Title: **Effects of three heavy metals on the bacteria growth kinetics. A bivariate model for toxicological assessment.**

Running title: **A bivariate model for toxicological assessment.**

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ABSTRACT

The effects of three heavy metals (Co, Ni and Cd) on the growth kinetics of five bacterial strains with different characteristics (*Pseudomonas* sp., *Phaeobacter* sp. strain 27-4, *Listonella anguillarum*, *Carnobacterium piscicola* and *Leuconostoc mesenteroides* subsp. *lysis*) were studied in a batch system. A bivariate model, function of time and dose, is proposed to describe simultaneously all the kinetic profiles obtained by incubating a microorganism at increasing concentrations of individual metals. This model combines the logistic equation for describing growth, with a modification of the cumulative Weibull's function for describing the dose-dependent variations of growth parameters. The comprehensive model thus obtained –that minimizes the effects of the experimental error– was statistically significant in all the studied cases and it raises doubts about toxicological evaluations that are based on a single growth parameter, especially if it is not obtained from a kinetic equation. In LAB cultures (*C. piscicola* and *L. mesenteroides*), Cd induced remarkable differences in yield and time-course of characteristic metabolites. A global parameter is defined ($ED_{50,\tau}$: dose of toxic chemical that reduces the biomass of a culture by 50% compared to that produced by the control at the time corresponding to its semimaximum biomass) that allows to compare toxic effects on growth kinetics using a single value.

Keywords: bacteria growth kinetics, logistic and Weibull equations, heavy metals, toxicity, dose-response modeling

INTRODUCTION

The microbial culture in a limited medium is a useful tool for assessing the biological activity of physical and chemical agents by dose-response (DR) analysis. This tool has been applied to goals as diverse as the study of the effect of electric pulses on cell viability (Peleg 1995), quantification of bacteriocins (Cabo et al. 1999; Vázquez et al. 2004a), probiotic tests (Vázquez et al. 2005a) or toxicological evaluations (Nyholm et al. 1992; Gikas 2007) The usual procedure in a DR analysis is based on the assumption that some quantity calculable from the growth data (some parameter of the growth equation, frequently the maximum specific growth rate, μ_m) varies depending on the dose according to a sigmoid model (Murado et al. 2002; Riobó et al. 2008a). Routine applications in toxicological assessments, such as those described in some legal norms, often replace the use of a growth equation with a simpler approach (ISO 1995; ISO 2006; Strotmann and Pagga 1996). Thus, it is common to estimate the specific growth rate from biomass measured at two points in the exponential phase, accepting that the appropriate interval for this measure is the same in the control units as in those treated with the chemical (ISO 2006). Although, in principle, the problem is simple and easy for standardize, it has several interrelated difficulties that can lead to questionable results and interpretations.

First, the variations in specific (μ_m) or absolute (v_m) maximum growth rate may not explain the differences between the kinetic profiles of the control and toxic-dosed cultures. This is because the kinetic profile also depends on factors such as yield (biomass production/substrate consumption) that the biological entity obtains from the carbon and energy sources available, and the duration of the lag phase, that is, the time required by the organism to adjust its enzymatic system to environmental conditions. Although these factors represent aspects of the same metabolic system, each factor defines a subsystem that can be independently affected by the toxic agent. In other words, a substance can affect, independently or not, three parameters of the growth equation: maximum growth rate (μ_m or v_m), maximum biomass (X_m) and lag phase

(λ). As a result, no assessment based on the variation of a single parameter can in general explain the disturbance that a toxic substance produces in the biological system tested.

Moreover, any change in these parameters produces variations in the kinetic profile that move in time the location of the exponential phase or change its duration. This requires the use of different time intervals with each dose even for the simplified calculation of μ_m. Since such intervals cannot be defined *a priori,* it is necessary that the corresponding kinetic profiles, with sufficiently defined phases, should be available. Under these conditions, the information required to calculate μ_m can be more efficiently used to describe the toxic effect with more realism and less error.

The term 'heavy metals' is «*used as a group name for metals and semimetals that have been associated with contamination and potential toxicity or ecotoxicity*» (Duffus 2002). Heavy metals can be classified as essential (*e.g.* Co, Ni, Cu) or non-essential (*e.g.* Cd, Hg, Pb) depending on whether they have a biological role for microorganisms (Bruins et al. 2000). Essential metals have growth stimulatory effects up to a limit concentration, with inhibitory effects from this level. Bacteria have adapted to the presence of heavy metals in the environment and have developed resistance mechanisms (Bruins et al. 2000). Although the type of mechanisms may be more or less homologous in all species of bacteria (Ji and Silver 1995), it is expected to obtain different responses to the same toxic concentration for several bacterial species (or bacterial groups). For example, lactic acid bacteria (LAB) have been proposed as a promising alternative to remove heavy metals from water (Halttunen et al. 2007) and *L. mesentoroides* proved to be an effective metal-binding species (Mrvčić et al. 2009).

In this work, we propose the use of a bivariate model, as a function of time and dose, which combines the logistic equation as a description of growth, with the cumulative function of the

Weibull distribution as a description of the dose-response relationships. This approach was applied to model the effect of three heavy metals (Co, Cd and Ni) on biomass production by five microorganisms (LAB and marine bacteria). Our results demonstrated the suitability and accuracy of these equations to describe and predict the experimental data and to supply parameters, with clear biological meaning, useful for toxicological evaluations.

MATERIALS AND METHODS

Microorganisms, culture media, reagents and incubation conditions

Microorganisms from different habitats (marine and terrestrial), metabolic characteristics (homo and heterofermentative), cell wall structure (Gram-positive and negative) and behaviour (free, opportunistic parasite, probiotic) have been used for toxicological assessment. Table 1 summarizes the basic features of all the evaluated bacteria. *Phaeobacter* sp. and *Listonella anguillarum* were kindly provided by Dr. Lone Gram (DTU Aqua, Denmark) and Dr. Harry Birkbeck (University of Glasgow, UK), respectively. *L. mesenteroides* was supplied by Dr. B. Ray (University of Wyoming, Laramie, USA).

Stock cultures of LAB and marine bacteria were kept at –80ºC in commercial MRS and marine medium, respectively, with 25% glycerol (Vázquez et al. 2004b; Cabo et al. 2001), respectively. Marine medium were provided by Difco (Becton, Dickinson and Company, MD, USA) and MRS medium by Pronadisa (Hispanlab S.A., Spain). Culture media were prepared as indicating on commercial formulation and sterilized at 121ºC for 15 min.

Chemicals, $Co(NO_3)_2.6H_2O$, $Ni(NO_3)_2.6H_2O$ and $Cd(NO_3)_2.4H_2O$, were in all cases purchased to Sigma (St. Louis, MO, USA). Concentrated solutions of these heavy metals were separately prepared and sterilized with steam flow at 101ºC for 1 h. Individual concentrations of these

chemicals on final culture media were (in mg l^{-1}): 0-control, 3, 6, 9, 15, 24, 40, 64, 100 and 150. For mathematical modelling, these concentrations were coded in [0, 1] interval.

Inocula (0.7% v/v) consisted of cellular suspensions from 14-h cultures on MRS and marine media adjusted to a 700 nm absorbance (A_{700}) of 0.600 for marine bacteria and 0.900 for LAB. Fermentations were carried out in triplicate using 300 ml Erlenmeyer flasks with 150 ml of culture medium containing 1 ml of the corresponding inoculum and the volume of the concentrated heavy metal solution necessary to obtain the fixed final concentration. Experiments were performed with orbital shaking at 200 rpm and 22ºC (*L. anguillarum*, *Phaeobacter* sp.), 27ºC (*Pseudomonas* sp.) and 30ºC (*L. mesenteroides*, *C. piscicola*).

Sampling and analytical determinations

At pre-established times, 2 ml samples were centrifugued at 4,000 g for 15 min. Sediments (biomass) were washed and resuspended in distilled water to the appropriate dilution for measuring the bacterial growth by A_{700}. In LAB cultures, supernatants were used for determining proteins (data not shown), glucose and characteristic metabolites from LAB fermentations.

Soluble proteins were quantified using the method of Lowry et al. (1951). Glucose, ethanol and lactic and acetic acids were measured by HPLC in membrane-filtered samples (0.22 µm Millex-GV, Millipore, USA) using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow = 0.4 ml min^{-1}) at 65ºC and a refractive-index detector.

