Modelling the inactivation of *Bacillus subtilis* spores by ethylene oxide processing

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Abstract

Ethylene oxide is currently a dominant agent in medical devices sterilization. This work intends to study the main effects and interactions of temperature (T), ethylene oxide (EO) concentration and relative humidity (RH) on commercial spore strips of *Bacillus subtilis,* var. *niger* (ATCC 9372) inactivation, the most common microorganism used in controlling the efficacy of the process. Experiments were carried out using a full factorial experimental design at two levels (2^3 factorial design). Limit target exposure conditions for EO concentration, temperature and relative humidity were 250 - 1000 mg EO/L, 40 - 60 °C and 50 - 90 %, respectively. Adopting a different approach from the first order kinetics, a Gompertz model was successfully applied in data fitting of the inactivation curves. *Bacillus subtilis* kinetic behaviour presented a sigmoidal inactivation with an initial shoulder (λ), followed by a maximum inactivation rate (k_{max}), these being model parameters. The influence of the referred environmental factors on lethality was assessed through λ and k_{max} estimates. It was concluded that, temperature and EO concentration were the most significant factors and consequently, additional experiments were carried out aiming at describing the parameters dependence on these process factors. Mathematical relations describing such dependences were successfully developed and included in the Gompertz kinetic model. The predictive ability of this integrated model was assessed, and its adequacy in predicting *B. subtilis* inactivation was proven.

Introduction

Sterility is a critical attribute of several medical devices (1). Nowadays, ethylene oxide (EO) is a dominant sterilization agent used in medical device industry. Its effectiveness and compatibility with most materials, together with the technical and technological advances that have been occurring in the last years, allowed to overlap the difficulties associated to this agent (2). The flexibility of EO sterilization process is one of its big advantages, resulting from its dependency on several factors, such as concentration, temperature, humidity and time (and their combinations).

Understanding the full dynamics of the sterilization allows the definition of appropriate process variables, thus contributing to its design optimization.

The microbial inactivation by EO sterilization has been considered to follow a first order kinetics, although model prediction was scarcely (or never) assessed. According to the first order kinetics approach, a plot of the logarithmic of the number of survival microorganisms vs. time is a straight line. The reciprocal of the slope is the well-known D-value (i.e. the required time for a 10-fold of the microbial load). However, a number of studies report deviations to the linear behaviour, therefore the extrapolations of predicted times based on reported D-values, may not be appropriate (3, 4).

Deviations from linearity can be assumed as a complete (or incomplete) sigmoidal behaviour with the following features: shoulder time (or lag) period, prior to a linear phase corresponding to a maximum growth/inactivation rate, followed by a tail (asymptotic phase). A number of models have been used to describe these

sigmoidal tendencies (5, 6): Weibull and logistic functions (7, 8), Gompertz equation (9), Baranyi and Geeraerd models (10, 11).

Gompertz function and its modified forms have the ability of modelling both linear and asymmetrical sigmoidal data. For the inactivation behaviour, the following Gompertz function (9, 12, 13, 14, 15, 16, 17) can be used:

$$\log\left(\frac{N}{N_0}\right) = A \cdot \exp\left[-\exp\left\{\frac{-k_{max} e}{A}(\lambda - t) + 1\right\}\right] \qquad (1)$$

where N is the microbial load at a particular process time t (the index 0 is related to initial microbial load). The model parameters are the maximum inactivation rate, k_{max}, and shoulder period, λ. The A value is the asymptotic response, being considered the tail (i.e. a resistant residual microbial population or an enumeration method limitation, according to different authors' opinions).

Studies on the influence of the process variables on microbial inactivation kinetics using ethylene oxide are lacking. Quantification of the kinetic parameters is obtained, as well as effect of relevant factors on their estimates. Consequently, the objectives of the present study were to: 1) screen the most significant variables on *B. subtilis* inactivation by EO sterilization, 2) model the inactivation kinetics of *B. subtilis* including the variables' effects and, 3) provide a method of integrating lethality, thus contributing to the design optimization and efficient control of the inactivation processes. This is certainly important when moving towards parametric release, i.e. the approval of the process relying merely on the measurement and assessment of process variables (18).

Materials and methods

Experimental design

Experiments were carried out in a standard EO sterilizer (21059C Sterichem, France) of approximately 3 m^3 with controlled temperature, EO concentration and humidity. The sterilization cycle was performed under vacuum and the referred conditions were maintained homogeneous inside the chamber due to forced recirculation, and were monitored by adequate equipment.

Temperature and relative humidity were monitored inside the load, using Kaye ValProbe® wireless data loggers, part numbers XVP-L-T and X2520, respectively. EO concentration was assessed by an infrared analyser in the sterilizer chamber and corresponds to a condition obtained in the sterilizer headspace, since the techniques currently available to the industry cannot measure this parameter inside the load, where the lethality of the process is being monitored.

