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Introducing a novel interaction model structure for the combined effect of temperature and pH on the microbial growth rate

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1 ABSTRACT

Efficient modelling of the microbial growth rate can be performed by combining the effects of individual conditions in a multiplicative way, known as the gamma concept. However, several studies have illustrated that interactions between different effects should be taken into account at stressing environmental conditions to achieve a more accurate description of the growth rate.

In this research, a novel approach for modeling the interactions between the effects of environmental conditions on the microbial growth rate is introduced. As a case study, the effect of temperature and pH on the growth rate of *Escherichia coli* K12 is modeled, based on a set of computer controlled bioreactor experiments performed under static environmental conditions. The models compared in this case study are the gamma model, the model of Augustin and Carlier (2000), the model of Le Marc et al. (2002) and the novel multiplicative interaction model developed in this paper. This novel model enables the separate identification of interactions between the effects of two (or more) environmental conditions. The comparison of these models focuses on the accuracy, interpretability and compatibility with efficient modeling approaches. Moreover, for the separate effects of temperature and pH, new cardinal parameter model structures are proposed.

The novel interaction model contributes to a generic modeling approach, resulting in predictive models that are (*i*) accurate, (*ii*) easily identifiable with a limited work load, (*iii*) modular, and (*iv*) biologically interpretable.

Keywords: Microbial growth rate; cardinal parameter model; environmental conditions; predictive microbiology.

1 INTRODUCTION

Combining different preservation factors to ensure microbial food safety and stability is a strategy that facilitates the production of foods with high sensory and nutritional quality (Leistner, 2000). However, building predictive models that accurately predict the growth rate at such stressing conditions has been found to be difficult. Membré and Lambert (2008) demonstrated that large deviations exist between predictions of the growth of *Listeria monocytogenes* obtained with different simulation packages when combining a stressing temperature, pH and water activity.

One of the most widely adopted methods to model the combined effect of environmental conditions (such as temperature T and pH) on the microbial specific growth rate relies on the gamma hypothesis (McMeekin et al. 1987; Zwietering et al. 1993). This hypothesis assumes that each of the environmental conditions has an independent effect on the reduction of the growth rate. Models built according to this hypothesis are composed of a multiplication of factors, each of which represents the influence of one of the environmental conditions on the growth rate. If this hypothesis is valid, models for the combined effect of environmental conditions on the growth rate can be built by only studying the separate effects of the environmental conditions.

This makes the gamma hypothesis very attractive because the experimental load required to study the individual effects is much less than the experimental load required to study the combined effect. Many studies also reported a good prediction quality when using the gamma concept (te Giffel, 1999; Pinon et al., 2004; Lambert and Biblas, 2007; Biblas and Lambert 2008; Leroi et al., 2012; Wijtzes et al., 2001). Additionally, the gamma models are compatible with the cardinal parameter models (Rosso et al., 1995; Ross and McMeekin, 2003), which contain biologically interpretable parameters, making them easy to use.

However, studies focusing on (combinations of) stressing environmental conditions revealed deviations from the gamma hypothesis. Publications in the domain of predictive microbiology often refer to these deviations as interactions. It should be noted that the gamma models, by construction, already contain interactions in the conventional sense of *additive interactions*. Indeed, when multiplying out the factors of a gamma model, terms (like $T \cdot pH$) will be found reflecting the combined effects of environmental conditions on the growth rate. The definition used for interactions in this paper is therefore one of so-called *multiplicative interactions*, i.e., those effects that cannot be found by only studying the separate effects of environmental conditions.

To account for such deviations from the gamma hypothesis, Augustin and Carlier (2000) integrated the calculation of the growth boundaries into a gamma model for *L. monocytogenes* and observed that this improved the prediction quality. Later, Le Marc et al. (2002) developed a factor to describe interactions between the effects of temperature, pH and organic acids on the growth rate of *L. monocytogenes*. This interaction factor was also inspired by the growth/no growth boundaries. Recently, Baka et al. (2013) demonstrated that the gamma concept is inadequate when describing the effect of temperature and pH on the growth rate of *E. coli* K12. This conclusion was drawn by illustrating that the parameters of the secondary model for temperature were dependent on pH. However, no adaptation of the gamma model concept was proposed in their research yet.

The initial objective of the current research is to demonstrate the need for secondary models that include multiplicative interactions for the effect of temperature and pH on the maximum specific growth rate. For this purpose, a dedicated experimental design is applied to bioreactor experiments with *E. coli* K12. The second objective, is to compare different model structures in their ability to describe the combined effect of

76 temperature and pH on the growth rate. The considered models are: the gamma model
77 without interactions, the model of Augustin and Carlier (2000), the model of Le Marc
78 et al. (2002) and a novel multiplicative interaction model. In addition, to describe the
79 individual effects of temperature and pH in these models, a set of new cardinal
80 parameter models is developed.
81
82

2 MODEL DEVELOPMENT

This section discusses the models that were obtained from literature and the new models that were developed. Growth curves were described using the primary model of Baranyi and Roberts (1994). The individual effects of environmental conditions on the maximum specific growth rate were modeled using cardinal parameter models. The advantage of this type of models compared to the square-root-type models (e.g., Ratkowsky et al., 1983) is that these only use biologically interpretable parameters. The parameters of these models represent the growth limits and the optimal conditions as parameters. It should be stressed that these parameters are in fact the theoretical growth limits and optimal conditions (McMeekin et al., 2013), which are only equal to the real values if the model describes the exact relationship between the environmental condition and the growth rate.

2.1 Primary model

To describe the evolution of the cell density N [CFU/mL] with time t [h], the widely used primary model of Baranyi and Roberts (1994) was implemented:

$$\frac{dN(t)}{dt} = \frac{Q(t)}{1+Q(t)} \cdot \mu_{max}(T, pH) \cdot \left(1 - \frac{N(t)}{N_{max}(T,pH)}\right) \cdot N(t) \quad (1)$$

$$\frac{dQ(t)}{dt} = \mu_{max}(T, pH) \cdot Q(t)$$

with $\mu_{max}(T, pH)$ [h^{-1}] the maximum specific growth rate and $N_{max}(T, pH)$ [CFU/mL] the maximum cell density for a specific temperature (T [°C]) and pH [-]. $Q(t)$ [$-$] is a measure for the physiological state of the cells and serves to describe the lag phase of the growth curve. For computational purposes, $N(t)$ and $Q(t)$ are replaced with their natural logarithms $n(t)$ [$\ln(CFU/mL)$] and $q(t)$ [$-$], resulting in (Baranyi and Roberts, 1994):

$$\frac{dn(t)}{dt} = \frac{1}{1+\exp(-q(t))} \cdot \mu_{max}(T, pH) \cdot \left[1 - \exp\left(n(t) - n_{max}(T, pH)\right)\right] \quad (2)$$

$$\frac{dq(t)}{dt} = \mu_{max}(T, pH)$$

The initial values of $n(t)$ and $q(t)$ are respectively n_0 and q_0.

2.2 Secondary models for independent effects

2.2.1 Temperature effect

CTMI. The individual effect of temperature on the maximum specific growth rate is often described with the Cardinal Temperature Model with Inflection (CTMI,

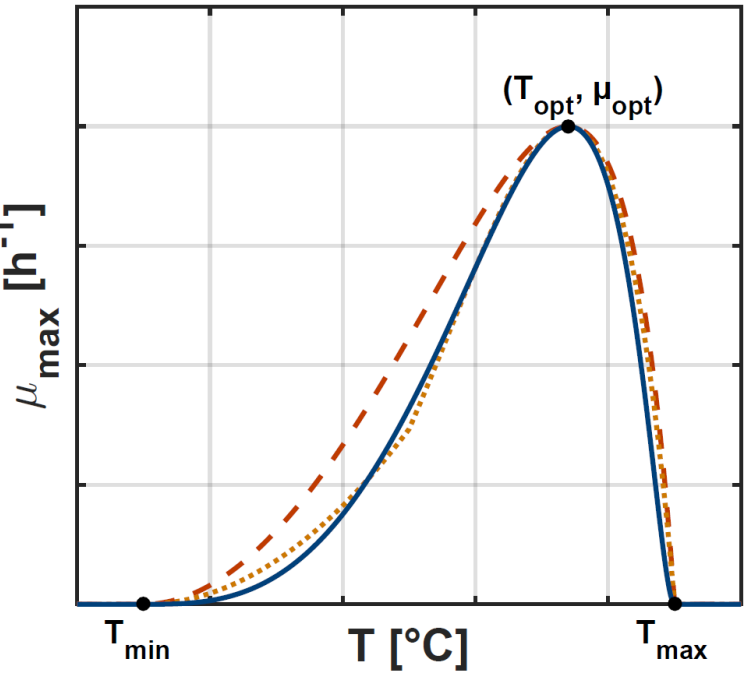


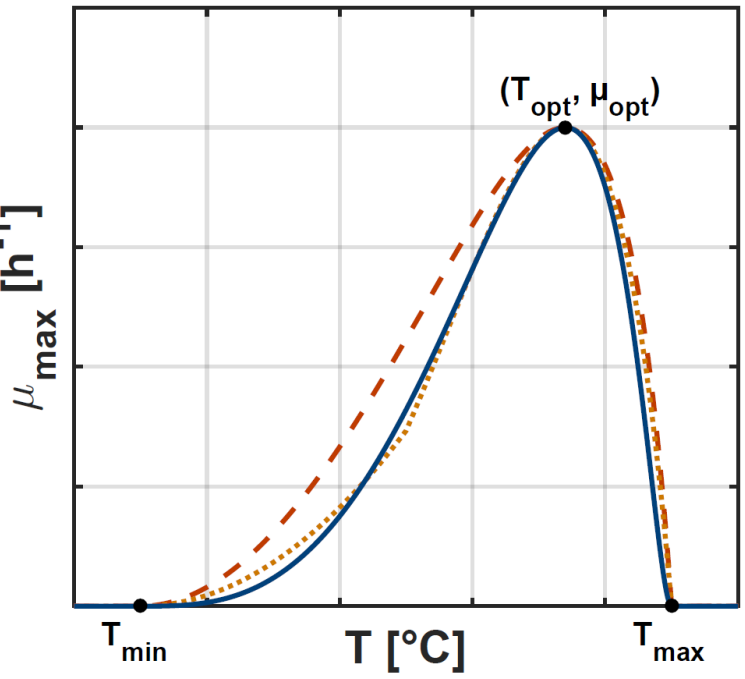
Fig. 1; Rosso et al., 1993). The advantage of this model is that it uses four interpretable parameters. The minimum temperature T_{min} [°C] and the maximum temperature T_{max} [°C] define the range of environmental conditions where growth is possible. The optimum temperature T_{opt} [°C] is the temperature at which the optimum growth rate μ_{opt} [h^{-1}] is reached. These parameters are combined in the following model structure:

$$\mu_{max}(T) = \mu_{opt} \cdot \gamma_T(T) \quad (3)$$

$$\gamma_T(T) = \frac{(T-T_{min})^2 \cdot (T-T_{max})}{(T_{opt}-T_{min}) \cdot [(T_{opt}-T_{min}) \cdot (T-T_{opt}) - (T_{opt}-T_{max}) \cdot (T_{opt}+T_{min}-2T)]}$$

with $\gamma_T(T)$ the reduction of the growth rate with respect to μ_{opt}, due to a non-optimal temperature.