Mathematical modelling

Dose-response model

In previous works we have argued in favour of our preference for the cumulative function of the Weibull distribution as a DR model (Riobó et al. 2008b; Murado and Vázquez 2010). If the

original function is multiplied by an asymptotic value *K*, it can account for the possibility of low toxic bioavailability, resistant subpopulations or other conditions, relatively frequent in DR tests that can produce less than 1 asymptotes. It is also appropriate to reparameterize the equation to make explicit the dose for semimaximum response (*m*), which simplifies the assignment of initial values and the calculation of the confidence interval using the appropriate statistical software. It should be noted that the ED_{50} or EC_{50} (effective dose or concentration for 50% of the tested population) only coincides with *m* when *K*=1. Assuming that the response increases with dose, we will use the following formula, which we will denote by ^{m}W:

$$R = K\left\{1-\exp\left[-\ln 2\left(\frac{D}{m}\right)^{a}\right]\right\}\ ;\ \text{briefly: } R = {}^{m}W\left(D;K,m,a\right) \qquad [1]$$

where *R* is the response (with *K* as maximum value), *D* is the dose, *m* is the dose corresponding to the semi-maximum response and *a* is a shape parameter related to the maximum slope of the response. Apart from the general method proposed, some biphasic profiles were fitted to a sum or difference of two equations [1]:

$$R = {}^{m}W\left(D;K_1,m_1,a_1\right) \pm {}^{m}W\left(D;K_2,m_2,a_2\right) \qquad [2]$$

Growth equation

A widely accepted model for the macroscopic description of the microbial growth kinetics is the logistic equation (Mercier et al. 1992; Wachenheim et al. 2003; Vázquez et al. 2005b), an advantage of which is the direct biological meaning of its parameters. This model describes the biomass variation versus time (growth rate *v*) by means of the following differential equation:

166 $$v = \frac{dX}{dt} = \mu_m \left(\frac{X_m - X}{X_m} \right) X$$

167

168 where X is the biomass (with X_m as asymptotic maximum), t the time and μ_m the maximum
169 specific growth rate or biomass increase per biomass unit and time unit (dimensions t^{-1}). When
170 this differential form is integrated with respect to time, for initial values t=0, X=X_0, the explicit
171 expression is obtained:

172

173 $$X = \frac{X_m}{1 + \exp\left[\ln\left(\frac{X_m}{X_0} - 1 \right) - \mu_m t \right]} \qquad [3]$$

174

175 For the purposes of our study, it is pertinent to reparameterize this basic form to make explicit
176 other parameters more appropriate in some cases. This requires taking into account that the
177 maximum rate (v_m) is the slope of the tangent to the function at the inflection point and that the
178 lag phase (λ) can be defined by the intersection of that tangent with the time axis (Zwietering et
179 al. 1990; Vázquez and Murado 2008a). The relationship between λ and v_m thus established (see
180 Appendix A) can involve a not very realistic restriction in some cases. An alternative time
181 parameter is the time (τ) required to achieve the half of the maximum biomass. Five
182 reparameterizations (L1 to L5) of [3] are shown in Table 2, and their calculation is detailed in
183 Appendix A.

184

185 *The joint dose-growth model*

186 When a parameter θ of the growth equation drops from a value θ_0 without toxic agent to a value
187 of θ in the presence of a given dose of the chemical, the response R_θ of this parameter can be
188 defined as:

189

$$R_\theta = \frac{\theta_0 - \theta}{\theta_0} = 1 - \frac{\theta}{\theta_0} \quad ; \text{ therefore: } \quad \theta = \theta_0 \left(1 - R_\theta\right) \qquad [4]$$

If the response increases the parametric value ($\theta > \theta_0$) we have:

$$R_\theta = \frac{\theta - \theta_0}{\theta_0} = \frac{\theta}{\theta_0} - 1 \quad ; \text{ and: } \quad \theta = \theta_0 \left(1 + R_\theta\right) \qquad [5]$$

In both cases, R_θ represents the equation [1]. Thus, when the parameters of the logistic equation are made dependent on the dose according to the equation [1], the result will be an expression that describes simultaneously all the kinetic series obtained in the presence of different concentrations of the toxic chemical. If the reparameterization L3 (Table 2) is taken as reference, the full model is:

$$X = X_{m\bullet} \left\{ 1 + \exp\left[2 + \frac{4 v_{m\bullet}}{X_{m\bullet}} \left(\lambda_\bullet - t \right) \right] \right\}^{-1} \quad ; \quad \text{where:} \qquad [6]$$

$$X_{m\bullet} = X_m \left[1 - {}^m W\left(D; K_x, m_x, a_x\right) \right]$$

$$v_{m\bullet} = v_m \left[1 - {}^m W\left(D; K_v, m_v, a_v\right) \right]$$

$$\lambda_\bullet = \lambda \left[1 + {}^m W\left(D; K_\lambda, m_\lambda, a_\lambda\right) \right]$$

This formulation assumes that the toxic action depresses the maximum biomass and the maximum growth rate, and prolongs the lag phase, but these assumptions, though reasonable, are not strictly necessary. The proposed DR models can describe other situations by changing the signs of the terms. If any of the parameter estimates (K_i, m_i, a_i) for the effect of the chemicals on a given parameter of the growth equation is not statistically significant, the effect involved is deleted and the model is recalculated. When the effects are inhibitory, it is advisable to include

the restriction $K_i \leq 1$ to fit to the experimental values. This limitation serves to prevent the possibility that, at high doses, the growth equation is solved with negative parameters, which has no physical meaning and can corrupt the system solution. Such a restriction is not necessary with stimulatory effects, since the asymptotes higher than 1 are not problematic here.

Numerical methods

Fitting procedures and parametric estimations from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro '*Solver*' of the *Microsoft Excel XP* spreadsheet. Subsequently, confidence intervals from the parametric estimations (Student's *t* test) and consistence of mathematical models (Fisher's *F* test) were determined using *DataFit 9* (Oakdale Engineering, Oakdale, PA, USA).

RESULTS

Preliminary approach

The response of *Pseudomonas* sp. to Cd is an example useful for discussing the proposed approach, which requires us to decide on: 1) the parametric form of the growth model regarding the use of maximum absolute (v_m) or specific (μ_m) growth rate as rate parameter; 2) the calculation method, with two options: 2a) individual fittings to the growth equation of the kinetic series corresponding to each dose, and use of model [1] to describe the effect of this dose on the growth parameters; 2b) simultaneous fitting to an equation [6] of all the kinetic series for obtaining the joint solution, once the effects that involve some parameter without statistical significance have been removed. The results were as follows (Figure 1 and Table 3):

Use of v_m (equations L3 and L5)

Individual fittings: Increasing concentrations of Cd caused a decrease of v_m and X_m values and an increase of λ (with L3) or τ (with L5). All the effects could be described (α=0.05) by means of the model [1]. The use of λ as time parameter provided better fittings than τ.

Simultaneous fitting: All the parametric estimates were significant (α=0.05), and the results of the individual fittings were confirmed.

These two fitting methods provided almost indistinguishable descriptions of the kinetic data, although with some differences in the profiles corresponding to the effects of the metal on the parameters of the growth equation. Since the simultaneous fitting involves the assumption that the toxic produces effects that satisfy DR models in all the cultures tested –which, in fact, must behave like a unitary system– this option was considered preferable to the individual fittings.

Use of μ_m (equation L4)

Individual fittings: Cd depressed the values of μ_m and X_m and increased λ. Nevertheless, the effect on μ_m did not obey the proposed model [1].

Simultaneous fitting: The parameters concerning the effect of Cd on μ_m (K_μ, m_μ and a_μ) were not statistically significant (α=0.05). The model still provided a statistically significant description after removing that effect, but there is a disadvantage: since $v_m=\mu_m X_m/4$ (expression [A2] in Appendix A), the elimination of μ_m involves equal percentual responses of v_m and X_m, which constitutes an artificial condition.

Although the variation of μ_m does not correspond with the profile defined by the equation [1], this does not mean that μ_m is constant. In fact, the effect of Cd on μ_m (obtained by individual fittings to L4, or calculated by means of [A2] from fitting to L3 or L5) could be described by means of a subtractive bi-sigmoid model [2]. However, the use of [2] would create an unnecessary and doubtful complication, since the value of μ_m is highly dependent on the kinetic

data at short times, which are very sensitive to the experimental error. Under these conditions, the use of v_m instead of μ_m as rate parameter seems to be a better solution. This fact does not prevent the use of other more appropriate parameters for specific cases.

