MOUSE BIOASSAY FOR PALYTOXIN: NOVEL DESCRIPTION OF SYMPTOMS AND DOSE- RESPONSE RELATIONSHIPS

Riobó P. *, Paz B. and Franco J.M.

Grupo de Fitoplancton Tóxico, Instituto Investigacións Mariñas (CSIC).

Eduardo Cabello 6, 36208 Vigo; Galicia (Spain).

Vázquez J.A. and Murado M.A.

Grupo de Reciclado y Valorización de Residuos, Instituto Investigacións Mariñas (CSIC).

Eduardo Cabello 6, 36208 Vigo; Galicia (Spain).

Cacho E.

Dependencia del Área Funcional de Sanidad, Ministerio de Administraciones Públicas (MAP).

Estación Marítima s/n, Vigo; Spain.

**Corresponding author: Pilar.riobo@vi.ieo.es*

Current address: Instituto Español de Oceanografía, Centro Oceanográfico de Vigo.

Apdo. 1552. 36200 Vigo, Spain. Tel.:+34-986-492111; fax: +34-986-498626

ABSTRACT

Nowadays, a variety of protocols are applied to quantitate palytoxin. However, there is not desirable agreement among them, the confidence intervals of the basic toxicological parameters are too wide and the formal descriptions lack the necessary generality to establish comparisons. Currently, the mouse bioassay is the most accepted one to categorize marine toxins and it must constitute the reference for other methods. In the present work, the mouse bioassay for palytoxin is deeply analyzed and carefully described showing the initial symptoms of injected mice which are presented here in the first time. These symptoms clearly differ from the more common marine toxins described up to now.

Regarding to the toxicological aspects two considerations are taking into account: (i) the empiric models based in the dose-death time relationships cause serious ambiguities and (ii) the traditional moving average method contains in its regular use any inaccuracy elements.

Herein is demonstrated that the logistic equation and the accumulative function of Weibull's distribution (with the modifications proposed) generate satisfactory toxicological descriptions in all the respects.

Key words: mouse bioassay, palytoxin, dose-response (DR), LD_{50}, mathematical models.

INTRODUCTION

Palytoxin is one of the most potent non-protein marine toxins known belonging to a group of closely related, very poisonous aliphatic molecules with high molecular weights of around 2600 Da (Habermann & Chhatwal 1982). It has been primarily isolated from the marine zoanthids *Palythoa*. Subsequently, it was also found to be present in benthic dinoflagellates of the genus *Ostreopsis* (Usami et al. 1995, Onuma et al. 1999, Lenoir et al. 2004, Riobó et al. 2004); which is exclusively marine and occurs in benthic or occasionally planktonic habitats. The *Ostreopsis* species are important components of subtropical and tropical marine coral reef-lagoonal environments. However, currently, they are also distributed worldwide probably as a result of global warming and trade globalization, since some species are transported by ships as part of the ballast water.

Palytoxin was confirmed as the causative agent in human seafood poisoning through the consumption of crabs (Alcala et al. 1988), mackerel (Kodama et al. 1989), triggerfish (Fukui et al. 1987), sardines (Yasumoto et al. 1986, Onuma et al. 1999) and parrotfish (Taniyama et al. 2003). Palytoxin seafood poisoning is characterized by nausea, a sharp, metallic or bitter taste, vomiting, hypersalivation, abdominal cramps, severe diarrhea, paresthesia of the extremities,

severe muscle spasms, respiratory distress, dispnea, tachycardia, chills, cyanosis, vertigo, progressive muscular paralysis, convulsions and respiratory failure (Yasumoto et al. 1986, Alcala et al. 1988). In severe cases, patients died within 30 minutes to a few days (2-4 days) of intoxication, while in mild cases they survived by treatment with endotracheal intubation (Kodama et al. 1989).

Since 1998, along the North Italy coasts and subsequently in North East Spain and Greece, noxious blooms of *Ostreopsis,* which can cause breathing difficulty in humans, have already been recorded (Ciminiello et al. 2006). On the other hand, two *Ostreopsis* species (*O. cf. siamensis* Smith and *O. ovata* Fukuyo) have been identified in the Mediterranean Sea, and both are shown to produce palytoxin (Penna et al. 2005). These blooms have caused benthic fauna mortality (possibly due to anoxia), and problems for humans (skin irritations, respiratory illness and in some cases fever).

Palytoxin can be detected and quantitatively measured by the use of biological assays, although chemical analytical methods are necessary to confirm its presence. Biological methods have the advantage of defining characteristic symptoms in models of different complexity (mice, cells...). Moreover they provide information about the total toxin content based on the measurement of a single biological or biochemical response which involves the activity of all the congeners present in the sample. The knowledge of potential global toxicity is priority in the monitoring programs to ensure the human health.

