MODELLING OF DIFFUSSION-LIMITED GROWTH TO PREDICT *Listeria* COLONIZATION OF STRUCTURED MODEL CHEESES

Estefanía Noriega, Adriana Laca and Mario Díaz*

Department of Chemical Engineering and Environmental Technology. University of Oviedo, Spain.

(*) Corresponding author:

Mario Díaz

Department of Chemical Engineering and Environmental Technology.

Faculty of Chemistry. University of Oviedo.

C/ Julián Clavería s/n.

33071. Oviedo. Spain.

Telephone number: +34-98-5103439

Fax number: +34-98-5103434

E-mail: mariodiaz@uniovi.es

ABSTRACT

Predicting microbial evolution in soft cheeses, which suffer the colonization of several pathogens, such as *Listeria monocytogenes*, is a main objective in the improvement of food safety conditions. In this work, a model that considers diffusional limitations during pathogen's growth in structured foods has been assayed. To test the feasibility of the model, *Listeria innocua* evolution with time and position was monitored in structured model cheeses prepared to mimic soft cheeses with different lactic acid contents. Prediction of biomass and substrate evolution requires to know the kinetics under different aeration conditions. *L. innocua* behaviour was also studied in broths prepared with similar composition to model model cheeses, evaluating the effect of oxygen concentration on cell growth. The assayed model provided quite accurate results for low concentration of acid lactic, whereas for lactic acid concentrations higher than 3 g l^{-1} cell development in structured media was inhibited.

Key Words: *food safety, Listeria, growth modelling, reaction and diffusion processes model foods, cheese, lactic acid.*

1. INTRODUCTION

Cheese intake is a fast and suitable way of assimilating significant amounts of proteins (vitamin A and B group vitamins, particularly riboflavin and B12, but also thiamine, niacin, B6 and folate) and most micronutrients (calcium, phosphorus, magnesium, potassium, iodine and zinc) (Madziva et al., 2006). Besides, the availability of hundreds of cheese types with the advantages of a ready to eat food makes this dairy product desirable for consumers (Pandey et al., 2003; Gameiro et al., 2006). About 18.3 million tons of cheese were produced worldwide in 2006 and an increase of 15 % above current levels is projected by 2014 (OECD-FAO, 2006).

Traditional raw-milk cheeses are strong-tasting foods with exceptional features due to the dynamic activity of the native microflora (Pandey et al., 2003; Millet et al., 2006). However, raw-milk products are labelled as a serious threat to the public health, owing to their involvement in outbreaks of pathogenic bacteria (De Buyser et al., 2001; Rogga et al., 2005).

Generally, raw-milk spoilage is caused by fecal or environmental cross-contamination involving poor quality of feeding stuffs, infrequent cleaning of farm facilities and animals, and unhygienic practices of milking (Lund et al., 2000; Schoder et al., 2003). The implementation of strict sanitary conditions on farm and raw-milk pasteurization prior to cheese-making becomes essential, even though the typical sensory quality of traditional cheeses is adversely affected (Pandey et al., 2003; Millet et al., 2006; Saubusse et al., 2007). However, the incidence of certain pathogens equally involves raw and pasteurized-milk cheeses, since post-pasteurization contamination can also arise as a result of uncontrolled manufacturing and ripening conditions (Rudolf et al., 2001; Silva et al., 2003; Saubusse et al., 2007;).

Among the high risk pathogens, *Listeria monocytogenes* is particularly troublesome to cheese industry, since outbreaks of listeriosis are responsible for high mortality rates. Besides, this pathogen represents a major cause of product recalls worldwide (Arqués et al., 2005; López-Pedemonte et al., 2007), such as 80 ton of cheese rejected in Germany in 2000 for being contaminated with listeria (Rudolf et al., 2001). In fact, cheeses may be contaminated at any stage from farm to table (Gameiro et al., 2006), since *L. monocytogenes* is endogenous to farm and cheese processing environments (Lund et al, 2000). In this sense, raw-milk has been widely recognised as a vehicle of listeriosis (Rogga et al., 2005) as consequence of either mastitic and asymptomatic animals shedding pathogen cells directly in milk (Arqués et al., 2005), or colonized stuffs, like water, soil, silage, feces, and animals skin coming into contact with it (Lund et al., 2000; Schoder et al., 2003). Thus, milk thermal processing seems to be almost mandatory to assure cheese safety (Pandey et al., 2003), although it has been reported that *L. monocytogenes* may survive pasteurization when it is located inside milk leukocytes (Doyle et al. 1987). Nevertheless, its natural presence in cheese processing facilities and its ability to grow at chilling temperatures and to tolerate salt and low pH have been established as the primary mechanism for contamination during manufacture, ripening and storage (Chambel et al., 2007).

Growth and survival of *L. monocytogenes* in cheeses are determined by the nature and activity of starter cultures, the rate and extent of acidification (pH and lactic acid content), the length, temperature and humidity of ripening, and the packaging and storage conditions (Morgan et al, 2001). In this sense, a higher incidence has been found in soft-white-fresh cheeses providing excellent growth conditions: high moisture (50-70 %), low acidity (pH 6) and low to moderate salt levels (Tsiotsias et al., 2002). Light cheeses based on the substitution of lipid components by proteins are also risk foods,

since microestructural modifications suffered by these cheeses provide a high nutrient and space availability for pathogen development (Parker et al., 1998).

Among the preventive strategies in "farm-to-fork" systems, the application of preservation procedures and the implementation of Good Manufacturing Practices (GMP), Good Hygienic Practices (GHP) and HACCP, represent the most widely practiced methods to control cheese spoilage (Saubusse et al., 2007). An emergent alternative is the development of mathematical models predicting the impact on cheese safety and quality of its formulation and processing, which allows to design the safest operational conditions and to avoid costly and time-wasting steps (Wilson et al., 2002). Most available models have been developed from experiments in liquid phase (McMeekin et al., 2002), but the absence of diffusional limitations in these broths leads to not entirely reliable predictions for real cheeses, where physical structure has been reported as a significant parameter (Parker et al., 1998). Models describing microbial growth evolution not only with time but also with position within the food are required, which implies that diffusional processes must be incorporated (Laca et al., 1998).

