**Mathematical description of yessotoxin production by Protoceratium
reticulatum in culture.**

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Abstract

The production dynamics of yessotoxin (YTX) by *Protoceratium reticulatum* and phosphate uptake in culture were investigated in relation to cell growth. The equations used were: the reparametrized logistic for cell production, Luedeking-Piret model for yessotoxin production and maintenance energy model for phosphate consumption. Thus, the YTX formation rate was proportional to producer biomass at the end of the asymptotic phase of culture as a secondary metabolite. Moreover, the equations proposed showed a high accuracy to predict these bio-productions in different experimental conditions and culture scales.

Keywords: yessotoxin production; dinoflagellate growth; *Protoceratium reticulatum*; phosphate consumption; unstructured mathematical model.

1. Introduction

Yessotoxins (YTXs) are disulphated polyether toxins, first isolated from the scallop *Patinopecten yessoensis* in Japan (Murata et al., 1987) and produced by the dinoflagellate *Protoceratium reticulatum* (Claparède et Lachmann) Bütschli (= *Gonyaulax grindleyi* Reinecke) (Murata et al., 1987; Satake et al., 1997; Satake et al., 1999; Ramstad et al., 2001; Samdal et al., 2004) and in smaller amounts by the dinoflagellate *Lingulodinium polyedrum* (Stein) Dodge (= *Gonyaulax polyedra* Stein) (Tubaro et al., 1998; Draisci et al., 1999; Paz et al., 2004). Although YTX does not produce diarrhoea to humans (Alfonso et al., 2003; De la Rosa et al., 2001) and its action mechanism is in part unknown it have been found to be potent cytotoxins (Bianchi et al., 2004; Pérez-Gómez et al., 2006). Moreover it has been demonstrated by i.p. injection in mice that YTXs causes cellular damage in myocardial tissues (Terao et al., 1990; Aune et al., 2002). YTXs have been detected in shellfish from different areas such as Norway (Aesen et al., 2005), Italy (Ciminiello et al., 2003a) or Spain (Arévalo et al., 2004; Mallat et al., 2006). YTXs have been associated with the diarrhetic shellfish poisoning (DSP), because it is often simultaneously extracted with DSP toxins, and gives positive results when tested in the conventional mouse bioassay for DSP toxins causing unnecessary shellfish farm closures. Because of this, European Union established a maximum level permitted in shellfish of 1 mg/Kg (EC 2004). These issues are now stimulating increasing interest in the study the YTXs and in the producing organisms.

Frequently *P. reticulatum* cultures are developed to obtain YTXs for different aims e.g. toxicological studies, YTX purification or extraction of YTX analogues (Satake et al.,

1999; Ciminiello et al., 2003b; Samdal et al., 2004; Miles et al., 2005; Paz et al., 2006; García-Camacho et al., 2007; Guerrini et al., 2007; Gallardo-Rodríguez et al., 2007).

Although the objectives of these cultures are different, experimental results show that both culture conditions and strain influence the dinoflagellate growth rate, the toxin yield and the profile of the YTXs produced.

Thus, an indispensable tool for the optimization, control, design and analysis of the yessotoxin production to industrial scale, derive of the development of mathematical robust models. These equations should be formulated with parameters of clear biological significance and they will have to be statistically consistent in order to be able to develop a suitable software for bioreactors. However, the mathematical description of this bioproduction, including growth, toxin production and substrates consumptions remain unstudied.

Taking this into account, we have focussed on describing YTX production and phosphate uptake in relation to growth by a strain of *P. reticulatum*. With this purpose, experimental cultures were grown under different conditions of irradiance, agitation and volume. The numerical results were evaluated in order to establish an unstructured mathematical model which can be used to describe the corresponding culture dynamics.

2. Materials and Methods

2.1. Cultures

A strain of *P. reticulatum* (GG1AM), originally from Southwest Spain, was obtained from the collection of phytoplankton cultures at the Centro Oceanográfico in Vigo. The

culture used for the inoculation was maintained at 19 ± 1 °C, at a salinity of 34, with an irradiance of $165\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ and under a 12:12 hrs light:darkness regime in L1 medium without silicate, which containing a phosphate level of $35\ \mu\text{mol PO}_4^{3-}\text{ L}^{-1}$, (Guillard and Hargraves, 1993). Cultures used were inoculated with 500 cells mL^{-1} in exponential growth phase, and were grown in three experiments under different conditions:

Experiment 1, culture was grown under conditions differing from the inoculum in: culture grown in 1000 mL flask containing 800 mL of L1 medium and was maintained at 19 ± 1 °C.

