Proposal for a simple and sensitive haemolytic assay for palytoxin. Toxicological dynamics, kinetics, ouabain inhibition and thermal stability.

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ABSTRACT

Benthic dinoflagellates of the genus *Ostreopsis* are important components of subtropical and tropical marine coral reef-lagoonal environments. Currently, as a result of global warming and trade globalization, they are also distributed worldwide. These microalgae are shown to produce palytoxin, one of the most potent non-protein marine toxins known.

The hemolytic assay is a very easy, rapid and sensitive method to determine palytoxin. However, under the conditions reported in previous works this assay is inadequate for a rigorous dose-response treatment, since: 1) it produces degenerate sigmoidal profiles, with a pronounced slope which makes the calculation of the ED_{50} very sensitive to the experimental error; 2) at the usual work temperature, the *in vitro* stability of the system is low, which accentuates the variability and ambiguity of the response. To resolve these problems hemolysis of sheep erythrocytes by palytoxin is studied including its toxicological dynamics, its kinetics, its inhibition by ouabain and its response to temperature. The results show that, to obtain a smoother, more stable and reproducible response, it is necessary to apply two resources simultaneously: operation at a moderate temperature and partial inhibition of the palytoxin by ouabain. It also produces highly reliable parameters and allows strict equivalencies to be established with the mouse bioassays, a traditional reference point, though bioethically questionable and 20 times less sensitive than the bioassay proposed here.

1. INTRODUCTION

Palytoxin is a polyol compound that shows extreme acute toxicity (Moore & Scheuer 1971). This toxin has been primarily isolated from marine Coelenterates of genus *Palythoa* and shows remarkable biological activity at an extremely low concentration. The intoxication produced by palytoxin represents a serious threat, as there have been a number of deaths associated with the ingestion of contaminated seafood. Deaths have reportedly occurred after comsumption of contaminated crabs in the Philipines (Alcala et al. 1988), sea urchins in Brazil (Granéli et al. 2002) and fish in Japan (Fukui et al. 1987; Onuma et al. 1999; Taniyama et al. 2003) etc…

As other neurotoxins, palytoxin can be quantified via traditional mouse bioassays, which were first carried out over 50 years ago (Thompson 1947, Thompson & Weil 1952, Weil 1952) and have basically remained unchanged ever since. These assays quantify the mortality of a small population of animals, which follows a violent and unusual contraction of its striated and smooth musculature, with increasing doses of the effector. As well as its neurotoxic character, another characteristic property of palytoxin is its capacity to bind, in mammal erythrocytes, to the external part of the Na^+/K^+ pump converting it into an ionic channel, which causes the rapid depletion of K^+, followed by delayed coloidosmotic hemolysis (Habermann et al. 1981, Artigas & Gadsby 2003, Hilgemann 2003). The hemolysis by palytoxin is specifically inhibited by Ouabain (Habermann & Chhatwal 1982), a glycoside that binds to the sodium pump in the same place as palytoxin does.

Mouse bioassays are criticised for bioethical and economic reasons, which have led to the development of other methods to minimise the death of animals, but which are less accurate. As in the case of the traditional method of moving averages (Thompson 1947, Thompson & Weil 1952, Weil 1952) or the more recent anaesthetic one (Holtrop et al. 2006). Abandoning these bioassays, however, is viewed reluctantly. Against chromatographic methods has been adduced their inability to assess the real biological activity when the toxin is accompanied by active structural analogues. Against methods based on delayed hemolysis –free from this previous problem and able to support a satisfactory bioassay– the objection is the lack of identification value of the symptoms prior to the mouse's death. However, the methods based on the hemolytic effect are dependent on the same molecular or toxoforic architecture as the neural fault of the mouse.

The use of empirical models is common in the evaluation of the activity of marine toxins. Regrettably, such models do not take into account the basic facts of the general relationships between the effector doses and the response produced in the target population (DR relationships). They usually search for simple linear forms throughout transformations (logarithmic and reciprocal). This reduce their accuracy, introduce biases and therefore cannot be justified nowadays adducing the complexity of the work with non-linear models. Furthermore, the parameters of these models lack of biological significance, which give rise to uncertainties in their use and can not be extrapolated to other systems. The advantage of the mechanistic and semimechanistic models is the establishment of a much stronger relationship of equivalence.

In this research, the toxicological dynamics of hemolysis by palytoxin and its inhibition by ouabain is studied using models whose foundations have been discussed in detail in previous studies, and whose form has been refined to adapt them rigorously to the description of DR relationships (Murado et al. 2002, Murado & Vázquez 2007). The thermal stability of the system is also investigated through the Arrhenius approximation, demonstrating that the formal methods applied produce coherent kinetic and dynamic descriptions. Information obtained in this way is used to define a sensitive and reliable assay which, under moderate temperature and partial inhibition of the palytoxin by ouabain, allows to obtain responses which give consistent and precise parametric estimations.

2. MATERIALS AND METHODS

2.1. Experimental methods

PBS solution: The vehicle for erythrocyte, palytoxin and ouabain dilutions was a solution containing 0.1 % bovine serum albumin (BSA), 1 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) and 1 mM boric acid (H_3BO_3) in phosphate buffered saline (PBS) 0.01 M, pH 7.4 (Sigma)

Palytoxin solution: The solution of palytoxin used, obtained from *Palythoa tuberculosa*, was provided by Wako Chemicals (Wako Chemical Industries, Ltd., Japan) as 100 µg of dry extract that was suspended in MeOH : Water (1:1) at 25 $ng \cdot \mu L^{-1}$ final concentration. Assays were performed by using the following series of dilutions in PBS: 2.500; 1.250; 1.000; 0.750; 0.660; 0.580; 0.500; 0.375; 0.250; 0.125; 0.050 and 0.025 ($pg \cdot \mu L^{-1}$)

Ouabain solution: Starting from1 mM solution, the following dilutions in PBS were prepared: 1, 2, 3, 4 and 5 µM. To carry out the experiment with ouabain the 1:99 erythrocytes dilution was

prepared with 50% of each one of the ouabain concentrations cited above and incubated to the specific temperature for 30 minutes before the assay.

Erythrocyte preparation: Sheep blood in Alsever was kindly provided by I. Manzano (CZ Veterinaria, S.A.; Porriño, Spain). Erythrocytes were separated from plasma by centrifugation (400 g, 10 ºC, 10 minutes), washed twice with PBS solution, then a first dilution (1:9) of erythrocytes in PBS at final concentration around 1.7 x 10^8 red cells·mL^{-1} was prepared.

Assay procedure: The Hemolytic assay is based on Bignami´s method (Bignami 1993). Two blood solutions with and without ouabain were prepared to the same final concentration of approx 1.7×10^7 erythrocytes·mL^{-1}as follows:

i) Blood solution without ouabain: 1 volume of the erythrocyte preparation was combined with 9 volumes of PBS solution.

ii) Blood solution with ouabain: 1 volume of the erythrocyte preparation was combined with 4 volumes of PBS solution and 5 volumes of the appropriate ouabain dilution and the mixture were incubated to the desired temperature during 30 minutes.

Next, 1 volume of each one of the previously blood solutions mentioned was mixed with 1 volume of the appropiate palytoxin dilution and incubated over pre-established times at the same temperature.

The control solutions were prepared as follows:

i) hemolysis 0% (Blank): was prepared by mixing both blood and PBS solutions (1:1)

ii) hemolysis 100%: was achieved by mixing blood solution and distilled water (1:1).

After the incubation period, erythrocytes were separated by centrifugation (300 x g, 10ºC, 10 min). A 200 µL portion of each supernatant was further transferred to a microwell plate to measure the absorbance at 405 nm into a microplate reader.

It must be taking into account that concentrations of erythrocytes, ouabain and palytoxin in the final reaction mixture are respectively equal to ½, ¼ and ½, of their values in the original preparations, but references will only made to the original concentrations to avoid confusions.

To study the effect of temperature on the reaction rate by means of the Arrhenius' model, the hemolytic activity induced by a 0.25 pg.μl^{-1} palytoxin solution at 15, 20, 25, 35, 40 and 45 ºC was checked over a period of 15 hours. Analyses were performed in centrifuge tubes with enough volume to transfer triplicate aliquots of 300 µl to a 96-well microtitre plate at intervals of 30 minutes or 1 hour depending on the reaction rate. Plate containing triplicate samples was centrifuged at 300 g and 10ºC for 10 minutes (Sigma K10 centrifuge equipped with a Sigma

11222 microplate rotor). After centrifugation a 200 μL portion of each supernatant was transferred to another plate to measure absorbance at 405 nm into a microplate reader (BioRad 550).

2.2 Numerical methods

Fitting procedures and parametric estimations from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro '*Solver*' of the *Microsoft Excel XP* spreadsheet. Subsequently, confidence intervals of the parametric estimations (Student's ***t*** test) and consistence of mathematical models (Fisher's ***F*** test) were determined using the non-linear section of *Statistica 6.0* pack (StatSoft, Inc. 2001).

