture) were obtained; 250/500 wells showed growth within the 3-day incubation period. The growth data were modelled using the composite equation and the regression parameters found are given in Table 4. The minimum pH for growth (4.54 +/-0.03) and MIC of acetic acid (343 +/-20 mg l^{-1}) were obtained using Eq.4. From plots of the calculated RTD against the observed RTD along with the error plot (calc. RTD – obs. RTD) the best fit regression lines were obtained as $RTD_{obs} = 0.996\ RTD_{calc} + 9x10^{-6}$, $r^2=0.996$; error = $0.0044\ RTD_{calc} - 9x10^{-6}$, $r^2 = 0.0046$ (data not shown).

3.3.4. *Ent. skazakii*; TTD data from 5 identical 10 x10 grids of Na acetate and pH each inoculated with a known amount of *Ent. sakazakii* (4 decimal dilutions from an initial $1x10^6$ cfu ml^{-1} culture) were obtained; 338/500 wells showed growth within a 5-day incubation period. The growth data were modelled using the composite equation and the regression parameters found are given in Table 4. The minimum pH for growth (4.09 +/-0.06) and MIC of acetic acid (529 +/-65 mg l^{-1}) were obtained using Eq.4. A plot of the calculated RTD against the observed RTD along with the error plot (calc. RTD – obs. RTD) gave the best fit regression lines as $RTD_{obs} = 0.982\ RTD_{calc} + 5x10^{-5}$, $r^2=0.985$; error = $-0.018\ RTD_{calc} - 5x10^{-5}$, $r^2 = 0.0215$.

3.4. *Effective Concentration*

One difficulty with multifactor data is graphically displaying the observed and modelled data. From Eq.2. the effect of pH and that of acetic acid on the growth of an organism can be separated, i.e. they are independent. This allows us to define the effective concentration (EffC) of the applied inhibitors as the summation term given in Eq.2. If a plot of the effective concentration calculated from the parameters given in Table 4, against the RTD for example for *A. hydrophila*, is made (Figure 9) then at low EffC the RTD_{ref} is obtained. As the EffC increases the RTD for a given inoculum size

436 decreases, but the ratio of RTD between different inoculum sizes, as predicted by the model, is
437 maintained. For the decimal dilution used the ratios of RTD between the highest an lowest inocula for
438 all EffC is 1: 0.767: 0.622:0.524: 0.452 (where the ratio is the TTD observed / TTD of the highest
439 inoculum for a given EffC); at EffC = 0.0357 (pH =6.49, no added Na acetate) the observed ratios are
440 1: 0.770: 0.628: 0.538:0.454 for $10^5 : 10^5 : 10^4 : 10^3 : 10^2 : 10^1$ respectively; at EffC = 1.301, (e.g. pH
441 5.2 & 240 mg l^{-1} Na acetate) the observed ratios are 1: 0.757: 0.594: 0.539: 0.447. From Figure 9 this
442 can be seen as a simple scaling of the curve obtained for $I = 10^5$ using these ratios as scaling factors.

4. Discussion

4.1. The standard inoculum size-incubation time curve

Under optimal conditions, or specified conditions (e.g. temperature and media,) the time to detection of decimal or other serial dilutions from a standard inoculum will give a linear relationship between the log of the initial inoculum size and the TTD. At very low cell densities (<10 per well), the probability of obtaining a well with no resident cell increases and the variance in the data increases with decreasing cell density. At very high cell densities, other factors such as quorum sensing may be in operation, but at the cell densities used in these studies no high inoculum deviation from the straight line regression was observed. For each bacterial species studied the simple linear model, Eq.1. was used to obtain the two growth parameters, m and C. Although the variance increased at the low cell densities no variance stabilisation was used on the linear regression as this had little impact on the results obtained. Furthermore at the low cell densities, although it might be expected to see a skewed distribution of TTD, in no case was this observed, although only ten replicates were done per inoculum size.

The use of OD = 0.2 at 600nm as the criterion for the time to detection was chosen for ease of data analysis. From figure 1, it can be seen that any specific OD can be chosen and indeed if, for example, with *Ent. Sakazakii* the TTD criterion is changed to OD = 0.55 at 600nm, then from the observed data TTD = 790.8 (+/-6.95) - 82.3 (+/-1.5) $\log_{10} I$. The gradients obtained at OD = 0.2 and OD = 0.55 are statistically equivalent (t-test; P = 0.38) whereas the intercept has increased to accommodate the new criterion. A lower OD value or a shorter wavelength could also be used -chosen for the convenience of the media and/or the added inhibitors being studied.

4.2. The composite Gamma function: inoculum size and inhibitors

Previous work (Lambert and Bidlas 2007a) had shown that a general Gamma model of which Eq.2, is the form using the reciprocal transformation to stabilise data variance, was able to model the affect of several combined inhibitors (pH, salt, weak acids). Those studies used a standard inoculum of 10^5 cfu

470 ml^{-1} and under optimal conditions (for a given temperature with no added inhibitors at an optimal pH)
471 the reference TTD was a characteristic of the organism used. It was further shown (Lambert and
472 Bidlas 2007c) that at different temperatures (range 25 – 41^{o}C) for *Ent. sakazakii*, although the TTD_{ref}
473 followed the expectations of the Cardinal Temperature Model (Rosso, Lobry and Flandrois 1993) the
474 inhibitor parameters were constant.
475
476 Since it was already known that TTD_{ref} was dependent on the initial inoculum size the
477 simplest alteration to the original Gamma model was to replace TTD_{ref} with Eq.1. This, at face value,
478 appeared to go against the work described in the introduction on the G/NG studies. Since the G/NG
479 boundary changed with inoculum size and since it has been shown that MIC was dependent on
480 inoculum size, the new model would be unable to reproduce these effects. Specifically, the parameters
481 P_{2i-1} of Eq.2. should be dependent on inoculum size since these parameters are akin to the MIC, indeed
482 Eq.4 defines MIC on the basis of the parameters P_{2i-1} and P_{2i} (the slope parameter).
483
484 The composite model, however, makes the prediction that for a given set of environmental
485 factors the inoculum size dependency of the TTD (Eq.1) will be simply multiplied by a factor given by
486 Eq.2. This implies that the linear relationship between TTD and $\log_{10}$ inoculum size will be preserved.
487 Furthermore, the inhibitory parameters P_{2i-1} and P_{2i} would be independent of inoculum size.
488
489 The analysis of the TTD data obtained using the composite Gamma model for combinations of
490 inoculum size and pH and for inoculum size, pH and Na acetate (the reciprocal of Eq.1 replacing
491 RTD_{ref} of Eq.2) has shown that the parameters P_{2i-1} and P_{2i} of Eq.2. are conserved and that the
492 inoculum size parameters (m and C) are also conserved. Figures 3, 4 and 6 directly show that as the
493 pH is lowered the gradient of the log inoculum against TTD increases, but that the linear nature of the
494 relationship between TTD and $\log_{10} I$ is preserved. The variance (Fig.3) also increases with decreasing
495 pH over the range of inoculum sizes used and does show an elevated variance at the lowest inoculum
496 levels used.
497

498 Figure 6 shows that at a given pH, there appears to be a simple relationship between inoculum size and
499 acetic acid concentration. With decreasing inoculum size, the intercept and the gradient of the
500 TTD/acetic acid concentration plots increase. For a given pH, and with an inoculum size dependency
501 of the TTD_{ref} given by Eq.1, Eq.2. can be written as

502
$$TTD = (C - m\log_{10} I)\exp\left\{K + \left(\frac{AcH}{P_3}\right)^{P_4}\right\}$$
$$= K'(C - m\log_{10} I)\left(1 + \left(\frac{AcH}{P_3}\right)^{P_4} + \ldots\right) \quad ,$$

503 where AcH is the acetic acid concentration (mg l^{-1}) and $K = (10^{-pH}/P_1)^{P2}$.
504
505 From this expansion we can see that the intercept of Fig.6 (when AcH = 0) is given by $K'(C\text{-}m\log_{10}I)$;
506 K' is simply a constant due to the inhibition caused by pH alone (pH = 6.505), which increases the
507 TTD_{ref} by a factor of 1.10. If the values of C and m in Table 4 are used, then for a given $\log_{10} I$,
508 multiplication by K' gives the approximate value for the intercept of the best fit regression lines given
509 in Figure 6. Hence the prediction of the model that for a given set of environmental factors, the
510 inoculum size dependency of the TTD (Eq.1) is simply multiplied by a factor given by Eq.2. Over the
511 acetic acid concentration range 0.5 – 140 mg l^{-1} the model gives an approximate linear relationship
512 between TTD and AcH, with an increasing gradient with decreasing inoculum size.
513
514 Figure 4 and 5 show that the observed and modelled RTD data are in excellent agreement over the pH
515 range studied. The former figure suggests a pH optimum between 6.52 and 7.09 for *S*.Poona, which
516 the model in its current guise does not allow for (the model is based on hydrogen ion concentration
517 rather than pH and does not have a pH optimum built in).
518
519 *4.3 Inoculum size dependency on the value of MIC*

520 The composite model for inoculum size and the effect of environmental factors described above leads
521 to the conclusion that the inhibitory parameters obtained from the non-linear regression analysis are

independent of the initial inoculum size. The demonstration that this conclusion is not contrary to the observations made by for example by Masana and Baranyi (2000), Koutsoumanis and Sofos (2005) and also by Robinson et al (2001) has to be made. The one thing that these fore mentioned studies have in common was that they were conducted over a specified timeframe.