Estimation of growth kinetics under different controlled limitations, diffusional parameters and cheese features is necessary to complete the model, but the use of real cheeses with heterogeneous properties makes difficult to determine independently the effect of several parameters (Wilson et al., 2002). The design of model cheeses, simulating the cheese structure and composition makes easier the experimental labour and the understanding of the results (Koutsoumanis et al., 2004; Lebert et al., 2004; Sebti et al., 2004; Antwi et al., 2006). With this same aim, *Listeria innocua*, an innocuous species of similar behaviour to *L. monocytogenes*, has been frequently used instead of the pathogen (Le Marc et al., 2002; Liu et al., 2003; Nakai et al., 2004). This

118 microorganism is also habitual in cheeses, where it has been isolated in the same
119 proportion or more than *L. monocytogenes* (Carvalho et al., 2007).

120 In this work, *L. innocua* growth was studied in culture broths with similar
121 composition to soft cheeses, but with different lactic acid content in order to simulate
122 low and high acid cheeses. The effect of oxygen concentration on cell growth was also
123 evaluated in both media. Microbial evolution with time and position was also
124 determined in structured model cheeses. Finally, a model that combines diffusion and
125 reaction terms was employed to predict the evolution of cells and substrates within
126 model cheeses and results were compared with the experimental data.

127

128 **2. MATERIALS AND METHODS**

129 **2.1. Microorganisms**

130 A commercial active-dry strain of *L. innocua* (ATCC 33090) was acquired from the
131 Spanish Collection of Type Cultures (CECT), and maintained frozen and lyophilized at
132 -20 ºC.

133 **2.2. Culture media and experimental conditions**

134 *2.2.1. Broth model cheeses*

135 These cheese broths are derived from Richard´s medium (Gay et al., 1996) and
136 consist of an aqueous solution containing: 10 g l^{-1} Tryptone, (Panreac); 10 g l^{-1}
137 Casamino acids (Merck); 2 g l^{-1} Yeast extract (Panreac) and 0.5 g l^{-1} Glycerol (Sigma).
138 Lactic acid concentrations were adjusted to the required values (99 %, Sigma). After
139 autoclaving, pH was aseptically adjusted to 5.9 using NaOH 10 N. At this pH, lactic
140 acid is almost fully dissociated (lactate).

141 Depending on the oxygen availability for cell growth, three types of experimental
142 conditions were assayed:

143 - *Aerobic conditions* (7.6 - 8.0 mg dissolved oxygen l^{-1}): 1 litre Erlenmeyer flasks
144 containing 200 ml of medium were incubated in an orbital shaker (New Brunswick
145 G25), at 250 rpm and 25 ºC.
146 - *Hypoxic conditions* (0.2 - 2.6 mg dissolved oxygen l^{-1})*:* 1 litre full bottles closed with
147 screw tops were cultured at 25 ºC without shaking.
148 - *Anoxic conditions* (< 0.01 mg dissolved oxygen l^{-1}): Initial dissolved oxygen was
149 removed from the medium by flushing with sterile nitrogen, and bottles were incubated
150 under the same conditions as those described for the hypoxic conditions.

151 Preinoculum was prepared from a frozen stock and cultured, in each case, under the
152 same conditions employed in the respective experiment. The inoculum was adjusted to
153 give an initial concentration of about 10^5 CFU ml^{-1}. This value is slightly higher than the
154 detection threshold of the analytical method applied to monitor cell concentration in the
155 structured food. It was experimentally probed that *L. innocua* growth curve was very
156 similar for inocula between 10^2 and 10^5 CFU ml^{-1}, and only the length of the lag phase
157 was affected. So, the results obtained with an inoculum size of 10^5 CFU ml^{-1} will be
158 representative of the microbial behaviour at lower initial concentrations, only by taking
159 into account the reduction/extension of the lag phase. Besides, this value is close to the
160 levels reached from the direct contamination by dairy cattle, 10^3 to 10^4 CFU ml^{-1} of
161 milk (Lund et al., 2000; López-Pedemonte et al., 2007).

162 *2.2.2. Structured model cheeses*

163 Structured model cheeses were prepared by adding a solidifying reagent to the
164 cheese broths above described. The most suitable hardening agent and its concentration
165 in the simulated food were selected so that the melting temperature of the gel does not
166 affect cell viability, a necessary condition to reach a homogeneous inoculation. After

testing several concentrations (0.8- 5 w v^{-1}), κ-carrageenan at 1.45 % (w v^{-1}) was selected as the hardening agent

Autoclaved cheese broths with κ-carrageenan were kept liquid in a shaking water bath at 40 ºC, inoculated and mixed. Inoculated medium was solidified into several sterile glass test tubes (16 cm length; 1.5 cm diameter) closed with cotton caps and incubated at 25 ºC. Sampled tubes were removed in order to avoid contamination of the model food.

These structured model cheeses were characterized by measuring their strength and water activity. For the selected κ-carrageenan concentration, media strength was 235 g cm^{-2}, similar to that determined in a commercial soft cheese, 220 g cm^{-2}, and water activity was 0.986, a value within the optimum range for microbial growth.

All experiments were carried out at least in duplicate.

2.3. Sample taking and analytical methods

2.3.1. Broth model cheeses

Samples to determine *Listeria* growth were taken not removing more than 10 % of the initial medium. Cells were harvested by centrifugation (Eppendorf 5415D) at 13200 g for 10 min, resuspended in sterile peptone salt water and the viable number of bacteria was determined by plating on BHI supplemented with agar, 1.2 % (w v^{-1}) (30 ºC for 48 h). Plates were prepared at least in triplicate. These values of viable cells were related to dry weight using a calibration curve.

A sterile pH electrode (Easyferm, Hamilton), a dissolved oxygen meter (model 58, YSI) and a water activity-meter (FA-st lab Prosoft, GBX) based on the hygrometric method (dew point) were employed for the measurements.

192 *2.3.2. Structured model cheeses*

193 A hollow cylindrical device was employed to take samples from tubes containing
194 the structured model food. A sterile scalpel was used to divide the removed gel
195 cylinders into small portions of approximately 0.5 cm in length. Experimental data of
196 cell growth were obtained by dissolving the sections corresponding to different axial
197 positions of solid cylinders (top, middle and bottom) in NaCl 0.9 % (w v^{-1}) at 40 ºC
198 with gently shaking for about 15 min, and plating on BHI plates supplemented with
199 agar, 1.2% (w v^{-1}). These plates were incubated at 30 ºC for 48 h and prepared at least in
200 triplicate.