The sporicidal activity of a specific EO sterilization cycle was assessed by placing the spore strip biological indicators with about 10^6 *B. subtilis* spores (SGMStrip™, SGM Biotech, Inc., Montana, USA) into the middle of peel-packs of drape material (488-103, Bastos Viegas S.A, Portugal) adjacent to temperature and humidity sensors. BI samples were removed after different exposure times to the sterilizing agent for enumeration and the recovery technique was validated to obtain reliable spores enumeration.

The conditions were defined according to a full factorial experimental design at two levels of three factors (2^3 factorial design) (19). The parameters used for each

condition, including the time of each step, with the exception of the gas exposure time, were kept constant. Three independent variables representing temperature, EO concentration and humidity were assumed, each variable tested in two levels: a high level (+) and a low level (-), according to Table 1 (totalling 8 experimental conditions, corresponding to runs 1 to 8). Target limits for the exposure conditions of EO concentration, temperature and relative humidity were 250 - 1000 mg EO/L, 40 – 60 °C and 50 - 90 %, respectively. The limits chosen for the process variables were based on literature review (20, 21, 22, 23) and operating conditions of the sterilizers. However, difficulties arise in stabilizing the process conditions and actual attained operating values are in Table 1.

The analysis of variance (ANOVA) allowed identifying the most significant parameters affecting microbial inactivation during EO sterilization and, additional experiments considering intermediate conditions of these parameters were defined in order to model their effects and combined effects on the lethality (runs 9 to 15 included in Table 1).

Modelling procedures

Equivalent time

Since standard-sized process chambers do not produce square wave cycles and significant lethality may occur throughout gas injection and exhaust phases, an equivalent exposure time, U, rather than exposure time, t, should be considered (in

equation 1). The following expression can be used for its estimation (24, 25, 21, 26):

$$U = \frac{t_{injection}}{2} + t_{nitrogen\ overlay} + t + \frac{t_{exhaust}}{2} \qquad (2)$$

assuming a constant rate of pressure increase (in the injection phase) and pressure decrease (in the exhaust phase).

Regression analysis and statistical assessment

The Gompertz model (equation 1) was used to fit experimental inactivation data normalized in relation to initial load and expressed as logarithms (*i.e.* $\log(N/N_0)$). The non-linear regression analysis was carried in Statistica© 6.0 software (StatSoft, USA), using the Levenberg-Marquardt algorithm to minimize the sum of the squares of the differences between the predicted and experimental values. Model parameters (i.e. k_{max} and λ) were estimated and their precision was evaluated by confident intervals by the standardised half width (SHW) at 95%. The quality of the regression was assessed by residuals analyses (normality and randomness) and by the coefficient of determination R^2.

Results from 2^3 factorial experimental design were analysed by ANOVA procedures.

Results and Discussion

Influence of environmental conditions on microbial inactivation kinetics

The influence of EO concentration, temperature and relative humidity on inactivation behaviour of *B. subtilis* spores was studied in a preliminary step using the conditions defined according to the 2^3 experimental design (Table 1 and Figure 1). The shape of the inactivation curves depends on the lethal agent intensity, but in general significant deviations from linearity are evident and the general shape is a concave downward curve. As observed, the data exhibit an initial shoulder prior to exponential phase of death. Forcing a straight line through the experimental non-linear survival curves is obviously an undesirable option, and can lead to a considerable error of underestimation of process time when combined with extrapolation.

A Gompertz model was chosen due to its versatility in describing different tendencies (from linear till pronounced sigmoidal shapes), depending on the magnitude of the model parameters. Results showed that experimental inactivation data were successfully fitted with the Gompertz model and a high precision of k_{max} and λ estimates was attained (evaluated by $SHW_{95\%}$, Table 1). The goodness of model fitting was assessed on the basis of residuals randomness and normality (which was verified in all cases) and on the coefficient of determination (R^2 was greater than 0.98, meaning that at least 98% of the observed variability was explained by the model).

The overall inactivation curves were divided into a slower first stage (i.e., a shoulder phase) and in a second stage for exponential inactivation (i.e. maximum death rate). A true tailing was not observed under the conditions tested. The residual final value could be defined due to an enumeration method limitation and does not correspond to a residual resistant population. Consequently, the tail was not defined as a model parameter, and an asymptotic value of -7.5 was assumed (reflects overall tendency in all experiments and avoids interference with the studied kinetic parameters).