3. RESULTS AND DISCUSSION

3.1. Dose equivalence in assays of the same effector on two different receptors

To evaluate the effect of palytoxin on mice *in vivo* using its hemolytic effect on *in vitro* sheep erythrocytes, a relationship between the functions that describe corresponding responses needs to be established: to define in assay 1 a dose D_1 that produces the same response R_1 as the dose D_2 in assay 2. Generally speaking, the descriptive functions can be expressed, in terms of dose D and three parameters, as:

$$R = f(D; K, m, \upsilon) \qquad [1]$$

K being the maximum response, m the dose that corresponds to the semi-maximum response and υ being a parameter related to the response slope, in a way which varies according to the specific function under consideration. In this way, we can write:

$$R_i = f_1(D_1; K_1, m_1, \upsilon_1) = f_2(D_2; K_2, m_2, \upsilon_2) \qquad [2]$$

Usually the two responses can be described by the same model; in other words, f_1 and f_2 will have the same functional form, although this is not an essential condition. In any case, once the parametric values of both models have been estimated, to obtain one of the doses as a function of the other, we can formulate a conversion function (δ) of the type:

$$D_1 = \delta(D_2; K_1, m_1, \upsilon_1, K_2, m_2, \upsilon_2) \qquad [3]$$

In this study, and for reasons discussed previously (Murado et al. 2002, Murado & Vázquez 2007), the descriptive functions used were the logistic equation and the cumulative function of the Weibull distribution, both modified to adapt them to DR analysis (See appendix A).

The modified logistic equation (hereinafter ^{m}L) has the form:

$$R = \frac{KA}{B}\left[\frac{1}{1+B\exp(-\mu D)} - \frac{1}{A}\right] \qquad [4]$$

where: $A = \exp(\mu m) - 1$; $B = \exp(\mu m) - 2$; and:

R response, with K as maximum value.

m dose corresponding to semi-maximum response.

μ maximum specific rate (increment of R by unit of R and unit of D).

and when the DR relationships involved in the transformation are described via this model, the conversion function δ which allows the determination of dose D_1 that produces in assay 1 the same response R_i as the dose D_2 in assay 2 is:

$$D_1 = \frac{-\ln\left(\frac{1-P}{B_1 P}\right)}{\mu_1} \text{ ; where:} \qquad [5]$$

$$P = \frac{A_2 B_1 K_2}{A_1 B_2 K_1} Q + \frac{1}{A_1} \quad ; \quad Q = \frac{1}{1+B_2 E_2} - \frac{1}{A_2} \quad ; \quad E_2 = \exp(-\mu_2 D_2)$$

On the other hand, the modified Weibull function (hereinafter ^{m}W) has the form:

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m}\right)^{a}\right]\right\} \qquad [6]$$

R response, with K as maximum value.

m dose corresponding to semi-maximum response.

a form parameter, related to the maximum slope of the response.

the conversion function δ being in this case:

$$D_1 = m_1\left(\frac{-\ln S}{\ln 2}\right)^{1/a_1} \quad ; \text{ where:} \qquad [7]$$

$$S = 1 + \frac{K_2\left(E_2 - 1\right)}{K_1} \quad ; \quad E_2 = \exp\left[-\ln 2\left(\frac{D_2}{m_2}\right)^{a_2}\right]$$

Figure 1 illustrates the main precaution that should be taken into account when applying any of the δ functions, a precaution that consists of avoiding the domains of the dose in which the reference response gets close to initial starting stage or to the asymptotic end. In other words: the conversion is more precise the closer the doses involved D_1 and D_2 are to the parametric values m_1 and m_2.

3.2. The hemolysis of palytoxin at 37ºC

The hemolysis kinetics at different levels of palytoxin at 37ºC produced the results seen in figure 2A, whose sigmoidal profiles in time are due to, in principle, two different reasons: 1) the action of some autocatalytic mechanism, similar to that produced if the hemolysis were accelerated by some of its products; 2) the populational nature of the DR phenomenon, since if the determining factors of sensitivity are random variables subject to some probability distribution, the response, *i.e.* the corresponding cumulative function will have to be sigmoidal with respect to dose and time (the entities that are more sensitive than average will respond to lower doses and a shorter time, and the opposite will occur for those that are less sensitive). Therefore, to explain the kinetic profile of the hemolysis, it is sufficient to accept that the number of palytoxin receptors by erythrocyte; the number of receptors that should be occupied by the toxin so that hemolysis takes place; or the affinity between receptor and toxin are not constant in all the erythrocytes, but variables with some probability distribution within the assay erythrocyte population.

Although later on we deal experimentally with this dilemma, here we should point out that in the second case the models ^{m}L and ^{m}W are adequate to describe the kinetics of the hemolysis, using time, instead of dose, as the independent variable. Taking into account that mammal erythrocytes are dead cells, the said kinetics can be assimilated to a time of failure of any complex system, conventionally described by the Weibull distribution (Canavos 1992, Shalel et al. 2002). Therefore, the model applied to the process was ^{m}W:

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{t}{t_{0.5}}\right)^{a}\right]\right\} \qquad [8]$$

R response as proportion of hemolysis with K as maximum value.

$t_{0.5}$ time corresponding to semi-maximum response.

a form parameter, related to maximum rate of hemolysis.

In all cases the model was consistent (Fisher's F, α=0.05) and led to significant estimates (Student's t, α=0.05) of all the parameters (figure 2B), with a high correlation between observed and expected results (r=0.999; figure 2C).

Naturally, every perpendicular to the time axis in figure 2B produces, at their intersections with the kinetic profiles, a series of values that represent, at a given time of exposure, the response to the dose series used, once again with a sigmoidal profile that fits the models ^{m}L or ^{m}W. If the responses at a given time are normalised, and are expressed as a fraction of the maximum of that time, then ideally any exposure time will lead to the same set of parameters, although in reality there can be deviations due to the different relative weight of experimental error at different times and at different doses.

Considering the series at 250, 300 and 350 minutes together, the fit to the models ^{m}L or ^{m}W (figure 2D and note b of Table 1) was consistent and the parameters statistically significant. However, the correlation between observations and predictions (despite an acceptable value: r=0.967; figure 2F) clearly showed groupings that reveal the lack of strict equivalence between the three series, a result connected with the fact that the value of parameter $t_{0.5}$ was not totally independent of the doses (figure 2E), dropping asymptotically from the lowest doses.

This situation makes it necessary to define some decision criteria to select the most appropriate incubation time. A problem which requires the following solutions:

S1. Use a time in which all kinetics have reached the asymptote (all the hemolyses have reached the equilibrium). At least for this present case, this criteria leads to the lowest value of m, which represents a factor of safety.

S2. Obtain a unified description of the response as a simultaneous function of time and dose, without rejecting any of the times studied. In this case, the set of experimental results may fit a model that is the product of the equations ^{m}L and ^{m}W, divided by the common asymptote (K) of the two dimensions of the response. One option is to use ^{m}W with time and ^{m}L with the dose ($^{m}W_t \times {}^{m}L_D$):

$$R = K\left\{1-\exp\left[-\ln 2\left(\frac{t}{t_{0.5}}\right)^{a}\right]\right\}\left\{\frac{A}{B}\left[\frac{1}{1+B\exp(\mu D)}-\frac{1}{A}\right]\right\} \qquad [9]$$

but any one of the four products ${}^{m}W_t \times {}^{m}L_D$, ${}^{m}L_t \times {}^{m}V_D$, ${}^{m}W_t \times {}^{m}W_D$ y ${}^{m}L_t \times {}^{m}L_D$ is in principle a feasible model.

Although unusual in DR analysis, this bidimensional model comprises the most complete description for the overall average of experimental data. Applied to the present case (figure 3A, Table 1), both the equation [9] and its alternative ${}^{m}W_t \times {}^{m}W_D$ were consistent and produced statistically significant parametric estimates. However, once again, the correlation between observations and predictions showed, within its linearity (r=0.996; figure 3B), groupings which reveal bias in the predictions (in which the value of m does not depend on time, when, in reality, the longer times tend to produce higher estimations of m). In fact, although the criteria applied in figures 2D and 3 generate undistinguishable estimates of m (25.3 ± 3.9 y 29.2 ± 7.0 pg.ml^{-1}), if the responses in the asymptotic stage of the kinetics are used, the value drops to 14.5 ± 3.0 pg.ml^{-1}.

This effect could be due to a spontaneous hemolysis over time, which exceeds that in the control without palytoxin, and which is not directly due to the concentration of the toxin, but to some liberating factor by damaged erythrocytes, *i.e.*, to a low intensity catalytic effect (we will come back to this problem later on). Furthermore, the high sensitivity of the erythrocytes to palytoxin, under assay conditions, generate DR curves with a degenerated sigmoidal profile, whose marked slope makes the calculation of parameter m unusually sensitive to experimental error. In the next section we shall see how the temperature of the assay can be an important source of variability.

Given the fact this study attempts to establish reference points to evaluate toxin activity, it could opt for the most prudent solution of the lowest m. This, however, would not eliminate the problems connected with the assay conditions that we have just mentioned. Therefore a better solution seemed to be to reduce the intensity of the response through two methods: operation at low temperatures and the addition of ouabain, which occupies the same receptors in the erythrocyte membrane as palytoxin, thus acting as a competitive inhibitor.

3.3. Influence of temperature and fit of hemolysis to the Arrhenius model.

To determine the temperature effect on the rate of hemolysis, a kinetic assay was carried out in which the same concentrations of erythrocytes and palytoxin were incubated at 7 temperatures between an interval of 15 and 45ºC. The results (figure 4), in terms of percentage of hemolysis fit a classic logistic equation:

$$H = \frac{H_m}{1+\exp\left(c-\mu_m \cdot t\right)} \;;\; \text{being } c = \ln\left(\frac{H_m}{H_0}-1\right) \qquad [10]$$

H percentage of hemolysis, with H_0 and H_m as initial and maximum values.