The bivariate model

The responses observed in the 15 studied cases showed characteristics dependent on the species and metals considered (Figures 2-4 and Tables 4-5). In all of them, the use of a model [6] led to a statistically significant description. Within the range of the tested doses, *C. piscicola* was remarkably insensitive to Co (which only prolonged the lag phase with an effect close to lack of significance), and *L. mesenteroides* was insensitive to Co and Ni (figures not shown). Five cases (Cd on *Pseudomonas* sp., *C. piscicola* and *L. mesenteroides*, Co and Ni on *Phaeobacter* sp.) involved changes in the three parameters (X_m, v_m and λ or τ) of the growth equation; in the remaining seven cases the changes affected X_m and v_m. Three cases showed peculiarities of interest, as detailed below.

The responses to Cd of *C. piscicola* and *L. mesenteroides* could not be described using the form L4 as the core of the model [6]. The reason, mentioned in mathematical modelling section, is that real relation between v_m and λ variations was not that assumed by model [6]. This problem can be solved by means of a reparameterization such as L2, where λ is not explicit, or L5, with τ as time parameter. The L5 option led to the best fit in both cases (Tables 4-5 and Figures 3-4).

It is obvious that the exposure to Cd accelerated the death phase of *L. mesenteroides* cultures (Figure 5), but we have not been able to develop an explicit algebraic expression that can describe these growth kinetics. Accordingly, the description of this case by means of a model of the type [6] was carried out excluding biomass values for times longer than 34 h.

290 The growth of *Phaeobacter* sp. in Ni-dosed cultures showed a value of X_m higher than the
291 control for concentrations up to 40 mg l^{-1}, with a marked drop from this level. The model [6]
292 adequately described this response assuming a negative value for the asymptote (K_m) of the
293 effect of Ni on X_m. This means to accept that low doses of Ni cause a slight increase in biomass
294 production, which is no longer detected at higher doses, where the effects on v_m and λ are of
295 greater intensity.
296
297 By representing biomass as a simultaneous function of dose and time (Figures 2-4), it was
298 possible to observe an interesting behaviour that it is not easily verifiable using 2D figures. The
299 biomass, especially at long times, falls in some cases with a stepped shape. Such a shape is
300 expected to be found when the toxic agent affects in different way the mechanisms that underlie
301 to the meanings of the different kinetic parameters (maximum growth rate, yield and lag phase).
302 Indeed, if the toxic action modifies a parameter θ_1 of the growth equation according to a DR
303 model with moderate values of K_1, m_1 and a_1 and a parameter θ_2 with high values of K_2, m_2 and
304 a_2, the effect on θ_1 will produce a smooth fall of the biomass at low doses and a sharp decline at
305 doses near the m_2 value. The model [6] adequately describes this response, not predictable with
306 estimates based on the effect of the chemical on a single parameter.
307
308 It should be noted that if the toxic effect is typified by means of the ED_{50}, it is necessary to
309 provide the values corresponding to all the affected parameters of the growth equation. Another
310 option is to consider a single index as a summary of all the effects on the biomass produced at a
311 given time. This is the main datum with practical interest in operational contexts (effluent
312 treatment, bio-silage, batch fermentation) and also provides the most reliable estimate of the
313 expected effects in problems of environmental assessment. Since an important time reference is τ
314 (time required to achieve semimaximum biomass), the summary index may be defined as $ED_{50,\tau}$,
315 or dose that reduces the biomass by 50% compared to that produced by the control at time τ (see

316 Table 6 and Appendix B). In summary, Cd was the most toxic heavy metal and Ni the least toxic
317 chemical in most cases.
318
319 **Effects on lactic acid fermentation**
320 In MRS medium, *C. piscicola* and *L. mesenteroides* produce lactic acid as the main metabolite
321 from glucose, which provides additional criteria to assess the effects of the metals tested (e.g.,
322 the yields of metabolite productions by substrate consumption). With regard to this production, it
323 is interesting to observe the cumulative variation of the following magnitudes with the meaning
324 of yields:
325
326 $$Y_{X/G} = \frac{-\Delta X}{\Delta G} \;\; ; \;\; Y_{L/G} = \frac{-\Delta L}{\Delta G} \;\; ; \;\; Y_{L/X} = \frac{\Delta L}{\Delta X} \qquad [7]$$
327
328 where ΔX, ΔG and ΔL are the increments along the time of biomass, glucose and lactic acid with
329 respect to the corresponding initial concentrations (biomass may be replaced by A_{700}, since
330 relationships are here of greater interest than absolute values).
331
332 Figure 6 shows the variation of these yields in *C. piscicola* cultures exposed to the three metals;
333 coincidentally, the affected growth parameters were different for the three cases (Table 4). The
334 occasional divergence in the profiles at short times is less important than its convergence (or lack
335 thereof) at middle and long times, because the analytical error is higher at the beginning of the
336 culture, and it is, moreover, amplified by the use of relations. In any case, the profiles are clearly
337 characteristic of the considered response.
338
339 Thus, with respect to Cd –which modified X_m, v_m and τ – the production of biomass and lactic
340 acid per unit of substrate consumption diminished progressively with increasing doses. This
341 result for lactic acid is in agreement with its definition as primary metabolite (Luedeking and

342 Piret 1959). However, the production of lactic acid per unit of biomass increased with the dose,
343 despite the involved higher energy cost. With regard to Co, which slightly extended the lag
344 phase, the three yields were essentially the same in control and Ni-dosed cultures. Concerning
345 Ni, which only altered the maximum growth rate, the behaviour of yields took an intermediate
346 position.
347
348 *L. mesenteroides* showed a heterofermentative metabolism with production of ethanol, acetic and
349 lactic acid (data not shown). When the sum (M) of the three metabolites was used for calculation
350 of the yields [7], the results were similar to those obtained with *C. piscicola*. Against Cd –which
351 modified X_m, v_m and τ – an increase of the dose generated a decrease of $Y_{X/G}$ and $Y_{M/G}$, but an
352 increase of $Y_{M/X}$. Against Co and Ni –without effect in the dose domain tested–, control and
353 dosed cultures showed no significant differences.
354
355 It may be noted that if the (constitutive) production of lactic acid were stimulated as a
356 mechanism of resistance, its relations with the concentration of chemical, substrate and biomass
357 would be as those found in our results. On the other hand, for both LAB the variations of $Y_{X/G}$ (as
358 well as $Y_{L/G}$ and $Y_{L/X}$, linked to primary metabolism) are associated to responses whose
359 description involved to admit an inhibitory effect on the parameter X_m. These features confirm
360 that the effects on yield, which are connected with the value of the parameter X_m, can be
361 independent (as in the mentioned cases) from those related to growth rate and lag phase.
362 Therefore, none of such effects can be neglected in predictive toxicological evaluations.
363
364 **DISCUSSION**
365 The use of sigmoidal equations for describing both microbial growth (Vázquez and Murado
366 2008b; Gernaey et al. 2010) and dose-response relationships (Vølund 1978; Faust et al. 2003;
367 Gennings et al. 2004) is an extensively accepted practice. However, the combination of both

368 approaches in a single mathematical equation, that enables the evaluation of the effects of a
369 chemical on all the growth parameters, have not been completely explored. The model proposed
370 here assumes that a toxic agent can determine independent variations satisfying DR relationships
371 on all the parameters of the growth equation. The alternative use of different reparameterizations
372 of this growth equation allowed us to solve a variety of particular cases and to accurately
373 describe the batch cultures kinetics of five bacteria as affected by three heavy metals.
374
375 This approach is very similar to that proposed, almost 30 years ago, by Kooijman et al. (1983).
376 We believe that our focus improves the treatment of the effects on the lag phase, provides
377 flexible reparameterizations and avoids, by using a global model able to solve simultaneously all
378 the possible effects on the parameters of the growth equation, some incoherences difficult to
379 explain, as we saw, if we deal with such effects individually. We also believe that it has not been
380 sufficiently underlined the fact that the evaluations based on the variation of a single parameter
381 of the growth equation can have limited predictive value (as it can be verified by applying the
382 global index $ED_{50,\tau}$ under different hypothesis). In our work, we have found that the maximum
383 growth rate –the most often affected parameter– only provides an adequate description for one of
384 the twelve cases in which an inhibitory response was detected.
385
386 Cabrero et al. (1998) have shown that the effects of Zn and Cu on activated sludge bacteria
387 modify the biomass yield coefficient and the growth rate. Nevertheless, the kinetics of growth
388 were individually fitted and equations for predicting the effect of metals on growth parameters
389 were not proposed. Recently, Giotta et al. (2006) have calculated for *Rhodobacter sphaeroides*
390 the concentration that inhibits 50% of μ_{m} and X_m for seven heavy metals; but the effect on the lag
391 phase was only evident in three of the seven cases and it was neglected for this calculation. On
392 the contrary, this last parameter was identified as responsible for the Ni, Co and Zn-induced
393 decreases on the growth of *Pseudomonas* sp. and mixed microbiota from a wastewater treatment

plant (Şengör et al. 2009). However, the experimental data of this report clearly showed that maximum biomass and growth rate should have been used for modelling the described processes.