Currently, mice bioassays are the only methods recognized internationally for determination of PSP (Paralytic shellfish poisoning), DSP (Diarrhetic shellfish poisoning) and NSP (Neurotoxic shellfish poisoning) toxins in sanitary controls. Moreover the careful observation of mice injected with crude extracts can help to characterize known toxins or indicate the presence of other –maybe new– ones. The mouse responds to the injected toxin by exhibiting several characteristic symptoms prior to death, and the dose-death time relationship observed in mice indicates that this toxin differs from the more commonly known marine toxins Table 1. The distinguishing initial symptoms recorded in mice after intraperitoneal (i.p.) injection of palytoxin are described here for the first time. These distinctive initial symptoms are really important because regardless of mice die or survive, they are going to show them.

At present, bibliography of mouse bioassay for palytoxins is not very clear (Table 2) in relation to definition of “mouse unit” (MU), detection limit, LD_{50} value (which ranges between 150 and

720 ng/Kg), and observation time of mice (from 4 to 48 hours). Additionally, some of the usual models in the toxicological evaluation of this and other marine toxins (Table 3) contain questionable aspects from the point of view of the dose-response (DR) theory.

Under these conditions, the present work examines (i) the conceptual problems linked to the use of the survival time for the determination of the dose for semi-maximum response; (ii) the reliability problems linked to the traditional moving average method and (iii) the results, appreciably more reliable, obtained applying the models that will be discussed in the subsequent sections.

Besides proving the accuracy, very superior, of the last approach, such results allow to recommend (in accordance with international general assent for lipophylic toxins) an observation time of 24 hours for the mouse bioassay, to define the MU for palytoxin as the amount of the toxin that kills a mouse 24 hours after i.p. injection, and to use the DR model proposed here as the base for a calibration curve through which equivalences can be established with the haemolysis method for palytoxin recently published (Riobó et al. 2007).

MATERIAL AND METHODS

Chemicals

Palytoxin standard isolated from the coelenterate *Palythoa tuberculosa* was provided by Wako Chemicals and was re-suspended in MeOH 50% at 25 ng/µL final concentration. An aliquot of methanolic palytoxin standard was dried under N_2 stream. Subsequently it was re-suspended in Tween 60 1% solution for their use in the mouse bioassay.

Mouse bioassay

The mouse bioassay for palytoxins is based on the neurotoxic effect caused by an organic extract obtained from a biological sample, which is dried and re-suspended in aqueous Tween 60 1% solution following the protocol described for lipophylic toxins (Yasumoto et al. 1978). In the current work healthy male Swiss mice NMRI, weight 20±1 g are used. The stock colony for routine assay is managed following the Council Directive (EC 2007) on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

Dilutions of palytoxin standard in Tween 60 1% solution, are prepared over the following range: 2.5, 5, 5.8, 6.6, 7.5, 10, 15, 20, 25 and 30 ng/mL equivalent to the following Dose: 125; 250;

290; 330; 375; 500; 750; 1000; 1250 and 1500 ng/kg. Initially, two groups of 5 and 7 mice were respectively injected with the two highest Dose and were carefully observed until death. Then 20 mice were injected with 250 ng/Kg and 10 mice for each one of the other doses of palytoxin were injected.

Toxicity determination is performed in relation to death time of the mice ip injected with 1 mL of Tween standard solution. After inoculation mice must be carefully observed paying attention to the symptoms in the initial 15 minutes and recording the times of the beginning of the stretching of hind limbs, lower back and the concave curvature of spinal column. The death time is determined as the time elapsed from completion of injection to the last gasping breath of the mouse. To establish it, mice must be observed continuously in one hour. Subsequently, observation is performed intermittently each 30 minutes. If mice survive for 12 hours, hold them for a total 24 hours and observe discontinuously each hour.

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).

RESULTS AND DISCUSSION

1. Symptoms associated to the mouse bioassay

The symptoms of the mice initially injected with the two highest doses of palytoxin started very fast in all of them (Table 4), after about two minutes, with characteristic stretching of hind limbs, lower backs and concave curvature of the spinal column. All these mice showed considerable damage from the beginning with their hair standing on end and possible blindness. The death times recorded with 1500 ng/Kg ranged between 42 min and 55 min and with 1250 ng/Kg ranged between 42 min and 84 min (Table 5). When the survival time of mice was still less than one hour, the mice showed convulsions, gasping for breath and finally death. When the mice survived more than one hour the death time varies considerably because the mice remained motionless with minimum energy consumption. This situation can go on for hours and the movements of the mice are just reflexes.