aCTMI. Le Marc et al. (2002) proposed an adaptation of the CTMI for *Listeria* strains



(aCTMI, Fig. 1. This adaptation involves the use of two additional parameters. Above and below the change temperature T_c [°C] a different mathematical expression is used. T_1 [°C] is the temperature where the equation for temperatures above T_c becomes equal to zero in the region below T_{opt}. The aCTMI is defined as follows:

$$\mu_{max}(T) = \mu_{opt} \cdot \gamma_T(T) \quad (4)$$

$$T \geq T_c;\ \gamma_T(T) = \frac{(T-T_1)^2 \cdot (T-T_{max})}{(T_{opt}-T_1)\cdot[(T_{opt}-T_1)\cdot(T-T_{opt})-(T_{opt}-T_{max})\cdot(T_{opt}+T_1-2T)]}$$

$$T < T_c;\ \gamma_T(T) = \frac{(T_c-T_1)^2 \cdot (T_c-T_{max})}{(T_{opt}-T_1)\cdot[(T_{opt}-T_1)\cdot(T_c-T_{opt})-(T_{opt}-T_{max})\cdot(T_{opt}+T_1-2T_c)]} \cdot \left(\frac{T-T_{min}}{T_c-T_{min}}\right)^2$$

Both in the CTMI and in the aCTMI the growth rate is equal to zero for temperatures below T_{min} and above T_{max}.

bCTMI. Literature suggests that an alternative to the CTMI (Eq. 3) is needed to describe the relationship between temperature and the maximum specific growth rate of *E. coli* K12 at suboptimal temperatures. Based on a large dataset, Van Derlinden and

Van Impe (2012) demonstrated that a more accurate description of the $\mu_{max}(T)$-relationship is provided by the aCTMI (Eq. 4). This is due to the ability of the aCTMI to predict higher growth rates at lower temperatures compared to the CTMI when they predict the same growth rates closer to the optimal temperature. These findings were further investigated by Stamati et al. (submitted) who implemented advanced optimal experimental design techniques to discriminate between the CTMI and aCTMI. These authors concluded that the improvement of the accuracy by using aCTMI was small compared to the added complexity. The aCTMI does not only contain two additional parameters but is also a piecewise smooth function with a change point in the suboptimal range. This makes that the aCTMI is not continuously differentiable and can therefore cause errors during numerical computations (e.g., solving a differential equation for microbial growth at dynamic temperatures).

Due to these observations, a new cardinal parameter model is proposed in this paper to model the $\mu_{max}(T)$-relationship (bCTMI,

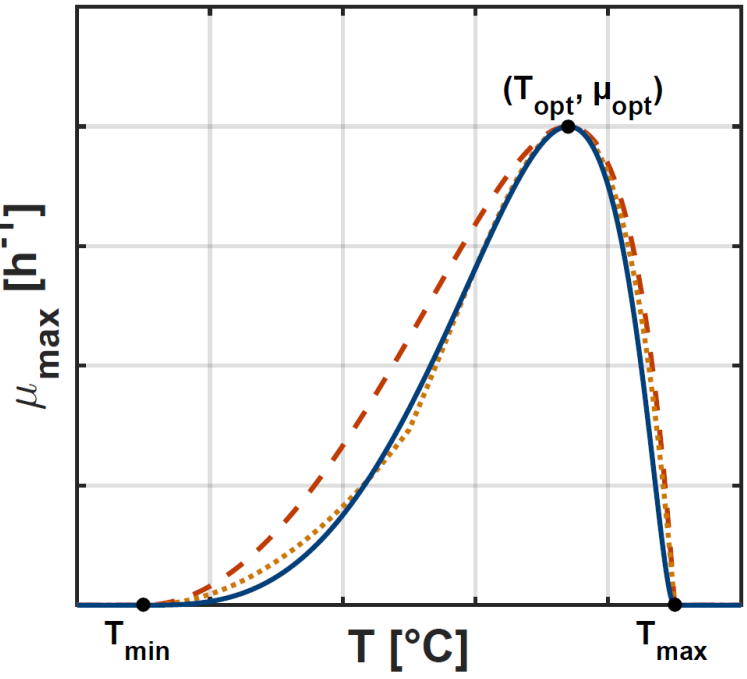


Fig. 1):

$$\mu_{max}(T) = \mu_{opt} \cdot \gamma_T(T) \quad (5)$$

$$\gamma_T(T) = \frac{(T-T_{min})^3 \cdot (T-T_{max})^2}{(T_{opt}-T_{min}) \cdot [(T_{opt}-T_{min}) \cdot (T-T_{opt}) - (T_{opt}-T_{max}) \cdot (T_{opt}+T_{min}-2T)] \cdot \left[(T-T_{min}) \cdot (T-T_{max}) - (T-T_{opt})^2\right]}$$

Similar to the CTMI, the bCTMI only contains 3 cardinal temperatures as parameters. For the same parameters, the bCTMI predicts lower growth rates than the CTMI. Hence, for the same predicted growth rates at near-optimal conditions, the bCTMI predicts higher growth rates than the CTMI at stressing conditions. Contrary to the aCTMI, the bCTMI is continuously differentiable.

2.2.2 pH effect

CPM. Similar to the CTMI for the effect of temperature, Rosso et al. (1995) proposed a Cardinal pH Model (CPM, Fig. 2) to describe the effect of pH on the microbial growth rate. Here, the minimum pH, $pH_{min}[-]$, and the maximum pH, $pH_{max}[-]$, are the growth boundaries. pH_{opt} $[-]$ is the pH at which the optimum growth rate μ_{opt} $[h^{-1}]$ is achieved. The CPM is formulated as:

$$\mu_{max}(pH) = \mu_{opt} \cdot \gamma_{pH}(pH) \quad (6)$$

$$\gamma_{pH}(pH) = \frac{(pH-pH_{min}) \cdot (pH-pH_{max})}{(pH-pH_{min}) \cdot (pH-pH_{max}) - (pH-pH_{opt})^2}$$

In the CPM, the growth rate is equal to zero for pH values below pH_{min} or above pH_{max}. This model structure contains a distinct optimum and has the mirror model structure for the suboptimal and superoptimal pH range. However, it has been reported that *E. coli* species are very sensitive to changes in internal pH in an alkaline environment and have a good ability to maintain pH homeostasis in the external pH range of 4.5 to 7.9 (Booth, 1985). This ability translates into a so-called plateau, where there is little or no change in the growth rate when the external pH changes. This observed plateau was previously accounted for in the model of Presser et al. (1997) for *E. coli* M23, though only in the suboptimal range.

srCPM. The CPM is easily adapted to obtain a flattened optimum by raising it to the power $1/\kappa$, with κ larger than or equal to 1:

$$\mu_{max}(pH) = \mu_{opt} \cdot \gamma_{pH}(pH) \quad (7)$$

$$\gamma_{pH}(pH) = \left(\frac{(pH-pH_{min})\cdot(pH-pH_{max})}{(pH-pH_{min})\cdot(pH-pH_{max})-(pH-pH_{opt})^2}\right)^{1/\kappa}$$

The effect of κ on the model output is illustrated by Fig. 3. In this paper, the value for κ was set equal to 2 based on the shape of the $\mu_{max}(pH)$-relationship that was found from the experimental results of Section 4.2, to avoid additional parameters. As such, this new cardinal pH model is calculated as the square root of the CPM (srCPM, Fig. 2).

aCPM. Another cardinal parameter model is suggested for situations where the response to a change in pH is structurally different in the suboptimal and superoptimal pH range. This model structure is obtained by raising the factors for the suboptimal range to the power η in numerator and denominator:

$$\mu_{max}(pH) = \mu_{opt} \cdot \gamma_{pH}(pH) \quad (8)$$

$$\gamma_{pH}(pH) = \left(\frac{(pH-pH_{min})^{\eta}\cdot(pH-pH_{max})}{(pH-pH_{min})^{\eta}\cdot(pH-pH_{max})-(pH-pH_{opt})^2}\right)^{1/\kappa}$$

Fig. 4 shows the effect of η on the model output. In this research, η and κ were equated respectively to 2 and 3 to obtain the aCPM (Fig. 2).