μ_m maximum specific rate (increase in H by unit of H and unit of t).

The meaning of the maximum specific rate (μ_m) can assimilate to that of an initial reaction rate, but in sigmoidal kinetics the criterion of maximum rate (v_m) can be more robust, in the sense of being less sensitive to experimental error. With the aim of comparing the descriptive aptitude of both parameters, a reparametrized version of [10], to make the maximum rate explicit (Vázquez et al. 2006), was also used:

$$H = \frac{H_m}{1+\exp\left[2+\frac{4v_m}{H_m}\left(\lambda-t\right)\right]} \qquad [11]$$

v_m maximum rate (increase of H by unit of t).

λ delay (intersection of the tangent at inflection point with the x axis).

In both cases (Table 2) the equations were consistent and the parametric estimates statistically significant.

Now then, according to the Arrhenius definition, the relationship between the rate of a chemical reaction and the temperature of a system is described by the equation:

$$k_r\left(T\right) = A \cdot \exp\left[-\frac{E_a\left(k_r\right)}{R \cdot T}\right] \;;\; \text{or, in linear version:} \qquad [12]$$

$$\ln k_r = \ln A - \frac{E_a\left(k_r\right)}{R \cdot T} \qquad [13]$$

with k_r being the kinetic constant, E_a the activation energy, R the constant of gases and T the absolute temperature (the meaning of the pre-exponential term A is limited here to only a fitting

parameter). Given that the substitution in [13] of the kinetic constant k_r for either of the rate parameters (μ_m or v_m) is dimensionally coherent, we can write:

$$\ln \mu_m = \ln A_1 - \frac{E_a(\mu_m)}{R \cdot T} \quad [14]$$

$$\ln v_m = \ln A_2 - \frac{E_a(v_m)}{R \cdot T} \quad [15]$$

The representations of both functions (figure 5) clearly showed that the rate increases with the temperature from 15 to 35ºC, and rapidly falls after the latter value, suggesting the disorganisation of the biological system under these conditions. Furthermore, the fittings of the ascending tracts (of activation) to the models [14] and [15] were consistent, significant and demonstrated that the maximum rate (v_m) provides a more robust criterion than the specific maximum rate (μ_m).

The values obtained for activation energies (E_a; α=0.05) were 18.89 ± 6.42 and 12.72 ± 2.16 kJ/mol, using μ_m and v_m respectively. Although slightly lower, they are within the range of those other authors have determined in human erythrocytes for other hemolytic agents, such as the Kanagawa hemolysin and hypochloric acid (Huntley & Hall 1994, Zavodnik et al. 2002). The difference can be due to: 1) differences in the potential barriers of the corresponding effectors, a barrier that without doubt is very low in palytoxin, given its notable activity; 2) differences dependent on the species (Brzezinska-Slebodzinska 2003, Riobó et al. Unpublished) in the mechanical and structural resistance of the erythrocytes; 3) the common use of initial rates or first order kinetic models, which involve, as we have just demonstrated, less statistically robust parameters, and even distortion of the reality of the process, which requires the use of asymptotic equations such as those applied here.

Finally, it should be mentioned that 35ºC was a temperature that was very close to the domain at which thermal destabilisation of the biological system starts. This, without doubt, results in a high sensitivity to small variations in experimental conditions in this environment. This fact could contribute to the difficulties found in the fit of the results obtained at 37ºC, and, in any case, suggests the need to operate at lower temperatures.

3.4. Autocatalytic process versus populational response. Influence of ouabain.

In section 2 we had pointed out the possibility of interpreting the sigmoidal character in time of the hemolysis as a result of an autocatalytic mechanism, in which the hypothetical release of some product from the cellular lysis acted as an activating cofactor of the process. In this case (See appendix B) the corresponding kinetics would follow the model:

$$H = \frac{H_m}{1+\left(\frac{H_m}{H_0}-1\right)\exp\left(-k_1 t\right)} \qquad [16]$$

H_0 and H_m being the initial and final values of the hemolysis, and k_1 the rate constant. This equation differs from ^{m}L [4], which describes the populational response, in its non null intercept, H_0. However, as the value of H_0 is necessarily low, comparing the fit of the kinetic data to [4] and [16] is a very unsafe criterion even with low experimental error, which makes the alternative between the autocatalytic process and populational response indescribable.

However, in the presence of a competitive inhibitor of hemolysis, such as ouabain, autocatalytic kinetics would follow the model (I being the concentration of ouabain):

$$H = \frac{H_m\left(\frac{k_1-k_2 I}{k_1}\right)}{1+\left(\frac{H_m}{H_0}\frac{k_1-k_2 I}{k_1}-1\right)\exp\left[-\left(k_1-k_2 I\right)t\right]} \qquad [17]$$

whereas in a populational response the ouabain effect could be described (Murado et al. 2002) introducing in any type [1] sigmoidal model a factor with the form:

$$F_i = 1+b_i I \quad ; \ (i=K, m, \upsilon) \qquad [18]$$

which would multiply or divide K, m o υ, according to its effect raise or reduce the value of the corresponding parameter, leading to specific alterations in the response profile.

Given that under these conditions the models differ more substantially, the presence of an inhibitor like ouabain enables us to decide between the autocatalytic and populational hypotheses. In this respect, the results obtained working with 0.25 ng.ml^{-1} of palytoxin in the presence of 2, 3, 4 and 5 μM of ouabain clearly supports the populational option. Indeed (figure

6), whereas the autocatalytic model with inhibition [17] resulted in very inadequate fits, both the equations ^{m}L [4] and ^{m}W [6] led to satisfactory simultaneous solutions of the set of kinetic profiles, once the factors F_i [18] had been introduced.

In the case of the equation ^{m}L, the factors F_K and F_μ divided the K and μ values, whereas the factor F_m multiplied the value of m. In the equation ^{m}W, however, a divisor F_K and a multiplier F_m were sufficient, F_a being null. It is interesting to note this characteristic, as it is the essential difference between the equations with regard to DR models: in ^{m}L the geometric elements of the curve vary independently with each parameter in ^{m}W, if m varies, the slope varies, too.

As far as we are interested here, these results showed that the inhibitory action of ouabain can be incorporated into the models that describe palytoxin activity without difficulty. Furthermore, the apparent parameters generated by the joint response do not alter (if the concentration of ouabain is known) the attribution of a specific dose of palytoxin to a specific hemolytic response. However, it is not so clear that the same results allow us to reject the hypothesis of a low intensity autocatalytic effect, as mentioned in section 2 with respect to a possible spontaneous hemolysis.

3.5. Evaluation at 25ºC in the presence of ouabain.

Given the effects of temperature and ouabain on the response under study, the simultaneous action of both factors are considered an adequate resource to reduce the apparent potency of the toxin. Consequently, three new kinetics were carried out at 25ºC, in the absence and presence of ouabain 1 and 5 μM, also redefining the series of palytoxin doses in order to obtain a greater number of values in the low concentration domain. The results, evaluated via the S1 and S2 crtieria (figures 7 and 8; Table 1), showed the following facts:

1: Both criteria provide consistent descriptions and statistically significant parametric estimates. In the more intense responses, however, also at 25ºC the estimated potency was higher (the value of m lower) when the results at asymptotic times were used than when all the information in bidimensional model was used.

2: The combined effect of the decrease in temperature and the presence of ouabain contributed not only to soften the response, but also to create the sigmoidal profile characteristic of DR relationships, to improve the correlation between observations and predictions in the bidimensional model and to make progressively convergent the estimates according to the

criteria S1 and S2. In fact, with ouabain 5 μM the confidence intervals (α=0.05) of the estimates of *m* for both criteria overlapped, making both values statistically indistinguishable.

3: Comparing the results at 25 and 37ºC in the absence of ouabain it is clearly seen that if higher temperatures slightly accelerate the response, the same does not happen to the potency: the *m* values obtained through the criteria S1 and S2 at the two temperatures were statistically indistinguishable, even with a lower average (greater potency) at 25ºC according to S1. This situation was confirmed in two independent and complete series of experiments, with different erythrocyte preparations. Though unable to make a detailed interpretation of this fact, the results from the Arrhenius approximation suggest that the erythrocyte system is, under *in vitro* assay conditions, at 37º rather unstable, which could partially disorganise the palytoxin response mechanism.

In a DR assay where the safety margin of the effector is very low (*i.e.,* the slope of the function is very high), the calculation of the parameter *m* (*i.e.*, the DL_{50}) is relatively sensitive to experimental error, especially if the potency of the effector is very high (the *m* parameter is very low). This is precisely what happens in the case of palytoxin, therefore to obtain reliable parametric estimates it is necessary to work under conditions that, without altering the mode of action, reduces the intensity of the response and transforms a degenerated sigmoidal profile (more similar to a von Bertalanffy equation than a logistic one) into the sigmoidal profile expected according to the foundations of the DR phenomenon. As such foundations also require, according to the facts demonstrated in the previous sections, that the response is sigmoidal as a function of the time and the dose, a good criterion to define reliable conditions is the convergence between the solutions derived from S1 and S2. In other words, reliable conditions are those in which the normalized response to the dose series assayed is independent of the time under consideration, at least in the interval between $t_{0.5}$ and the asymptotic response time.