Experiment 2, culture was grown under conditions differing from the inoculum in: culture grown in 2000 mL borosilicate spinner flasks with two vertical sidearms containing 1000 mL of L1 medium, with an agitation of 80 rpm and a low irradiance of $70\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$.

Experiment 3, was a large culture of 100 L maintained in L1 medium under the same conditions as the inoculum, but with an irradiance ranging between 80 and $160\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$, with mechanical agitation at 40 rpm for 1 hour per day and with continuous aeration at $20\text{-}30\text{ L minute}^{-1}$.

2.2. Toxin and phosphate analysis

For toxin analysis, 10 mL aliquots of each culture were harvested at pre-established times and filtered through $1.4\ \mu\text{m}$ GF/C glass fibre filters (25 mm diameter) (Whatman,

Maidstone, England). YTX in both medium and cells was extracted and determined by liquid chromatography with fluorescence detection (HPLC-FLD) following Paz et al (2004). The total production of YTX by *P. reticulatum* in $\mu\text{g mL}^{-1}$, was determined as the sum of the partial productions in the medium and in the cells.

For phosphate determination in water, 10 mL aliquots of each culture were collected in parallel with toxin samples and filtered through 1.4 μm GF/C glass fibre filters (25 mm diameter) and were frozen at -20°C until analysis. The PO_4^{3-} determination was performed with an automatic Scan Plus System Skalar analyzer.

2.3. Cell number

For determining biomass production as cell concentration (cells mL^{-1}), 5 mL samples were collected at pre-established times, fixed with Lugol's solution and cell counts were made using a Sedgwick-Rafter counting chamber by optical microscopy.

2.4. Numerical methods

Fitting procedures and parametric estimates from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values. The non-linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel XP spreadsheet was used for it. Statistica 6.0 software (StatSoft, Inc. 2001) was used to evaluate the significance of the estimated parameters by fitting the experimental values to the proposed mathematical models, and the consistency of these equations.

3. Results and Discussion

3.1. Unstructured mathematical models

In the kinetic models that next are schematized the production rate equations of dinoflagellate cells ($\underline{C}$), yessotoxin ($\underline{Y}$) and phosphate uptake ($\underline{P}$) were used. The meaning and definition of the group of model parameters as well as its units are summarized in the symbolic notation table 1.

Cell production

The logistic equation is easily managed and suitable for the adjustment of the typical sigmoid profiles of cell production, also facilitating the calculation of parameters of biological and geometrical significance (Vázquez et al., 2006; Guerrini et al., 2007):

$$\frac{dC}{dt} = \mu_m \cdot C \cdot \left(\frac{C_m - C}{C_m} \right) \quad (1)$$

which, integrated between $\underline{C}_0 \rightarrow \underline{C}$ and $0 \rightarrow t$, gives:

$$C = \frac{C_m}{1 + \exp(b - \mu_m \cdot t)}, \text{ with } b = \ln\left(\frac{C_m}{C_0} - 1\right) \quad (2)$$

Or, in reparametrized form (Vázquez and Murado, 2008):

$$C = \frac{C_m}{1 + \exp\left[2 + \frac{4 \cdot v_m}{C_m} \cdot (\lambda - t)\right]} \quad (3)$$

Yessotoxin formation

The kinetic of the yessotoxin production was based on the Luedeking and Piret model (Luedeking and Piret, 1959a; Luedeking and Piret, 1959b). Applied to the problem under study, said criteria permits the following assumptions: 1) *P. reticulatum* growth ($\underline{C}$) can be described according to the logistical equation (3); 2) the yessotoxin formation rate obeys the Luedeking and Piret equation:

$$\frac{dY}{dt} = \alpha_y \cdot \frac{dC}{dt} + \beta_y \cdot C \quad (4)$$

developing this differential equation among $\underline{0} \rightarrow \underline{Y}$, $\underline{C_0} \rightarrow \underline{C}$ and $\underline{0} \rightarrow \underline{t}$, we obtain (Vázquez and Murado, 2008):

$$Y = Y_0 + \frac{\alpha_y \cdot C_m}{1 + \left(\frac{C_m}{C_0} - 1 \right) \cdot e^{-\frac{4 \cdot v_m \cdot t}{C_m}}} - \alpha_y \cdot C_0 + \frac{\beta_y \cdot C_m^2}{4 \cdot v_m} \cdot \ln \left[\frac{C_0 \cdot \left(e^{\frac{4 \cdot v_m \cdot t}{C_m}} - 1 \right) + C_m}{C_m} \right] \quad (5)$$

This mathematical model permits us to classify the toxin as primaries, if the formation rate depends solely on the rate of growth ($\alpha_y \neq 0$; $\beta_y = 0$); secondaries, if this depends solely on the cells present ($\alpha_y = 0$; $\beta_y \neq 0$), and mixed, if this depends simultaneously on the rate of biomass production and on the biomass present ($\alpha_y \neq 0$; $\beta_y \neq 0$).