The factorial experimental design allowed concluding about the process variables (and combination of them) that significantly affected the inactivation kinetics of *B. subtilis* behaviour (assessed by k_{max} and λ parameters). Results showed that temperature had the most significant effect on k_{max} and λ, followed by EO concentration (at a significance level of 15%). The temperature and the EO concentration have a negative effect on λ and a positive effect on k_{max}. This means that higher temperatures and EO concentration imply narrow shoulder times and higher inactivation rates. On the contrary, lower inactivation rates and more evident shoulder phases were observed at the lowest temperature and EO concentration. Effects resulting from the combination of temperature and EO were not significant. Also, the relative humidity (and its combined effects with the remaining variables) did not influence significantly the inactivation of *B. subtilis.*

Based on these achievements, seven additional experimental conditions were tested using intermediate conditions of temperature and concentration, combined with the limits previously defined: one temperature (about 50 °C) and two more EO concentrations (about 470 mg/L and 700 mg/L). These experiments provided

important data aiming at describing more accurately the dependence of kinetic parameters on environmental conditions. These results are also included in Table 1 (runs 9 to 15) and inactivation curves in Figure 2. Model adequacy and goodness of fits were assessed as previously mentioned. A good precision of parameter estimates was observed, since low errors were attained (evaluated by $SHW_{95\%}$, Table 1).

Assessment of model prediction

Temperature and EO concentration were the most significant factors affecting *B. subtilis* inactivation parameters. Consequently, equations that describe these influences on k_{max} and λ were developed aiming at obtaining a mathematical inactivation model expressed in terms of the relevant processing variables. It was assumed that k_{max} varied linearly with EO concentration, for given temperatures ($k_{max}=a_k+b_k[EO]$) (Figure 3). Concerning λ parameter, the relation $\lambda=a_\lambda+ b_\lambda \ln[EO]$ provided the best fits (Figure 4).

The parameters a_k and b_k, as well as a_λ and b_λ, were estimated by regression analysis procedures, and the influence of temperature on these estimates were studied. Linear relations of these parameters on temperature were defined (Figure 5 and 6). The quality of the model fits were attained by residual analysis and R^2 magnitude, that in all cases were above 0.90.

Merging all the equations developed, the final expressions that relate k_{max} and λ with temperature and EO concentration are the following:

$$k_{max} = \left(1.42\times10^{-4}\,T - 4.96\times10^{-3}\right) + \left(5.54\times10^{-8}\,T + 1.25\times10^{-6}\right)[EO] \qquad (3)$$

$$\lambda = \left(1.63\times10^{1}T - 1.06\times10^{3}\right)\ln([EO]) + \left(-1.25\times10^{2}T + 8.23\times10^{3}\right) \quad (4)$$

The final objective of this work was to express the inactivation data (i.e. $\log(N/N_0)$) in terms of the most significant processing variables (i.e. T and EO concentration). Consequently, equations 3 and 4 were integrated in the Gompertz model (eq. 1). The final expression obtained was:

$$\log\left(\frac{N}{N_0}\right) = (-7.5)\exp\left\{-\exp\left[\frac{-\left[\left(1.42\times10^{-4}T - 4.96\times10^{-3}\right) + \left(5.54\times10^{-8}T + 1.25\times10^{-6}\right)[EO]\right]e}{-7.5}\times\right.\right.$$
$$\left.\left.\times\left[\left(1.63\times10^{1}T - 1.06\times10^{3}\right)\ln([EO]) + \left(-1.25\times10^{2}T + 8.23\times10^{3}\right) - U\right] + 1\right]\right\}$$

(5)

Or deducted for U,

$$U = \lambda - \frac{(-7.5)}{k_{max}\times e}\left\{1 - \ln\left(-\ln\left(\frac{SAL}{(-7.5)}\right)\right)\right\} \quad (6)$$

where SAL is the sterility assurance level.

As already discussed, the tail parameter was assumed to be -7.5.

The prediction of *B. subtilis* inactivation by the newly developed model can be visualized in Figure 1 (for the seven first runs). The grey line was obtained considering the average values of temperature and EO concentration. One should be aware about difficulties in reproducing (and/or stabilizing) experimental conditions. Based on experimental results, it was found a variability of 4% for temperature and 14% for EO conditions. Thus, the predictive ability of the final model was assessed considering a band of prediction (upper and lower limits

defined by considering the maximum fluctuations of temperature and EO concentration and calculated using eq. 5).

These bands include the experimental data for all the conditions tested, which demonstrates that the inactivation of *B. subtilis* under EO sterilization can be successfully predicted using a model that only takes into account the process variables.