3.6. The method proposed

Taking into account the results described in the previous sections, the method proposed here to determine the equivalent activity of palytoxin in a given matrix can be summarised in the following terms:

3.6.1. Reagents (in solution or PBS suspension)

A. Erythrocyte preparation: 1.7×10^{8} cells·ml^{-1} (dilution 1/10 from original blood)
B. Ouabain (5 μM)

C. Palytoxin dilution series: 8 to 10 values in the interval 10-1.200 $pg.ml^{-1}$

D. Dilution series from the extract that must be evaluated.

3.6.2. Procedure

Have a number of experimental units available (tubes or microplate dishes) sufficient for the triple assay of the dilution series included in **C** and **D**.

3.6.2.1. Combine 1 volume of **A**, 4 volumes of PBS and 5 volumes of **B** (getting a dilution 1/100 of original blood, 1.7×10^7 $cells\cdot ml^{-1}$). Incubate 30 minutes at 25ºC.

3.6.2.2. Combine 1 volume of solution in 3.6.2.1 with 1 volume of **D**. Incubate 18-24 hours at 25ºC.

3.6.2.3. Eliminate the cell residues by centrifuge (400 or 300 g depending on if they are tubes or microplates, 10 minutes, 10ºC) and measure absorbance at 405 nm in the supernatant.

3.6.3. Blank, total hemolysis and calibration curve

For the blank assay (0% hemolysis) the procedure described above is used substituting **D** for PBS in 3.6.2.2. To determine the total hemolysis (100% hemolysis value), the procedure described above is used substituting **D** for distilled water in 3.6.2.2. The calibration curve is performed substituting **D** dilution series for **C** dilution series in 3.6.2.2.

Under these conditions, the semi-maximum response time is ~400 minutes, and the asymptotic stage of the kinetics is reached at ~1,000 minutes, with ~70% hemolysis. The parameters obtained when the calibration data obtained from the above conditions are fitted to the models ^{m}L and ^{m}W are given in Table 3. The corresponding functions with normalised responses (K=1) at 960 minutes are shown in figure 8. These results represent the corresponding reference curves of hemolysis by palytoxin in the presence of ouabain (PHO), and not just the concentration of palytoxin, which, as mentioned in the introduction can be misleading.

CONCLUSIONS

In the previous sections the reasons for the reliability and legitimacy of a method for evaluating the toxicity of palytoxin have been discussed, establishing working conditions that allow the application of statistically robust dose-response model, whose parameters possess unequivocal biological significance.

Leaving aside the development required for its justification, this method is very simple. In fact, once the protocol described in section 6 has been applied to an extract obtained from a sample of known weight, it is enough to: 1) fit the responses of the extract's dilution series to an ^{m}L or ^{m}W equation, to obtain the parameter m_{dil} or the dilution that corresponds to the semi-maximum response; 2) Fit the PHO calibration curve to an ^{m}L or ^{m}W equation to obtain the parameter m_H or palytoxin concentration that corresponds to the semi-maximum response. In this way, the dilution activity m_{dil} will be equivalent to the concentration m_H of palytoxin.

Taking into account that the mouse bioassay is the traditional reference for neurotoxins of the kind studied here, it can be useful to establish the equivalence between both assays, which is possible if both are described by the same models applied here. With regard to this, Table 3 and figure 9 define, next to the PHO curve mentioned in the previous section, the results, in terms of ^{m}L and ^{m}W equations, of a previous mouse assay (Riobó et al. Unpublished) with the following specifications: mortality in 24 hours of NMRI males with weights of 19.5 to 22g; 124 individuals; dose expressed as concentration of ($ng \cdot ml^{-1}$) the solution intraperitonally injected. This shows that taking into account the dose for semi-maximum response, the hemolysis assay is approximately 20 times more sensitive.

The parameters of the mouse models can be used as permanent references to avoid more animal deaths. The parameters of the PHO models can be used, together with the conversion functions [5] and [7], to calibrate the curves obtained for each batch of erythrocytes and therefore enable us to express the results, if required, in terms of toxicity to the mouse.

Actually, however, the best option would be to dispense with the mouse assay, using the toxicity to this animal to establish the maximum acceptable dose (MAD) in humans, expressed in terms of palytoxin equivalents, and determine such equivalents through the hemolysis assay in the terms described above.

ACKNOWLEDGEMENTS

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Appendix A. The dose-response models used.

1. Modified logistic equation (^{m}L)

Logistic equation can be transferred from its habitual formulation (as a model for describing an autocatalytic kinetics, or a biological growth) to the context of the DR relationships, where it would have the form:

$$R = \frac{K}{1+\exp\left(c-\mu D\right)} \;;\; \text{with} \;\; c = \ln\left(\frac{K}{R_0}-1\right), \;\text{where:} \qquad [A1]$$

R response, with R_0 and K as minimum and maximum values, respectively.

D dose.

μ maximum specific rate of response (maximum increment of the R per unit of R and per unit of D).

Although [A1] is sometimes used directly as DR model, in this application it is important to introduce two modifications:

1. To eliminate the intercept (to make R_0=0), so that the model obeys the condition of null response at null dose. Besides a basic fact of the DR relationships, the condition R_0=0 is useful for the calculation of the remaining parameters by means of non lineal fitting methods. Indeed, with real data, affected of experimental error, the calculation can lead to unacceptably high values of R_0. The problem decreases including restrictions that limit R_0 to very low values, but it can create biases in the value of μ, very sensitive to the experimental error, in particular to overestimations –frequent in the practice– of the response at low doses.

2. To reparametrize the equation, so that it includes explicitly the dose for semi-maximum response (ED_{50}, LD_{50}, m in our notation), an essential parameter in the DR analysis. It allows the direct calculation of the corresponding confidence interval by means of computer applications as *Statistica* or *MatLab*.

Beginning with the reparametrization, if we make R=K/2 in [A1], we have $c = m$, and therefore:

$$R = \frac{K}{1+\exp\left[\mu\left(m-D\right)\right]} \qquad [A2]$$

Now, since the intercept (R for D=0) of [A2] is:

$$R_0 = \frac{K}{1+\exp(\mu m)}$$

the logistic equation without intercept is:

$$R = K\left(\frac{1}{1+\exp\left[\mu(m-D)\right]} - \frac{1}{1+\exp(\mu m)}\right) \qquad [A3]$$

In this last equation, however, K and m do not represent the maximum response and the dose for semi-maximum response, respectively, the real values of which (K_r and m_r) can be obtained from [A3]:

$$K_r = \lim_{D\to\infty} R = K\left[\frac{\exp(\mu m)}{1+\exp(\mu m)}\right] \qquad [A4]$$

$$m_r = \frac{1}{\mu}\ln\left[2+\exp(\mu m)\right] \qquad [A5]$$

So that the model includes such real values, K and m could be isolated from [A4] and [A5], and the resulting expressions to be introduced in [A3]. A simpler resource, however, is to reorder [A5] in the form:

$$\exp(\mu m) = \exp(\mu m_r) - 2$$

and to substitute, in [A3] and [A4], the term $\exp(\mu m)$ for its equivalent one, what leads to the form:

$$R = \frac{K_r\left[\exp(\mu m_r)-1\right]}{\exp(\mu m_r)-2}\left\{\frac{1}{1+\exp(-\mu D)\left[\exp(\mu m_r)-2\right]} - \frac{1}{\exp(\mu m_r)-1}\right\} \qquad [A6]$$

For simplifying the notation, it can be made:

$A = \exp(\mu m_r) - 1$; $B = \exp(\mu m_r) - 2$; and therefore:

$$R = \frac{KA}{B}\left[\frac{1}{1 + B\exp(-\mu D)} - \frac{1}{A}\right] \qquad [A7]$$

which is the ^{m}L model used in this work.

2. Modified accumulative function of the Weibull's distribution (^{m}W)

In terms of DR model, the original accumulative Weibull's function would be (α and β being parameters of form and scale, respectively):

$$R = 1 - \exp\left[-\left(\frac{D}{\beta}\right)^{a}\right] \qquad [A8]$$

This form has the advantage on the logistic model of its null intercept. However, its use as a DR model makes convenient two modifications:

1. Multiplication of the second member for the maximum response *K*, so that the asymptote can take values different from 1:

$$R = K\left\{1 - \exp\left[-\left(\frac{D}{\beta}\right)^{a}\right]\right\} \qquad [A9]$$

2. Reparametrization of the equation, to make explicit the dose (*m*) for semi-maximum response. This way, if we make *R*=*K*/2 in [A9], we have:

$$m = \beta(\ln 2)^{1/a} \; ; \; \beta = \frac{m}{(\ln 2)^{1/\alpha}}$$

what leads to the definitive form:

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m}\right)^{a}\right]\right\} \; ; \text{ where:} \qquad [A10]$$

R response, with K as maximum value.

m dose for semi-maximum response.

a form parameter, related with the maximum slope of the response.

which is the ^{m}W model used in this work.

Appendix B. Description of an autocatalytic process with competitive inhibition.