201 Strength of model cheeses was measured by means of a penetrometer with 1 cm^2 of
202 surface, placing different weights on it. The strength of the material is the force applied
203 during 20 seconds necessary to break a 3-centimeter-thick gel, stabilized at 20 ºC. Water
204 activity was estimated with the previously described hygrometric method. These
205 analytical measurements were developed in triplicate.

206

207 **3. RESULTS AND DISCUSSION**

208 **3.1. Broth model cheeses**

209 *L. innocua* is a facultative anaerobic bacterium, and dissolved oxygen concentration
210 in the culture medium governs its metabolism. Due to its relatively low solubility in
211 water, oxygen is the substrate most likely to limit microbial growth in dairy liquid
212 products, but especially in solid foods, where the presence of diffusional limitations
213 play an important role.

214 In order to know the effect of oxygen on bacteria development. *L. innocua* growth
215 was monitored in the cheese broths under different oxygen concentrations ranging from
216 almost 100 % air saturation to oxygen absence. Figures 1 and 2 show microbial growth

217 under aerobic, hypoxic and anoxic conditions in the cheese broths prepared with
218 different concentrations of lactic acid.

FIGURE 1 **FIGURE 2**

219 Microbial growth took place under all assayed aeration levels. However, cell growth
220 kinetics were strongly influenced by the oxygen availability. A higher oxygen
221 concentration in the culture medium increased the cell concentration achieved at
222 stationary phase, the specific growth rate at exponential phase and lag time (Tables 1
223 and 2). On the other hand, significant differences were not found between maximum
224 cell concentration under hypoxic and anoxic conditions, which shows that the initial
225 presence of low oxygen concentrations in the medium almost do not change the
226 development of *Listeria*.

227 In addition, a delay in lag phase and lower specific growth rates were observed in
228 the cheese broth with higher lactic acid concentration, under all studied conditions. This
229 ability of lactic acid to inhibit microbial growth (Choi et al., 2003, Deumier et al., 2003)
230 may be attributed to the reduction of water activity in the product (Stekelenburg et al.,
231 2001), and the acidification of internal pH that interferes with metabolic processes such
232 as proton motive force, ATP synthesis, enzyme activities, and uptake of substrates such
233 as amino acids entering the cells by active transport (Deumier et al., 2003).
234 Nevertheless, *Listeria* shows high acid tolerances when it is exposed to a gradual
235 decrease in pH (Rogga et al., 2005), as occurs in cheese fermentation and it can grow in
236 the presence of significative concentrations of lactic acid (Nakai et al., 2004).

237

238 **3.2. Structured model cheeses**

239 *L. innocua* was inoculated on the surface of structured model cheeses prepared as
240 explained in Materials and Methods with different concentrations of lactic acid (10.6, 7,

241 5, 4, 3, 1 and 0.5 g l^{-1}) and incubated at 30 ºC. Colonies grew on media with lactic
242 concentration ≤ 3 g l^{-1}, but there was no growth for concentrations ≥ 4 g l^{-1}. In fact, the
243 minimum inhibitory concentration of lactic acid at pH 5.25 for *L. innocua* has been
244 reported as 6.33 g l^{-1} (Nakai et al., 2004). On the opposite, as explained before, cell
245 growth took place in liquid media even for lactic acid concentrations of 10.6 g l^{-1}. The
246 structured environment together with a relatively high concentration of lactic acid seems
247 to play a protector role against Listeria contamination.

248 Two concentrations of lactic acid (10.6 and 3 g l^{-1}) were selected for a deeper study,
249 and *L. innocua* was homogeneously inoculated in the two selected structured media.
250 Concentration profiles along test tubes will be representative of those obtained in foods
251 with similar size and structure. Evolution of cell concentration with time and position in
252 the structured model cheeses at 25 ºC is shown in Figures 3 and 4.

FIGURE 3 **FIGURE 4**

253 Microbial growth was observed throughout the solid medium with low lactic content
254 (Figure 3), although biomass levels on the surface were thirteen percent higher than cell
255 concentration found inside the solid. Concentrations achieved at 3 cm from the surface
256 were the same as that found at the bottom of the tube (6 cm). This means that further a
257 few centimetres from the surface, cell development was independent of location.
258 Specific growth rate and lag time values were practically the same for all positions.
259 These concave profiles of cell concentration can be correlated with high values of the
260 Thieles modulus for the substrate (Laca et al., 1998) and they are probably due to
261 different availability of oxygen throughout the food. This substrate coming from the
262 environment is at much higher concentrations on the surface than inside the solid, and
263 the diffusion of the oxygen is likely to be a key parameter for development of this
264 facultative bacterium in foods. Comparing these results with those obtained in the

265 respective cheese broth, the specific growth rate and lag time values practically
266 remained constant in all cases. Maximum cell concentrations achieved in solid and
267 liquid phase were in the same order except for aerobic conditions, where a more
268 pronounced growth was observed with respect to the rest.

269 As expected, the structured model cheese with high lactic acid concentration showed
270 a totally opposite behaviour, since a substantial decrease in cell concentration with time
271 was monitored for all positions (Figure 4).

272

273 **3.3. Modelling**

274 *3.3.1. Broth model cheeses*

275 Growth curves in the cheese broths under aerobic, hypoxic and anoxic conditions
276 were fitted to Ricatti's equation (Bailey & Ollis, 1986).

277 $$r_x = \frac{dC_x}{dt} = K \cdot C_x \cdot (1 - \tau \cdot C_x) \quad (1)$$

278 where K and τ ($1/C_{x\ \text{stationary phase}}$) are kinetic parameters. Parameter values
279 obtained from fitting are shown in Tables 1 and 2 and the solid lines of Figures 1 and 2
280 show the theoretical data of cell growth in each broth, respectively.

TABLE 1 **TABLE 2**

281 Values of the parameter τ decreased as oxygen concentration in the culture media
282 rose and this trend was more pronounced for the cheese broth with high lactic acid
283 content. Anyway, these values were always lower in the cheese broth with 3 g l^{-1}, which
284 means that a higher cell development was achieved. The K parameter value decreased
285 with oxygen concentration, being similar for both media, although slightly lower in the
286 cheese broth with high lactic concentration. These kinetic parameters will be used to
287 model the microbial behaviour in solid medium.