Conclusions

The *B. subtilis* EO inactivation did not follow a first order kinetics (i.e. linear inactivation). Experimental data showed an initial shoulder and a maximum inactivation rate period, and a Gompertz model was successfully applied in data fitting. An inactivation model that described the process kinetics only in terms of the relevant process variables (temperature and EO concentration) was achieved. The predictive ability of this integrated model was assessed, and its adequacy in predicting *B. subtilis* inactivation was verified.

The results of this work are certainly a contribution for an efficient control, design and optimization of the EO sterilization process.

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References

1. **Rodriguez, A. C., B. Young, K. Caulk, J. Zelewski, S. Kwasnica, and S. Aguirre.** 2001. Calculating accumulated lethality and survivorship in EtO sterilisation processes. Med Devices Diagn Industry. **23**(9):100-107.

2. **Mendes, G. C., T. R. S. Brandão, and C. L. M. Silva.** 2007. Ethylene Oxide Sterilization of Medical Devices: A Review. Am J Infect Control. **35**(9):574-581.

3. **Juneja, V. K., B. S. Eblen, and H. M. Marks.** 2001. Modelling non-linear survival curves to calculate thermal inactivation of *Salmonella* in poultry of different fat levels. Int J Food Microbiol. **70**:37-51.

4. **Peleg, M.** 2000. Microbial survival curves – the reality of flat “shoulders” and absolute thermal death times. Food Res Int. **33**:531-538.

5. **Chen, H.** 2006. Use of linear, Weibull, and log-logistic functions to model pressure inactivation of seven foodborne pathogens in milk. Food Microbiol. **24**(3):197-204.

6. **Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van't Riet**. 1990. Modeling of the Bacterial Growth Curve. Appl Environ Microbiol. **56**(6):1875-1881.

7. **Cole, M. B., K. W. Davies, G. Munro, C. D. Holyoak, and D. C. Kilsby**. 1993. A vitalistic model to describe the thermal inactivation of *Listeria monocytogenes.* J Ind Microbiol Biotechnol. **12**(3-5):232-239.

8. **Peleg, M., and M. B. Cole.** 1998. Reinterpretation of microbial survival curves. Crit Rev Food Sci Nutr. **38**(5):353-380.

9. **Bhaduri, S., P. W. Smith, S. A. Palumbo, C. O. Turner-Jones, J. L. Smith, B. S. Marmer, R. L. Buchanan, L. L. Zaika, and A. C. Williams.** 1991. Thermal destruction of *Listeria monocytogenes* in liver sausage slurry. Food Microbiol. **8**:75–78.

10. **Baranyi, J., and T. A. Roberts**. 1994. A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol. **23**:277-294.

11. **Geeraerd, A. H., C. H. Herremans, and J. F. Van Impe**. 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int J Food Microbiol. **59**(3):185-209.

12. **Chhabra, A. T., W. H. Carter, R. H. Linton, and M. A. Cousin**. 1999. A predictive model to determine the effects of pH, milkfat, and temperature on thermal inactivation of *Listeria monocytogenes*. J Food Prot. **62**:1143–1149.

13. **Garthright, W. E.** 1991. Refinements in the prediction of microbiol growth curves. Food Microbiol. **8**:239–248.

14. **Gibson, A. M., N. Bratchell, and T. A. Roberts.** 1987. The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. J Appl Bacteriol. **62**(6):479–490.

15. **Gil, M. M., T. R. S. Brandão, and C. L. M. Silva**. 2006. A modified Gompertz model to predict microbial inactivation under time-varying temperature conditions. J Food Eng. **76**(1):89-94.

16. **Kim, S. R., M. S. Rhee, B. C. Kim, H. Lee, and K. H. Kim.** 2007. Modelling of the inactivation of *Samonella typhimurium* by supercritical carbon dioxide in

physiological saline and phosphate-buffered saline. J Microbiol Methods. **70**(1):132-141.

17. **Linton, R. H., W. H. Carter, M. D. Pierson, and C. R. Hackney.** 1995. Use of a modified Gompertz equation to model non-linear survival curves for *Listeria monocytogenes* Scott A. J Food Prot. **58**:946–954.

18. ISO technical committee 198. 2007. ISO 11135-1, Sterilization of health care products - Ethylene oxide, Part 1: Requirements for development, validation and routine control of a sterilization process for medical devices.

19. **Box, G. E. P., W. G. Hunter, and J. S. Hunter.** 1978. *In* J. Wiley & Sons (ed.). Statistics for Experiments: an Introduction to Design, Data Analysis and Model Building. New York.

20. **Heider, D., J. Gomann, B. U. Junghann, and U. Kaiser.** 2002. Kill kinetics study of *Bacillus subtilis* spores in ethylene oxide sterilisation processes. Zentr Steril. **10**(3):158-167.