To describe in a formal way the possible autocatalytic formation of the hemolysis products (H) due to palytoxin (P) in the presence of ouabain (I), we can admit an autocatalytic mechanism (that, for further generality, we will consider reversible, although this hypothesis is not pertinent in this case), simultaneous with a competitive irreversible inhibition:

$$r_{H1}:\ H + P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} nH$$

$$r_{H2}:\ H + I \xrightarrow{k_2} HI$$

Applying the mass action law, the process is defined by the following differential equations:

$$r_{H1} = \frac{dH_1}{dt} = k_1 \cdot H \cdot \left(\frac{H_m - H}{H_m} \right) - k_{-1} \cdot H^n$$

$$r_{H2} = \frac{dH_2}{dt} = k_2 \cdot H \cdot I$$

$$r_H = r_{H1} - r_{H2} = \frac{dH_1}{dt} - \frac{dH_2}{dt} = k_1 \cdot H \cdot \left(\frac{H_m - H}{H_m} \right) - k_{-1} \cdot H^n - k_2 \cdot H \cdot I \qquad \text{[B1]}$$

In the non trivial simplest (n=2) case, we have:

$$r_H = \frac{dH}{dt} = k_1 \cdot H \cdot \left(\frac{H_m - H}{H_m} \right) - k_{-1} \cdot H^2 - k_2 \cdot H \cdot I = H \cdot \left(k_1 - k_2 \cdot I \right) - H^2 \cdot \left(\frac{k_1}{H_m} + k_{-1} \right)$$

or, by making $\alpha = k_1 - k_2 \cdot I$ and $\beta = \frac{k_1}{H_m} + k_{-1}$ [B2]

$$r_H = \frac{dH}{dt} = \alpha \cdot H - \beta \cdot H^2 = H \cdot \left(\alpha - \beta \cdot H \right)$$

By separation of variables and subsequent integration:

$$\int_{H_0}^{H} \frac{dH}{H\cdot(\alpha-\beta\cdot H)} = \int_{0}^{t} dt \qquad [B3]$$

The integral in the first member can be solved by means of the decomposition:

$$\frac{1}{H\cdot(\alpha-\beta\cdot H)} = \frac{A}{H} + \frac{B}{\alpha-\beta\cdot H}$$; that leads to:

$$\left.\begin{matrix} H\cdot(\beta\cdot A - B) = 0 \\ \alpha\cdot A = 1 \end{matrix}\right\}$$ por lo que: $A = \frac{1}{\alpha}$; $B = \frac{\beta}{\alpha}$

In this way, [B3] can be reformulated as:

$$\int_{H_0}^{H} \frac{1}{\alpha}\cdot\frac{dH}{H} + \int_{H_0}^{H} \frac{\beta}{\alpha}\cdot\frac{dH}{(\alpha-\beta\cdot H)} = t$$; that is to say:

$$\frac{1}{\alpha}\cdot\ln H\Big|_{H_0}^{H} - \frac{1}{\alpha}\cdot\ln(\alpha-\beta\cdot H)\Big|_{H_0}^{H} = t \quad ; \quad \frac{1}{\alpha}\cdot\ln\left(\frac{H}{H_0}\right) - \frac{1}{\alpha}\cdot\ln\left(\frac{\alpha-\beta\cdot H}{\alpha-\beta\cdot H_0}\right) = t$$

Reordering and isolating *H*:

$$H = \frac{H_0\cdot\alpha\cdot e^{\alpha\cdot t}}{\alpha-\beta\cdot H_0+H_0\cdot\beta\cdot e^{\alpha\cdot t}} = \frac{\alpha/\beta}{\frac{\alpha\cdot e^{-\alpha\cdot t}}{\beta\cdot H_0} - e^{-\alpha\cdot t}+1} = \frac{\alpha/\beta}{1+\left(\frac{\alpha}{H_0\cdot\beta}-1\right)e^{-\alpha\cdot t}}$$

Finally, restoring to α and β their values according to [B2], we obtain:

$$H = \frac{H_m\left(\frac{k_1-k_2\cdot I}{k_1+k_{-1}\cdot H_m}\right)}{1+\left\{\left[\frac{H_m}{H_0}\frac{k_1-k_2\cdot I}{k_1+k_{-1}\cdot H_m}\right]-1\right\}e^{-(k_1-k_2\cdot I)t}} \qquad [B4]$$

It should be observed that if the autocatalytic reaction r_{H1} is irreversible (k_{-1}=0), we obtain the expression [17], used in the section 4 as a contrast against the populational hypothesis:

$$H = \frac{H_m\left(\frac{k_1 - k_2 I}{k_1}\right)}{1 + \left\{\left[\frac{H_m}{H_0}\ \frac{k_1 - k_2 I}{k_1}\right] - 1\right\} e^{-(k_1 - k_2 I)t}}$$

Whereas the additional condition of absence of inhibitor (I=0) produces:

$$H = \frac{H_m}{1 + \left(\frac{H_m}{H_0} - 1\right) e^{-k_1 t}}$$

That is to say, the logistic equation as a description of an autocatalytic process.

REFERENCES

Alcala AC, Alcala LC, Garth JS, Yasumura D, Yasumoto T (1988) Human fatality due to ingestion of the crab *Demania reynaudii* that contained a palytoxin-like toxin. Toxicon 26:105-107

Artigas P, Gadsby DC (2003) From the Cover: Na+/K+-pump ligands modulate gating of palytoxin-induced ion channels. PNAS 100:501-505

Bignami GS (1993) A rapid and sensitive hemolysis neutralization assay for palytoxin. Toxicon : Official Journal of the International Society On Toxinology 31:817-820

Brzezinska-Slebodzinska E (2003) Species differences in the susceptibility of erythrocytes exposed to free radicals in vitro. Vet. Res. Comm. 27:211-217

Canavos GC (1992) Probabilidad y estadística. Aplicaciones y métodos, Vol. Mc Graw Hill, Madrid

Fukui M, Murata M, Inoue A, Gawel M, Yasumoto T (1987) Ocurrence of palytoxin in the Trigger fish *Melichtys vidua*. Toxicon 25:1121-1124

Granéli E, Ferreira CEL, Yasumoto T, Rodrigues E, Neves MHB (2002) Sea urchins poisoning by the benthic dinoflagellate *Ostreopsis ovata* on the Brazilian coast. Book of abstracts Xth International Conference on Harmful algae (Florida)

Habermann E, Ahnert-Hilger G, Chhatwal GS, Beress L (1981) Delayed haemolytic action of palytoxin. Biochemica et Biophysica Acta 649:481-486

Habermann E, Chhatwal GS (1982) Ouabain inhibits the increase due to Palytoxin of cation permeability of erythrocytes. Naunyn-Schmiedeberg´s Archives of Pharmacology 319:101-107

Hilgemann DW (2003) From a pump to a pore: How palytoxin opens the gates. PNAS 100:501-505

Holtrop G, Petrie J, McElhiney J, Dennison N (2006) Can general anaesthesia be used for the paralytic shellfish poison bioassay? Toxicon 47:336-347

Huntley JS, Hall AC (1994) Aspects of the haemolytic reaction induced by kanagawa hemolysin of *Vibrio parahaemolyticus*. Toxicon 32:1397-1412

Moore RE, Scheuer PJ (1971) Palytoxin: A new marine toxin from a coelenterate. Science 172:495-498

Murado MA, González MP, Vázquez JA (2002) Dose response relationships: an overview. A generative model and its application to the verification of descriptive models. Enzyme and Microbial Technology 31:439-455

Murado MA, Vázquez JA (2007) The notion of hormesis and the dose-response theory: A unified approach. Journal of Theoretical Biology 244:489-499

Onuma Y, Satake M, Ukena T, Roux J, Chanteau S, Rasolofonirina N, Ratsimaloto N, Naoki H, Yasumoto T (1999) Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37:55-65

Riobó P, Vázquez JA, Murado MA, Cacho E, Paz B, Franco JM (Unpublished) Mouse bioassay for palytoxin: Dose-Response and Dose-Death time Relationships.

Shalel S, Streichman S, Marmur A (2002) Modeling surfactant-induced hemolysis by Weibull survival analysis. Colloids and Surfaces B: Biointerfaces 27:223-229

Taniyama S, Arakawa O, Terada m, Nishio S, Takatani T, Mahmud Y, Noguchi T (2003) *Ostreopsis sp.*, a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42:29-33

Thompson WR (1947) Use of moving averages and interpolation to estimate median-effective dose. Bact. Rev. 11:115-145

Thompson WR, Weil CS (1952) On the construction of tables of moving average interpolation. Biometrics march:51-54

Vázquez JA, Docasal SF, Mirón J, González MP, Murado MA (2006) Proteases production by two *Vibrio* species on residual marine media. J. Ind. Microb. Biotech 33:661-668

Weil CS (1952) Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}) and instructions in their use. Biometrics september:249-263

Zavodnik IB, Lapshina EA, Zavodnik LB, Soszynski M, Bartosz G, Bryszewska M (2002) Hypochlorous acid-induced oxidative damage of human red blood cells: effects of tert-butyl hydroperoxide and nitrite on the HOCl reaction with erythrocytes. Bioelectrochemistry 58:127-135

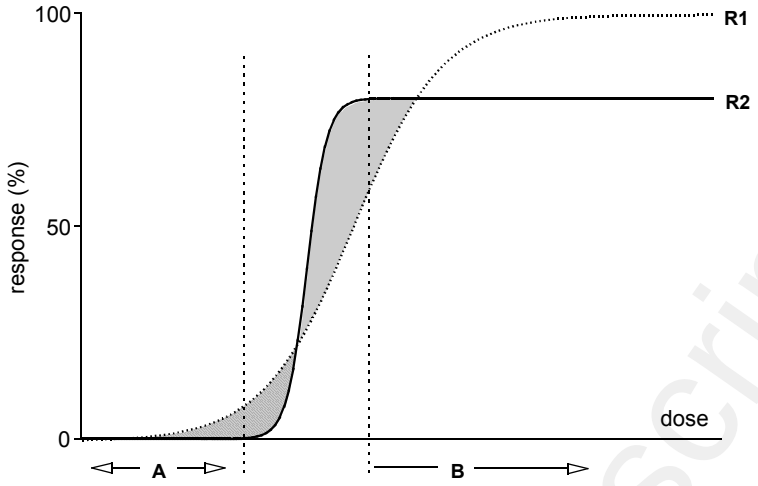

Figure 1: Relationships between DR curves for the two effectors as against the same receptor or one effector as against two different receptors (equivalent to the assessment of one effector by two different methods). The represented case is the most general, where the maximum response, the slope and the correspondent dose to the semi maximum response vary. Take notice that to calculate the D_1 dose which produces in R1 an equivalent response to the D_2 dose in R2, it must be taking into account that: 1) in the domains of the highlighted surfaces the relative magnitudes of the responses are reversed; 2) the conversion function δ is only applied in the abscissa interval between the domains A and B. That is, from the end of the starting to the beginning of the asymptotic stretch of the reference curve (herein R2).