The calculated MIC is defined by Eq.(4) and is independent of time, being a concentration value calculated for a specific level of inhibition. The minimum inhibitory concentration, MIC, as defined in the general microbiological literature, however, is the concentration required to inhibit growth at a specified time, e.g. 18 hours (e.g. Andrews 2000). The composite Gamma model can be rearranged to give an expression relating the concentration of acetic acid required to achieve a given level of inhibition in a specified time for a given pH and initial inoculum size, Eq.5;

$$TTD = (C - m\log_{10} I)\exp\left(\frac{H^+}{P_1}\right)^{P_2}\left(\frac{AcH}{P_3}\right)^{P_4}$$

$$let\ TTD = a\ specified\ time, e.g. = 24hrs,\ TTD_{MIC} \qquad (5)$$

$$[AcH] = P_3\left(\ln\left(\frac{TTD_{MIC}}{C - m\log_{10} I}\right) - \left(\frac{[H^+]}{P_1}\right)^{P_2}\right)^{(1/P_4)}$$

This form of the equation will, for a given pH and initial inoculum size, give the concentration of acetic acid [Ac] needed to obtain TTD = TTD_{MIC}. Above this concentration no growth (to the OD standard used, e.g. visual growth) will be observed in the time given.

Figure 10 shows a plot of the initial inoculum size against the $TTD_{MIC\ =24hr}$ of Na acetate at three pH conditions using the data for *L. innocua* (Table 4). At an initial inoculum of $1x10^3$ cfu ml^{-1} at pH = 4.9 no growth is calculated within 24hrs (hence no MIC is recorded). At $1x10^4$ and at $1x10^5$cfu ml^{-1} 156 and 400 mg l^{-1} of Na acetate are required to achieve the 24 hr MIC respectively. A ten-fold increase in the initial inoculum increases the 24hr MIC by a factor of 2.56 in this case. At pH 5.4, growth is

observed within 24hrs at all initial inocula, the pH is by itself not inhibitory enough to prevent growth within 24 hrs. With $\log_{10} I$ = 2, 3 and 4, the total concentration of Na acetate required to inhibit growth at 24 hrs was calculated as 677, 1023 and 1446 mg l^{-1} respectively. This shows is that the MIC for a fixed time is dependent on the inoculum size used.

Figure 11 shows a similar use of Eq.5, but in this case a fixed pH (pH = 5.1) has been used and three different MIC used: MIC at 24, 48 and 72 hours. The calculation shows that as the time is extended the amount of Na acetate required to inhibit the system at specified times increases and the amount is dependent on the size of the inoculum. For example if $\log_{10} I$ = 1, then at 24hrs, there is no growth recorded (the pH is inhibitory enough to slow the growth) whereas at 48 and 72 hrs, 603 and 1051 mg l^{-1} are required to prevent growth at those times. If $\log_{10} I$ = 3, then 360 mg l^{-1} of Na acetate is required at 24hrs, and 1130 and 1578 at 48 and 72 hrs respectively. This shows that the MIC is dependent on both the inoculum size used and the incubation time given.

4.4. Possible explanation of literature studies.

Masana and Baranyi (2000) described the changes in the growth /no growth boundary of *Brochothrix thermosphacta* as a function of pH (4.20 – 5.8) and salt concentration (0 – 10%) at 25°C (the optimum growth temperature for the organism). They demonstrated that the boundary changed with inoculum size and stated (a point which has direct relevance to Robinson et al. 2001) “It was apparent that, in most cases, once a replicate from a combination presented growth eventually all the others also grew. The changes of probability over time also showed that under more extreme conditions the time to growth, as a kinetic parameter, exhibited increasing variability.”

The model described here gives a very simple explanation for the changes in the boundary – for a fixed time experiment the boundary line for a lower inoculum will always be inside that of the higher inoculum. Modelling from the published data was problematic since the data were discrete (the growth was recorded in whole number of days, and therefore there was no differentiation between conditions recorded as Growth =1 day). Using the composite model (Eq.1. & Eq.2), an initial set of parameters

were used to generate continuous data based on the pH and salt grid given by Masana and Baranyi. The modelled data were then transformed to TTD and rounded-up to the nearest whole number of days. An upper limit of TTD of 60 days was used, such that if the calculated TTD≥ 60days, the TTD was set to 60 days; no growth data from the published work was also treated as TTD = 60 as this was approximately the experimental duration. From this the deviances between the model and the published work were minimised; the following parameters were obtained, C = 708, m = 109, the pH parameters P_1 and P_2 were 5.75×10^{-6}, and 1.17 respectively and the salt parameters, P_3 and P_4, were 4.15 and 2.3 respectively. Figure 12 shows the change in the TTD with respect to pH and salt when the inoculum size was reduced. Qualitatively, the change in the boundary with reducing inoculum size described by the model is very similar to that described by Masana and Baranyi; the large difference between the G/NG border at $10^{6.18}$ with those of $10^{3.18}$ and $10^{1.18}$ reflects the observed data, as well as the similarity between the G/NG of the latter two lower inocula. Quantitatively there are some differences, especially close to the G/NG border of 60 days, but this is to be expected given the nature of the data, the fitting and the declaration concerning the variability. At $I = 10^{6.18}$, with salt concentrations at 10% both the observed and model agree - no growth in 60 days, similarly at all pH = 4.4. The model suggests that at pH 4.6 and with salt levels less than 3.5%, growth will be observed within 15 days, this was observed except at the lowest salt level (observation of no growth). At pH 5 with salt = 8%, the observed TTD = 5 days, the model suggests growth within 15 days; at 9% salt there was no observed growth at pH 5, in agreement with the model. With $I = 10^{3.18}$, the observed and modelled pH boundary = 4.6, with the salt boundary decreasing to 8% (observed) or 8.5% (modelled). At pH 5 and 6% salt, TTD_{obs} = 7 days, modelled <15days; increasing the salt by 1% results in NG (observed), whereas the model suggests a TTD between 45 and 60 days. At $I = 10^{1.18}$, the observed and modelled pH boundary = 4.6, and the salt boundary = 8% (observed and modelled). At pH 5.2 with 7% salt the TTD_{obs} = 10 days, whereas the model suggests 30-45 days; a reduction by 0.2 pH units or an increase of 1% salt results in both the observed and modelled giving NG.

When the 231 observations were ranked and reclassified as G or NG, out of 111 observations of NG, the model labels 12 of these as G (10 of which are TTD_{calc} >15days, only two conditions (1)

pH 4.6, 0.5% salt, I = $10^{6.12}$ and (2) pH 4.8, 6% salt and I = $10^{6.12}$ are very different from the modelled TTD of 7 days); of the 120 observations of G, the model labels all as G.

Masana and Baranyi (2001) explained the inoculum size effect of *Brochothrix thermosphacta* on the probability of growth and the location of the growth/no growth boundary by invoking the hypothesis that population differences in resistance to environmental factors were responsible. The use of the composite model suggests that the observed changes in the G/NG boundaries are due to using different inoculum sizes only.

Koutsoumanis and Sofos (2005) examined the effect of multiple temperatures, aw, pH and inoculum size on the 60 day G/NG boundary of *Listeria monocytogenes*. The authors state that "The growth limits of the pathogen and hence the position of the growth boundary were found to be affected by the size of the inoculum." They further stated that their study "indicates the importance of inoculum size for microbial growth initiation and provides quantitative data that show how the combinations of hurdles which prevent growth vary with inoculum size."

Data for the effect of combinations of aw and pH (10 x10 grid) at a fixed temperature (15^{o}C) for four initial inoculum sizes were given in Figure 2 of their publication. These data were extracted from the figures and for each combination of pH, aw and $\log_{10} I$ assigned either G or NG. The data were modelled using a composite model with initial values for C, m, P_{2i-1} and P_{2i}. The model produced TTD values for each combination of factors, these values were then degraded to nominal values of G or NG based on a G/NG boundary of 60 days. The initial parameters were adjusted to reduce the total number of mismatched G/NG labels between the observed and modelled data. The parameters obtained were C = 7951, m = 556.9, $P_1 = 10^{-4.761}$, $P_2 = 1.168$, $P_3 = 6.53$, $P_4 = 1.38$ (15 G/NG mismatches- 3.75%).Figure 13 displays the results of the model along with the published data. Table 6 shows the results of a simple contingency analysis; of the 15 mismatches, 11 had modelled TTD of between 40 and 80 minutes. The other four mismatches ((1) aw 0.997, pH 4.24, I = $10^{4.2}$; (2) aw 0.997, pH 3.94, I = $10^{6.81}$;

(3) aw 0.997, pH 4.24, I = $10^{6.81}$; (4) aw 0.983, pH 4.24, I = $10^{6.81}$) were observed to grow, whereas the model showed NG (modelled TTD = 233, 26663, 173 and 256 days respectively).

The initial values used for the inoculum size dependency and the pH parameters were taken from the data of *L .innocua* (Tables 1 and 2). The values for C and m given in Table 2 were obtained at 30°C. By using the Cardinal Temperature model with T_{min} = -0.4°C , T_{opt} = 37°C and T_{max} = 45°C, the Gamma factors at 30°C and at 15°C (0.826 and 0.217 respectively) were obtained; the ratio of the two (3.81) was used to estimate the initial values of C and m at 15°C (4600 and 533 respectively). The pH parameters were used directly, the initial salt parameters used were obtained considering the lower a_w value found by Koutsoumanis and Sofos ($a_w \cong 0.9 \cong$ 14% salt) and using Eq.4 to estimate an initial value for P_{2i-1}, using an estimation of P_{2i} = 2. The derived values obtained are not surprising – they reflect the general values expected of such parameters; indeed the calculated pH_{min} = 4.4, MIC_{salt} = 13.8 % reflect the literature pH_{min} values and the a_w found by Koutsoumanis and Sofos (note the model and observed mismatch at the lowest pH values, especially at pH 3.94).