Bearing in mind these results, the rest of the mice were injected for one of each mentioned doses of palytoxin (ranging between 125-1000 ng/Kg) specified in Methods section. The difficulty and complexity of mice bioassay is revealed by the high variability of death times, which also overlap for different concentrations (Table 5). All the mice injected with the toxin, regardless of whether they died or stayed alive, showed, within 15 minutes (Table 4), the characteristic initial symptoms described above, *i.e.* stretching of hind limbs, lower backs and concave curvature of the spinal column.

This assay is definitely a very useful tool for palytoxin, since its high sensitivity reaches 250 ng/Kg, a value considered as the detection limit according to many authors. The distinguishing initial symptoms recorded in mice after intraperitoneal (i.p.) injection of palytoxin are not showed after the injection of other lipophylic toxins and they do not interfere with any of hydrophilic toxins (Table 1). Besides being exclusive for palytoxin, these symptoms are showed always after i.p. injection of palytoxin, regardless of mice will death or will survive symptoms and they reveal unmistakably the presence of palytoxin in a short period of time, between 2 and 15 minutes after i.p. injection (Table 4).

2. Time course of survival at different doses

The death (or survival) time is a magnitude frequently used for the toxicological evaluation of the palytoxin by means of the mouse bioassay. Accepting this approach (that later on we will criticize), we studied in the first place the variability of the death time in 8 groups of mice treated with increasing doses of palytoxin (250; 290; 330; 375; 500; 750; 1,000 and 1,250 ng/kg). The results, in terms of mortalities, were fitted to the ^{m}L and ^{m}W models. (equations [B7] and [B10]; Appendix B).

In all the cases the parametric estimations were statistically significant (Student's ***t***; α=0.05), and the models were consistent (Fisher's ***F***; α=0.05). With very slight differences, however, the best correlations between observed and expected results were obtained with the ^{m}W equation (figure 1) and, consequently, the corresponding $t_{0.5}$ values were those applied to the analysis that we discuss next. This way, we will represent the variability of the survival time through a value which offers the minimum sensitivity to the experimental error.

3. The relation between dose and survival time

As we have pointed out in the precedent section, this relation shows two serious inconveniences even though its frequency of use in the field of the marine toxins:

(i) Dose for semi maximum effect (mortality or another quantifiable characteristic of the population; in any case m in ^{m}W or ^{m}L) is the essential parameter of the DR analysis. The time passed until the manifestation of the measured effect is another datum of interest, but scarcely relevant in connection with the measure of the effect in the strict sense. In other words: if the dose D_n kills the 50% of one target population in one hour, we will say that D_n is the lethal dose 50% in one hour; if it kills the 50% in 10 hours we will specify this period, but we will follow labelling such dose as lethal 50%. On the contrary, the death time linked to a given effector contains very little information if the dose is not enunciated.

(ii) On the other hand, the survival (or death) time cannot be permissibly used to calculate the dose for semi-maximum effect, because such a time is not delimited: at null dose –even at sufficiently low doses– the survival time is undetermined.

An additional form of this second inconvenience arises in our case when one examines the dose-survival time relationship using the $t_{0.5}$ values obtained through the ^{m}W model. Indeed, as it is shown in figure 1, the asymptote of the response to the two lower doses is lower than 1 (what indicates that a fraction of the population is immune to these doses). This way, the corresponding $t_{0.5}$ values have a different meaning under such conditions and they cannot be used jointly with the remaining ones. Figure 2 shows the dose-$t_{0.5}$ relationship limited to the interval in which the whole population dies.

To attribute a functional form to the experimental results of the figure 2, several transformations could be assayed without another justification that the achievement of the best fitting. The clearest option is probably to work with natural values, that can be described by means of a negative exponential model:

$$t_{0.5} = a \cdot \exp(-rD) \quad ; \text{ where:} \qquad [1]$$

D dose (ng/kg)

a numeric fitting parameter (dimensions: time)

r numeric fitting parameter (dimensions D^{-1})

the dose for semi-maximum response being: $m = \frac{\ln 2}{r}$

Now then, this equation can describe the situation with a reasonable numerical accuracy (the correlation coefficient between expected and observed results was 0.990), but it is seriously problematic from the toxicological point of view. Indeed, the values resulting from [1] for the semi-maximum response and the corresponding dose are 20.3 hours and 229.7 ng/kg, respectively. However, these values imply to admit that the intercept of the function is 40.6 hours, what represents an inadmissible extrapolation: the biological meaning of a (or $t_{0.5}$ at null dose) is half of the average life of the test animal, without a doubt bigger than 40.6 hours. Obviously, any other model applied to the relationship between dose and survival or death time will be also equally ambiguous, or even more, if the natural values are subjected to logarithmic or reciprocal transformations.