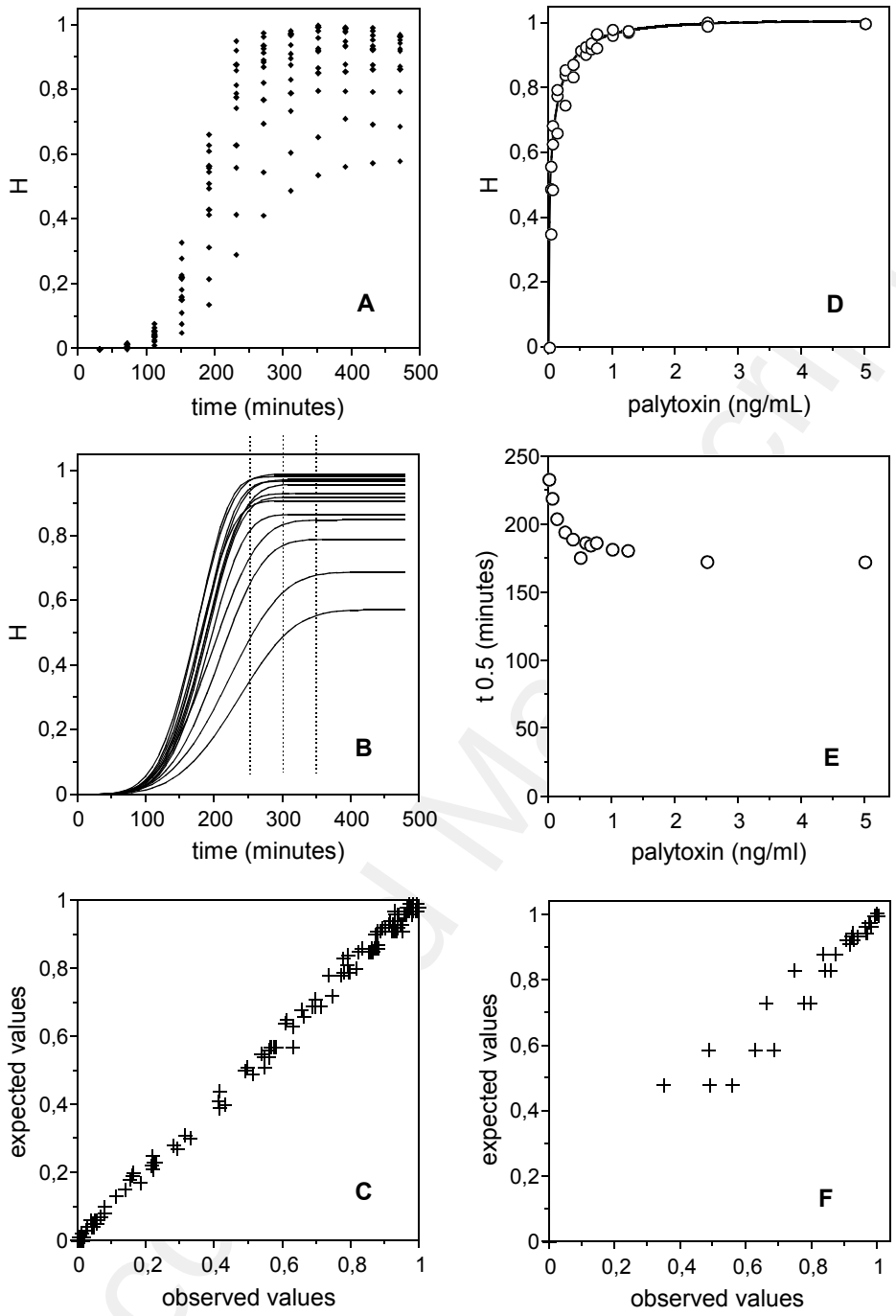

Figure 2: Palytoxin hemolytic kinetics at 37ºC. For further details, see text.

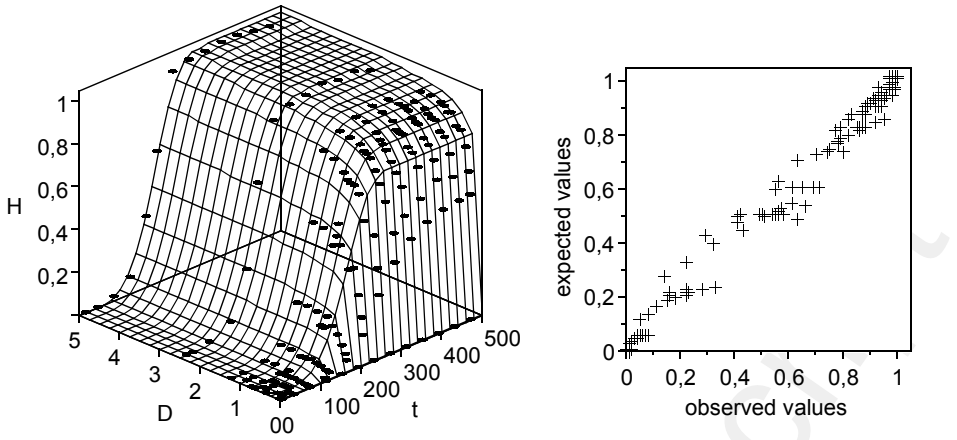

Figure 3: Surface of dose-time response (S2 criteria) belonging to the palytoxin hemolysis at 37ºC. For further details, see text.

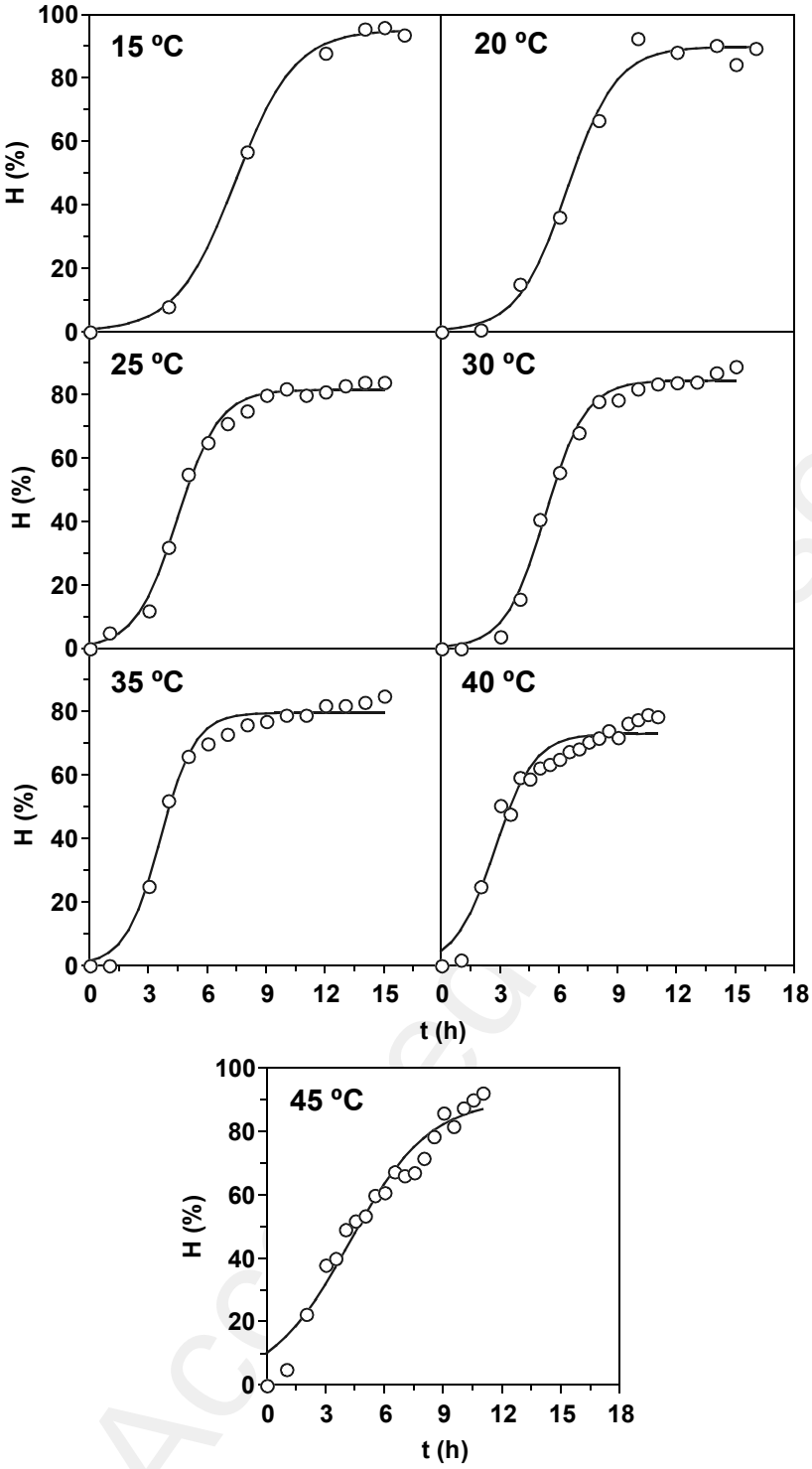

Figure 4: Palytoxin hemolysis kinetic at different temperatures. Fit of the experimental results (dots) according to the logistic models (continuous lines). For the estimation of the speed parameters , see text.