The most important point being made here is that the inhibitory function of the composite model is independent of inoculum size and that changes in the G/NG boundary with inoculum size can be explained as being due to the inhibitory function applying a factor (gamma factor) to the linear model of inoculum size dependency on the TTD, i.e. the change in shape of the G/NG boundary due to changes in initial inoculum size (for a given set of environmental conditions) is due to the change of inoculum size alone.

Robinson et al. (2001) showed that in replicates the variance of *Listeria monocytogenes* cells increased with increasing concentrations of NaCl. Further they described experiments which showed that as the concentration increased the "number of cells required to initiate growth increased from one cell under optimum conditions to 10^5 cells in medium with 1.8 M NaCl" (approx 9.35%, a_w = 0.939). From their work (Figure 1 of their publication) an inoculum/TTD plot at 37°C, zero added salt, gave an inoculum size dependency (Eq.1) with C = 858.5 and m = 109.95 (r^2= 0.9898). Comparison of these values to

655 those of *Listeria innocua* (Table 1) shows them to be comparable (given the differences in
656 temperatures). The work described herein would suggest that on addition of inhibitory levels of salt,
657 the observed TTD values with respect to inoculum size would increase by a factor given
658 by $\exp\left(\left(\frac{salt}{P_1}\right)^{P_2}\right)$. When the data from the exponentially growing wells were analysed (taken from
659 Figure 1 of Robinson et al. 2001), using a composite model of inoculum size and salt inhibition (using
660 the logarithmic transform to partially stabilise the variance – see Lambert and Bidlas 2007a), the
661 following parameters were obtained; C = 852.2 (SE 69.0), m = 110.7 (SE 9.3), salt parameters, P_1 =
662 6.84 (SE 0.22), P_2 = 2.979 (SE 0.28), for 37 observations. A plot of the log of the modelled detection
663 time against the log of the observed gave a linear relationship with ln(obs) = 1.033 ln(modelled) -
664 0.236, r^2 = 0.947.
665
666 Again the model suggests that the changes observed are due to the multiplication of a linear
667 relationship between TTD and log *I* by a factor dependent on the added stress. Interestingly, in this
668 case the variance in the data is much more severe than that observed in our studies with pH and Na
669 acetate. The MIC of salt can be calculated using Eq. 4 and in this case is 9.6%, hence the use of 1.6M
670 NaCl is quite close to this G/NG boundary value and a large variation so close to the boundary would
671 therefore be expected as is observed.
672
673 Robinson et al. (2001) concluded on the basis of their observations that growth under severe salt stress
674 appeared "to depend on the presence of a resistant sub-fraction of the population, although high cell
675 densities assist adaptation of those resistant cells to the unfavourable growth conditions by some
676 unspecified medium conditioning effect." The study done using the composite model would suggest
677 that there is no need to invoke a hypothesis of resistant sub-fractions, nor by suggesting the presence
678 of an unknown conditioning effect; the data are consistent with the idea that the inoculum size and the
679 applied inimical procedure are independent.
680

In our studies described herein, identical chequerboards (or grids) of pH and acetate were prepared; a plot of the log of the initial inoculum against TTD for *any* given set of pH and Na acetate was found to be linear and extrapolation to the inoculum axis gave, for example with *E.coli* an Io = 7.79 +/-0.26. In these experiments, therefore, the lag was constant, although the specific growth rate decreased with increasingly harsher conditions. We would conclude using the hypothesis of Robinson et al. that there was no resistant sub-fraction of the population present. Nor would we consider quorum sensing to be operating at the inoculum levels used in our experiments, since this would also lead to deviations from the model.

The experiments we have performed challenge the idea that the IE is a 'real' phenomenon, i.e. anything other than a consequence of using a different inoculum level. Although we recognise that where the organism can alter the concentration of the inhibitor are special cases (usually at high cell densities) – this includes certain antibiotic resistant organisms (Thomson and Moland 2001) and also some spoilage yeasts which can destroy (metabolise) certain preservatives (Casas, Ancos, Valderrama, Cano and Peinado 2004). We also recognise the so-called inoculum effect used to describe studies of the variance of single cells, especially those where a pre-inhibitory step has been carried out; we would suggest that these be called low (or single cell) inoculum effect studies to separate them from studies where higher inocula are used.

Our experiments have shown that only the reference time to detection (TTD_{ref}) is affected by inoculum size, and this is an easily modelled function. That the data required to model this function requires only a maximum of 2 days to procure for rapid growing bacteria (see Figure 1) and that this can be done independently of any other environmental factor suggests that this will readily allow future predictive models to incorporate inoculum size as a common feature. Conversely, a response surface model already in the literature could be augmented with an inoculum size dependency by invoking the Gamma hypothesis.

Conclusion

709 The hypothesis used in this study was the null-hypothesis - that the apparent IE and the changes in the
710 G/NG boundary with respect to inoculum size were due to the time taken for a specific inoculum size
711 to achieve growth under the given environmental conditions. The model developed to study the
712 experimental data obtained in our laboratory and from the literature appears to have validated this
713 hypothesis.

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Tables

Table 1. Parameters for the time to detection (defined as the time to an OD = 0.2 at 600nm) of initial inocula at 30°C at pH 6.5 (TSB) produced from the decimal dilutions of a standard inoculum.

Organism	Standard Inoculum (log_{10} I, cfu ml^{-1}) for cultures with OD = 0.5 at 600nm)	m (St.Err) (mins $log_{10}n^{-1}$)	C (St.Err) (mins)
Aeromonas hydrophila **ATCC 7966**	**8.81**	**80.0**	**674.1**
		(0.44)	**(2.2)**
Listeria innocua **ATCC 33090**	**8.83**	**147.0**	**1186.1**
		(2.1)	**(8.7)**
Enterobacter sakazakii **FSM 263**	**8.88**	**80.7**	**685.8**
		(1.0)	**(4.6)**
Escherichia coli **ATCC 25922**	**8.90**	**91.1**	**788.5**
		(2.4)	**(11.1)**
Salmonella **Poona NCTC 4840**	**8.80**	**95.2**	**807**
		(1.2)	**(6.1)**

Microbial counts are the average count from three agar plates; m is the time (in minutes) for a ten fold change in microbial numbers (n).

862 Table 2. Parameters for the inoculum and pH dependency on TTD (Eq.2)

	Parameter (St.Err)			
Organism	**m (mins $\log_{10}n^{-1}$)**	**C (mins)**	**P_1 (mol l^{-1})**	**P_2**
A. hydrophila	**78.9**	**654.5**	**1.03 E-05**	**0.930**
	(1.80)	**(9.3)**	**(2.43 E-07)**	**(0.034)**
L. innocua	**143.7**	**1162**	**1.39 E-05**	**0.996**
	(0.66)	**(4.8)**	**(8.2 E-08)**	**(0.009)**
Ent. sakazakii	**82.5**	**676.7**	**1.973 E-05**	**0.722**
	(2.73)	**(14.8)**	**(5.72 E-07)**	**(0.025)**
E. coli	**92.0**	**763.5**	**1.445 E-05**	**0.598**
	(1.87)	**(10.9)**	**(3.24 E-07)**	**(0.015)**
***S.* Poona**	**89.6**	**780.0**	**3.14 E-05**	**0.706**
	(1.20)	**(6.1)**	**(4.16 E-07)**	**(0.011)**

863 m is the time (in minutes) for a ten fold change in microbial numbers (n)

864

865

Table 3. Modelled and observed TTD ratios between different pH and inoculum size relative to pH 6.505 and $I = 10^{5.4}$ for *E.coli*.

Modelled ratio								
		pH						
		6.505	6.169	5.835	5.537	5.216	4.828	4.502
$\log_{10} I$	5.400	1.00	1.06	1.17	1.33	1.64	2.50	4.45
	5.099	1.10	1.17	1.29	1.46	1.81	2.76	4.91
	4.798	1.21	1.28	1.41	1.60	1.98	3.02	5.37
	4.497	1.31	1.39	1.53	1.74	2.15	3.28	5.84
	4.196	1.42	1.50	1.65	1.88	2.32	3.54	6.30
	3.895	1.52	1.61	1.77	2.01	2.49	3.80	6.76
	3.594	1.62	1.72	1.89	2.15	2.66	4.06	7.22

Observed Ratio								
		pH						
		6.505	6.169	5.835	5.537	5.216	4.828	4.502
$\log_{10} I$	5.400	1.00	1.05	1.14	1.30	1.64	2.48	4.18
	5.099	1.12	1.17	1.32	1.48	1.81	2.79	4.66
	4.798	1.23	1.28	1.42	1.60	1.97	3.10	4.93
	4.497	1.36	1.41	1.55	1.74	2.18	3.51	5.83
	4.196	1.41	1.49	1.61	1.89	2.33	3.73	6.29
	3.895	1.50	1.57	1.75	1.99	2.53	4.00	6.80
	3.594	1.61	1.67	1.82	2.17	2.69	4.22	7.17

For a given initial inoculum size, the value in the table for a given pH is the factor by which the observed TTD at pH = 6.505, for $I = 10^{5.4}$ is multiplied to obtain the TTD at that inoculum size and pH.