A way to avoid such an ambiguity would be, as is suggested in Appendix A, using the equation [1] in the role of the link expression [7A] into the frame of an expanded or generalized DR model. Supposing that the response is described as a simultaneous function of the time and the dose by means of the product of two equations ^{m}W –as in [6A]–, such an expanded model would have the general form:

$$R = K \times {}^{m}W\left(t_D, a_1 ; t\right) \times {}^{m}W\left(m, a_2 ; D\right) \qquad [2]$$

Where t_D has the meaning of semi-maximum response time, depending on the dose, and does not generate problems at null dose because in this case the response (measured in terms of any characteristic of the target population) is null. That is: the dose-response time relationship is only useful when is included into a bivaried model, where the time, as the dose, has the character of an independent variable. This situation is represented in the simulation of figure 3A.

Although the model [2] is without doubt the more complete to apply to the temporal progression of the response to an increasing series of doses, here we will disregard him, because its use demands a very high number of values, not suitable for an assay such as the mouse bioassay.

4. The moving averages method and its problems.

Applying this method –as described in Appendix A, section 2– to an assay performed with 4 doses of palytoxin (125; 250; 500; 1,000 ng/kg; q=2) and n=10, we obtained the vector of death (0, 2, 10, 10), to which the Thompson and Weil tables assigned the values f=0.3 and σ_f=0.133. Therefore the equations [8A] and [9A] lead to (note that the LD_{50} is not the center of the 95% confidence interval):

LD_{50} = 307.79 (255.84 to 370.27) ng/kg

Figure 4 shows what is implied by this result, together with what would be implied if the central values were permuted (and which would lead to the same conclusion). If the first case does not appear over conclusive, the second only reasonably induces a re-start. Certainly, in order to that the smoothing of a sigmoid profile should not appear abusive we need more than 4 points, as shown in the same figure 4C (in other words, the equation for a sigmoidal curve requires at least 3 parameters, such that 4 points supposes only one degree of freedom).

5. The direct formulation of dose-response relationships.

In the context of the toxicological analysis of marine toxins expressions which parameters are only adjust coefficients, without biological meaning, are often proposed, avoiding the convenient adaptation of the functional form to the determinant factors of the DR phenomenon. As well, the best adjust is looked for throughout transformations of the variables (inverse, logarithmic) that alter the variance relations and introduce biases in the parametric estimates. Therefore, the toxicology of these effectors is abundant in models that, without a doubt, translate correctly the observations that suggest them, but they lack of theoretical justification and mechanistic content (Table 3).

We do not deny that these models are adjusted to the experimental data. We solely state that they are only applicable in particular cases (some authors warn this explicitly), that their forms do not allow to compare parameters really relevant in the DR phenomenon, and that the confidence intervals of their estimates are often –when their rigorous calculation is possible–unacceptably wide (Table 3). It is true that these approaches can reduce (but not too much) the sacrifice of animals. However, it would be convenient that this desirable reduction can be gotten through alternatives that does not violate the basic suppositions of the dose-response theory. A typical case in this respect is the mouse bioassay, applied to several marine neurotoxins (Table 3), strongly attacked by ethical and economic considerations, and however it constitutes an unavoidable referent, even in those cases when alternatives bioassays for the same toxophore and action mode are possible. Maybe, the lack of optimum models to establish rigorous equivalences make difficult its substitution.

When, as occurs today, a linear fitting is performed in seconds with a personal computer, the use of an algebraic model is justifiably the best option for describing a DR relationship. To this

respect, as it was already said, the equations ^{m}L [4A] and ^{m}W [5A] are specially appropriate, for the reasons adduced in the Appendix A, as well as for their ability to translate distributions of populational sensitivities to an effector more realistic that the normal one.

The verification of these models was performed by means of an assay with 8 dose which included the geometric progression of the precedent one, and 4 additional doses distributed within the same domain, quantifying the response as mortality at 24 hours. Both functional forms led to satisfactory fittings (Table 6 and figure 5), and the tests of Student and Fisher (both for α=0.05) allowed to conclude the statistical significance of all the parametric estimates, as well as the consistency of the models. The values obtained for *m* (^{m}L: 293.5 ± 7.299; ^{m}W: 294.6 ± 5.384 ng/kg) showed a good agreement with the one derived of the moving averages method [LD_{50}=307.79 (255.84 to 370.27 ng/kg)], with the advantage of substantially smaller confidence intervals.