Phosphate uptake

The consumption of phosphate (P) with the time can be modelled using a Luedeking and Pirt like equation, in which one keeps in mind the quantity of phosphate that is metabolized to form cellular biomass and for the cellular maintenance (maintenance energy model, (Pirt, 1965)):

$$\frac{dP}{dt} = - \left(\frac{1}{Z_{c/p}} \cdot \frac{dC}{dt} + m_c \cdot C \right) \quad (6)$$

that, integrating between $\underline{P}_0 \rightarrow \underline{P}$, $\underline{C}_0 \rightarrow \underline{C}$ and $\underline{0} \rightarrow \underline{t}$, allows to establish (Vázquez and Murado, 2008):

$$P = P_0 + \frac{C_0}{Z_{c/p}} - \frac{1}{Z_{c/p}} \cdot \frac{C_m}{1 + \left(\frac{C_m}{C_0} - 1 \right) \cdot e^{-\frac{4 \cdot v_m \cdot t}{C_m}}} - \frac{m_c \cdot C_m^2}{4 \cdot v_m} \cdot \ln \left[\frac{C_0 \cdot \left(e^{\frac{4 \cdot v_m \cdot t}{C_m}} - 1 \right) + C_m}{C_m} \right] \quad (7)$$

3.2. Growth, yessotoxin production and phosphate consumes by Protoceraatium reticulatum

In order to demonstrate the usefulness of the mathematical model, the kinetic of production and consumptions of the P. reticulatum were studied under the conditions which are described in the materials and methods section. Figure 1 represents the dinoflagellate growth and the chemical changes during culture in the experiment 1 with time. The cell production showed a classical growth trend, that is, sigmoid profiles. Subsequently to a fast and not very defined lag phase ($\lambda=6$ days), the cells began the exponential growth phase until achieving the asymptotic phase about 16 days. In the

same way, phosphate uptake took place simultaneously with the biomass growth. The formation of yessotoxin started when the growth was between the end of the exponential phase and the beginning of the stationary phase. This tendency is typical of a secondary metabolites, with values for Luedeking and Piret parameters of $\alpha_y=0$ and $\beta_y=8\times 10^{-5}$ ng cells⁻¹ day⁻¹.

The results in other experimental conditions (experiment 2) are shown in figure 2. In this case, the kinetic profiles were similar as described previously. However, the lag phase was longer ($\lambda=13$ days) and the yessotoxin formation behaved as a mixed metabolite ($\alpha_y=1\times 10^{-6}$ ng cells⁻¹ and $\beta_y=4.5\times 10^{-4}$ ng cells⁻¹ day⁻¹). Moreover, the maximum cell production (C_m), maximum cell production rate (v_m) and yield coefficient for phosphate uptake (Y_{cp}) were higher. Table 2 summarises the numerical values of the kinetic parameters obtained from fitting the experimental data to the unstructured mathematical models as well as their corresponding statistical analysis.

Finally, we tested the capability of these equations to predict the behaviour of *P. reticulatum* in a 100 L bioreactor (figure 3). This scale-up culture maintained the profiles obtained in the other experiments, with $\lambda=10$ days and secondary productions of the toxin ($\alpha_y=0$ and $\beta_y=3.8\times 10^{-4}$ ng cells⁻¹ day⁻¹).

In all cases, the fitting of results was satisfactory graphical and statistically. The mathematical equations were consistent (Fisher's F test) and the parametric estimations were significant (Student's t -test). Furthermore, all the values foreseen in the non-linear

adjustments produced high coefficients of linear correlation with the values really observed ($r > 0.97$).

Though the initial concentration of phosphate in the L1 medium is not suitable to obtain the largest production of dinoflagellate' cells (Gallardo-Rodríguez et al., 2009), however it is appropriated for a study of modelling as we proposed. The maximum production of biomass was lower than the outcomes released by Gallardo-Rodríguez et al (2007) but higher than Guerrini et al (2007) growths. Furthermore, yessotoxin concentrations are in agreement with observation by Guerrini et al (2007). Nevertheless, in these articles the mathematical descriptions of the kinetics are unexplored. The main objective of present work is focused on modelling of YTX, cell and phosphate dynamics and not to maximize and optimize YTX and cell productions. Our results demonstrated the suitability and accuracy of these equations to describe the experimental data and to supply parameters with biological meaning that are indispensable for scale-up the productions.