874 Table 4. Parameters for the effect of inoculum size, pH and Na acetate on the TTD

Organism	Parameter (St.Err)					
	Inoculum effect		pH effect		Na acetate effect	
	C (mins)	m (mins $\log_{10}n^{-1}$)	P_1 (mol l^{-1})	P_2	P_3 (mg l^{-1})	P_4
A. hydrophila	**651.7**	**78.53**	**1.015E-5**	**0.967**	**103.7**	**0.834**
	(4.6)	**(0.84)**	**(1.28E-7)**	**(0.019)**	**(1.2)**	**(0.012)**
L. innocua	**1208**	**152.7**	**1.576E-5**	**1.638**	**347.9**	**1.003**
	(7.0)	**(1.51)**	**(1.28E-7)**	**(0.031)**	**(4.12)**	**(0.016)**
Ent. sakazakii	**621.7**	**91.21**	**2.41E-5**	**0.831**	**140.1**	**0.752**
	(8.4)	**(1.5)**	**(6.73E-7)**	**(0.029)**	**(3.50)**	**(0.021)**
E. coli	**865.6**	**109.0**	**1.454E-5**	**0.613**	**208.7**	**0.786**
	(6.1)	**(1.0)**	**(1.62E-7)**	**(0.008)**	**(2.03)**	**(0.008)**
S.poona	**862.6**	**110.8**	**3.469E-5**	**0.660**	**222.4**	**0.706**
	(9.6)	**(1.8)**	**(5.81E-7)**	**(0.014)**	**(3.1)**	**(0.010)**

875 m is the time (in minutes) for a ten fold change in microbial numbers (n)

876 Table 5. *Listeria innocua*: derived regression parameters from Eq.2 for the pH and Na acetate
877 inhibition of various initial inocula

	Regression Parameters (standard errors)					Calculated values (Eq.3)	
		pH effect		Na acetate effect			
Initial Log_{10} I	P_0 (min^{-1})	P_1 (mol l^{-1})	P_2	P_3 (mg l^{-1})	P_4	min pH	MIC acetic
5.00	0.00224 (2.31E-5)	1.621E-05 (5.68E-7)	1.511 (0.069)	349.7 (13.6)	1.011 (0.051)	4.50	940.5
4.70	0.00205 (1.55E-5)	1.560E-05 (3.60E-7)	1.601 (0.082)	341.8 (10.6)	1.035 (0.044)	4.53	898.3
4.40	0.00183 (1.56E-5)	1.559E-05 (3.39E-07)	1.728 (0.092)	351.8 (11.24)	1.013 (0.043)	4.56	943.8
4.10	0.00171 (1.32E-5)	1.589E-05 (3.27E-7)	1.589 (0.089)	349.2 (10.8)	1.018 (0.042)	4.52	932.4
3.80	0.00158 (1.14E-5)	1.582E-05 (3.43E-7)	1.594 (0.077)	344.2 (11.6)	0.965 (0.042)	4.53	970.1
3.49	0.00149 (1.12E-5)	1.582E-05 (3.34E-7)	1.621 (0.075)	360.6 (12.9)	0.950 (0.045)	4.53	1032.8
3.19	0.00141 (1.10E-5)	1.589E-05 (3.43E-7)	1.666 (0.080)	350.5 (114.9)	0.949 (0.048)	4.54	1005.1
2.89	0.00132 (1.055E-5)	1.582E-05 (3.48E-7)	1.643 (0.086)	359.2 (14.3)	0.955 (0.048)	4.54	1023.3

878

879 Table 6. Contingency Table. Comparison of Observed (Koutsoumanis and Sofos 2005) and Modelled
880 data for *Listeria monocytogenes* for the 60 day growth/no-growth boundary

		Observed		
		G	**NG**	**Totals**
Model	**G**	**170 (42.5%)**	**8 (3.59%)**	**178(44.5%)**
	NG	**7 (1.75%)**	**215 (53.75%)**	**222 (55.5%)**
	Totals	**177 (44.25%)**	**223 (55.75%)**	**400 (100%)**

881

Legends to Figures

Figure 1. The optical density/incubation time curves for successive decimal dilutions of an initial inoculum (OD = 0.5) of *Aeromonas hydrophila* (ATCC 7960) incubated at 30°C for 2 days. Each curve is the average of ten replicates. Nine decimal dilutions were performed on the standard culture; the ninth decimal dilution showed no growth in any of the ten replicate wells within the 2-day incubation time.

Figure 2. Time to detection (TTD) against $\log_{10}$ initial inoculum size (I, cfu ml^{-1})of *Enterobacter sakazakii*. Best fit regression line (no variance stabilisation used) TTD = 685.8-80.69 $\log_{10} I$ cfu ml^{-1}, $r^2 = 0.999$. Error bars give the standard deviation for ten replicates per initial inoculum size.

Figure 3. *Listeria innocua* (ATCC 33090): Observed (symbols) and modelled (solid lines) effect of inoculum size on the time to detection at different pH values; pH 4.8, x ; pH 5.2, △; pH 5.5, □; pH 7.0, ■. Ten repeats per pH and per inoculum size. Best fit regression lines for the observables (not shown): pH 7.0, TTD = 1163.4 – 143.5 $\log_{10} I$; pH 5.5, TTD = 1402.6 – 171.6 $\log_{10} I$; pH 5.2, TTD = 1630.0 – 197.8 $\log_{10} I$; pH 4.8, TTD = 3445.8 – 420 $\log_{10} I$.

Figure 4. *Salmonella* Poona (NCTC 4840): The effect of pH and initial inoculum size on the observed RTD (average of 2 replicates) at 30°C; pH 4.08, ■; pH 4.22, □; pH 4.52, ◆; pH 4.82, ◇; pH 5.20, ▲; pH 5.53, △ ; pH 5.77, ● ; pH 6.22, ○ ; pH 6.52, + ; pH 7.09, ×; solid horizontal line (no symbols) marks the incubation time limit of the experiment (1/4320mins).

Figure 5. *Salmonella* Poona (NCTC 4840): The effect of pH and initial inoculum size on the modelled RTD (Eq.2) at 30°C; pH 4.08, ■; pH 4.22, □; pH 4.52, ◆; pH 4.82, ◇; pH 5.20, ▲; pH 5.53, △; pH 5.77, ● ; pH 6.22, ○; pH 6.52, + ; pH 7.09, ×; solid line (no symbols) marks the incubation time limit of the experiment conducted (1/4320mins).

Figure 6. *Escherichia coli*: observed TTD at pH 6.50 for different initial inoculum sizes challenged with acetic acid, calculated from the total Na acetate present; initial $\log_{10}$ inoculum size: 4.02 ■; 4.32, □; 4.62, ◆; 4.92, ◇; 5.22, ▲; 5.52, △. The best fit linear regression lines (solid lines) were $\log_{10} I$ = 4.02, TTD = 484.3 + 3.368 [acetic] ($r^2 = 0.995$); $\log_{10}$ I = 4.32,TTD = 446.6 + 3.226[acetic] (r^2 = 0.999); $\log_{10}$ I = 4.62, TTD = 407.2 + 2.917[acetic] ($r^2 = 0.998$); $\log_{10}$ I = 4.92, TTD = 368.8 + 2.707[acetic] ($r^2 = 0.999$); $\log_{10}$ I = 5.22, TTD = 337.8 + 2.283[acetic] ($r^2 = 0.996$); $\log_{10}$ I = 5.52, TTD = 298.4 + 2.167[acetic] ($r^2 = 0.997$).

Figure 7. Iso-pH plots (pH 6.50 top, pH 5.83 bottom, observed on left and modelled on right) for the effect of Na acetate and initial inoculum size on the RTD at 30°C. Initial $\log_{10}$ inoculum size: D5, 4.02; D4, 4.32; D3, 4.62; D2, 4.92; D1, 5.22; D0, 5.52.

Figure 8. Calculated RTD against the observed RTD for the effect of inoculum size, pH and Na acetate (as acetic acid) on the time to detection of different inoculum sizes of *E. coli* (315 observations, filled symbols) {$RTD_{obs} = 1.002\ RTD_{calc} - 4x10^{-6}$, r^2=0.997 and the error (calculated RTD-observed RTD) against the calculated RTD (open symbols){$error = -0.002\ RTD_{calc} + 4x10^{-6}$, r^2 = 0.001}.

Figure 9. *Aeromonas hydrophila*: observed (symbols) and modelled (solid lines) RTD against the effective concentration, $\sum_{i=1}^{n}\left(\frac{inhibitor_i}{P_{2i-1}}\right)^{P_{2i}}$, calculated for pH (i = 1) and acetic acid (i = 2) with P_{2i-1} and P_{2i} given in Table 4 for five different initial inocula; $I = 10^5$, ■; 10^4, □; 10^3, ◆; 10^2, ◇; 10^1, ▲.