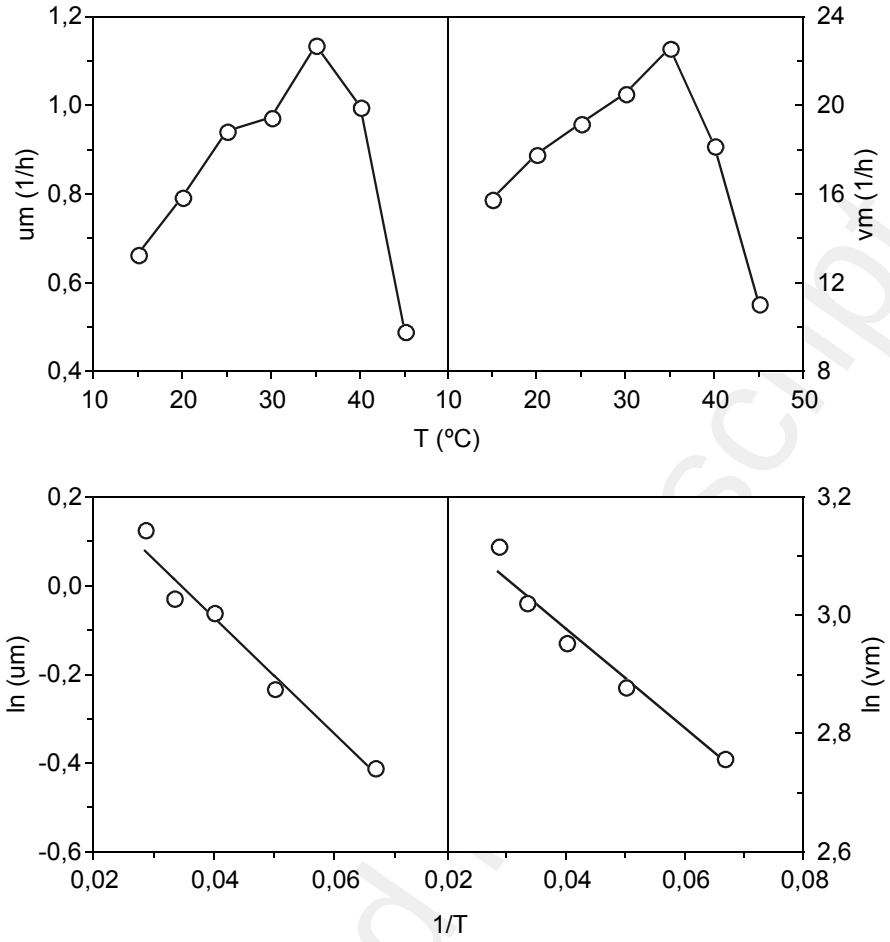

Figure 5: On the top, temperature effect over the kinetics parameters (on the left: μ_m, on the right: v_m) of the palytoxin hemolysis. On the bottom , fit of the Arrhenius equation of the kinetic parameters belonging to the increasing speed stench, for the calculus of the activation energy.

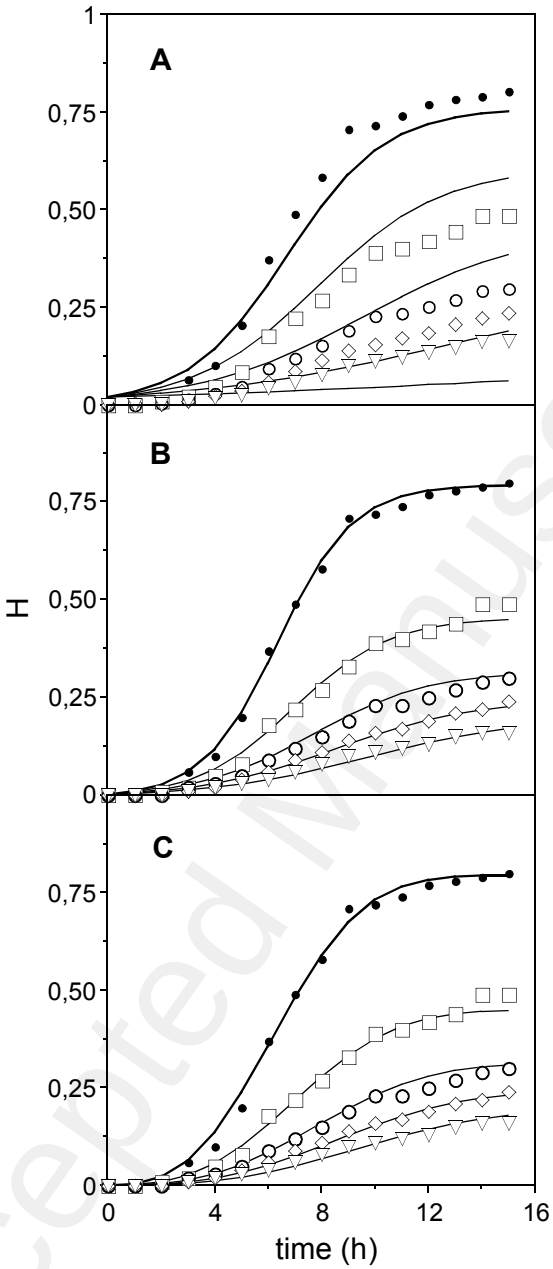

Figure 6: Catalytic hypothesis (on the top) in contrast to populational hypothesis, according to the models ^{m}L (in the middle) and ^{m}W (on the bottom), in the Ouabain inhibition of the palytoxin hemolysis. Absence (●)and presence of 5 (▽), 4 (◇), 3 (○) and 2 (□) μM of ouabain.