288

289 *3.3.2. Structured model cheeses*

290 To carry out the modelling of *L. innocua* behaviour in the structured model cheeses,
291 a homogeneous food with cylindrical geometry and constant properties was supposed
292 and oxygen was considered as limiting substrate of cell growth. It can be also thought
293 that the surface and the bottom of the cylinder can simulate the effects on the surface
294 and the core of spherical shape-cheeses. Considering a differential volume element,
295 mass balances within model cheeses were set out for all involved solutes, besides initial
296 and boundary conditions for biomass and substrate:

297 - Biomass balance

298 $$\frac{\partial C_x}{\partial t} = D_x \cdot \frac{\partial^2 C_x}{\partial z^2} + r_x \quad (2)$$

299 - Substrate balance

300 $$\frac{\partial C_s}{\partial t} = D_s \cdot \frac{\partial^2 C_s}{\partial z^2} + r_s \quad (3)$$

301 - Initial conditions:

302 $$t = 0, C_s = C_{s_{sat}}, C_x = C_{x_o} \quad (4)$$

303 - Boundary conditions:

304 $$z = L \text{ (surface)}, C_s = C_{s_{sat}} \quad (5)$$

305 $$z = 0 \text{ (bottom)}, \frac{\partial C_x}{\partial z} = 0, \frac{\partial C_s}{\partial z} = 0 \text{ (symmetry condition)} \quad (6)$$

306 C_x and C_s are cell and oxygen concentrations respectively in the solids, $C_{s_{sat}}$ is the
307 saturation oxygen concentration in the solids at 25 ºC, C_{x_o} is the initial cell
308 concentration in the solids, r_x and r_s are cell growth and oxygen consumption rates, t is
309 the time, z is the position. D_s is the oxygen diffusion coefficient in solid phase and D_x is
310 the cell pseudodiffusion coefficient in solid phase defined by equation 2. A FORTRAN
311 program was used to solve this system of differential equations.

312 Kinetic parameters, K and τ, of Ricatti´s equation obtained from experimental data
313 in the cheese broths under several aeration conditions (see Tables 1 and 2) were used in
314 the simulation. K hardly changed with oxygen concentration, so it was assumed to be a
315 constant (Tables 3 and 4). τ values were correlated (equation 7) as a function of

316 $$\tau = a - b \cdot C_s \quad (7)$$

317 oxygen concentration (an average value was taken in each case). The values of a and b
318 in this equation are given in Tables 3 and 4 from the results in liquid phase.

319 Oxygen uptake rate was expressed as $r_s = -(Y_{o/x} \cdot r_x + m_o \cdot C_x)$, where $Y_{o/x}$ is the
320 inverse of the biomass yield on oxygen and m_o is the maintenance coefficient for
321 oxygen. These parameters were obtained from average values in literature (Bailey,
322 1986).

323 For initial conditions, it was assumed that solid media were completely saturated
324 with oxygen at the beginning, and it was also supposed that this maximum
325 concentration of oxygen was maintained on the surface all time. Owing to the lack of
326 bibliographic data, saturation oxygen concentration, $C_{s_{sat}}$, in the model cheeses was
327 determined as a first approach from saturation oxygen concentration in water and gel
328 porosity (ε_g) as follows: $C_{s_{sat}} = C_{s_{sat\ (water)}}\, \varepsilon_g$. Gel porosity was assumed 95 % (Wijffels,
329 Gooijer, Schepers, Beuling, Mallée & Tramper, 1995), giving a $C_{s_{sat}}$ of 7.9 mg l^{-1}.

330 Oxygen diffusion coefficient in κ-carrageenan has been reported to fluctuate
331 between 1.5-2.1·10^{-9} m^2 s^{-1}, in a range of gel concentrations of 0.5-5 % (w v^{-1}) (Gooijer,
332 Wijffels & Tramper, 1991; Wijffels et al., 1995; Varzakas, Leach, Israilides &
333 Arapoglou, 2005). In this system, diffusion coefficient was estimated by interpolating κ-
334 carrageenan concentration used in the model cheeses, 1.45 % (w v^{-1}). Cell diffusion
335 coefficient in κ-carrageenan was supposed similar to this established for *Serratia*

336 *marcescens* immobilized in alginate beads (Laca et al., 1998). As expected, this value of
337 pseudodiffusion is very low. Other parameters to be considered in the simulation were
338 initial cell concentration and length of solid cylinders inside test tubes. In Tables 3 and
339 4, parameters employed in modelling and initial conditions are summarized.

TABLE 3 **TABLE 4**

340 Regarding the structured model cheese with high lactic content, because of its
341 inhibitory effect, theoretical biomass profiles (Figure 5) obtained from simulation were
342 very different from the real behaviour, since the model is based on kinetics from liquid
343 medium, where microbial growth was still observed. Growth absence in this solid
344 medium, with a death constant of 3.1×10^{-5} s^{-1}, could be caused by lactic inhibition, as
345 mentioned before, by gradients of oxygen concentration and by cell environment.

346 **FIGURE 5**

347 With low lactic acid content, theoretical biomass profiles for the structured model
348 cheese were qualitatively similar to the experimental results, achieving a good fitting
349 with regard to lag phase and growth rate. Only some differences were found between
350 cell concentrations reached on the surface, the model predicts a higher value for the
351 stationary state. Nevertheless, this difference significantly reduces if the rest of the solid
352 is considered. The hollow symbols of Figure 6 show the agreement between the real and
353 experimental data.

354 **FIGURE 6**

355 These differences may be related to the cell confinement in a solid matrix, since it is
356 well-known that, the solid environment surrounding the cells may cause itself
357 alterations in cell development, morphology and membrane permeability, as well as, in
358 surface tension and osmotic pressure (Dervakos & Webb, 1991). All this may have
359 important repercussions to such an extent that microorganisms immobilized in a solid

360 support can suffer changes in their metabolism and growth rate (Meldrum,
361 Brocklehurst, Wilson & Wilson, 2003) and consequently in the kinetic parameters of
362 equation 1.