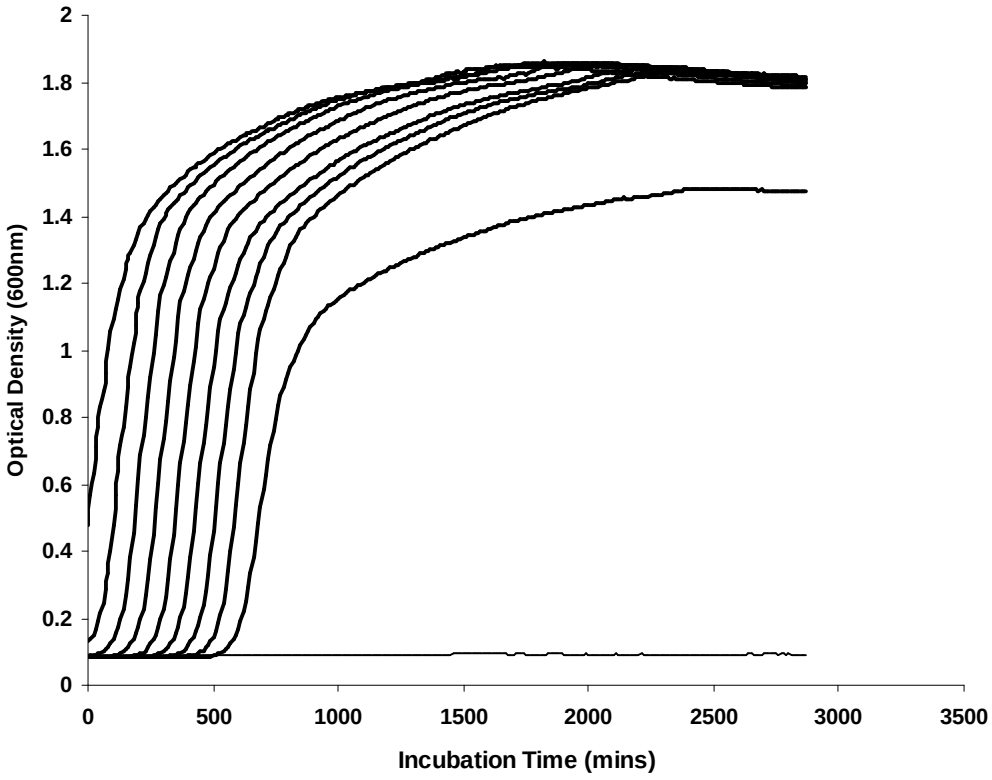
Figure 10. Calculated MIC of acetic acid dependent on the initial inoculum size at 24 hours at pH 4.9, ■ ; pH 5.2, ◆; pH 5.4, ○ .

Figure 11. Calculated MIC of acetic acid dependent on the initial inoculum size at pH 5.1 for a TTD = 24hrs, ■ ; 48 hrs, ◆; pH 72hrs, ○ .

Figure 12. Modelled time to detection for *Brochothrix thermosphacta* at 25°C for combinations of salt and pH with respect to initial inoculum size (from top to bottom $I = 10^{6.18}$, $I = 10^{3.18}$, $I = 10^{1.18}$). The contours are given in steps of 15 days with the outermost region (top left) having TTD ≥ 60days, with the innermost region (bottom right) having a TTD: 0 < TTD <15 days.

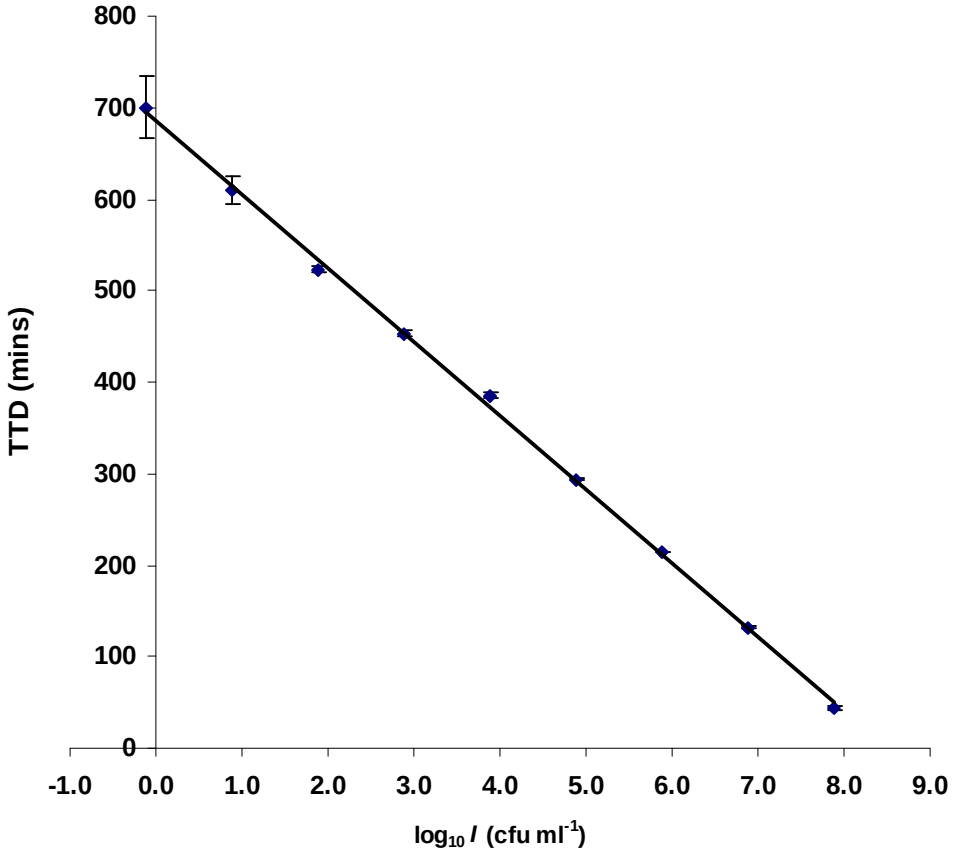
Figure 13. Comparison between the observed 60 day G/NG data of Koutsoumanis and Sofos (2005) for *Listeria monocytogenes* with the modelled data for identical grids of pH and a_w, for four different initial inocula (Top left to bottom right: $10^{6.81}$, $10^{4.2}$, $10^{2.58}$ and $10^{0.9}$ respectively). Modelled data are shown by filled circles, G; open circles, NG. Symbols (open or closed) with a surrounding box indicate those conditions where observed data disagrees with the modelled fit.

948 *Figure 1*



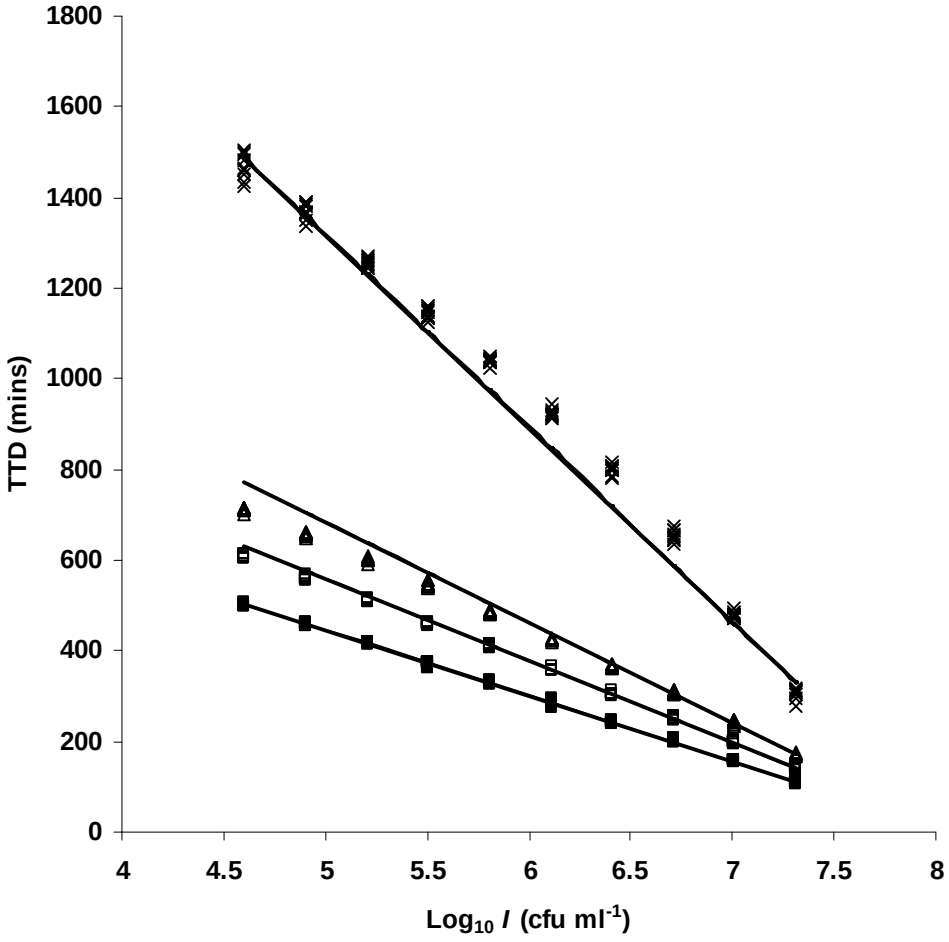
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950 *Figure 2.*