2.3 Secondary models for a combined effect

2.3.1 Existing models

Gamma model. When adopting the hypothesis that the reduction of the growth rate caused by one environmental condition $\gamma_{E_i}(E_i)$ is independent of the rest of the set of considered environmental conditions e, the combined effect can be described by a gamma model (Zwietering et al., 1993):

$$\mu_{max}(e) = \mu_{opt} \cdot \gamma_{E_1}(E_1) \cdot \gamma_{E_2}(E_2) \cdot \gamma_{E_3}(E_3) \cdot \ldots \quad (9)$$

Applying the gamma hypothesis to the case study of temperature and pH results in the following expression:

$$\mu_{max}(T, pH) = \mu_{opt} \cdot \gamma_T(T) \cdot \gamma_{pH}(pH) \quad (10)$$

Model of Augustin and Carlier (2000). Augustin and Carlier (2000) included the influence of environmental conditions on the growth limits into the gamma model by describing the effect of these conditions on the minimum cardinal parameters. According to this model, the growth limits with respect to temperature and pH are interrelated as follows:

$$\left[\frac{T_{opt}-T_{min}(pH)}{T_{opt}-T_{min}}\right]^{\beta} + \left[\frac{pH_{opt}-pH_{min}(T)}{pH_{opt}-pH_{min}}\right]^{\beta} = 1 \quad (11)$$

where $pH_{min}(T)$ is the minimum growth pH at a specific temperature and $T_{min}(pH)$ is the minimum growth temperature at a specific pH. β is a shape parameter related to the extent of the interactions and should be larger than or equal to 1. Very large positive values of β represent a situation of limited interactions and values close to 1 indicate extensive interactions. The effect of the parameter β on the growth limits in the suboptimal range of temperature and pH is illustrated by Fig. 5. Augustin and Carlier (2000) rather arbitrarily set the value of β equal to 3, based on a set of published growth/no growth data. In this research, β was estimated to obtain the best possible fit of growth data at combinations of stressing temperatures and pH values.

In this model, the gamma factors describing the effects of temperature and pH become also dependent on pH and temperature:

$$\mu_{max}(T, pH) = \mu_{opt} \cdot \gamma_T(T, pH) \cdot \gamma_{pH}(pH, T) \quad (12)$$

Model of Le Marc et al. (2002). The model of Le Marc et al. (2002) was built using only kinetic data and aimed to predict the interactions at the growth boundaries. This is in contrast with the approach of Augustin and Carlier (2000), which aimed at improving the prediction of the growth rate by including information on the growth boundaries. To this end, the model of Le Marc et al. (2002) used an interaction factor. This model included the effect of temperature, pH and organic acid concentrations on the growth kinetics of *Listeria*. For the present research, the model was simplified since only the effects of temperature and pH were assessed:

$$\mu_{max}(T, pH) = \mu_{opt} \cdot \gamma(T, pH) = \mu_{opt} \cdot \gamma_T(T) \cdot \gamma_{pH}(pH) \cdot \gamma_i(T, pH) \quad (13)$$

$$\begin{cases} \gamma_i(T, pH) = 1, & \text{if } \xi(T, pH) \leq \delta \\ \gamma_i(T, pH) = 2 \cdot \left(1 - \xi(T, pH)\right), & \text{if } \xi(T, pH) < 1 \\ \gamma_i(T, pH) = 0, & \text{if } \xi(T, pH) \geq 1 \end{cases}$$

$$\xi(T, pH) = \frac{1}{2} \cdot \left(\frac{\varphi_T(T)}{1 - \varphi_{pH}(pH)} + \frac{\varphi_{pH}(pH)}{1 - \varphi_T(T)} \right)$$

$$\varphi_T(T) = \left(1 - \sqrt{\gamma_T(T)}\right)^2$$

$$\varphi_{pH}(pH) = \left(1 - \gamma_{pH}(pH)\right)^2$$

In this model $\gamma_i(T, pH)$ is an interaction factor. The parameter δ was set equal to 1/2 based on theoretical assumptions in the original publication. Fig. 6 illustrates the calculation of the gamma factor $\gamma(T, pH)$ in the model of Le Marc et al. (2002). Both Eq. 13 and Fig. 6 demonstrate that the model distinguishes between three regions of environmental conditions: (*i*) the growth rate can be derived from the independent effects, (*ii*) the growth rate is smaller than predicted from the independent effects, and (*iii*) the combination of stressing conditions prevents growth altogether.

2.3.2 Modeling interactions

Before attempting to develop a new model structure that is capable of describing the combined effect of temperature and pH on the growth rate of *E. coli*, the requirements of such a model should be decided upon. A sound model structure is more likely to be applicable to other microorganisms and/or environmental conditions.

First of all, the model should be structured in such a way that it can be applied in a sequential modeling approach, i.e., an approach that allows separate identification of the individual effects and possible interactions. This property of the model structure can be referred to as *modularity*. Modularity is very important to obtain a model that is easy to understand and to enable the use of efficient experimental methods for gathering data and dedicated numerical methods for the identification of the model parameters. In the simplest case, the gamma approach results in such a model. This approach allows to independently determine the effects of temperature and pH and to build a model for the combined effect afterwards. The models of Augustin and Carlier (2000) and Le Marc et al. (2002) also satisfy this criterion, since interaction effects are included in such a way that these models reduce to the gamma model when only one environmental condition is not optimal. On the other hand, the more general polynomial models, which are often constructed based on traditional factorial designs, are not compliant with this criterion.

Secondly, the model structure should be applicable to various situations where the interactions may be more or less pronounced. The model of Augustin and Carlier (2000) allows for different extents of interactions by changing the shape parameter of the growth/no growth interface. The shape parameters can, however, only be linked to the environmental conditions themselves and not to the effects of a combination of environmental conditions. The model of Le Marc et al. (2002) contains no parameters

to change the extent of interactions, but computes these interactions based on the values of the gamma factors, in a fixed manner.

Thirdly, the model should be parsimonious (Ratkowsky, 1993). This is closely related to the previous requirement since additional model parameters are needed to make the interaction model applicable in various situations. The model of Augustin and Carlier (2000) only requires one additional parameter to specify the extent of the interactions linked to a certain environmental conditions. The model of Le Marc et al. (2002) requires no additional parameters with respect to a gamma model, but this is of course at the expense of the flexibility of the model.

Lastly, it is preferred that the description of the interactions is (biologically) interpretable. Both the models of Augustin and Carlier (2000) and Le Marc et al. (2002) link the interactions to the growth/no growth boundary. The description of the model structure seems less arbitrarily defined in the model structure of Augustin and Carlier (2000), which is based on the interpretation of the cardinal parameters. The shape parameter used in this model is not biologically interpretable but should be interpreted as the extent of the interactions linked to a certain environmental condition.

2.3.3 Novel interaction model

Based on the requirements of Section 2.3.2, the following general model structure is proposed:

$$\mu_{max}(e) = \mu_{opt} \cdot \gamma(e) = \mu_{opt} \cdot \left[\prod_{E_j \in e} \gamma_j(E_j)\right] \cdot \gamma_i(e) \quad (14)$$

$$\gamma_i(e) = \left[1 - \left(1 - \gamma_1(E_1)\right) \cdot \left(1 - \gamma_2(E_2)\right)\right]^{\beta_{1,2}} \cdot \left[1 - \left(1 - \gamma_1(E_1)\right) \cdot \left(1 - \gamma_3(E_3)\right)\right]^{\beta_{1,3}} \cdot \left[1 - \left(1 - \gamma_2(E_2)\right) \cdot \left(1 - \gamma_3(E_3)\right)\right]^{\beta_{2,3}} \cdot \ldots$$

$$\cdot [1-(1-\gamma_1(E_1))\cdot(1-\gamma_2(E_2))\cdot(1-\gamma_3(E_3))]^{\beta_{1,2,3}}\cdot \ldots$$

with e the set of environmental conditions considered and $\beta_{1,2}$ the shape parameter that expresses the extent of the interactions between the effects of the environmental conditions E_1 and E_2. If β is equal to zero, no interactions are present between the corresponding environmental conditions. Large positive values of β coincide with strong interactions. Eq. 14 also demonstrates that interactions between the effects of more than two environmental conditions can easily be described. For the case study with temperature and pH, the model is written as:

$$\mu_{max}(T,pH) = \mu_{opt}\cdot\gamma(T,pH) = \mu_{opt}\cdot\gamma_T(T)\cdot\gamma_{pH}(pH)\cdot\gamma_i(T,pH) \quad (15)$$

$$\gamma_i(T,pH) = \left[1-\left(1-\gamma_T(T)\right)\cdot\left(1-\gamma_{pH}(pH)\right)\right]^{\beta}$$

The proposed model structure, which is illustrated in Fig. 7, is applicable in a sequential modeling approach. It is possible to study the effect of temperature independently from the pH effect by working at an optimal pH, since both $\gamma_{pH}(pH)$ and $\gamma_i(T,pH)$ would be equal to 1, and vice versa. The model is also reduces to a model without interactions if the parameter β is found to be equal to 0. After identifying the independent effects of temperature and pH, β is estimated in order to obtain an accurate estimate of the combined effect.

The general model structure (Eq. 14) allows the modeler to define different interactions between specific sets of conditions by using different shape parameters. This is in contrast with the gamma model, the model of Le Marc et al. (2002) and even the model of Augustin and Carlier (2000). The shape parameters lack biological interpretation but can be interpreted as a measure for the extent of interactions between the effects of a specific set of environmental conditions.

3 MATERIALS AND METHODS

The mathematical models described in the experimental and computational methods that were applied in this research.

3.1 Experimental methods

3.1.1 Microorganisms and inoculum preparation

E. coli K12 MG1655 (CGSC#6300) was obtained from the *E. coli* Genetic Stock Center at Yale University. A stock culture was stored at -80°C in Brain Hearth Infusion broth (BHI, Oxoid), supplemented with 20% (w/v) glycerol (Acros Organics). The inoculum was prepared in a three step procedure: (i) a loopful of stock culture was spread onto a BHI agar plate (BHIA, BHI supplemented with 14 g/L technical agar nr. 3, Oxoid) and incubated overnight at 37°C. (ii) Then, a single colony was transferred into a 50 mL Erlenmeyer containing 20 mL BHI and stored at 37°C for 9 h. (iii) Finally, 20 µL of the stationary phase culture was inoculated into 20 mL fresh BHI and incubated at 37°C for 15 h before inoculation.