21. **Mosley, G. A., J. R. Gillis, and J. E. Whitbourne.** 2002. Calculating equivalent time for use in determining the lethality of EtO sterilization processes. Med Devices Diagn Industry. **24**(2):54-63.

22. **Rutala, W. A., and D. J. Weber**. 1999. Infection control: the role of disinfection and sterilization. J Hosp Infect. **43**(Suppl):S43-55.

23. **Sordellini, P. J., S. E. Satter, and V. A. Caputo**. 1998. EtO sterilization: principles of process design. Med Devices Diagn Industry. **20**(12):47.

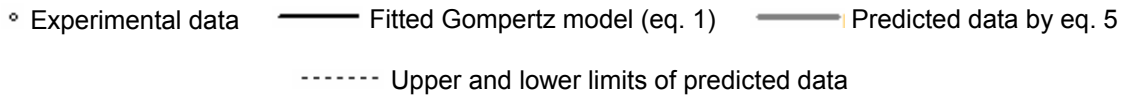
24. **Mosley, G. A., and J. R. Gillis.** 2004. Factors affecting tailing in ethylene oxide sterilization Part 1: When tailing is an artifact … and scientific deficiencies in ISO 11135 and EN 550. PDA J Pharm Sci Technol. **58**(2):81-95.

25. **Mosley, G. A., J. R. Gillis, and G. Krushefski.** 2005. Evaluating the formulae for integrated lethality in ethylene oxide sterilization using six different endospores forming strains of bacteria, and comparisons of integrated lethality for ethylene oxide and steam systems. PDA J Pharm Sci Technol. **59**(1):64-86.

26. **Mosley, G. A., and C. W. Houghtling.** 2005. Interpreting and understanding microbial data in validation of ethylene oxide sterilization processes. Biomed Instrum Technol. **39**:466-482.

Figure Captions

Fig. 1 Inactivation of *B. subtilis* spores by EO sterilization at conditions defined according to the 2^3 factorial design.



a) Run1: T=60 °C; EO=233 mg/L; RH=63 %

b) Run2: T=44 °C; EO=257 mg/L; RH=86 %

c) Run3: T=34 °C; EO=222 mg/L; RH=60 %

d) Run4: T=40 °C; EO=980 mg/L; RH=90 %

e) Run5: T=59 °C; EO=266 mg/L; RH=85 %

f) Run6: T=33 °C; EO=940 mg/L; RH=61 %

g) Run7: T=59 °C; EO=1004 mg/L; RH=98 %

Fig. 2 Inactivation of *B. subtilis* spores by EO sterilization at the additional experimental conditions.

Run9: T=37 °C; EO=674 mg/L; RH=73%

Run10: T=37 °C; EO=456 mg/L; RH=80%

Run11: T=51 °C; EO=247 mg/L; RH=80%

Run12: T=51 °C; EO=447 mg/L; RH=67%

Run13: T=50 °C; EO=675 mg/L; RH=72%

Run14: T=60 °C; EO=738 mg/L; RH=71%

Run15: T=62 °C; EO=498 mg/L; RH=77%

Fig. 3 Influence of EO concentration on k_{max} at 37.0, 50.5 and 60.0 °C.

Fig. 4 Influence of EO concentration on λ at 38.0, 50.5 and 60.0 °C.

Fig. 5 Influence of T on a_k and b_k parameters.

Fig. 6 Influence of T on a_λ and b_λ parameters.

Table Captions

Table 1 Estimated k_{max} and λ parameters of *B. subtilis* inactivation at the temperature, EO concentration and relative humidity conditions tested.