363 The K parameter of Ricatti´s equation does not appear to be influenced by the
364 environment, whereas cell confinement might justify a reduction in the τ value. In fact,
365 a small increase of the value in the τ function (from 16 to 40 ml^2 mg^{-2}) gives a
366 theoretical maximum cell concentration on the surface in agreement with experimental
367 data, as it is shown by the solid symbols of Figure 6. The cell and oxygen
368 concentrations evolution with time in the different points of the solid can be now
369 obtained for the new τ value. Results are given in Figure 7.

370 **FIGURE 7**

371 In agreement with the model, oxygen was theoretically taken up faster than it
372 diffused during the first hours, so the hypothetical concentration after eighteen hours
373 was practically null throughout the solid, except for the surface. As the microbial
374 growth rate decreased, the metabolic oxygen requirements also decreased and
375 diffusional supply of oxygen was higher than its consumption. As a consequence,
376 oxygen concentration increased at longitudinal positions $z/L > 0.4$, as shown in Figure
377 7. These oxygen concentration profiles gave rise to a higher cell development on surface
378 and nearest zones, whereas growth in the rest of the solid was almost the same,
379 independently of location.

380 It is convenient to point out that the uncertainty of other parameters could also
381 influence the results of modelling. For example, saturation concentration of oxygen in
382 κ-carrageenan has been estimated from the respective value in water and gel porosity,
383 and this value could be not maintained on the surface during the culture. Biomass yield
384 on oxygen, maintenance coefficient for oxygen and diffusivity parameters also

385 influence considerably model's results. Presence of some inhibitory substances, for
386 example lactate, could also affect the kinetic equation (equation 1).

387 Taking into account the complexity of the analysed systems, the model describes
388 quite properly the experimental behaviour shown in the structure model cheese with
389 lactic acid concentration of 3 g l^{-1} or below. Accurate predictions of microbial behaviour
390 in these cases can be reached from kinetics in liquid medium and considering oxygen as
391 the key substrate for cell development.

392 Regarding the structured model cheese with lactic acid concentration equal to 4 g l^{-1}
393 or above, to predict the behaviour in solid media from studies in liquid media it is
394 necessary to introduce an inhibitor term for growth kinetics, since the behaviour is
395 whole the opposite, decreasing the cell concentration with a death constant of 3.1 10^{-5} s
396 in the studied case.

397

398 **4. CONCLUSIONS**

399 *Listeria innocua* growth was significantly affected in cheese broths by oxygen
400 availability and lactic concentration. Ricatti's kinetics suitably described microbial
401 growth under all aeration conditions tested.

402 In the solid structured medium, lactic acid concentration is a key parameter to
403 determine the absence / presence of *Listeria* growth. For low concentrations ($\leq$ 3 g l^{-1}), a
404 preferential growth on surface was observed, being inside quite independent of location.
405 This behaviour is likely caused by diffusional limitations that gave rise, at different
406 depth, to different oxygen concentrations, determining the maximum cell concentration.
407 On the opposite, higher lactic acid concentrations ($\leq$ 4 g l^{-1}) completely inhibited
408 microbial development in the solid medium.

To model *L. innocua* growth in structured cheeses, the use of kinetics from liquid medium as a function of oxygen concentration gave rise to a quite proper simulation of experimental data for low lactic concentrations (≤ 3 g l^{-1}), and significant differences were only detected in predictions for the surface. In order to develop a model also suitable for higher lactic acid concentrations, an inhibition term should be incorporated.

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419 **SYMBOLS**

420	A	Slope of the straight line relating lag time and temperature
421	a, b	Constants of τ function
422	BHI	Brain Heart Infussion
423	CECT	Spanish Collection of Type Cultures
424	CFU	Colony forming units
425	C_s	Substrate (oxygen) concentration
426	$C_{s_{sat}}$	Saturation oxygen concentration in κ-carrageenan at 25 ºC
427	C_x	Cell concentration
428	C_{x_o}	Initial cell concentration
429	OD	Optical density
430	D_s	Substrate (oxygen) diffusion coefficient
431	D_x	"Cell diffusion" coefficient
432	K	Kinetic parameter of Ricatti´s equation
433	L	Distance between food core and surface
434	m_o	Maintenance coefficient for oxygen
435	r_s	Oxygen uptake rate
436	r_x	Cell growth rate
437	t	Time
438	t_d	Lag time
439	t_{do}	Origin ordinate of the straight line relating lag time and temperature
440	T	Temperature
441	τ	Kinetic parameter of Ricatti´s equation (1/Cx stationary phase)
442	μ	Specific growth rate at exponential phase of growth

443	$Y_{o/x}$	Inverse of the biomass yield on oxygen, mg O_2 consumed (mg biomass)$^{-1}$
444	z	Longitudinal coordinate

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575 **FIGURE CAPTIONS**

576 Figure 1. *L. innocua* growth in the cheese broth with 3 g l^{-1} lactic acid (25 ºC, pH 5.9),
577 under aerobic (△), hypoxic (◭) and anoxic (▲) conditions. Experimental (symbols) and
578 fitted (line) data to Ricatti´s equation. Inoculum size 4.9 10^{-4} mg ml^{-1}.

579 Figure 2. *L. innocua* growth in the cheese broth with 10.6 g l^{-1} lactic acid (25 ºC, pH
580 5.9), under aerobic (△), hypoxic (◭) and anoxic (▲) conditions. Experimental
581 (symbols) and fitted (line) data to Ricatti´s equation. Inoculum size 4.9 10^{-4} mg ml^{-1}.

582 Figure 3. *L. innocua* growth in the structured model cheese with 3 g l^{-1}. z/L = 0
583 (bottom); z/L = 0.5 (middle); z/L = 1 (surface). Inoculum size 4.9 10^{-5} mg ml^{-1}.

584 Figure 4. *L. innocua* growth in the structured model cheese with 10.6 g l^{-1}. z/L = 0
585 (bottom); z/L = 0.5 (middle); z/L = 1 (surface). Inoculum size 1.9 10^{-4} mg ml^{-1}.

586 Figure 5. Theroretical profiles of cell (a) and oxygen (b) concentration in the structured
587 model cheese with 10.6 g l^{-1} (z/L = 0, bottom; z/L = 1, surface) considering parameters
588 shown in Table 4 and kinetics from liquid medium.