3.1.2 Bioreactor experiments

Experiments were performed in a set of bioreactors (BioStat B, Sartorius Stedim GmbH). The reactor vessels were filled with 3.5 L of BHI. Temperature was measured with a PT100 resistance temperature detector. A circulation chiller enabled temperature control below room temperature. pH measurement was performed with a gel-filled pH electrode (Hamilton Company) and the measurement was corrected for temperature. pH was controlled by addition of acid (1 N H_2SO_4, Sigma-Aldrich) or base (1 N KOH, Thermo Fisher Scientific) by a PID controller. The bioreactor was aerated at 0.2 L/min after autoclaving and the oxygen concentration was controlled at the stabilized

oxygen level during the experiment. The reactor content was stirred at 75 rpm with Rushton impellers. To avoid foaming, 500 µL of an antifoaming agent (Y-30 emulsion, Sigma-Aldrich) was added prior to every experiment.

3.1.3 Sampling and microbiological analysis

Depending on the specific experimental conditions, a sample was taken from the bioreactor every hour or every two hours during daytime. The appropriate dilutions were made in BHI and 49.2 µL of sample was plated onto BHIA plates, in triplicate, using a spiral plater (Eddy Jet, IUL Instruments s.a.). These plates were incubated at 37°C for about 15 h and then colonies were counted to obtain viable cell numbers (CFU/mL). The average over the three plates was used as the measured cell density of a sample. Experiments lasted between 12 and 200 h, depending on the growth rate.

3.2 Experimental design

The experimental design is represented in Fig. 8. Firstly, the effects of temperature and pH on the maximum specific growth rate were investigated separately. In these experiments, all but one environmental condition were kept close to optimal. The effect of temperature was studied at a pH of 7.50 (Dataset 1, 12 experiments) and the effect of pH at a temperature of 37.0°C (Dataset 2, 17 experiments). Secondly, experiments were performed at conditions that were suboptimal for both temperature and pH (Dataset 3, 8 experiments). Experiments focused on suboptimal conditions because these are more relevant to the food industry than superoptimal conditions. Lastly, a set of four validation experiments was performed (Dataset 4, 4 experiments). Fig. 8 also shows the environmental conditions where replicates were performed.

3.3 Parameter estimation and confidence intervals

Parameter estimations of the secondary models were performed in a *one-step* parameter estimation, i.e., directly on the measured growth curves by including the secondary models into the primary model. This is in contrast with the *two-step* method often used in predictive microbiology in which the parameters of the secondary model are estimated on the (computed) growth rates (Versyck et al., 1999). To aid in the comparison of the secondary models, growth rates were also calculated and plotted against the model prediction. However, the objective function of the parameter estimation was not the minimization of the difference between these measured and predicted growth rates.

The optimal combinations of parameters were calculated using the *lsqnonlin* routine of the Optimization Toolbox of Matlab version 7.14 (The Mathworks Inc.). This routine was always combined with a multi-start method that generated multiple sets of uniformly distributed random initial values of the parameters to be optimized. The objective function of the parameter estimation was the minimization of the sum of squared errors (SSE) for N_m measurements:

$$\mathrm{SSE} = \sum_{i=1}^{N_m} \left(n_{m,i}(t_i) - n_{p,i}(t_i, p) \right)^2 \quad (16)$$

with $n_{m,i}(t_i)$ the logarithm of the measured cell density and $n_{p,i}(t_i, p)$ the logarithm of the predicted cell density for a set of parameter p at time t_i. The 95% confidence interval of every parameter p_i was calculated based on the Student's t-distribution (Van Impe et al., 2001):

$$\left[p_i \pm t_{0.975, N_m - N_p} \cdot \sqrt{s_{p_i}^2} \right] \quad (17)$$

where N_p is the number of parameters and consequently $N_m - N_p$ is the number of degrees of freedom. $s_{p_i}^2$ is the variance on the parameter p_i and is found on the main

diagonal of the variance covariance matrix V, which is approximated as the inverse of the Fisher Information Matrix F (Walter and Pronzato, 1997):

$$s_{p,i}^2 = V(i,i) \quad (18)$$

$$V = F^{-1} \quad (19)$$

$$F = \frac{1}{MSE} \cdot J^T \cdot J \quad \text{with} \quad MSE = \frac{SSE}{N_m - N_p} \quad (20)$$

with J the Jacobian matrix and MSE the mean sum of squared errors. The MSE is used in this research as a measure for the quality of fit. Low MSE values represent a good quality of fit.

The accuracy factor A_f and bias factor B_f were calculated for the validation study (Baranyi et al., 1999):

$$A_f = \exp\left(\sqrt{\frac{\sum_{i=1}^{N_m}\left(\ln\left(\mu_{p,i}(p)\right) - \ln\left(\mu_{m,i}\right)\right)^2}{N_m}}\right) \quad (21)$$

$$B_f = \exp\left(\frac{\sum_{i=1}^{N_m}\left(\ln\left(\mu_{p,i}(p)\right) - \ln\left(\mu_{m,i}\right)\right)}{N_m}\right) \quad (22)$$

with $\mu_{m,i}$ the growth rate estimated with Eq. 2 and $\mu_{p,i}(p)$ the predicted growth rate for a set of parameter p. Models with smaller A_f values yield more accurate predictions. Overestimations of the growth rate result in a positive B_f and underestimations in a negative B_f.

4 RESULTS AND DISCUSSION

The four models compared in this section to describe the combined effect of temperature and pH should comply with a sequential modeling approach. Therefore, this study starts by selecting the most suitable model structures to describe the individual effects of temperature and pH in Sections 4.1 and 4.2. These models are implemented in Section 4.3 to model the combined effect of temperature and pH. If present, the shape parameter is estimated. The models with known parameter estimates are assessed using validation data in Section 4.4.

4.1 Modeling the effect of temperature

A set of 12 experiments was performed to model the effect of temperature on the maximum specific growth rate of *E. coli* (Dataset 1, Fig. 8). All these experiments were conducted at a pH of 7.50. Studied temperatures ranged from 13.0 to 44.0°C. The CTMI (Eq. 3), aCTMI (Eq. 4) and bCTMI (Eq. 5) were all fitted to the measurements in a one-step parameter estimation. The resulting parameter estimates and their 95% confidence intervals are listed in

	CTMI		aCTMI		bCTMI	
T_{min} [°C]	7.3	± 0.2	5.6	± 0.6	2.3	± 0.4
T_1 [°C]	/		8.9	± 0.5	/	
T_c [°C]	/		16.1	± 0.8	/	
T_{opt} [°C]	42.0	± 1.1	41.0	± 0.7	40.6	± 0.4
T_{max} [°C]	44.4	± 0.5	44.9	± 0.5	45.5	± 0.6
μ_{opt} [h^{-1}]	2.47	± 0.29	2.46	± 0.16	2.49	± 0.15
MSE	0.130		0.080		0.084	

Table 1, along with the MSE values as a measure of the quality of fit. The model fits are compared with the experimental growth rates in Fig. 9. From visual comparison, it

is clear that the CTMI is less capable of providing a good fit at both suboptimal and near-optimal conditions than the aCTMI or bCTMI. There is little difference between the approximations of the $\mu_{max}(T)$-relationship by the aCTMI and bCTMI. The same comparison can be made based on the MSE values, which are lower for the aCTMI and bCTMI than for CTMI. As explained in Section 2.2, the aCTMI has two disadvantages in comparison with the bCTMI: the model has two additional parameters and is described by a piecewise function. Since the MSE of the aCTMI is only slightly lower than the one of the bCTMI, the latter model is preferred. Therefore, the bCTMI was used to describe the combined effect of temperature and pH on the growth rate in Section 4.3.

A difference of several degrees Celsius is found between the estimated values of the parameter T_{min} for the different secondary models. However, care should be taken with the interpretation of these values (McMeekin et al., 2013). The parameter T_{min} is an extrapolation of the observations, since there is no information available in this study to indicate how well the model fits the effect of temperature on the growth rate for values below the lowest experimental temperature. Reported values for the minimum growth temperature of *E. coli* are generally based on a similar extrapolation of experimental data and therefore no comparison of these values was made with experimentally determined minimum temperatures.

4.2 Modeling the effect of pH

To model the effect of pH on the maximum specific growth rate, 17 experiments were available in Dataset 2 (Fig. 8). All these experiments were performed at a temperature of 37.0°C. Three model structures were available to model this effect: the CPM (Eq. 6), srCPM (Eq. 7) and aCPM (Eq. 8). The model fits are illustrated in Fig. 10, along with the experimental growth rates. Parameter estimates and MSE values are gathered in

	CPM		srCPM		aCPM	
pH_{min} [-]	4.38	± 0.05	4.48	± 0.01	4.46	± 0.02
pH_{opt} [-]	6.89	± 0.10	7.30	± 0.09	7.10	± 0.13
pH_{max} [-]	10.00[1]	± 0.27	9.40	± 0.14	9.02	± 0.01
μ_{opt} [h^{-1}]	2.49	± 0.09	2.32	± 0.05	2.19	± 0.03
MSE	0.132		0.058		0.041	

[1] This parameter reached the upper bound of the parameter value during the parameter estimation.

Table 2. As expected, based on the qualitative description in literature of the response of *E. coli* species to an external pH (Booth, 1985), the model structure of the CPM is not suitable to describe the effect of pH on the growth rate of *E. coli*. The MSE of the srCPM is only 44% of that of the CPM. This illustrates that the quality of fit is greatly improved by the simple adaptation of the srCPM. A further improvement in the quality of fit is made by the aCPM. This is due to the different response of *E. coli* in the sub- and superoptimal pH range. Since the aCPM provides the best fit of all three models, it was selected to describe the combined effect of temperature and pH on the growth rate in Section 4.3.