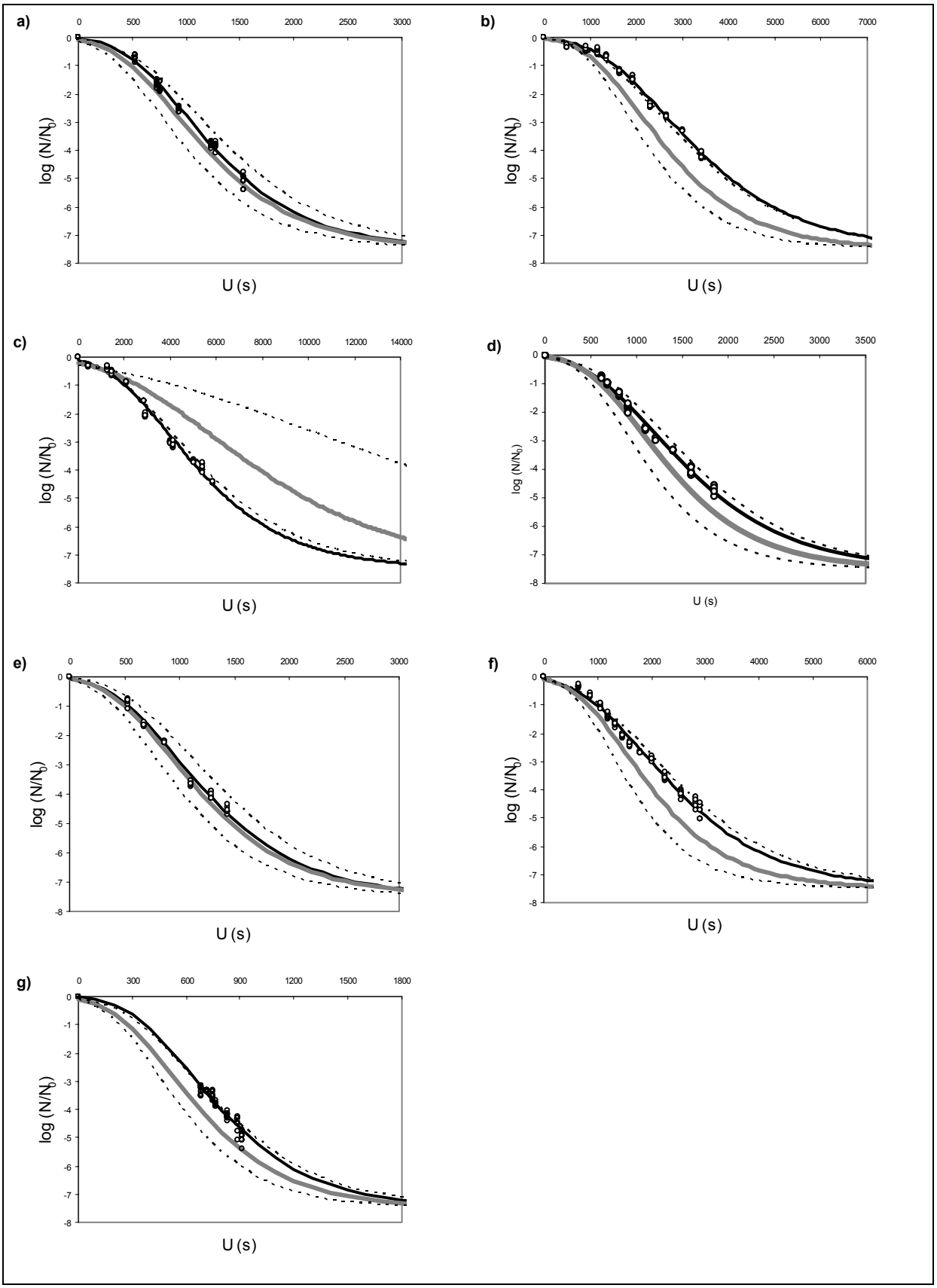


Fig. 1

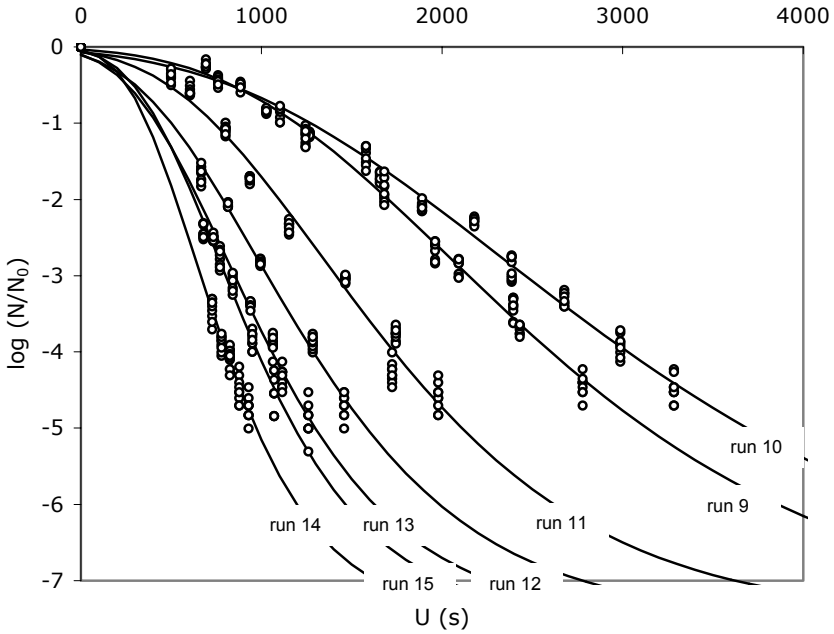

0
1000
2000
3000
4000
0
-1
-2
-3
-4
-5
-6
-7
log (N/N0)
U (s)
run 10
run 9
run 11
run 14
run 13
run 15
run 12