589 Figure 6. Comparison between experimental and model results of *Listeria* growth on the
590 surface (z/L = 1, ■□), in the middle (z/L = 0.5, ▲△) and at the bottom (z/L = 0, ◆◇)
591 of the structured model cheese with 3 g l^{-1}, considering kinetics from liquid medium
592 (hollow symbols) and kinetics modified for solid medium (solid symbols).

593 Figure 7. Theroretical profiles of cell (a) and oxygen (b) concentration throughout the
594 structured model cheese with 3 g l^{-1} (z/L = 0, bottom; z/L = 1, surface) considering
595 parameters shown in Table 3 and kinetics modified for solid medium.

596 **FIGURES**

597 Figure 1

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609 Figure 2

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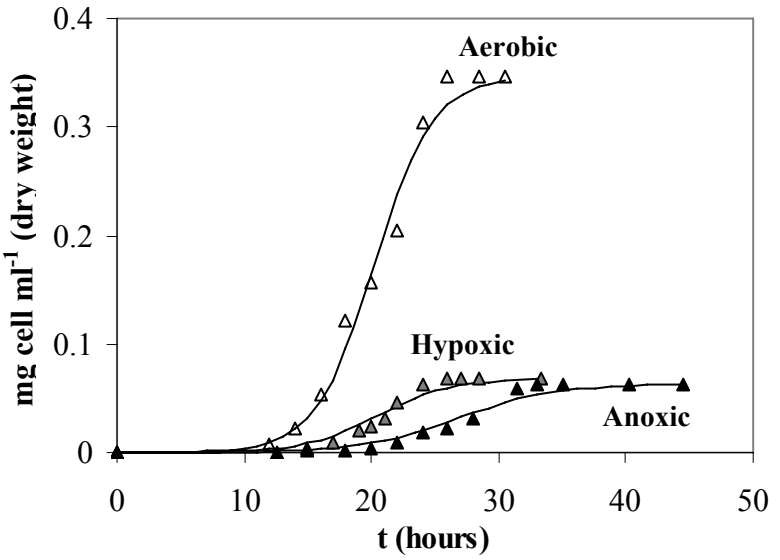


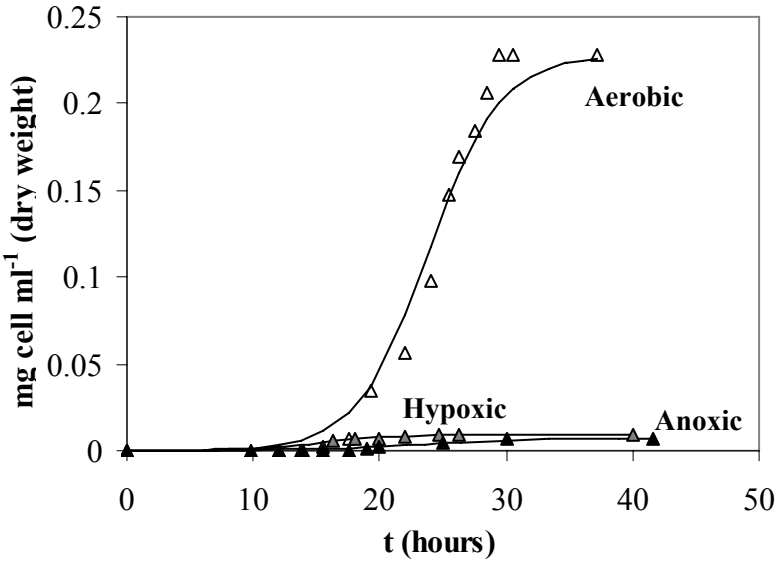
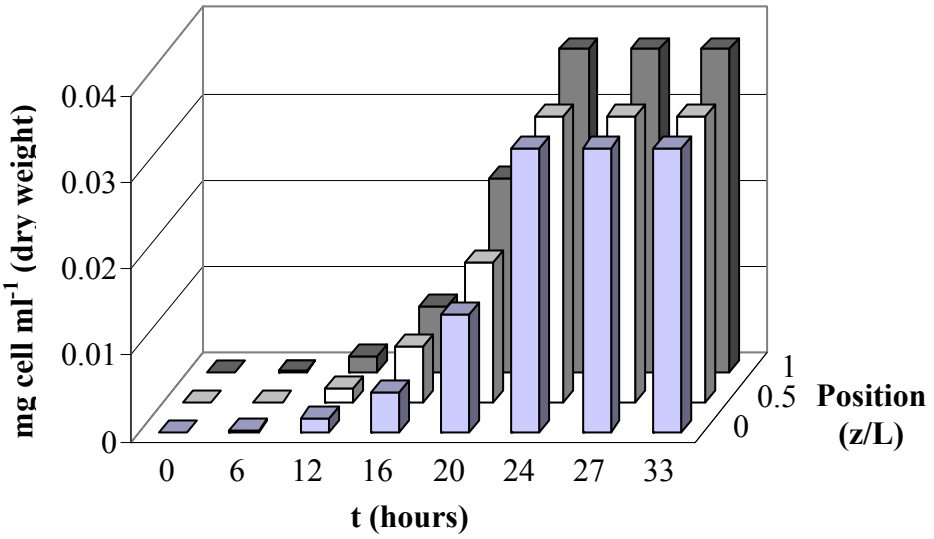


Figure 3



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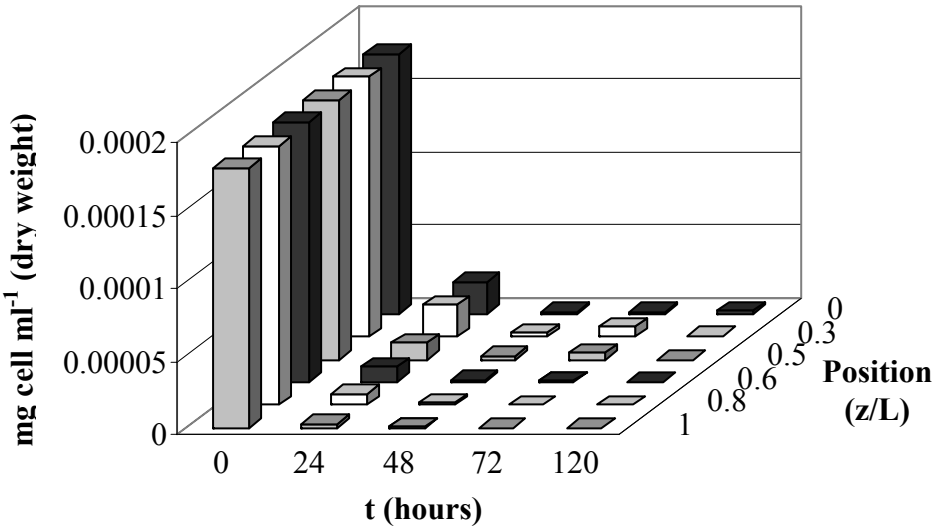