4.3 Modeling the combined effect of temperature and pH

First, a parameter estimation of the gamma model on the data at optimal temperature and the data at optimal pH was performed (Dataset 1 and 2, Fig. 8). This parameter estimation only differs from the separate parameter estimations of Section 4.1 and 4.2 in the sense that the model equations of the bCTMI and aCPM need to intersect in the point where $T = 37.0°C$ and $pH = 7.50$, since these models are combined in the gamma model (Eq. 10). Consequently, the estimates of the cardinal parameters were almost equal to those of the bCTMI in

	CTMI		aCTMI		bCTMI	
T_{min} [°C]	7.3	± 0.2	5.6	± 0.6	2.3	± 0.4
T_1 [°C]	/		8.9	± 0.5	/	
T_c [°C]	/		16.1	± 0.8	/	
T_{opt} [°C]	42.0	± 1.1	41.0	± 0.7	40.6	± 0.4
T_{max} [°C]	44.4	± 0.5	44.9	± 0.5	45.5	± 0.6
μ_{opt} [h^{-1}]	2.47	± 0.29	2.46	± 0.16	2.49	± 0.15
MSE	0.130		0.080		0.084	

481

482 Table 1 and those of the aCPM in

	CPM		srCPM		aCPM	
pH_{min} [-]	4.38	± 0.05	4.48	± 0.01	4.46	± 0.02
pH_{opt} [-]	6.89	± 0.10	7.30	± 0.09	7.10	± 0.13
pH_{max} [-]	10.00[1]	± 0.27	9.40	± 0.14	9.02	± 0.01
μ_{opt} [h^{-1}]	2.49	± 0.09	2.32	± 0.05	2.19	± 0.03
MSE	0.132		0.058		0.041	

[1] This parameter reached the upper bound of the parameter value during the parameter estimation.

Table 2. The value of μ_{opt} was equal to 2.48 h^{-1} and the MSE was 0.065.

Note that the four competing models have the same model structure at these conditions since the three models with interactions (Eq. 12, 13 and 15) reduce to the gamma model (Eq. 10) when only one environmental condition is not optimal. This implies that the cardinal parameters of a gamma model, which were identified on the basis of these two datasets, can be used for all four models.

Using these parameter estimates, the description of the combined effect of temperature and pH of each of the four models was evaluated, based on Dataset 3. In Fig. 11 (a) and (b), the models outputs are calculated for a set of linearly changing temperature and pH combinations, along with the experimental growth rates at these conditions. Two additional experimental conditions are depicted in Fig. 11 (c). The estimated shape parameters and their 95% confidence bounds are collected in Table 3 along with the MSE values.

For the gamma model (Eq. 10), all model parameters needed to describe the combined effect of temperature and pH are already known. The calculated gamma model at stressing conditions in Fig. 11 (b), makes a large overestimation of the experimental growth rates. For the three most stressing conditions, the growth rates were overestimated between 52 and 79 %. This confirms the existence of multiplicative

interactions between the effects of temperature and pH, i.e., the inability to describe the combined effect by only studying the separate effects. Consequently, models that describe these interactions are needed. When employing the model of Augustin and Carlier (2000) (Eq. 12), the growth rate is reduced in the region of stressing conditions by optimizing the shape parameter. Compared with the gamma model without interactions, this results in a decrease of the MSE of 53%, which means that a large improvement in the model accuracy is obtained. In the model of Le Marc et al. (2002) (Eq. 13), no additional parameters can be chosen, meaning that the description of the interactions is entirely defined, based on the individual effects of the environmental conditions. Comparing the model in Fig. 11, clarifies that the output changes little with respect to the gamma model by implementing interactions as described by the model of Le Marc et al. (2002). As concluded from comparing the MSE values, the interactions of this model provide no improvement of the model fit for the considered environmental conditions. This is because the interaction factor in the model of Le Marc et al. (2002) is equal to 1 for most of the environmental conditions (Fig. 6). On the other hand, the shape parameter of the gamma-interaction model (Eq. 15) provides the possibility to decrease the predicted growth rate over a large range of environmental (Fig. 7). The effect of this interaction factor is clearly visible in Fig. 11 (b) and (c) and causes a decrease of the MSE with 75% compared to the gamma model without interactions. This confirms that the best approximation of Dataset 3 was obtained by the gamma-interaction model.

4.4 Validation

Since all parameters of the four secondary models were determined in Section 4.3, the models can be validated with Dataset 4. The validation study is limited to experiments

529 in broth, since the aim is to assess the ability of the considered models to describe the
530 combined effect of temperature and pH. The model complexity has to be increased
531 before any of these models can be applied to real food products.
532 Fig. 12 compares the experimental growth rates with the values predicted by the four
533 secondary models. To obtain a measure for the agreement between the secondary
534 models and the experimental data, parameter estimations were performed on the
535 available growth curves using the calculated growth rates, meaning that only n_0, q_0
536 and n_{max} were estimated (Eq. 2). Also the Af and Bf were calculated for each model
537 based on the growth rates estimated with Eq. 2 and predicted with the secondary
538 models. The resulting MSE, A_f and B_f values are summarized in Table 4. For the
539 experiments performed at $pH = 6.50$ and $T = 21.0°C$ and at $pH = 7.00$ and $T =$
540 $25.0°C$ there is almost no difference between the predicted growth rates of the various
541 models and the experimental growth rates were predicted very accurately. Since only
542 temperature was not optimal during these experiments, an accurate approximation of
543 the growth rate was achieved with the simple gamma model. The interactions are absent
544 for the model of Le Marc et al. (2002) and negligible for the model of Augustin and
545 Carlier (2000) and the gamma-interaction model. For the two other experiments, the
546 gamma model and the model of Le Marc et al. (2002) provided the largest
547 overestimation of the growth rate since these models do not consider any interactions.
548 Both the model of Augustin and Carlier (2000) and the novel gamma-interaction model
549 predict lower growth rates, but the latter provides a prediction error that is significantly
550 lower than the former one. The MSE and A_f are also lowest for the gamma-interaction
551 factor, indicating the most accurate prediction. The B_f demonstrates that all models still
552 overestimate the growth rate and are therefore fail safe. As a result, it is concluded that

553 also in the validation study the novel gamma-interaction model outperformed the
554 competing models.

555

556

5 CONCLUSION

A novel gamma-interaction model was proposed in this research for the description of the combined effect of multiple environmental conditions. This model was compared with the simple gamma model and the models of Augustin and Carlier (2000) and Le Marc et al. (2002). A case study was considered on the basis of 39 bioreactor experiments describing the effect of temperature and pH on the growth rate of *E. coli* K12. Firstly, two new cardinal parameter models were developed to capture the independent effects of temperature and pH on the growth rate. Secondly, the combined effect of temperature and pH was modeled with all four models based on a dataset at suboptimal conditions. The parameter estimation results confirmed that interactions should be accounted for and that the best approximation of the data was obtained by the new gamma-interaction model. Also when performing a validation on new data, the gamma-interaction model predicted the growth rates most accurately. Due to the very general model structure, it is expected that the gamma-interaction model will also accommodate the improvement of predictions when working with different micro-organisms or different environmental conditions. The shape parameter used in this model has no biological interpretation but is interpretable in the sense that it expresses the extent of interactions between the effects of two environmental conditions.

By basing the gamma-interaction model on the gamma model, it is still easily identifiable with a limited work load and straightforward to add new environmental conditions and interaction effects to an existing model. By including the new interaction factor with a shape parameter, the prediction quality at combinations of multiple stressing environmental conditions can be significantly improved.

6 ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

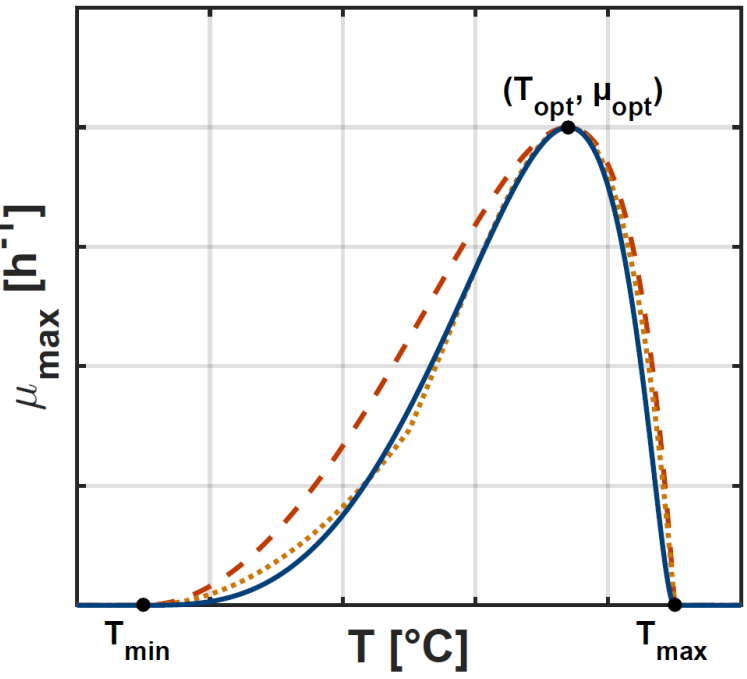
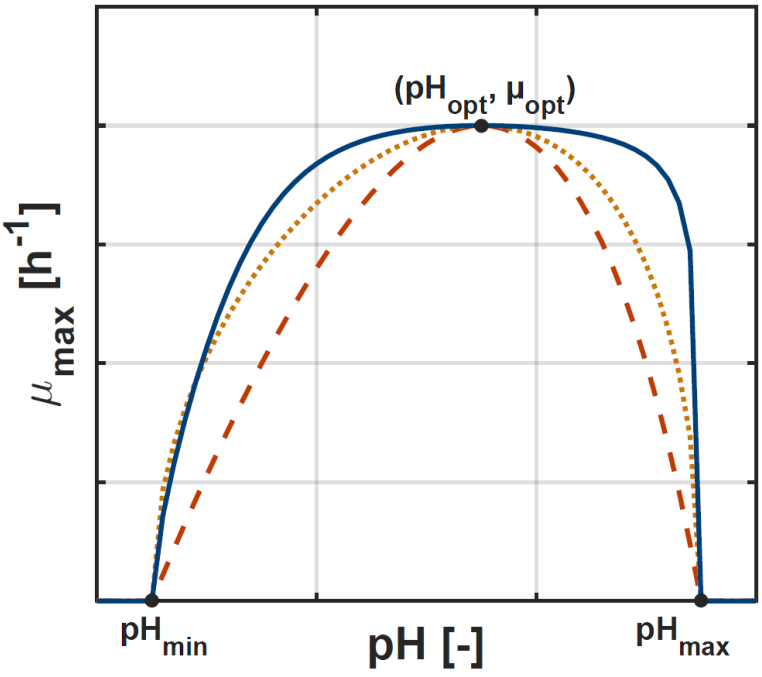


Fig. 1. Comparison of the model structures for the effect of temperature on the maximum specific growth rate: CTMI (---), aCTMI (···) and bCTMI (—). The three models are calculated using the same parameter values for T_{min}, T_{opt}, T_{max} and μ_{opt}.