Fig. 2

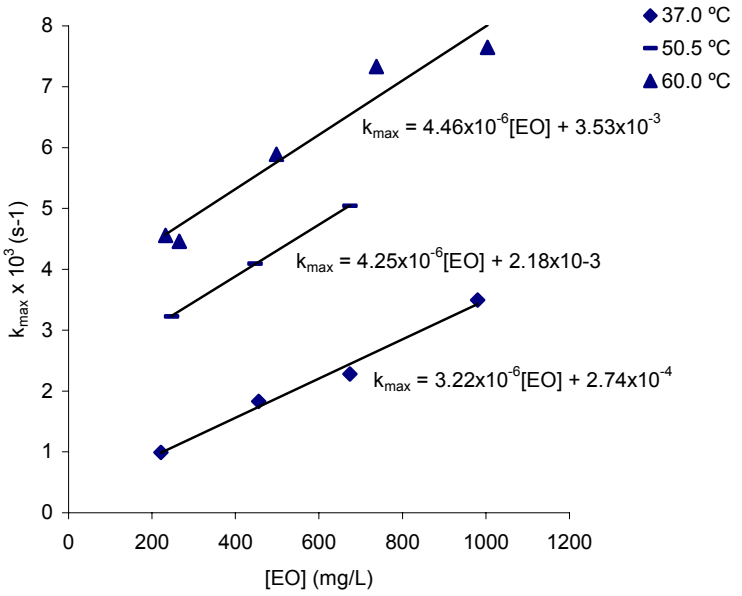

37.0 ºC
50.5 ºC
60.0 ºC
kmax = 4.46x10-6[EO] + 3.53x10-3
kmax = 4.25x10-6[EO] + 2.18x10-3
kmax = 3.22x10-6[EO] + 2.74x10-4
kmax x 10^3 (s-1)
[EO] (mg/L)
0
200
400
600
800
1000
1200
1
2
3
4
5
6
7
8

Fig. 3

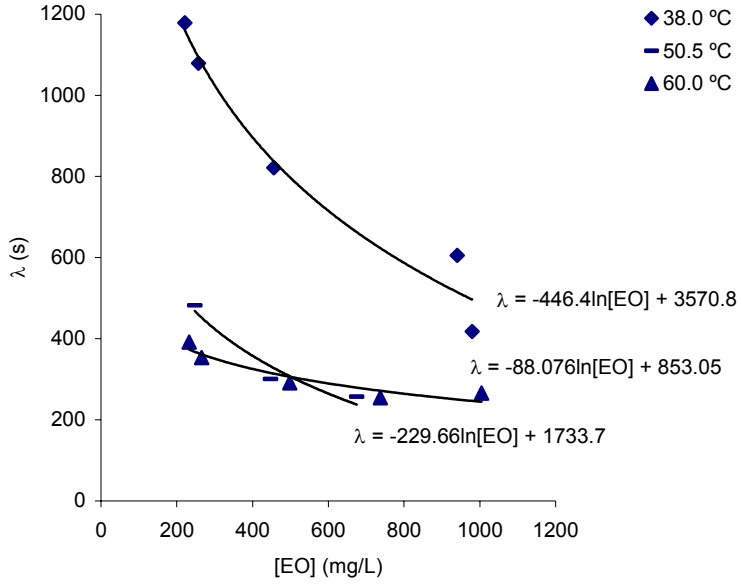

38.0 ºC
50.5 ºC
60.0 ºC
λ = -446.4ln[EO] + 3570.8
λ = -88.076ln[EO] + 853.05
λ = -229.66ln[EO] + 1733.7
λ (s)
[EO] (mg/L)
0
200
400
600
800
1000
1200