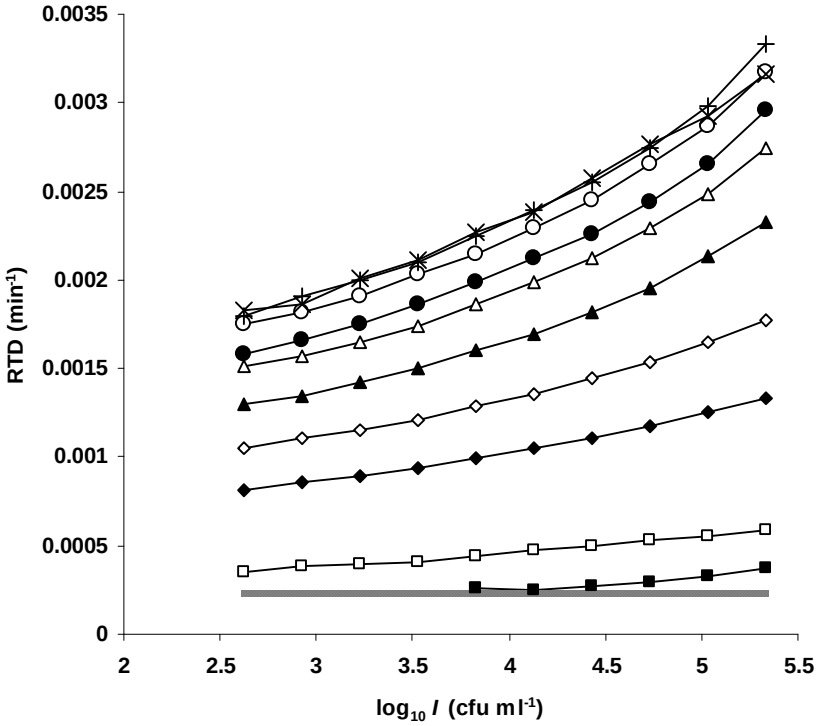
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952 *Figure 3.*



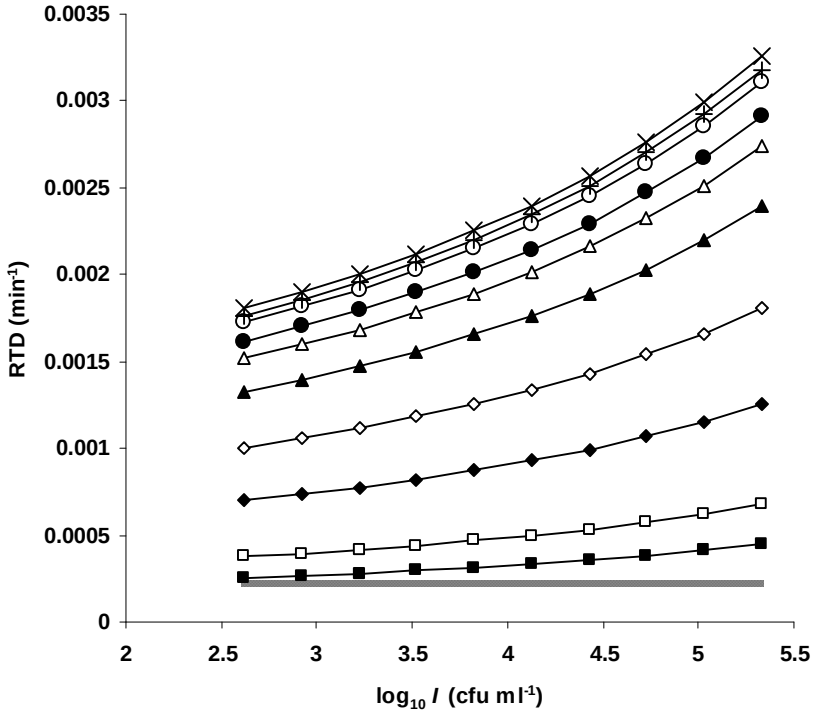
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954 Figure 4.



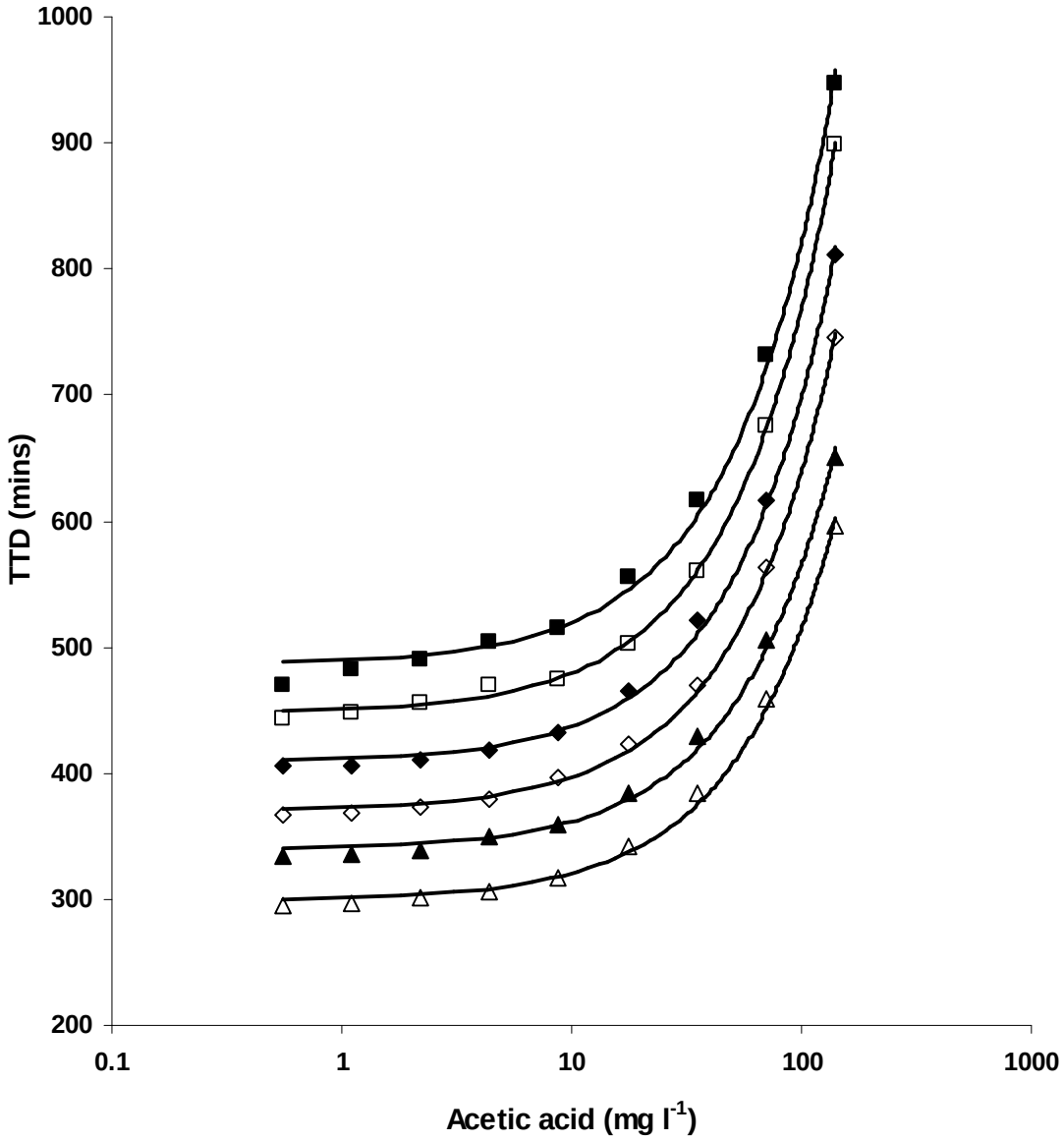
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956 *Figure 5.*



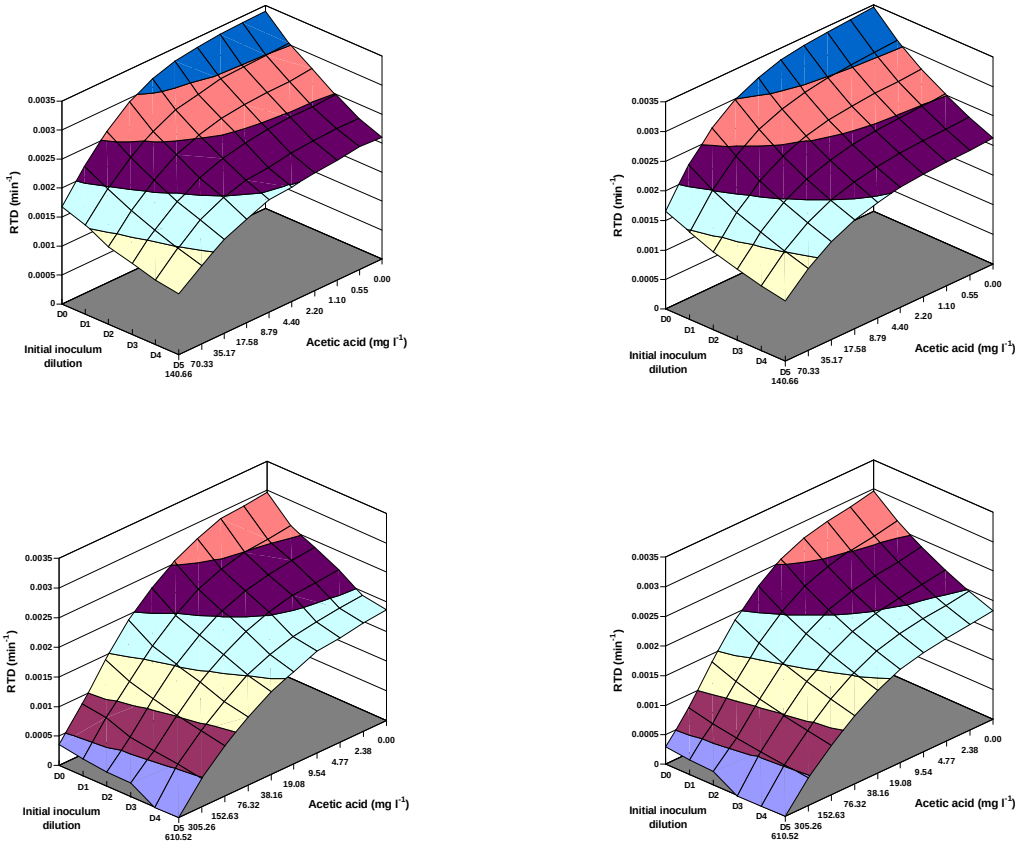
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958 *Figure 6.*