682

683 **Fig. 2.** Comparison of the model structures for the effect of pH on the maximum specific

684 growth rate: CPM (---), srCPM (·--·) and aCPM (—). The three models are calculated using

685 the same parameter values for pH_{min}, pH_{opt}, pH_{max} and μ_{opt}.

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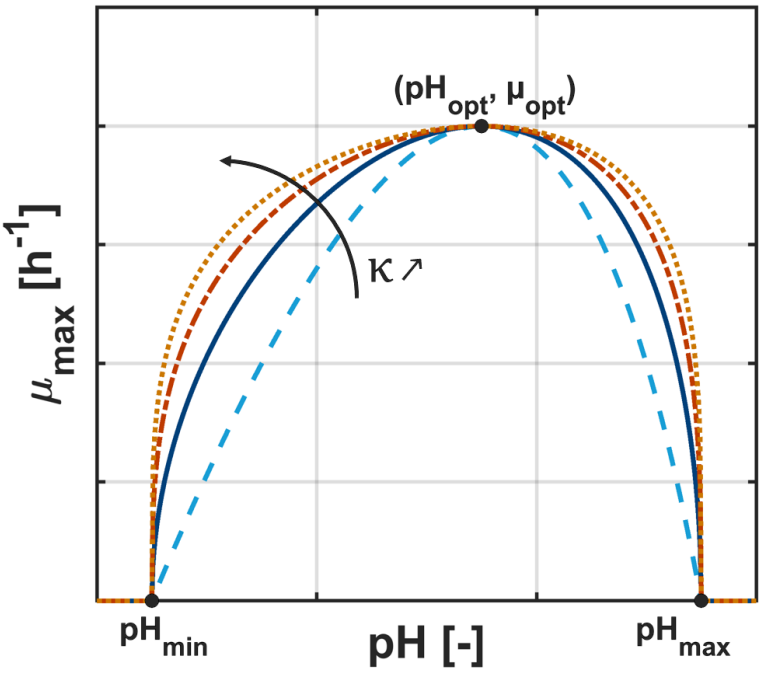

688 **Fig. 3:** The effect of the parameter **κ** on the output of the model represented by Eq. 7.

689 The values of **κ** are1 (---), 2 (—), 3 (·--) and 4 (···).

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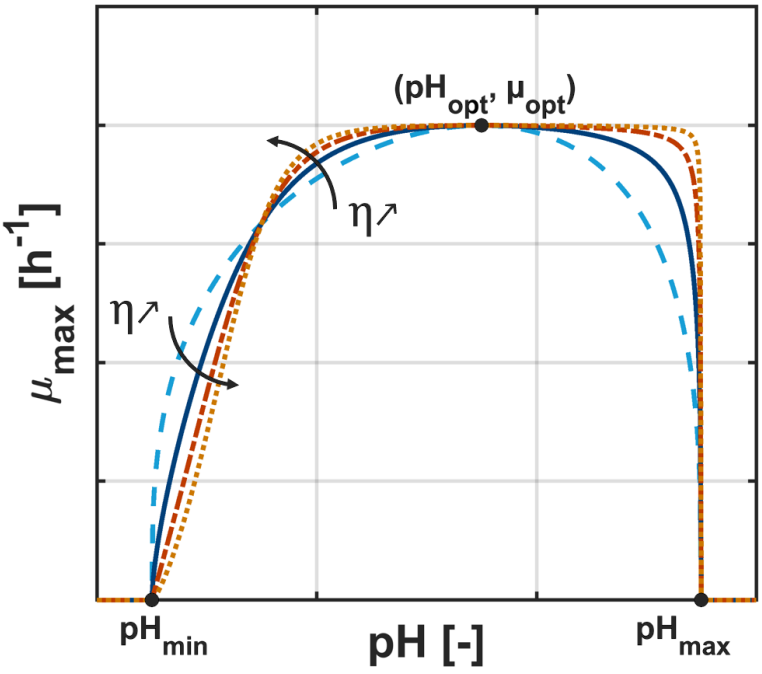
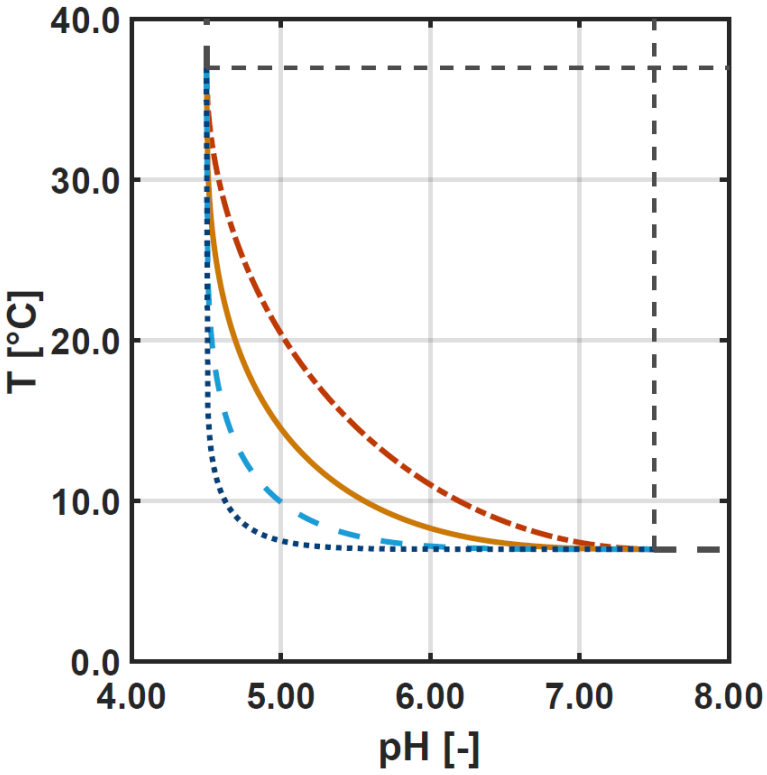


691

692 **Fig. 4:** The effect of the parameter η on the output of the model formulized in Eq. 8. The

693 values of η are1 (---), 2 (—), 3 (··-) and 4 (···).

694

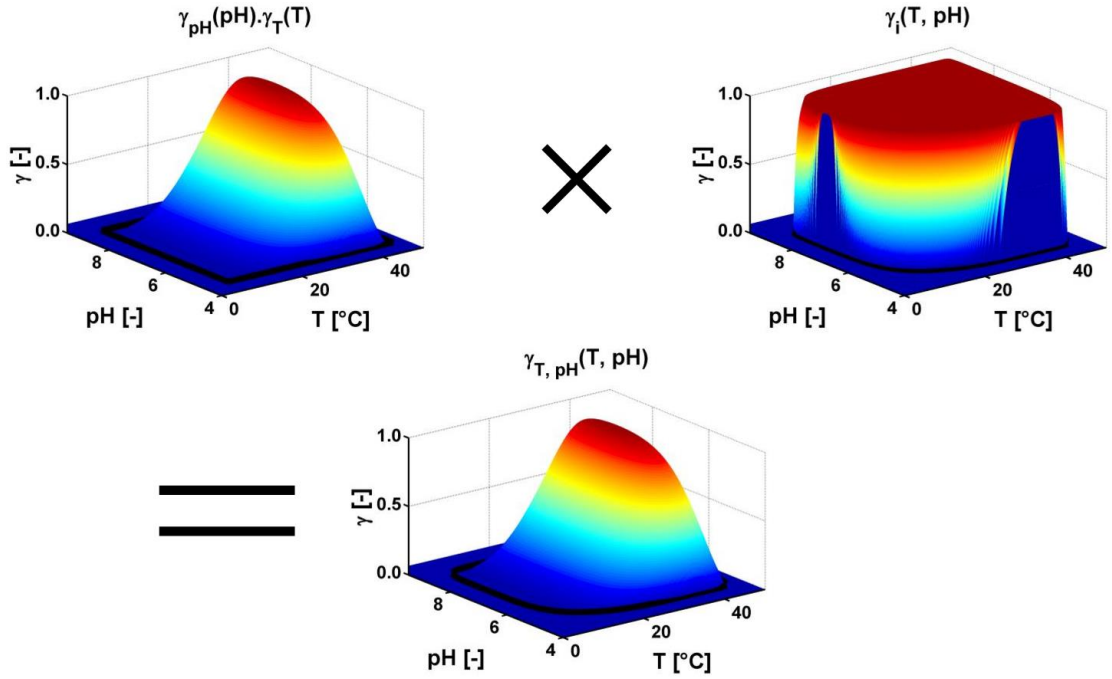


695

696 **Fig. 5.** The growth boundaries (cardinal parameters) of temperature and pH in the

697 suboptimal range (---) for different shape parameters β: 2 (·--·), 3 (—), 5 (---), 10 (···).

698



699

700 **Fig. 6.** Illustration of the interaction factor of the model of Le Marc et al. (2002).