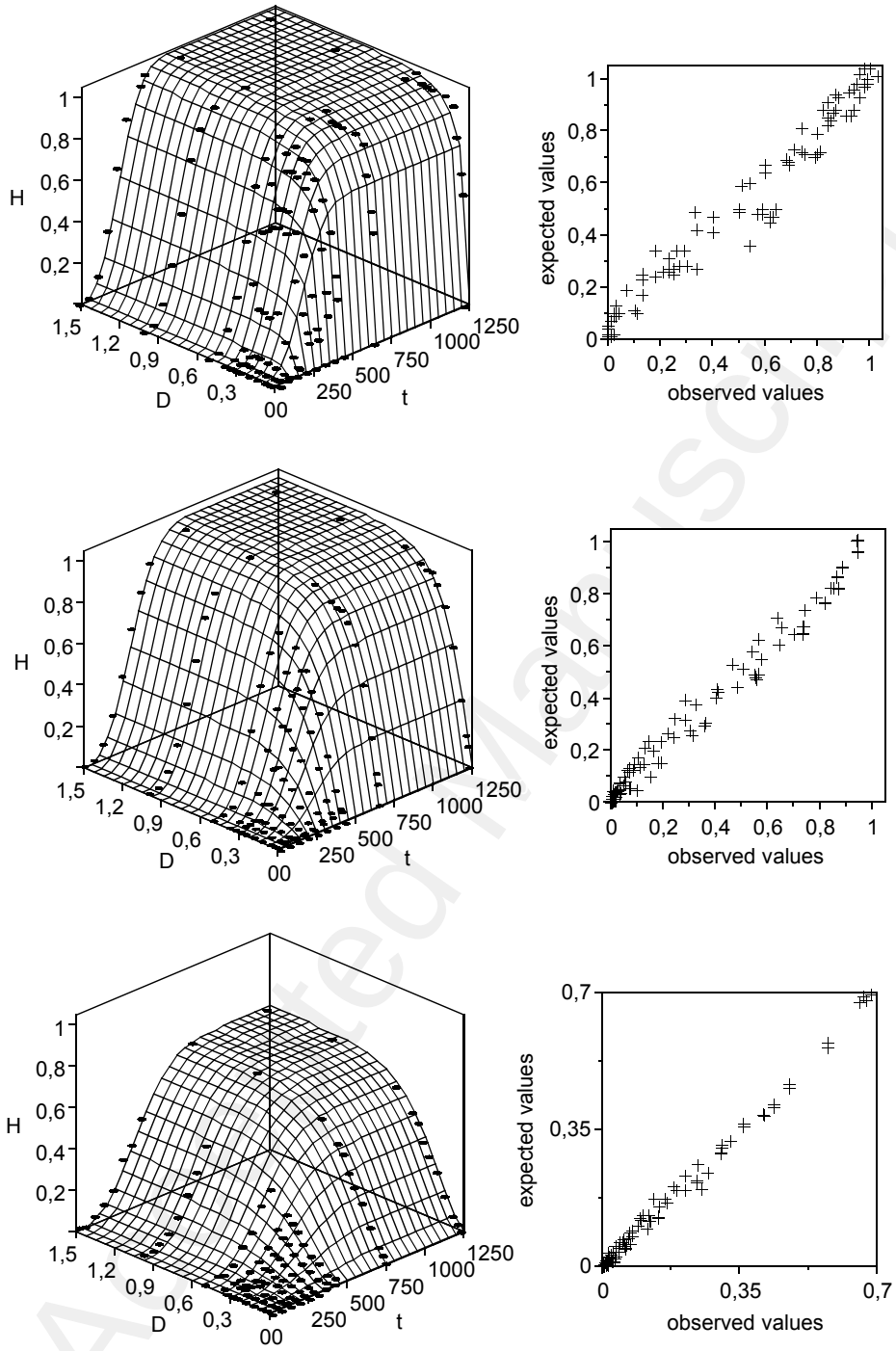

Figure 7: Dose-time response surfaces (S2 criteria) for the palytoxin hemolysis at 25ºC, absence (on the top) and presence of 1 μM (in the middle) and 5 μM of Ouabain (on the bottom). For further details, see text.

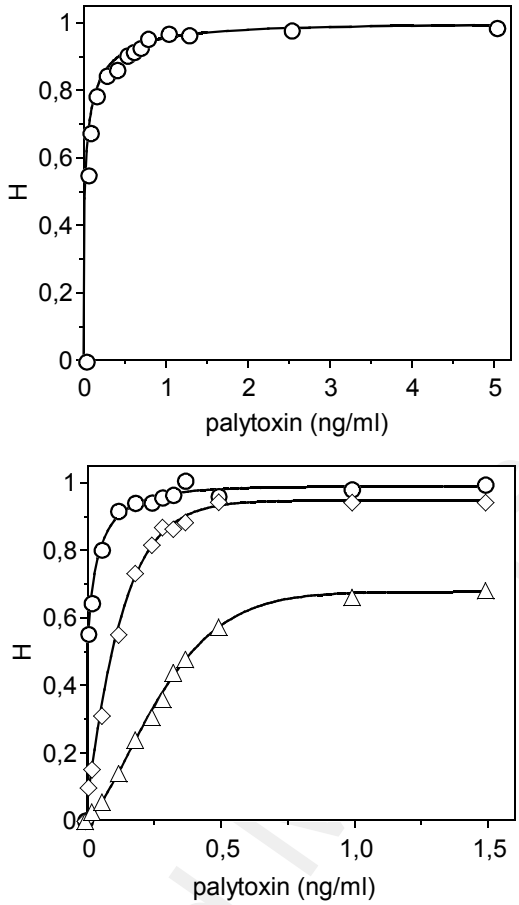

Figure 8: DR curves of asymptotic response times. On the top: at 37ºC; on the bottom: at 25 ºC in absence (○) and presence of 1 (◇) and 2 (△) μM of ouabain.

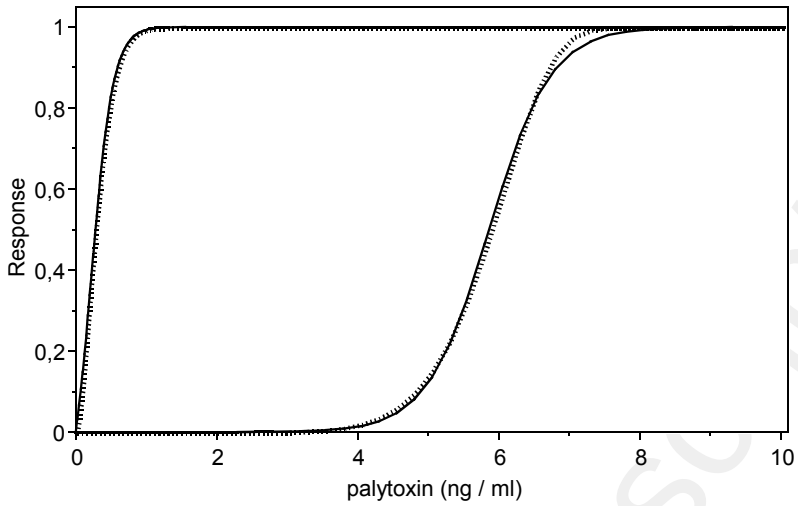

Figure 9: Fitting of the palytoxin responses to the models ^{m}L (continuous lines) and ^{m}W (dotted lines) according to the hemolytic assays (PHO: at 25ºC with 5 μM of Ouabain (on the left)) and to the mouse bioassay (on the right).

TABLE 1: Basic parameters of the hemolysis by palytoxin, under the specified conditions, as estimated by means of the criteria discussed in the text. $t_{0.5}$ and m: time and dose for semi-maximum response, in minutes and pg.ml^{-1}, respectively; OU: ouabain. In equation [8], we give maximum and minimum $t_{0.5}$ obtained when adjusting separately the kinetic series; in the rest of the cases, the confidence intervals for α=0.05 are specified.

		Evaluation criteria		
conditions		eq. [8]	S1 [a]	S2
37ºC	$t_{0.5}$	173-233	-	185 ± 2
	m	-	14.5 ± 3.0 [b]	25.3 ± 3.9
25ºC	$t_{0.5}$	222-399	-	245 ± 8
	m	-	10.3 ± 1.8	27.6 ± 5.0
25ºC; OU 1 μM	$t_{0.5}$	272-450	-	303 ± 7
	m	-	94.1 ± 6.8	132.3 ± 9.4
25ºC; OU 5 μM	$t_{0.5}$	375-454	-	395 ± 7
	m	-	260.2 ± 11.4	277.0 ± 7.3

([a]) At asymptotic response time.
([b]) 29.2 ± 7.0 if the results at 250, 300 and 350 minutes are jointly considered (as in figure 3D).

TABLE 2: Parametric estimates of the hemolysis rate at different temperatures, as obtained by fitting of the experimental results to the equations [10] and [11]. Confidence intervals (α=0.05) and correlation coefficients (r) between observations and predictions are specified ([10] and [11] are parametric forms of the same equation and, consequently, the corresponding r are coincident).

T (ºC - K)	μ_m [eq. 10]	v_m [eq. 11]	r
15 - 288	0.664 ± 0.169	15.772 ± 3.783	0.999
20 - 293	0.793 ± 0.255	17.796 ± 5.411	0.996
25 - 298	0.942 ± 0.169	19.188 ± 3.286	0.997
30 - 303	0.973 ± 0.176	20.533 ± 3.517	0.997
35 - 308	1.136 ± 0.313	22.589 ± 6.007	0.994
40 - 313	0.996 ± 0.297	18.181 ± 5.090	0.981
45 - 318	0.490 ± 0.141	11.057 ± 2.412	0.982

TABLE 3: Parameters of the DR relationships concerning palytoxin, according to the mouse bioassay, and hemolysis assay at 25ºC in the presence of ouabain, calculated through the fitting of the experimental results to the models ^{m}L and ^{m}W. See also figure 10.

		mouse bioassay	hemolysis assay (PHO)
eq. ^{m}L [4]	K	1.008 ± 0.040	0.987 ± 0.023
	μ	2.258 ± 0.687	6.989 ± 0.862
	m	5.870 ± 0.146	0.263 ± 0.009
	r	0.996	0.999
eq. ^{m}W [6]	K	1.003 ± 0.029	0.990 ± 0.026
	a	9.340 ± 1.943	1.508 ± 0.126
	m	5.892 ± 0.108	0.260 ± 0.011
	r	0.998	0.999

Table of symbolic notations used.

H :	Normalized hemolysis. Dimensions: percent (or unitary fraction) of hemolysis.
t :	Time.
H_m :	Maximum hemolysis. Dimensions: percent (or unitary fraction) of hemolysis.
μ_m :	Maximum specific rate of hemolysis. Dimensions: t^{-1}
H_0 :	Initial hemolysis. Dimensions: %H
v_m :	Maximum growth rate of hemolysis. Dimensions: %H.t^{-1}
λ:	Hemolysis lag phase. Dimensions: t
k_r:	Kinetic constant. Dimensions: Arbitraries (for example, 1st order kinetic: t^{-1})
T :	Absolute temperature. Dimensions: Kelvin (K)
A:	Arrhenius pre-exponential factor. Dimensions: Arbitrary (*e.g.* 1st order kinetic: t^{-1})
E_a (k_r):	Activation energy (k_r). Dimensions: J.mol^{-1}
A_1:	Arrhenius pre-exponential factor (μ_m). Dimensions: t^{-1}
A_2:	Arrhenius pre-exponential factor (v_m). Dimensions: %H.t^{-1}
R:	Gas-law constant. Value and dimensions: 8.315 J.mol^{-1}.K^{-1}
E_a (μ_m):	Activation energy (μ_m). Dimensions: J.mol^{-1}
E_a (v_m):	Activation energy (v_m). Dimensions: J.mol^{-1}