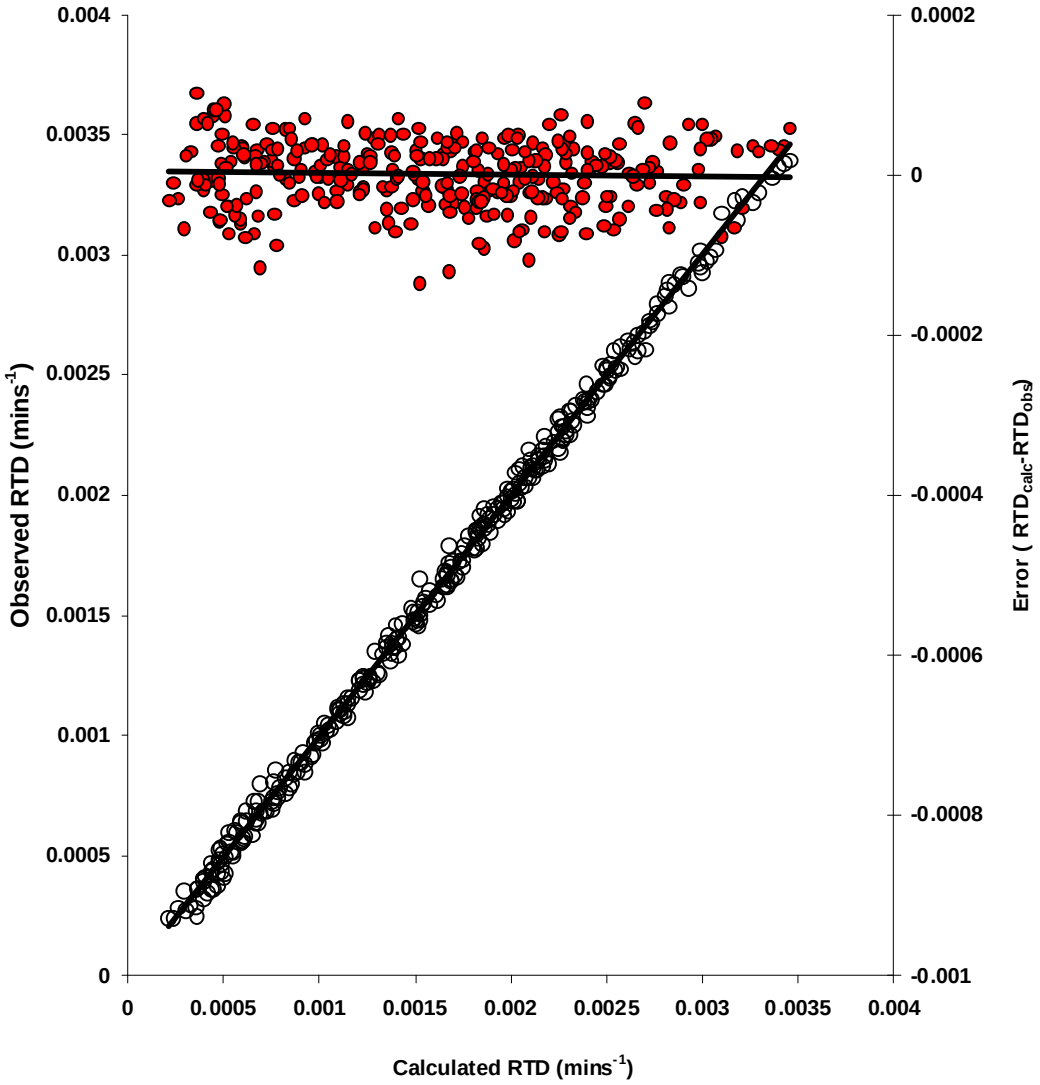
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960 *Figure 7.*



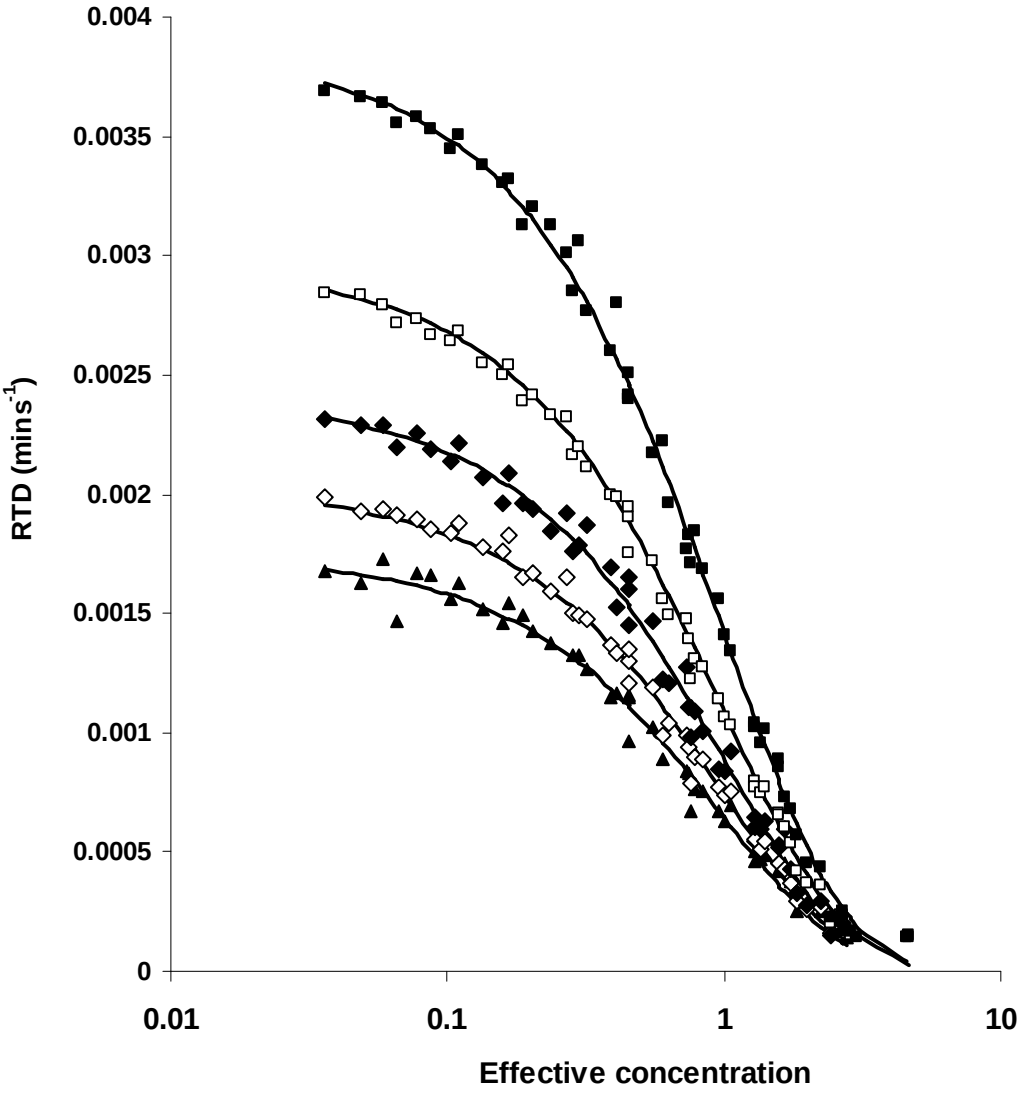
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962 *Figure 8.*



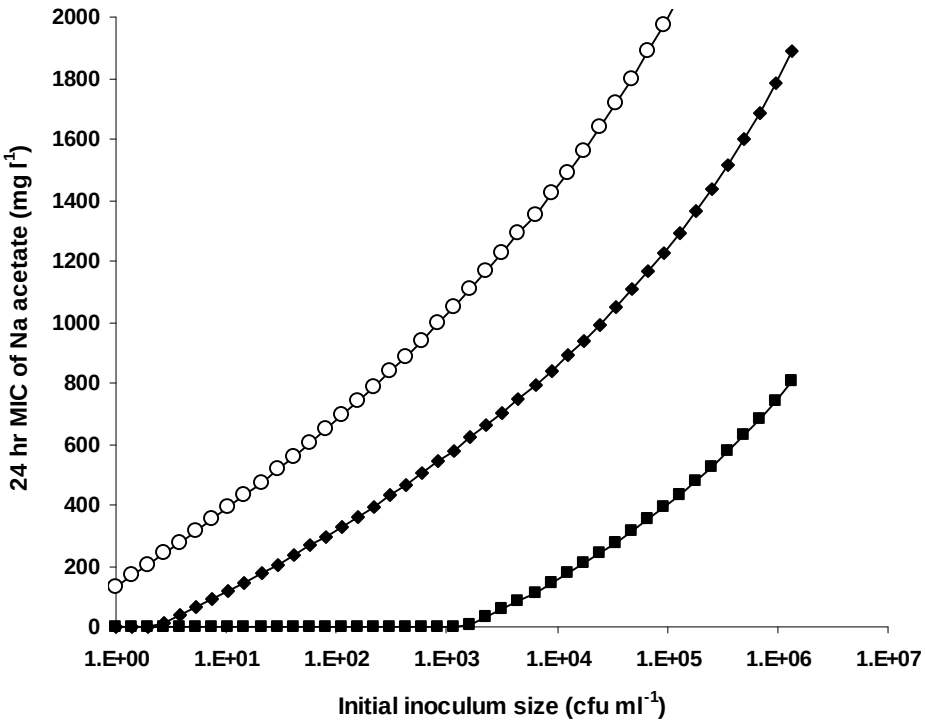
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964 Figure 9.