3.3. Testing the mathematical models

In literature, few authors have studied the production of YTX and substrate consumption by *P. reticulatum* on batch culture (Gallardo-Rodríguez et al., 2007; Guerrini et al., 2007; Gallardo-Rodríguez et al., 2009), but works using modelling approach have not been reported. However, several interesting articles have been published on modelling of other marine plankton and nutrient dynamics in chemostat or enviro

- , 2000; Stolte et al., 2002; Flynn, 2003; Flynn et al., 2008). Thus, in

order to evaluate the cell production, toxin formation and nutrient uptake models with data from bibliography, experimental results of nodularin formation obtained by Stolte et al. (2002) were used.

Figure 4 shows the dynamics of chlorophyll-a (for growth description) and nodularin production as well as phosphorus consumption in batch culture of *Nodularia spumigena*. The models employed were equation (3) for chlorophyll-a (Chla), equation (5) for nodularin-toxin (Nod) and equation (7) for phosphorus (P). As it can be observed the equations adjusted closely experimental data of Chla and Nod with coefficients of linear correlation between predicted and measured values of 0.997 and 0.999, respectively. Fisher's *F* test also corroborated the robustness of both equations ($p < 0.0001$). Nevertheless, phosphorus modelled was poorer ($r = 0.829$) and less consistent ($p < 0.005$) than the other ones. All Chla-parameters (maximum Chla production in $\mu\text{g L}^{-1}$, maximum Chla production rate in $\mu\text{g L}^{-1}\text{days}^{-1}$ and Chla production lag phase in days^{-1}) were significant (Student's *t*-test), with numerical values of 180.86 ± 8.56 , 27.29 ± 3.71 , 2.34 ± 0.49 , respectively. Coefficients from Luedeking and Piret equation for nodularin production were also significant and the formation of this toxin was able to define as mixed metabolite ($\alpha \neq 0$; $\beta \neq 0$).

In figure 5, two cultures with different inorganic nitrogen source are depicted. In both cases, experimental patterns of Chla and Nod productions were accurately described by equations (3) and (5), respectively. Statistically, in all cases fittings were consistent ($r > 0.994$; $p < 0.0001$). Finally, Chla-parameters were always significant and the toxin

production was mixed in terms of Nod-parameters according Luedeking and Piret definition.

4. Conclusions

The equations presented in this manuscript are suitable to fit the experimental data of *P. reticulatum* cultures in different experimental conditions. These models are also appropriate to describe the production of nodularin, chlorophyll and phosphorus uptake reported by Stolte et al. (2002). Furthermore, the metabolic behaviour of the yessotoxin production can be described as secondary or slightly mixed. From the viewpoint of industrial applications, the group of equations establishes a useful tool for the control of yessotoxin production in bioreactors.

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FIGURE CAPTIONS

Figure 1: Time course of total YTX formation (sum of particulate and dissolved), growth of *Protocerratium reticulatum* and consumption of phosphate in the culture conditions of Experiment 1: Culture grown in 1000 mL flask containing 800 mL of L1 medium without silicate with a phosphate level of $35 \mu\text{mol PO}_4^{3-} \text{ L}^{-1}$, temperature 19 ± 1 °C, salinity of 34 psu and irradiance of $165 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ under a 12:12 hrs light:darkness regime. Cultures were gently shaken once a day. Continuous lines represent the mathematical models used to fit experimental data represented by points: ●, Cells; ○, Yessotoxin; □, Phosphate.

Figure 2: Time course of total YTX formation (sum of particulate and dissolved), growth of *Protocerratium reticulatum* and consumption of phosphate in the culture conditions of Experiment 2: Culture grown in 2000 mL spinner flask with two vertical sidearms containing 1000 mL of L1 medium without silicate with a phosphate level of $35 \mu\text{mol PO}_4^{3-} \text{ L}^{-1}$, temperature 19 ± 1 °C, salinity of 34 psu, agitation of 80 rpm and a low irradiance of $70 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ under a 12:12 hrs light:darkness regime. Notations as in figure 1.

Figure 3: Time course of total YTX formation (sum of particulate and dissolved), growth of *Protocerratium reticulatum* and consumption of phosphate in the culture conditions of Experiment 3: Culture grown in a 100 L tank containing L1 medium without silicate with a phosphate level of $35 \mu\text{mol PO}_4^{3-} \text{ L}^{-1}$, temperature $19 \pm 1 \text{ }^\circ\text{C}$, salinity of 34 psu, agitation of 40 rpm for 1 hour per day, continuous aeration at 20-30 L minute^{-1} and irradiance between 80 and $160 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ under a 12:12 hrs light:darkness regime. Notations as in figure 1.