In LAB cultures, not significant effects on kinetic parameters were observed for Ni and Co. It corroborated the high capacity of these bacteria to accumulate some heavy metals without inhibitory effects on biomass production (Halttunen et al. 2007; Mrvčić et al. 2009). Nevertheless, Cd-dosed cultures of both LAB species tested were significantly affected in all the parameters of the growth equation.

Our results and mathematical proposal defined a global dose-growth model that: 1) constitutes the simultaneous solution of the series of kinetic profiles obtained by incubating a microorganism in the presence of increasing concentrations of a toxic agent; 2) allows to quantify the effects of such an agent on all the parameters of the growth equation, as well as to determine directly the corresponding confidence intervals; 3) considers the time-dose matrix as a whole, which minimizes the effects of experimental error, both random and systematic; 4) generated consistent descriptions when it was applied to study the effects of three heavy metals (Cd, Co and Ni) on five bacteria whose responses showed marked differences within the dose and time domains tested.

Appendix A. Reparameterizations of the logistic equation

The explicit form of the logistic equation [2] can be written as follows:

$$X = \frac{X_m}{1+\exp\left(c-\mu_m t\right)} \quad ; \quad c = \ln\left(\frac{X_m}{X_0}-1\right) \qquad \text{[A1]}$$

For determining the maximum growth rate it is necessary: 1) to obtain the abscissa (τ) of the inflection point, by isolating it from the expression that results by equating the second derivative of the function to zero; 2) to insert the value τ in the first derivative of the function. The results are:

$$\tau = \frac{c}{\mu_m} \quad ; \quad v_m = \frac{X_m \mu_m}{4} \qquad [A2]$$

For determining the lag phase, it must be kept in mind that the ordinate of τ is $K/2$. Thus, the equation of the tangent at the inflection point and its intersection (λ) with the abscissa axis are:

$$X = \frac{X_m}{2} + v_m (t - \tau) \quad ; \quad \lambda = \frac{c - 2}{\mu_m} \qquad [A3]$$

Thus, the reparametrized logistic equation, with explicit v_m and λ, requires to isolate μ_m and c in [A2] and [A3] respectively, and to insert the corresponding values into [A1]:

$$X = \frac{X_m}{1 + \exp\left[2 + \frac{4 v_m}{X_m}(\lambda - t)\right]} \qquad [A4]$$

Moreover, by inserting $X = X_m/2$ in [A1], we obtain $c = \mu_m \tau$, where τ (abscissa of the inflection point) is the time needed to reach the semimaximum biomass. By replacing c by $\mu_m \tau$ in [A1] we obtain another reparameterized form:

$$X = \frac{X_m}{1 + \exp\left[\mu_m (\tau - t)\right]} \qquad [A5]$$

Or, in general, to make explicit the time τ_q necessary to achieve a proportion q of the maximum biomass:

$$X = \frac{X_m}{1+\exp\left[\ln\left(\frac{1}{q}-1\right)+\mu_m\left(\tau_q - t\right)\right]} \qquad \text{[A6]}$$

Appendix B. Calculation of $DE_{50,\tau}$

Once the solution of the examined system is obtained by means of a model [6], the $ED_{50,\tau}$, or dose that reduces the biomass to 50% of that produced by the control in time τ, can easily be calculated as follows:

1. Fit the kinetic data of the control to the growth equation in the parametric form L5 (Table 1) to obtain the semimaximum biomass ($X_{m,0}/2$) and the time needed to reach it (τ_0). For another proportion q of the maximum biomass, use the form [A6].

2. Set an arbitrary initial value ($^{I}ED_{50,\tau}$) (see next point 5).

3. Calculate the value of biomass (X) that results from applying the model [6], by assigning the values $^{I}ED_{50,\tau}$ and τ_0 to the variables D and t.

4. Calculate the absolute value of the difference $H = \left| X - \frac{X_{m,0}}{4} \right|$

5. Calculate, using the *Solver* macro in *Microsoft Ex*cel, the value of $ED_{50,\tau}$ that minimizes *H*. For ensuring that the algorithm finds the absolute minimum, it is advisable to start with an ${}^{I}ED_{50,\tau}$ value associated with a reasonably small value of *H*.

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REFERENCES

Bruins MR, Kapil S, Oehme FW (2000) Microbial resistance to metals in the environment. Ecotoxicol Environ Saf 45:198-207

Cabo ML, Murado MA, González MP, Pastoriza L (1999) A method for bacteriocin quantification. J Appl Microbiol 87:907-914

Cabo ML, Murado MA, Gonzalez MP, Vazquez JA, Pastoriza L (2001) An empirical model for describing the effects of nitrogen sources on nisin production. Lett Appl Microbiol 33:425-429

Cabrero A, Fernandez S, Mirada F, Garcia J (1998) Effects of copper and zinc on the activated sludge bacteria growth kinetics. Water Res 32:1355-1362

Duffus JH (2002) "Heavy metals" - A meaningless term? (IUPAC technical report). Pure Appl Chem 74:793-807

Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, Hamer V, Scholze M, Vighi M, Grimme LH (2003) Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquat Toxicol 63:43-63

Gennings C, Carter Jr. WH, Carney EW, Charles GD, Gollapudi BB, Carchman RA (2004) A novel flexible approach for evaluating fixed ratio mixtures of full and partial agonists. Toxicol Sci 80:134-150

Gernaey KV, Lantz AE, Tufvesson P, Woodley JM, Sin G (2010) Application of mechanistic models to fermentation and biocatalysis for next-generation processes. Trends Biotechnol 28:346-354

Gikas P (2007) Kinetic responses of activated sludge to individual and joint nickel (Ni(II)) and cobalt (Co(II)): An isobolographic approach. J Hazard Mater 143:246-256

Giotta L, Agostiano A, Italiano F, Milano F, Trotta M (2006) Heavy metal ion influence on the photosynthetic growth of *Rhodobacter sphaeroides* . Chemosphere 62:1490-1499

Halttunen T, Salminen S, Tahvonen R (2007) Rapid removal of lead and cadmium from water by specific lactic acid bacteria. Int J Food Microbiol 114:30-35

Hjelm M, Bergh Ø, Riaza A, Nielsen J, Melchiorsen J, Jensen S, Duncan H, Ahrens P, Birkbeck H, Gram L (2004) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units Syst Appl Microbiol 27:360-371

International Organization for Standardization (2006) Water quality - Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum* ISO 10253:2006.

International Organization for Standardization (1995) Water quality - *Pseudomonas putida* growth inhibition test ISO 10712:1995.