Fig. 4

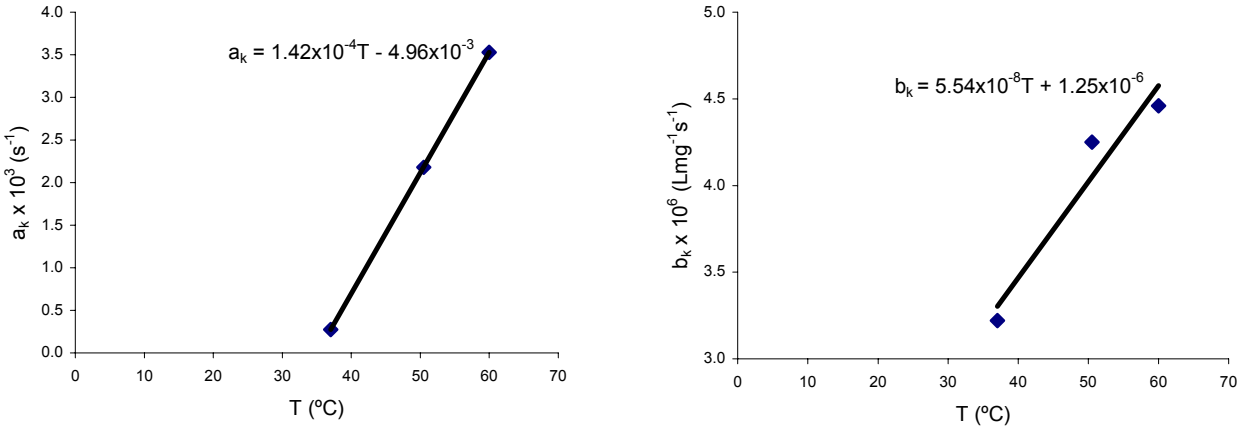

$a_k = 1.42x10^{-4}T - 4.96x10^{-3}$
a_k x 10^3 (s^{-1})
T (ºC)
$b_k = 5.54x10^{-8}T + 1.25x10^{-6}$
b_k x 10^6 ($Lmg^{-1}s^{-1}$)
T (ºC)

Fig. 5

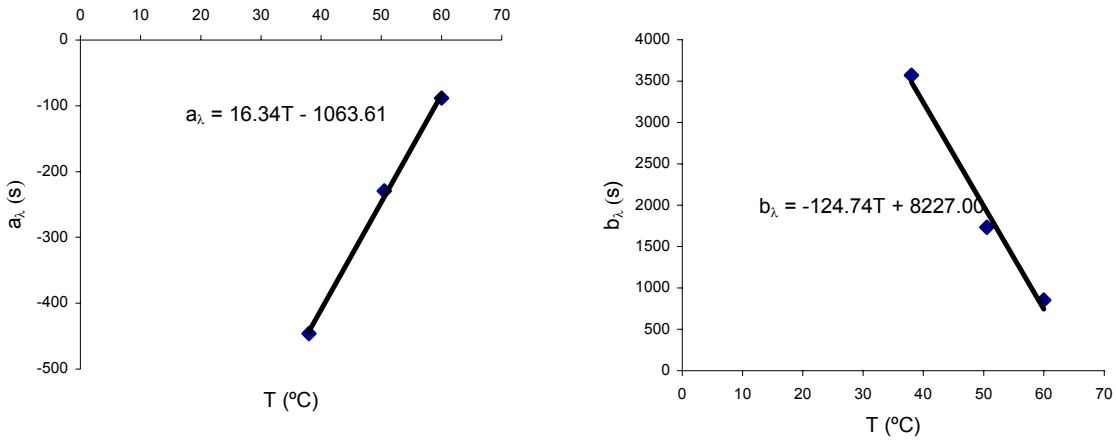

0
10
20
30
40
50
60
70
0
-100
-200
-300
-400
-500
a_λ = 16.34T - 1063.61
a_λ (s)
T (ºC)
4000
3500
3000
2500
2000
1500
1000
500
0
b_λ = -124.74T + 8227.00
b_λ (s)
T (ºC)

Fig. 6

Table 1

Run	Variables						Parameters				Regression analysis
	T (°C)		[EO] (mg/L)		RH (%)		k_{max} x10^3 (s^{-1})	$SHW_{95\%}$	λ (s)	$SHW_{95\%}$	R^2
1	60	(+)	233	(-)	63	(-)	4.56	3.01	391.22	5.24	0.992
2	44	(-)	257	(-)	86	(+)	1.78	2.42	1079.26	3.25	0.993
3	34	(-)	222	(-)	60	(-)	0.989	2.93	1178.85	7.45	0.988
4	40	(-)	980	(+)	90	(+)	3.49	2.66	417.73	5.26	0.991
5	59	(+)	266	(-)	83	(+)	4.46	3.56	353.18	7.19	0.991
6	33	(-)	940	(+)	61	(-)	2.16	2.50	605.12	5.63	0.989
7	59	(+)	1004	(+)	98	(+)	7.65	8.59	265.92	16.75	0.983
8	60	(+)	977	(+)	46	(-)	10.00	*	0.00	*	*
9	37		674		73		2.28	2.72	831.31	4.05	0.991
10	37		456		80		1.83	2.57	821.44	4.68	0.991
11	51		247		80		3.23	3.60	481.61	7.10	0.985
12	51		447		67		4.09	3.37	300.04	8.67	0.994
13	50		675		72		5.04	4.94	256.72	14.53	0.992
14	60		738		71		7.33	8.01	254.67	17.83	0.994
15	62		498		77		5.89	6.25	291.40	12.25	0.988

* Meaningless value