Figure 4: Time course of chlorophyll-a (Chla, ●) and nodularin (Nod, ○) concentrations during growth of *N. spumigena* in phosphorus-limited (P, □) batch culture with nitrate as only source of nitrogen. Experimental data from Stolte et al. (2002) were fitted to the equations depicted in the text.

Figure 5: Time course of chlorophyll-a (Chla, ●) and nodularin (Nod, ○) concentrations during growth of *N. spumigena* without any dissolved inorganic nitrogen (A) and with ammonium (B) in batch cultures. Experimental data from Stolte et al. (2002) were fitted to the equations depicted in the text.

TABLE 1. Symbolic notations used

C :	Cell production, cells mL ⁻¹
t :	Time, days
C_m :	Maximum cell production, cells mL ⁻¹
C_0 :	Initial cell concentration, cells mL ⁻¹
μ_m :	Specific maximum cell production rate, days ⁻¹
v_m :	Maximum cell production rate, cells mL ⁻¹ days ⁻¹
λ_x :	Cell production lag phase, days
Y :	Yessotoxin concentration, ng mL ⁻¹
Y_0 :	Initial yessotoxin concentration, ng mL ⁻¹
α_y :	Luedeking and Piret parameter (cell production-associated constant for yessotoxin formation), ng (yessotoxin) cells ⁻¹
β_y :	Luedeking and Piret parameter (non cell production-associated constant for yessotoxin formation), ng (yessotoxin) cells ⁻¹ days ⁻¹
P :	Phosphate concentration, $\mu\text{mol L}^{-1}$
P_0 :	Initial phosphate concentration, $\mu\text{mol L}^{-1}$
$Z_{c/p}$:	Yield coefficient for cell production on phosphate, cells nmol ⁻¹ (phosphate)
m_c :	Maintenance coefficient, nmol (phosphate) cells ⁻¹ days ⁻¹

TABLE 2. Parametric estimations corresponding to the equations (2), (4) and (6), applied to production of yessotoxin by *Protoceraatium reticulatum*. CI: confidence intervals ($\alpha=0.05$). F : F-Fisher test (df_1 =model degrees freedom and df_2 =error degrees freedom). r =correlation coefficient between observed and predicted data. (-) meaning that zero value was obtained

VARIABLES	Experiment 1	Experiment 2	Experiment 3
CELLS (C)	values $\pm$ CI	values $\pm$ CI	values $\pm$ CI
C_m	43011 $\pm$ 3541	49005 $\pm$ 9816	35881 $\pm$ 7968
C_0	191.1 $\pm$ 4.832	3.150 $\pm$ 1.322	210.3 $\pm$ 6.455
v_m	6066 $\pm$ 2332	6812 $\pm$ 4526	2761 $\pm$ 280
λ	6.048 $\pm$ 1.525	13.763 $\pm$ 2.661	10.181 $\pm$ 0.768
F ($df_1=3$, $df_2=3-5$; $\alpha=0.05$)	529.18	128.46	2467.21
p -value	<0.001	<0.001	<0.001
r (obs-pred)	0.995	0.989	0.999
YESSOTOXIN (Y)	values $\pm$ CI	values $\pm$ CI	values $\pm$ CI
Y_0	-	-	-
α_Y	-	$1.0 \times 10^{-6} \pm 1 \times 10^{-7}$	-
β_Y	$8 \times 10^{-5} \pm 1 \times 10^{-8}$	$4.5 \times 10^{-4} \pm 7 \times 10^{-5}$	$3.8 \times 10^{-4} \pm 8 \times 10^{-5}$
F ($df_1=3$, $df_2=3-5$; $\alpha=0.05$)	386.57	207.08	179.04
p -value	<0.001	<0.001	<0.001
r (obs-pred)	0.989	0.985	0.979
PHOSPHATE (P)	values $\pm$ CI	values $\pm$ CI	values $\pm$ CI
P_0	34.686 $\pm$ 7.884	33.704 $\pm$ 5.386	37.117 $\pm$ 5.246
$Z_{c/p}$	1507 $\pm$ 642	2959 $\pm$ 1518	784 $\pm$ 198
m_c	$7 \times 10^{-6} \pm 6 \times 10^{-7}$	$1.5 \times 10^{-5} \pm 4 \times 10^{-6}$	$5 \times 10^{-6} \pm 3 \times 10^{-7}$
F ($df_1=3$, $df_2=3-5$; $\alpha=0.05$)	49.099	61.954	172.38
p -value	0.002	<0.001	<0.001
r (obs-pred)	0.975	0.968	0.990

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5