Ji G, Silver S (1995) Bacterial resistance mechanisms for heavy metals of environmental concern. J Ind Microbiol 14:61-75

Kooijman SALM, Hanstveit AO, Oldersma H (1983) Parametric analyses of population growth in bio-assays. Water Res 17:527-538

513 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin
514 phenol reagent. J Biol Chem 193:265-275
515 Luedeking R, Piret EL (1959) Kinetic study of the lactic acid fermentation. Batch process at
516 controlled pH. J Biochem Microbiol Tech Eng 1:393-412.
517 Martens T, Heidorn T, Pukal R, Simon M, Tindall BJ, Brinkhoff T (2006) Reclassification of
518 *Roseobacter gallaeciensis* Ruiz-Ponte et al. 1998 as *Phaeobacter gallaeciensis* gen. nov., comb.
519 nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* (Lafay
520 et al. 1995) Uchino et al. 1999 as *Marinovum algicola* gen. nov., comb. nov., and emended
521 descriptions of the genera *Roseobacter*, *Ruegeria* and *Leisingera* . Int J Syst Evol Microbiol
522 56:1293-1304
523 Mercier P, Yerushalmi L, Rouleau D, Dochain D (1992) Kinetics of lactic acid fermentation on
524 glucose and corn by *Lactobacillus amylophilus* . J Chem Technol Biotechnol 55:111-121
525 Mrvčić J, Prebeg T, Barišić L, Stanzer D, Bačun-Drùina V, Stehlik-Tomas V (2009) Zinc
526 binding by Lactic acid bacteria. Food Technol Biotechnol 47:381-388
527 Murado MA, Gonzalez MP, Vázquez JA (2002) Dose-response relationships: an overview, a
528 generative model and its application to the verification of descriptive models. Enzyme Microb
529 Technol 31:439-455
530 Murado MA, Vázquez JA (2010) Biphasic toxicodynamic features of some antimicrobial agents
531 on microbial growth: A dynamic mathematical model and its implications on hormesis. BMC
532 Microbiol 10:220
533 Nyholm N, Sorensen PS, Kusk KO (1992) Statistical treatment of data from microbial toxicity
534 tests. Environ Toxicol Chem 11:157-167
535 Peleg M (1995) A model of microbial survival after exposure to pulsed electric-fields. J Sci Food
536 Agric 67:93-99

Riobó P, Paz B, Franco JM, Vázquez JA, Murado MA, Cacho E (2008a) Mouse bioassay for palytoxin. Specific symptoms and dose-response against dose-death time relationships. Food Chem Toxicol 46:2639-2647

Riobó P, Paz B, Franco JM, Vázquez JA, Murado MA (2008b) Proposal for a simple and sensitive haemolytic assay for palytoxin. Toxicological dynamics, kinetics, ouabain inhibition and thermal stability. Harmful Algae 7:415-429

Şengör SS, Barua S, Gikas P, Ginn TR, Peyton B, Sani RK, Spycher NF (2009) Influence of heavy metals on microbial growth kinetics including lag time: Mathematical modeling and experimental verification. Environ Toxicol Chem 28:2020-2029

Skov MN, Pedersen K, Larsen JL (1995) Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar O1 Appl Environ Microbiol 61:1540-1545

Strotmann UJ, Pagga U (1996) A growth inhibition test with sewage bacteria -results of an international ring test 1995. Chemosphere 32:921-933

Vázquez JA, González MP, Murado MA (2004a) Peptones from autohydrolysed fish viscera for nisin and pediocin production. J Biotechnol 112:299-311

Vázquez JA, González MP, Murado MA (2004b) A new marine medium: Use of different fish peptones and comparative study of the growth of selected species of marine bacteria. Enzyme Microb Technol 35:385-392

Vázquez JA, González MP, Murado MA (2005a) Effects of lactic acid bacteria cultures on pathogenic microbiota from fish. Aquaculture 245:149-161

Vázquez JA, Mirón J, González MP, Murado MA (2005b) Bacteriocin production and pH gradient: Some mathematical models and their problems. Enzyme Microb Technol 37:54-67

Vázquez JA, Murado MA (2008a) Mathematical tools for objective comparison of microbial cultures. Application to evaluation of 15 peptones for lactic acid bacteria productions. Biochem Eng J 39:276-287

563 Vázquez JA, Murado MA (2008b) Unstructured mathematical model for biomass, lactic acid and
564 bacteriocin production by lactic acid bacteria in batch fermentation. J Chem Technol Biotechnol
565 83:91-96
566 Volund A (1978) Application of the four-parameter logistic model to bioassay: comparison with
567 slope ratio and parallel line models. Biometrics 34:357-365
568 Wachenheim DE, Patterson JA, Ladisch MR (2003) Analysis of the logistic function model:
569 Derivation and applications specific to batch cultured microorganisms. Bioresour Technol
570 86:157-164
571 Zwietering MH, Jongenburger I, Rombouts FM, VAN'T Riet K (1990) Modeling of the Bacterial
572 Growth Curve. Appl Environ Microbiol 56:1875-1881
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TABLE CAPTIONS

Table 1: Marine and LAB bacteria used.

Table 2: Five reparametrizations of the logistic equation as growth model. X_0: initial biomass, X_m: maximum biomass, μ_m: maximun specific growth rate, v_m: maximum growth rate, λ: lag phase, τ: time for semimaximum biomass.

Table 3: Parametric estimates and confidence intervals (α=0.05) corresponding to the response of *Pseudomonas* sp. to Cd, according to the specified forms of the growth equation (see Table 2). Rate (v_m or μ_m) and time (λ or τ) parameters that are pertinent in each case are the explicit ones in the used reparametrization. ns: not significant; adj. r^2: adjusted coefficient of multiple determination.

Table 4: Parametric estimates and confidence intervals (α=0.05) corresponding to the specified responses fitted to the model [6]. Notations as in Table 3.

Table 5: Parametric estimates and confidence intervals (α=0.05) corresponding to the specified responses fitted to the equation [6]. Notations as in Table 3.

Table 6: Parametric estimates of ED50,τ values.

TABLES

Table 1

TABLE 1: Marine and LAB bacteria used

Bacteria	Strain	Characteristics
Pseudomonas sp.	CECT 4355	Marine / Gram (-) / free
Phaeobacter sp.	27-4*	Marine / Gram (-) / free / probiotic
Listonella anguillarum	90-11-287**	Marine / Gram (-) / opportunistic parasite
Leuconostoc mesenteroides subsp. *lysis*	HD-IIM_1	LAB / Gram (+) / free / heterofermentative
Carnobacterium piscicola	CECT 4020	LAB / Gram (+) / free / homofermentative

CECT: Spanish Type Culture Collection (University of Valencia, Spain).

HD-IIM: Department Animal Science, University of Wyoming (Wyoming, USA)

**Phaeobacter* 27-4 was initially identified as *Roseobacter* 27-4 (Hjelm et al., 2004; Martens et al., 2006).

***Listonella anguillarum* was isolated from rainbow trout and initially defined as *Vibrio anguillarum* (Skov et al., 1995).

Table 2

TABLE 2: Five reparametrizations of the logistic equation as growth model. X_0: initial biomass, X_m: maximum biomass, μ_m: maximun specific growth rate, v_m: maximum growth rate, λ: lag phase, τ: time for semimaximum biomass.

L1	$X = X_m \left\{1+\exp\left[\ln\left(\frac{X_m}{X_0}-1\right)-\mu_m t\right]\right\}^{-1}$
L2	$X = X_m \left\{1+\exp\left[\ln\left(\frac{X_m}{X_0}-1\right)-\frac{4v_m}{X_m}t\right]\right\}^{-1}$
L3	$X = X_m \left\{1+\exp\left[2+\frac{4v_m}{X_m}(\lambda-t)\right]\right\}^{-1}$
L4	$X = X_m \left\{1+\exp\left[2+\mu_m(\lambda-t)\right]\right\}^{-1}$
L5	$X = X_m \left\{1+\exp\left[\frac{4v_m}{X_m}(\tau-t)\right]\right\}^{-1}$

Table 3

TABLE 3: Parametric estimates and confidence intervals (α=0.05) corresponding to the response of *Pseudomonas* sp. to Cd, according to the specified forms of the growth equation (see table 2). Rate (v_m or μ_m) and time (λ or τ) parameters that are pertinent in each case are the explicit ones in the used reparametrization. ns: non significant; adj. r^2: adjusted coefficient of multiple determination.