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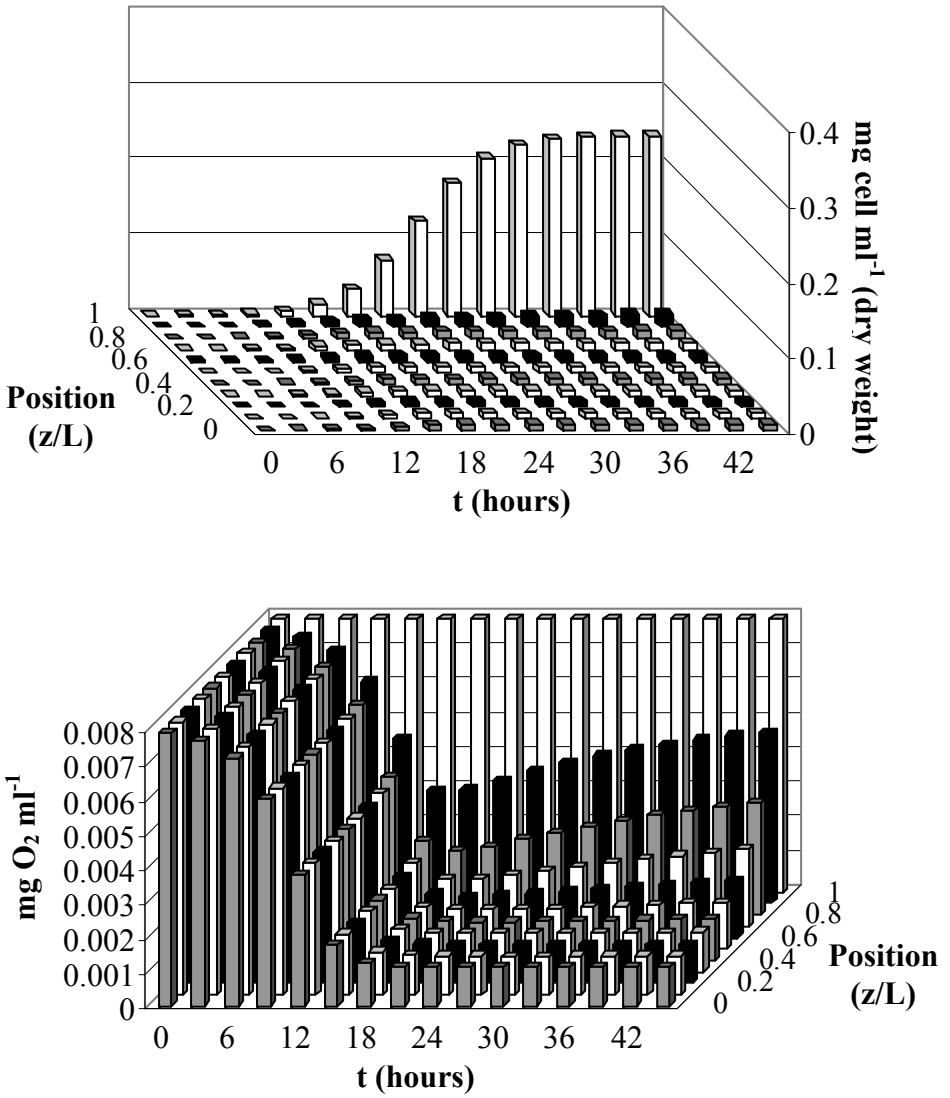

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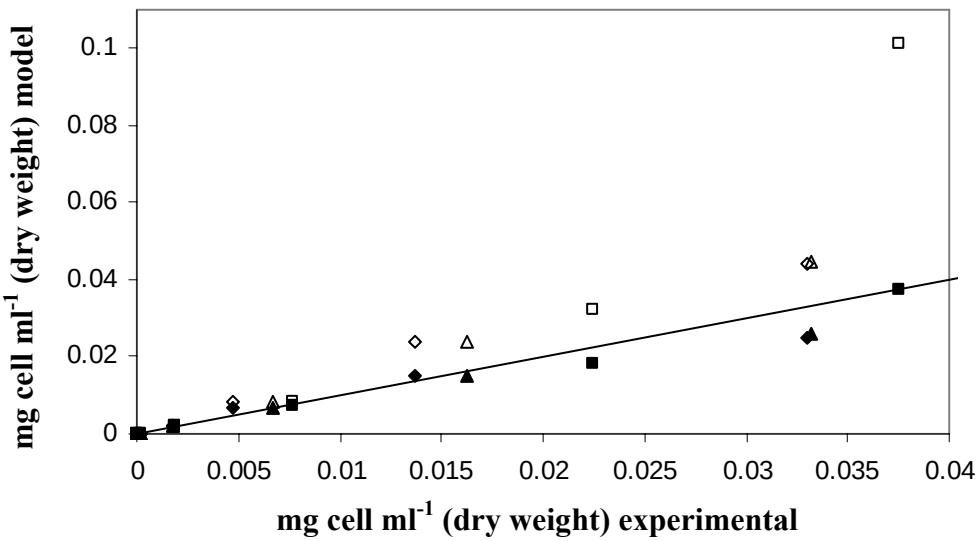