701

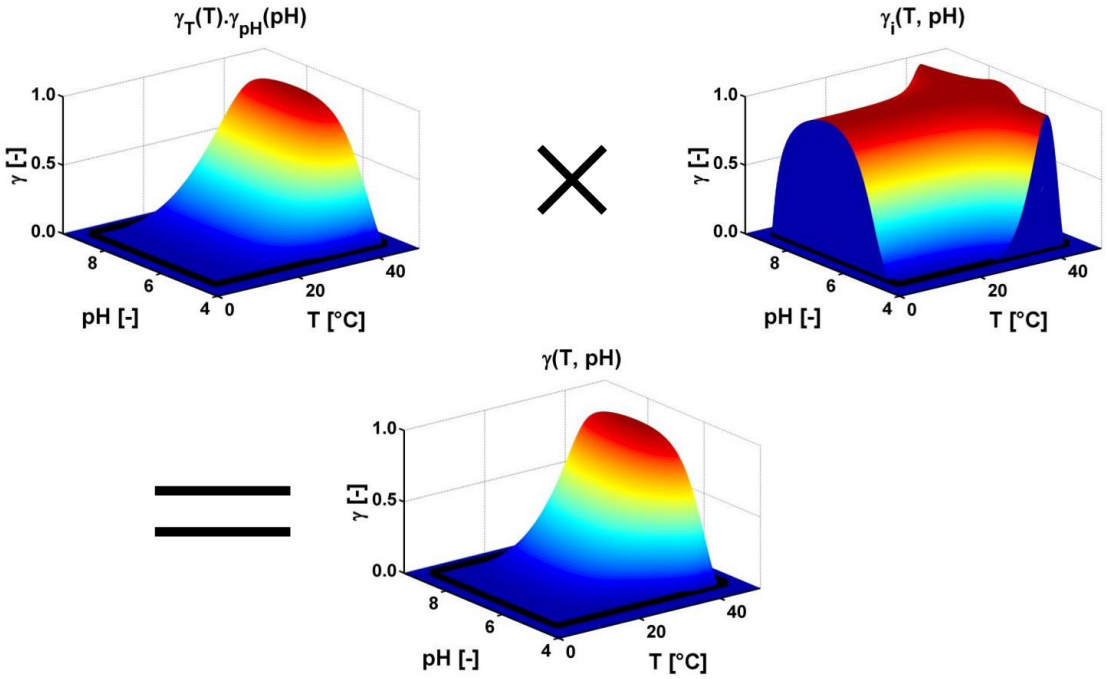


Fig. 7. Illustration of the interaction factor of the gamma-interaction model.

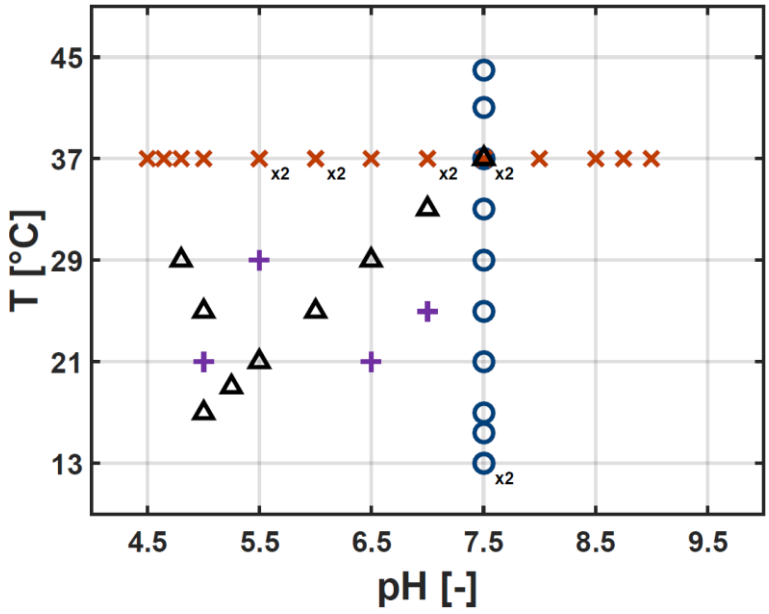
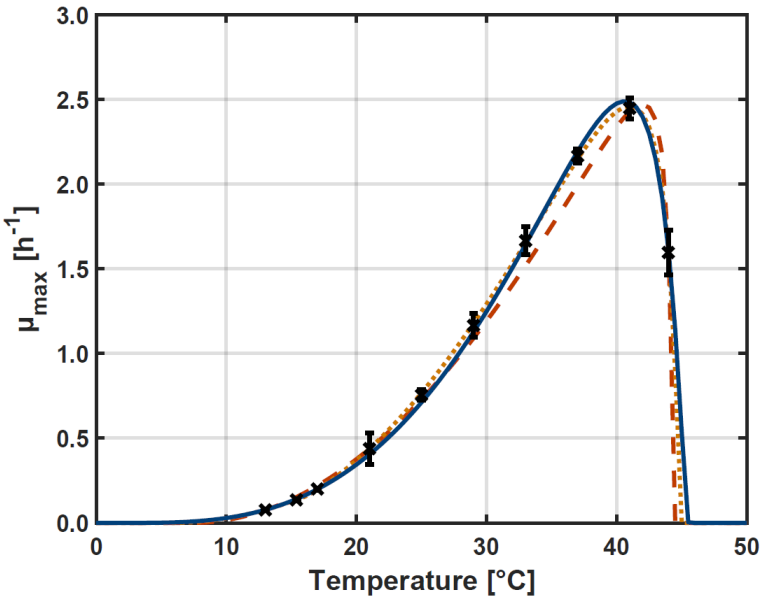


Fig. 8. Environmental conditions applied in bioreactor experiments. Dataset 1: temperature effect (**O**). Dataset 2: pH effect (**x**). Dataset 3: interactions (**Δ**). Dataset 4: validation (**+**). In case duplicates were performed, this is indicated with (**x2**).



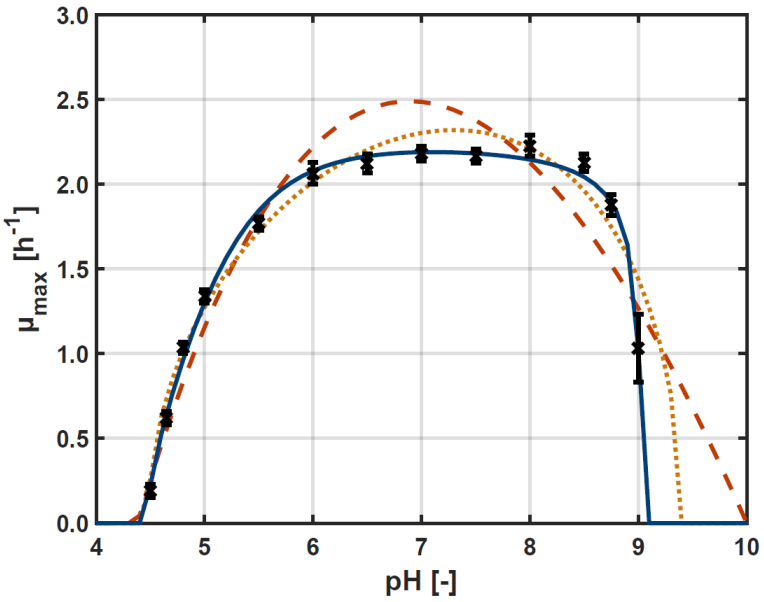
709

710 **Fig. 9.** Secondary models for the effect of temperature on the maximum specific growth

711 rate. Comparison of experimental growth rates with 95% confidence bounds (**x**) with

712 the CTMI (---), aCTMI (···) and bCTMI (—).

713



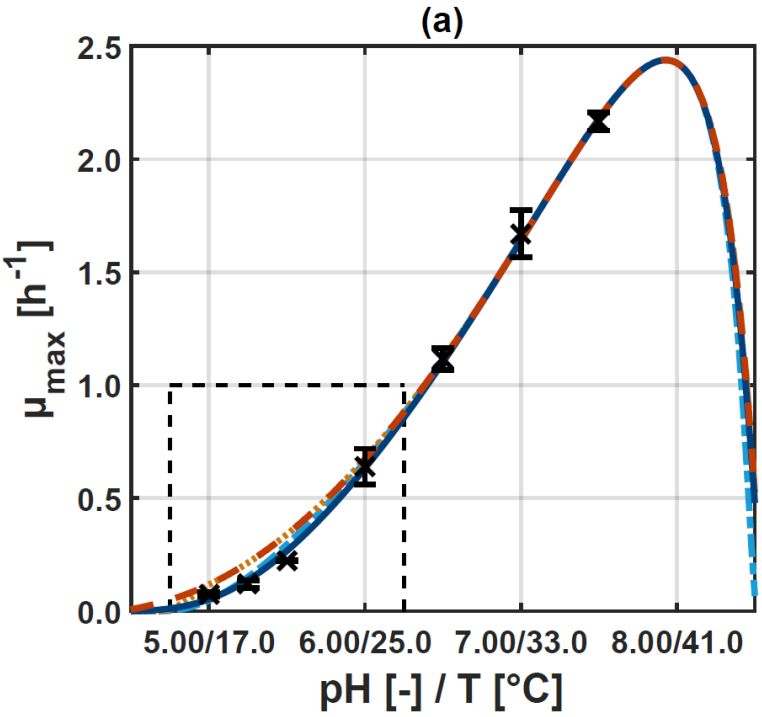
714

715 **Fig. 10.** Secondary models for the effect of pH on the maximum specific growth rate.