		L3 individual	L3 in [6]	L4 in [6]	L5 in [6]
growth model	X_m	0.869±0.049	0.852±0.026	0.846±0.026	0.865±0.034
	v_m - μ_m	0.058±0.011	0.057±0.005	0.265±0.024	0.058±0.007
	λ - τ	5.565±1.581	5.220±0.731	4.911±0.787	12.523±0.630
	adj. r^2	0.992	-	-	-
effect on X_m	K_x	0.600±0.152	0.383±0.053	0.405±0.049	0.929±0.039
	m_x	0.097±0.066	0.052±0.004	0.057±0.003	0.121±0.016
	a_x	0.911±0.664	6.738±3.073	7.800±6.124	1.476±0.266
	adj. r^2	0.889	-	-	-
effect on v_m or μ_m	K_v - K_μ	0.989±0.095	1.000±0.032	ns	0.996±0.017
	m_v - m_μ	0.125±0.027	0.142±0.021	ns	0.118±0.029
	a_v - a_μ	1.602±0.616	2.239±0.772	ns	1.787±0.627
	adj. r^2	0.975	-	-	-
effect on λ or τ	K_λ - K_τ	6.511±0.398	2.779±0.772	7.464±1.800	1.102±0.311
	m_λ - m_τ	0.172±0.018	0.090±0.007	0.159±0.016	0.089±0.010
	a_λ - a_τ	2.546±0.735	4.390±1.345	2.478±0.440	4.006±1.386
	adj. r^2	0.991	-	-	-
	adj. r^2	-	0.992	0.988	0.987

Table 4

TABLE 4: Parametric estimates and confidence intervals (α=0.05) corresponding to the specified responses fitted to the model [6]. Notations as in table 3.

		Pseudomonas sp.	*L. anguillarum*			*C. piscicola*		
		Co-L3	Cd-L3	Co-L3	Ni-L3	Cd-L5	Co-L3	Ni-L3
growth model	X_m	0.764±0.022	1.383±0.023	1.499±0.044	1.527±0.033	3.056±0.079	2.725±0.023	2.971±0.037
	v_m - μ_m	0.069±0.007	0.254±0.049	0.166±0.022	0.168±0.013	0.330±0.045	0.249±0.010	0.403±0.039
	λ - τ	6.578±0.539	5.337±0.425	3.383±0.538	3.640±0.323	10.604±0.419	6.801±0.279	5.289±0.353
effect on X_m	K_x	0.229±0.038	0.984±0.020	0.508±0.029	0.455±0.045	0.810±0.260	ns	ns
	m_x	0.115±0.023	0.315±0.012	0.052±0.003	0.308±0.040	0.298±0.237	ns	ns
	a_x	3.021±1.928	5.401±0.901	3.917±1.171	1.814±0.476	0.693±0.180	ns	ns
effect on v_m or μ_m	K_v - K_μ	1.000±0.032	0.970±0.099	0.976±0.017	0.705±0.564	0.892±0.063	ns	0.692±0.048
	m_v - m_μ	0.591±0.034	0.054±0.028	0.106±0.024	0.632±0.414	0.027±0.014	ns	0.515±0.057
	a_v - a_μ	3.272±0.956	0.477±0.104	1.004±0.183	2.165±2.011	0.470±0.140	ns	2.855±1.229
effect on λ or τ	K_λ - K_τ	ns	ns	ns	ns	1.269±0.203	0.302±0.260	ns
	m_λ - m_τ	ns	ns	ns	ns	0.061±0.015	0.557±0.418	ns
	a_λ - a_τ	ns	ns	ns	ns	1.030±0.172	1.902±1.851	ns
	adj. r^2	0.989	0.992	0.986	0.992	0.992	0.997	0.989

Table 5

TABLE 5: Parametric estimates and confidence intervals (α=0.05) corresponding to the specified responses fitted to the equation [6]. Notations as in tables 3 and 4.

		Phaeobacter sp.			*L. mesenteroides*		
		Cd-L3	Co-L3	Ni-L3	Cd-L5	Co-L3	Ni-L3
growth model	X_m	2.297±0.146	2.541±0.072	2.415±0.052	3.932±0.090	3.879±0.055	3.976±0.025
	v_m - μ_m	0.343±0.149	0.278±0.024	0.284±0.018	0,544±0.062	0.532±0.026	0.510±0.017
	λ - τ	8.664±1.402	6.564±0.359	6.501±0.275	8.866±0.240	5.291±0.203	5.360±0.150
effect on X_m	K_x	1.000±0.000	1.000±0.000	-0.253±0.053	1.000±0.000	ns	ns
	m_x	0.088±0.034	0.661±0.434	0.094±0.017	0.363±0.193	ns	ns
	a_x	1.642±0.923	0.768±0.284	2.395±1.076	0.261±0.066	ns	ns
effect on v_m or μ_m	K_v - K_μ	1.000±0.000	0.911±0.065	0.872±0.120	0.913±0.043	ns	ns
	m_v - m_μ	0.020±0.010	0.143±0.021	0.348±0.049	0.120±0.022	ns	ns
	a_v - a_μ	0.916±0.241	1.716±0.513	1.956±0.498	2.094±0.780	ns	ns
effect on λ or τ	K_λ - K_τ	ns	3.391±1.025	7.665±3.931	4.622±1.195	ns	ns
	m_λ - m_τ	ns	0.216±0.035	0.483±0.053	0.218±0.032	ns	ns
	a_λ - a_τ	ns	6.302±2.551	8.065±4.495	2.413±0.271	ns	ns
	adj. r^2	0.959	0.997	0.997	0.995	0.995	0.997

Table 6

TABLE 6: Parametric estimates of $ED_{50,\tau}$ values

Strain	Toxic	$ED_{50,\tau}$ (mg/L)
Pseudomonas sp.	Cd	10.8
	Co	84.3
Phaeobacter sp.	Cd	1.5
	Co	20.5
	Ni	49.4
Listonella anguillarum	Cd	10.0
	Co	17.8
	Ni	123.7
Carnobacterium piscicola	Cd	4.3
	Ni	121.9
Leuconostoc mesenteroides subsp. *lysis*	Cd	8.2

FIGURE CAPTIONS

Figure 1: Top (*t* as independent variable): growth kinetics of *Pseudomonas* sp. exposed to the specified doses of Cd (mg l^{-1}). Doses of 100 and 150 mg.l^{-1} were omitted because growth was completely inhibited. Experimental results (points) and individual (dashed line) or simultaneous (solid line) fittings to model [6]. Bottom: effect of Cd (coded doses as independent variable) on the parameters of the growth equation in its parametric form L3 (responses –R– as dependent variables calculated by means of equations [4] or [5]). Parameter values (points), and fittings to equations [1] (solid line) and [2] (dashed line). Natural dose (mg.l^{-1})=coded dose×150. For clarity, confidence intervals (in all cases less than 5% of the experimental mean value; α=0.05; n=3) were omitted.

Figure 2: Left: Experimental data of the growth kinetics for the tested bacteria (points), and fittings to equation [6] (surface). *D*: coded dose; *t*: time in hours. For clarity, confidence intervals (in all cases less than 5% of the experimental mean value; α=0.05; n=3) were omitted. Right: correlation between observed and predicted values. Numerical results are summarized in Table 4.

Figure 3: Left: Experimental data of the growth kinetics for the tested bacteria (points), and fittings to equation [6] (surface). For clarity, confidence intervals (in all cases less than 5% of the experimental mean value; α=0.05; n=3) were omitted. Keys as in Figure 2. Numerical results in Table 4.

Figure 4: Left: Experimental data of the growth kinetics for the tested bacteria (points), and fittings to equation [6] (surface). For clarity, confidence intervals (in all cases less than 5% of the

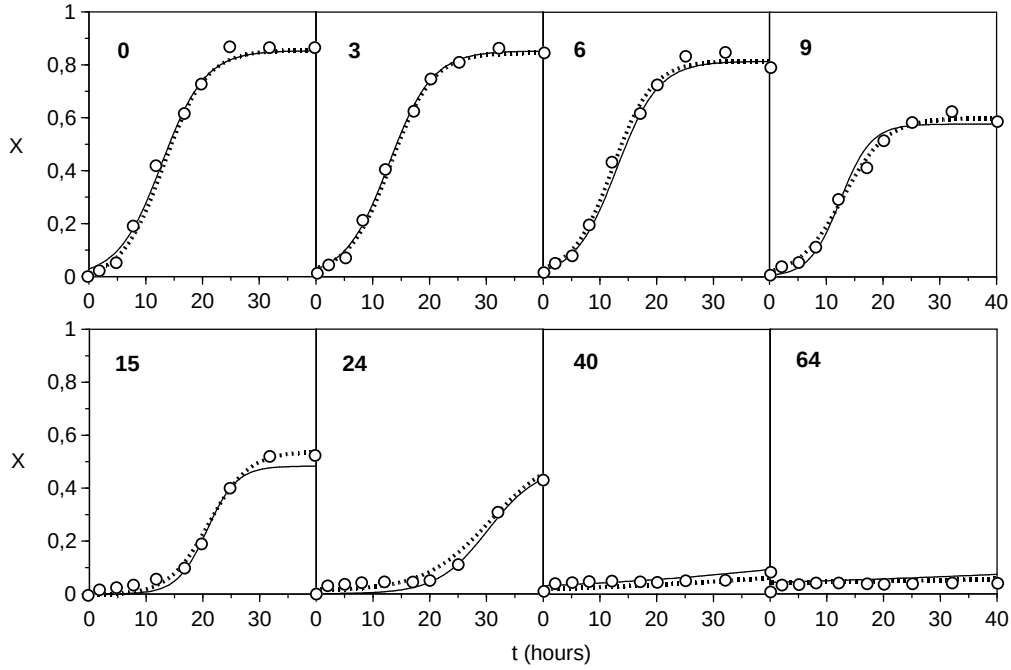
experimental mean value; α=0.05; n=3) were omitted. Keys as in Figure 2. Numerical results in Table 5.