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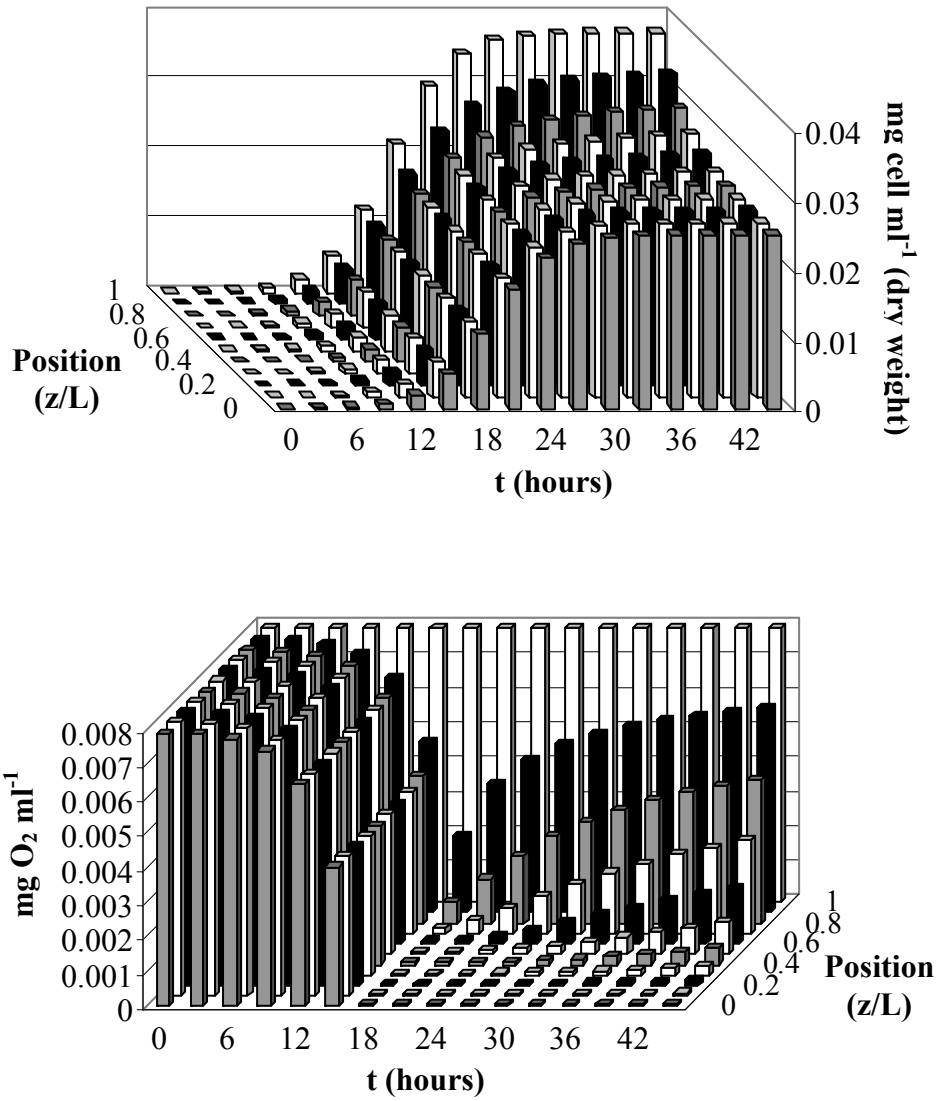
mg cell ml-1 (dry weight)
0.04
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Position (z/L)
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0.6
0.4
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0 6 12 18 24 30 36 42
t (hours)
mg O2 ml-1
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0.007
0.006
0.005
0.004
0.003
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0 6 12 18 24 30 36 42
t (hours)
Position (z/L)
1
0.8
0.6
0.4
0.2
0

696 **TABLES**

697 Table 1. Kinetic parameters of *L. innocua* growth in cheese broth (3 g l^{-1})

698

Conditions	**Parameters**			
	μ (s^{-1})	$\mathbf{t_d}$ (h)	**K** (s^{-1})	**τ** (ml mg^{-1})
Aerobic (7.6-8.0 mg l^{-1} O_2)	1.1 10^{-4}	12	1.2 10^{-4}	2.9
Hypoxic (0.2-2.6 mg l^{-1} O_2)	8.6 10^{-5}	13	9.8 10^{-5}	14.7
Anoxic (< 0.01 mg l^{-1} O_2)	6.4 10^{-5}	15	7.4 10^{-5}	15.8

699

700 Table 2. Kinetic parameters of *L. innocua* growth in cheese broth (10.6 g l^{-1})

701

Conditions	**Parameters**			
	μ (s^{-1})	$\mathbf{t_d}$ (h)	**K** (s^{-1})	**τ** (ml mg^{-1})
Aerobic (7.6-8.0 mg l^{-1} O_2)	8.8 10^{-5}	16	9.9 10^{-5}	4.4
Hypoxic (0.2-2.6 mg l^{-1} O_2)	7.1 10^{-5}	17	9.5 10^{-5}	110.4
Anoxic (< 0.01 mg l^{-1} O_2)	4.9 10^{-5}	18	5.9 10^{-5}	136.9

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703 Table 3. Parameters and initial conditions considered for simulating *L. innocua* growth
704 in structured model cheese (3 g l^{-1})

705

Parameters	**Initial conditions**
$D_x = 3.0\ 10^{-14}\ m^2\ s^{-1}$	$C_{x_o} = 4.9\ 10^{-5}\ mg\ ml^{-1}$
$D_s = 1.6\ 10^{-9}\ m^2\ s^{-1}$	$C_{s_{sat}} = 7.9\ 10^{-3}\ mg\ ml^{-1}$
$K = 9.8\ 10^{-5}\ s^{-1}$	
$a = 16\ ml\ mg^{-1}$, $b = 1689\ ml^2\ mg^{-2}$	
$L = 6\ cm$	

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707

708 Table 4. Parameters and initial conditions considered for simulating *L. innocua* growth

709 in structured model cheese (10.6 g l^{-1})

710

Parameters	**Initial conditions**
$D_x = 3.0\ 10^{-14}\ m^2\ s^{-1}$	$C_{x_o} = 1.9\ 10^{-4}$ mg ml^{-1}
$D_s = 1.6\ 10^{-9}\ m^2\ s^{-1}$	$C_{s_{sat}} = 7.9\ 10^{-3}$ mg ml^{-1}
$K = 8.4\ 10^{-4}\ s^{-1}$	
$a = 135$ ml mg^{-1}, $b = 16617\ ml^2\ mg^{-2}$	
L = 6 cm	

711