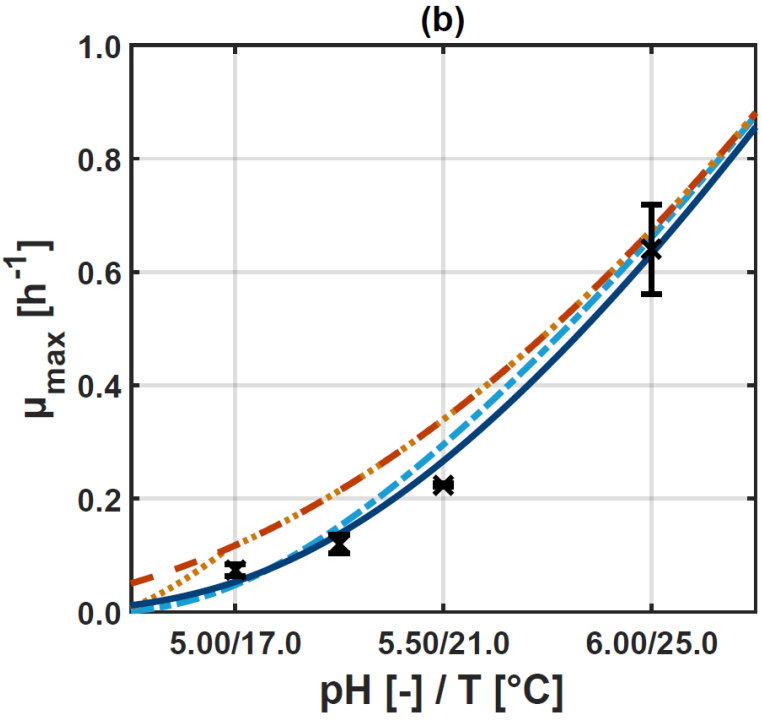
716 Comparison of experimental growth rates with 95% confidence bounds (**x**) with the

717 CPM (---), srCPM (·--·) and aCPM (—).

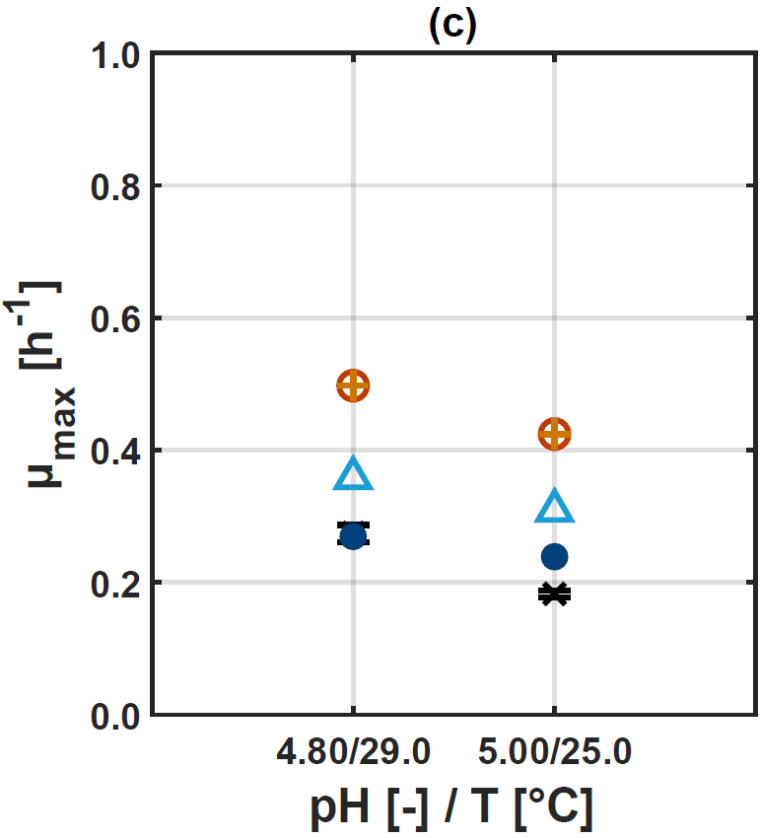
718


(a)
2.5
2.0
1.5
1.0
0.5
0.0
μmax [h-1]
5.00/17.0
6.00/25.0
7.00/33.0
8.00/41.0
pH [-] / T [°C]

719


(b)
1.0
0.8
0.6
0.4
0.2
0.0
μmax [h-1]
5.00/17.0
5.50/21.0
6.00/25.0
pH [-] / T [°C]

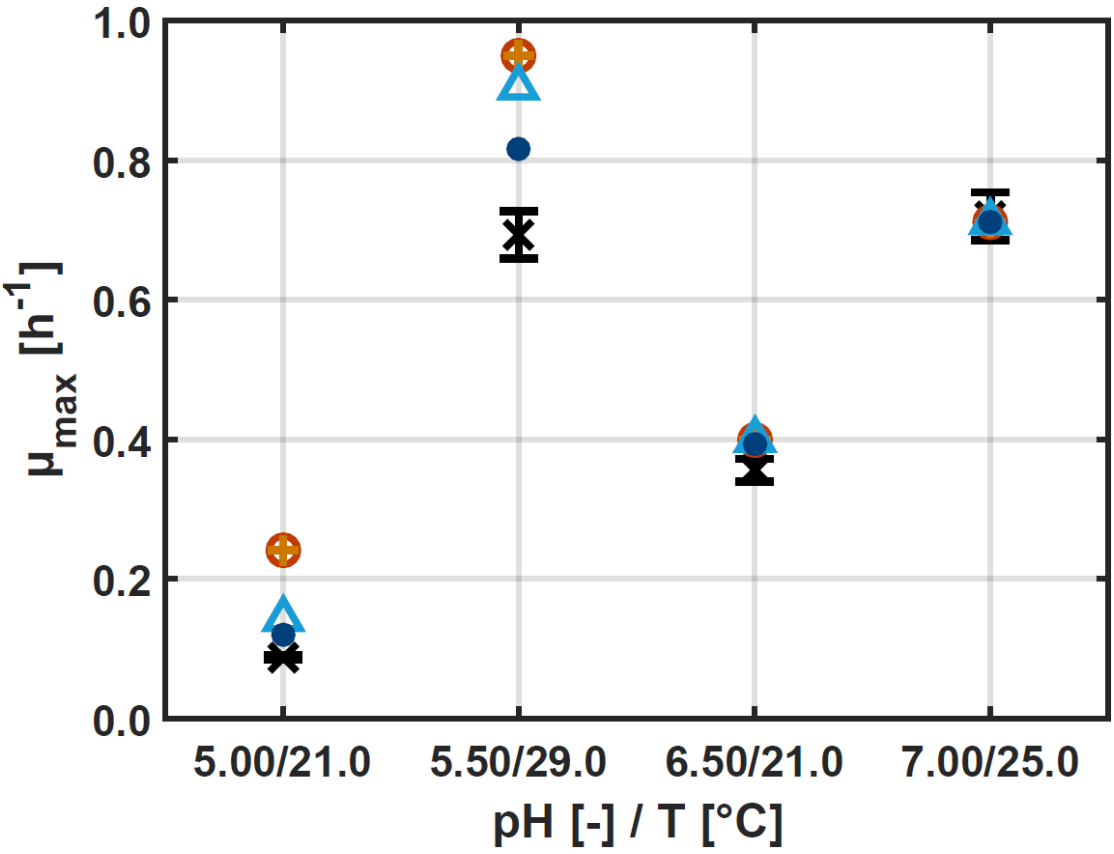
720



721

722 **Fig. 11.** Secondary models for the effect of temperature and pH on the maximum specific
723 growth rate. Comparison between the maximum specific growth rates of Dataset 3 (**x**) and
724 the four secondary models: the gamma model (**---**/**O**), the model of Le Marc et al. (2002)
725 (⋯/+), the model of Augustin and Carlier (2000) (·–·/Δ) and the gamma-interaction
726 model (–/●), (b) represents a close view at stressing conditions of (a). The two conditions
727 of Dataset 3 not included in (a) are illustrated in (c).

728



729

730 **Fig. 12.** Validation. Comparison between experimental growth rates (**x**) and the growth

731 rates calculated with the four secondary models: the gamma model (**O**), the model of Le

732 Marc et al. (2002) (+), the model of Augustin and Carlier (2000) (Δ) and the gamma-

733 interaction model (●).

734 # TABLE CAPTIONS

	CTMI		aCTMI		bCTMI	
T_{min} [°C]	7.3	± 0.2	5.6	± 0.6	2.3	± 0.4
T_1 [°C]	/		8.9	± 0.5	/	
T_c [°C]	/		16.1	± 0.8	/	
T_{opt} [°C]	42.0	± 1.1	41.0	± 0.7	40.6	± 0.4
T_{max} [°C]	44.4	± 0.5	44.9	± 0.5	45.5	± 0.6
μ_{opt} [h^{-1}]	2.47	± 0.29	2.46	± 0.16	2.49	± 0.15
MSE	0.130		0.080		0.084	

735

736 **Table 1**: Parameter estimates and 95% confidence intervals of the three cardinal
737 temperature models estimated on Dataset 1.

738

	CPM		srCPM		aCPM	
pH_{min} [-]	4.38	± 0.05	4.48	± 0.01	4.46	± 0.02
pH_{opt} [-]	6.89	± 0.10	7.30	± 0.09	7.10	± 0.13
pH_{max} [-]	10.00[1]	± 0.27	9.40	± 0.14	9.02	± 0.01
μ_{opt} [h^{-1}]	2.49	± 0.09	2.32	± 0.05	2.19	± 0.03
MSE	0.132		0.058		0.041	

[1] This parameter reached the upper bound of the parameter value during the parameter estimation.

Table 2: Parameter estimates and 95% confidence intervals of the three cardinal pH models estimated on Dataset 2.

	Gamma model	Model of Augustin and Carlier (2000)	Model of Le Marc et al. (2002)	Gamma-interaction model
β [-]	/	3.94 ± 0.22	/	1.68 ± 0.13
MSE	0.882	0.415	0.900	0.217

745
746

747 **Table 3**: Parameter estimation results and 95% confidence intervals of the four models

748 for the combined effect of temperature and pH estimated on Dataset 3.

749

	Gamma model	Model of Augustin and Carlier (2000)	Model of Le Marc et al. (2002)	Gamma-interaction model
MSE	1.522	0.576	1.522	0.319
A_f	1.703	1.327	1.703	1.203
B_f	1.430	1.238	1.430	1.153

750
751

752 **Table 4**: MSE, A_f and B_f values of the four models for the combined effect of temperature

753 and pH estimated on Dataset 4.