Figure 5: Growth kinetics of *L. mesenteroides* exposed to Cd, which shows the effect of metal on the death phase at times longer than 40 hours (removed in Figure 4). ○: control, •: increasing concentrations of Cd (in reverse order to the final values of the ordinate). Lines are merely indicative and do not represent fits to any model. For clarity, confidence intervals (in all cases less than 5% of the experimental mean value; α=0.05; n=3) were omitted.

Figure 6: Biomass (*X*), lactic acid production (*L*) and glucose consumption (*G*) relationships for *C. piscicola* at different times (hours). ○: control; •: increasing concentrations of heavy metal (when there is not overlapping, profiles move away from control as toxic concentration increases). Note that there is a correspondence between the grouping mode of the profiles and the parameters of the model [6] affected by the metals (Table 4).

FIGURES

Figure 1



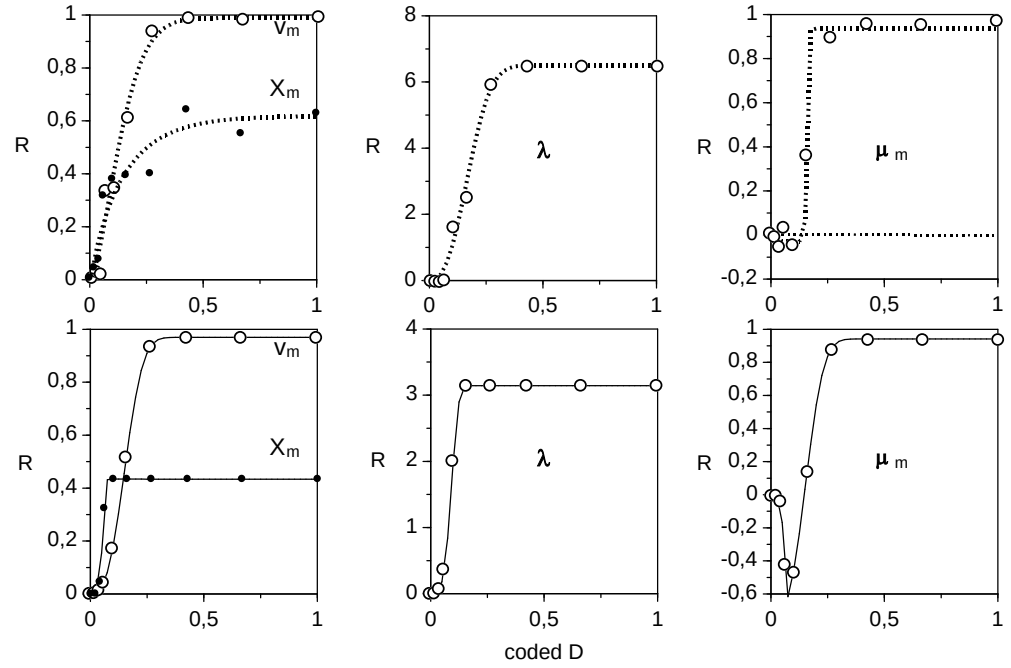


Figure 2

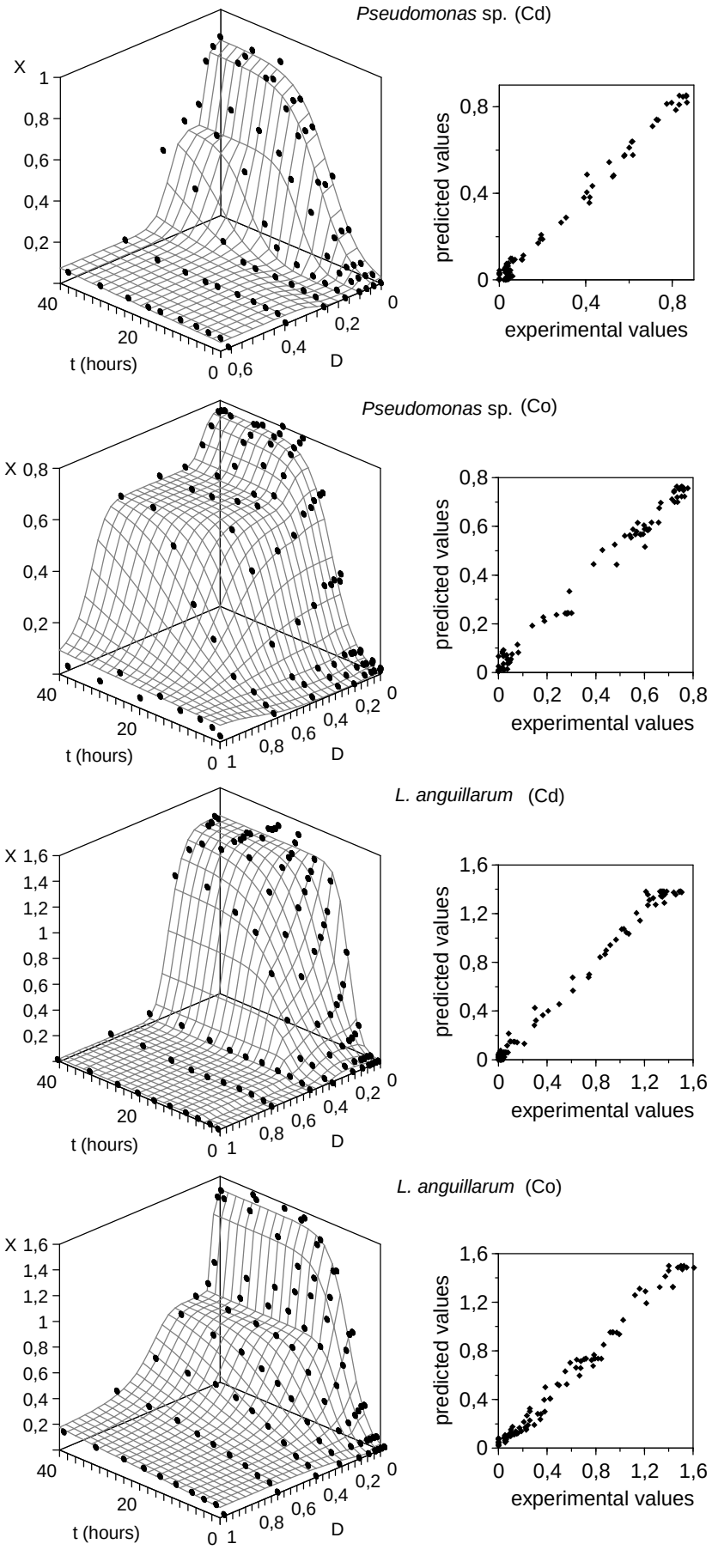

Pseudomonas sp. (Cd)
X
t (hours)
D
predicted values
experimental values
Pseudomonas sp. (Co)
X
t (hours)
D
predicted values
experimental values
L. anguillarum (Cd)
X
t (hours)
D
predicted values
experimental values
L. anguillarum (Co)
X
t (hours)
D
predicted values
experimental values