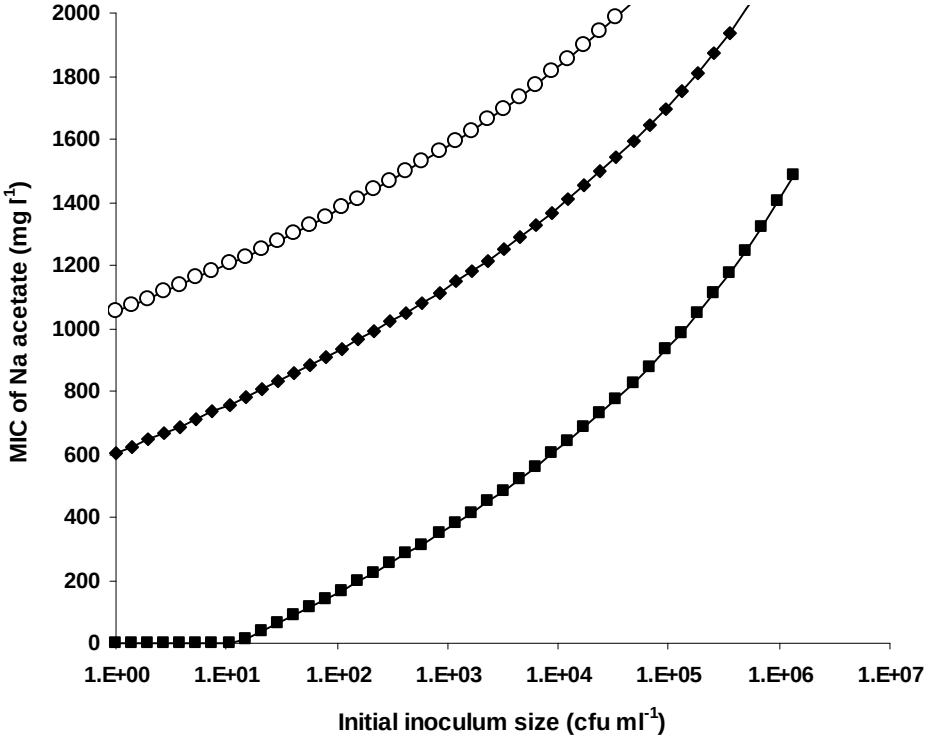
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966 Figure 10.



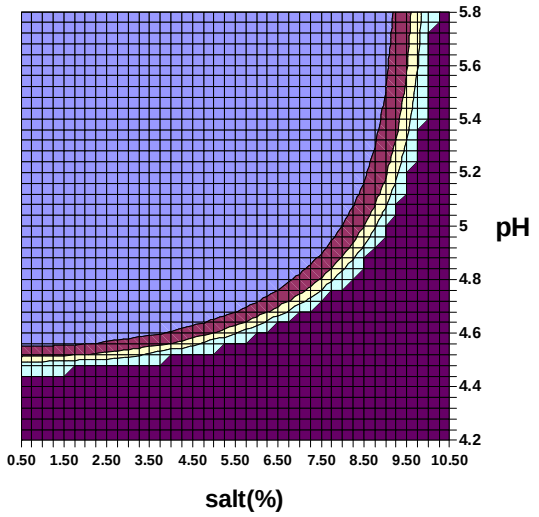
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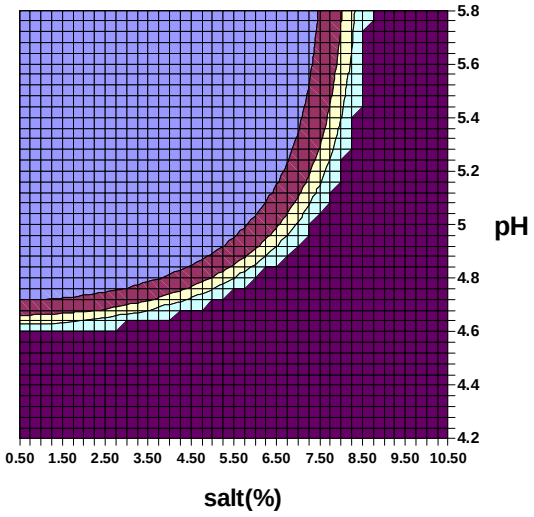
968 *Figure 11.*

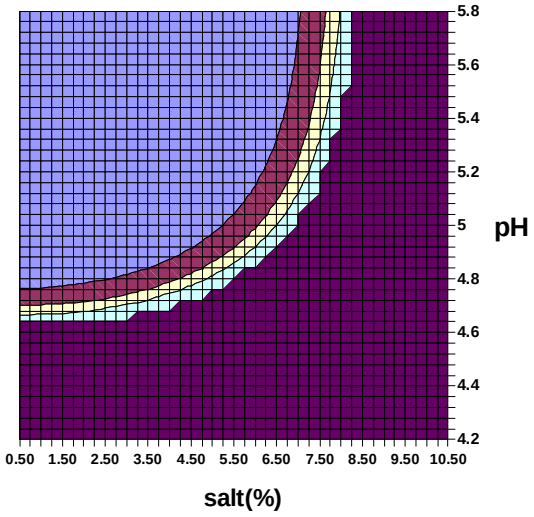


969

970 *Figure 12.*

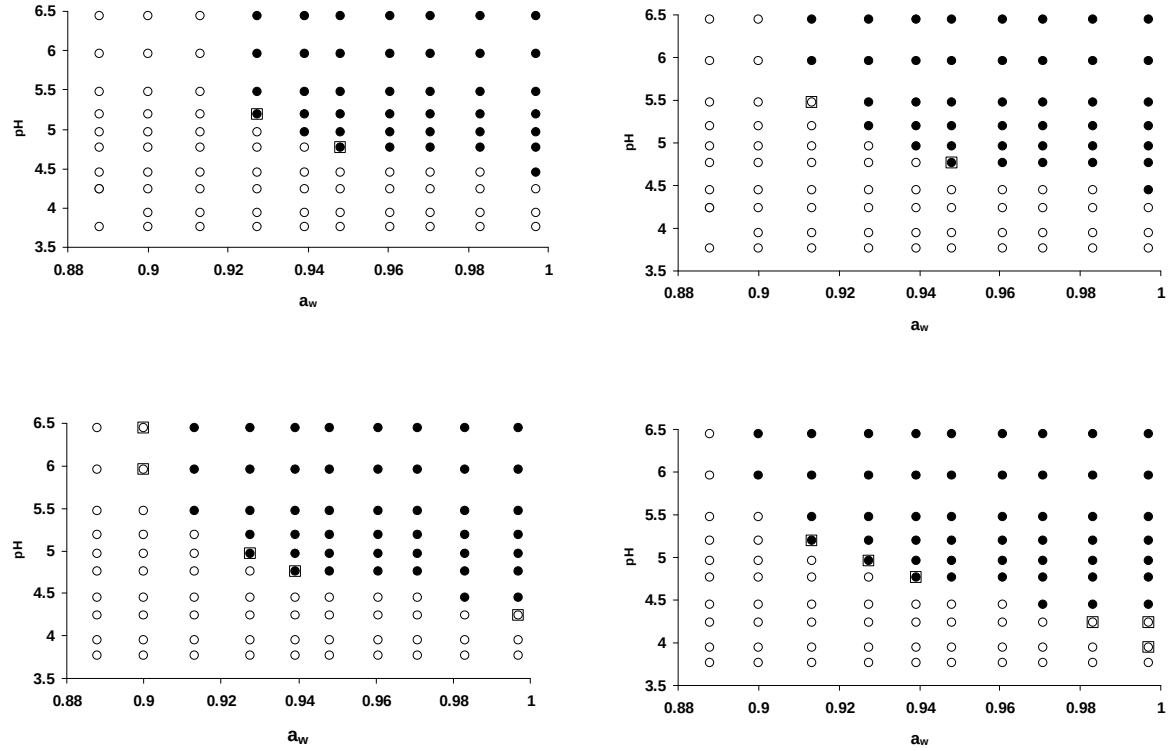






971

972 *Figure 13.*



973



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Bidlas, Eva; Du, Tingting; Lambert, Ronald J. W.

2008-08-15
Elsevier
http://dx.doi.org/10.1016/j.ijfoodmicro.2008.05.023
Eva Bidlas, Tingting Du, Ronald J.W. Lambert, An explanation for the effect of inoculum size on MIC and the growth/no growth interface, International Journal of Food Microbiology, Volume 126, Issues 1-2, 15 August 2008, Pages 140-152

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