Figure 3

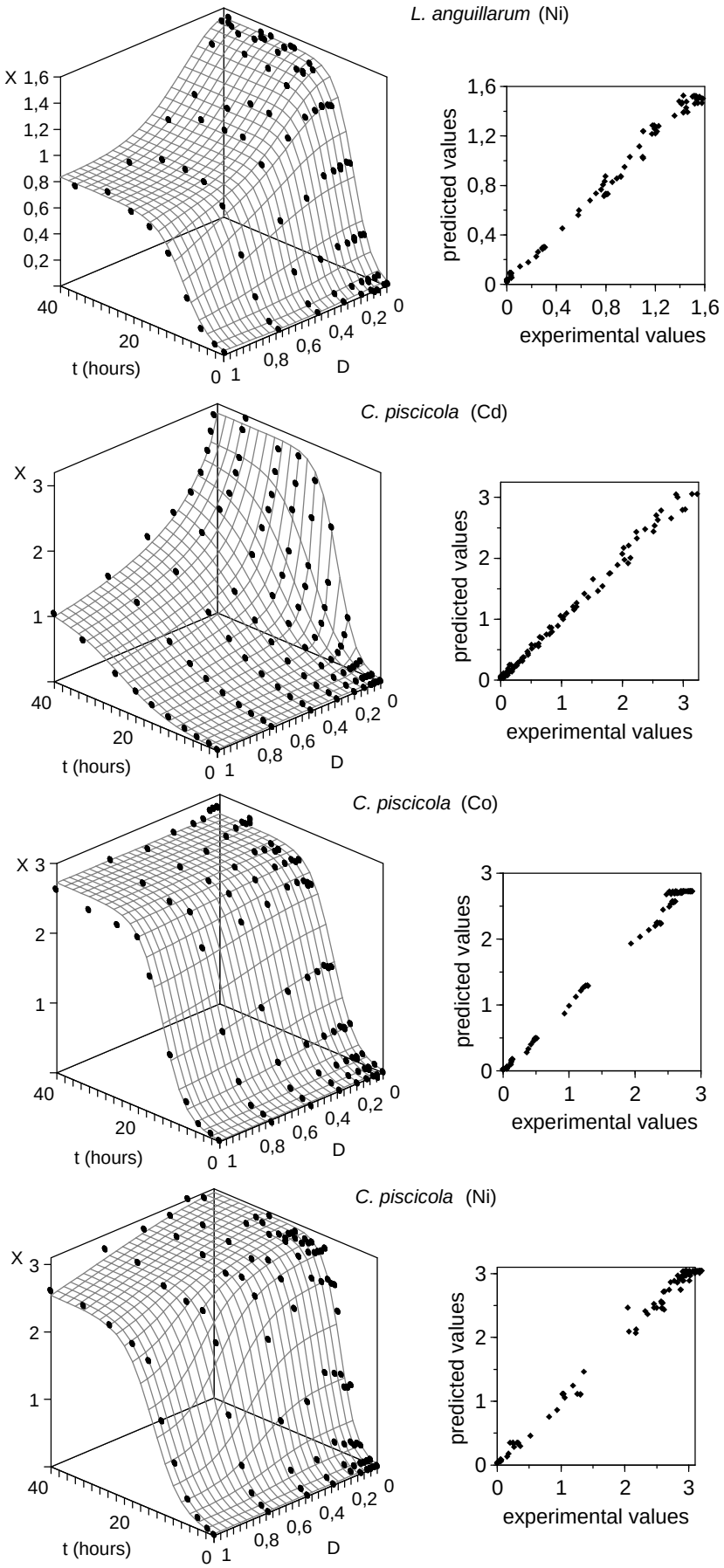

L. anguillarum (Ni)
X
t (hours)
D
predicted values
experimental values
C. piscicola (Cd)
C. piscicola (Co)
C. piscicola (Ni)

Figure 4

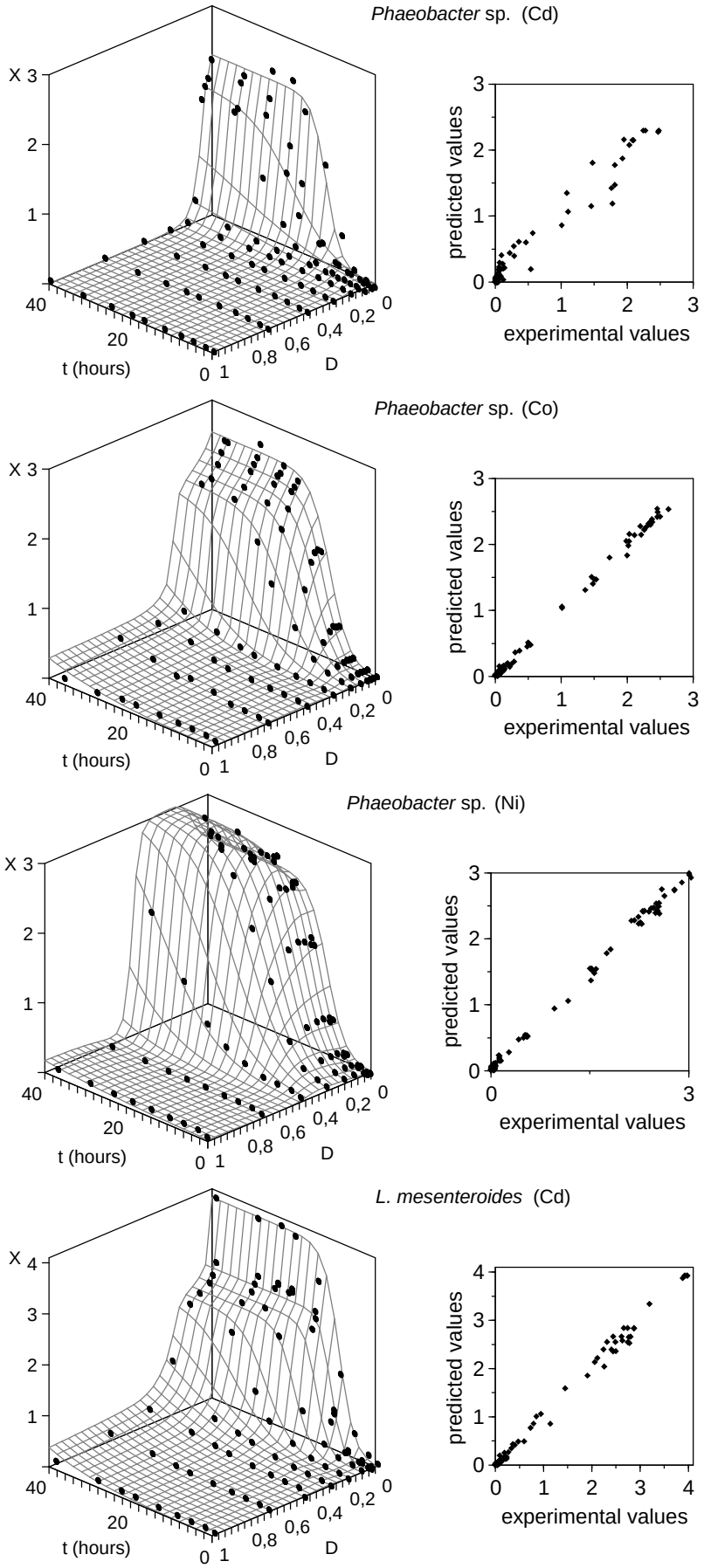

Phaeobacter sp. (Cd)
X
t (hours)
D
predicted values
experimental values
Phaeobacter sp. (Co)
X
t (hours)
D
predicted values
experimental values
Phaeobacter sp. (Ni)
X
t (hours)
D
predicted values
experimental values
L. mesenteroides (Cd)
X
t (hours)
D
predicted values
experimental values

Figure 5

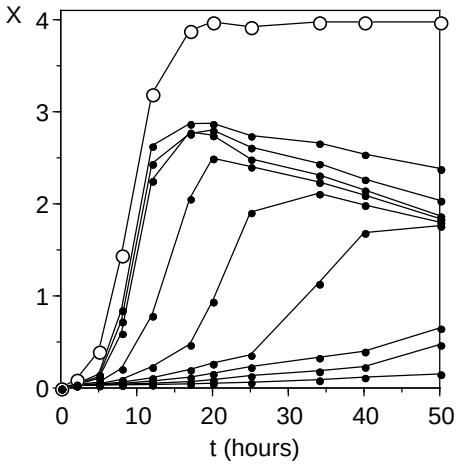


Figure 6