CONCLUSIONS

With the specific aim of establishing in a rigorous way the toxicity of the palytoxin, we have described in detail the characteristic symptoms of its effects on the mouse, and evaluated different resources for quantifying the biological response to an effector from the point of view of the adaptation to the basic features of the DR phenomenology. Despite of its common use in the field of the marine toxins, it is concluded that empiric models based on the dose-survival time or dose-death time relationships generate serious ambiguities and make difficult to obtain reasonably general descriptions.

The traditional moving average method contains, in the usual application of the Thompson and Weil tables, inaccuracy elements that involve confidence intervals too wide and make doubt about the tolerance to the permutation of the central values of the death vector.

Logistic and Weibull's models (modificated to adequate them to the DR context) can be applied in a consistent way to the toxicological dynamics of the palytoxin. Such descriptions provide parameters with very satisfactory confidence intervals, with unequivocal biological meanings and suitable for performing standardizations, transferences and toxicologically relevant comparisons among different systems and evaluation methods.

The LD_{50} value for palytoxin in the mouse bioassay by i.p. injection using a 24 h reference time is herein established in 294.6 ± 5.384 ng/Kg according to Weibull model

The utility of this assay is highlighted in the routinely mouse bioassay for lipophylic toxins because (regardless of the present of another toxins) in the initial 15 minutes could be identified the presence of palytoxin in the sample paying attention to the initial symptoms described in the current work. Furthermore, the death time could be used as semi-quantitative estimation of palytoxin and/or analogs presence.

ACKNOWLEDGEMENTS

Funded through project AGL2005-07924-CO4-02 and CCVIEO supported this work. Dr. José Antonio Vázquez Álvarez was under postdoctoral contract (CSIC-I3P-PC 2003, financed by the European Social Fund).

Appendix A

1. Theoretical considerations about the dose-response (DR) analysis

As it was pointed out at the end of the precedent section, our approach requires to consider here two important aspects of the DR analysis, as well as the current application of such analysis in the marine toxins field.

1.1. The two basic dimensions of the response to an effector.

In the response of a population to an effector, it is key the fact that the populational sensitivity is a random variable subjected to any probability distribution. Thus, if the populational sensitivity varies according to a unimodal distribution function, the response at increasing doses of the effector (*i.e.*, the corresponding cumulative function) is necessarily sigmoidal. For the same reason, the response is sigmoidal throughout the time, because a greater sensitivity to the effector is not only translated as responses at lower doses, but also at shorter times. However, the elements that respond at lower doses are not necessarily the same that respond at shorter times (the time that one element "resists" is a different concept of the dose that one element "resists"). Consequently, a description of the response including both aspects will be a bivariate function of the type represented in the figure 3B. A way to establish that function would be the following:

i. To describe the response R as a function of the dose D by means of an expression of the type:

$$R = f\left(K, p_i; D\right) \qquad [1A]$$

K being the maximum (asymptotic) response and p_i an additional group of parameters that now is not necessary to define.

ii. To describe the response R as a function of the time t by means of an expression of the type:

$$R = g\left(K, q_i; t\right) \qquad [2A]$$

K being the maximum (asymptotic) response and q_i an additional group of parameters that now is not necessary to define.

iii. Since the real maximum response is the same at doses high enough and times large enough (*i.e.*, R=K when $D\rightarrow\infty$ and $t\rightarrow\infty$), the function which describes the surface in the figure 3B will have, in the simplest case, the general form:

$$R = K \times f\left(p_i; D\right) \times g\left(q_i; t\right) \qquad [3A]$$

Regardless of its specific meaning, the p_i parameters describe the response on the domain of the dose, whereas the q_i parameters describe the response on the domain of the time. Although in both cases the profile is sigmoidal, and the asymptote (K) is the same, a time-response experiment would not allow conclusions about the parameters (p_i) that define the effect of the dose on the response (dose for semi-maximum response and safety margin, or slope, are toxicologically the most relevant). In the same way, an experiment dose-response would be useless to evaluate the parameters (q_i) that define the time-course of the response (time for semi-maximum response and maximum rate are the most relevant in this kinetics perspective).

1.2. The appropriate functions to model DR relationships.

Another essential aspect of the DR analysis concerns to the specific functional forms of the generic expressions [1A] and [2A]. In previous works (Murado et al. 2002, Murado & Vázquez 2007), this extreme has been discussed with detail and it has been concluded that the logistic and the accumulative function of the Weibull's distribution are the most suitable equations (both modified to make them consistent with the essential facts of the DR analysis: see appendix).

Modified logistic equation (from now on ^{m}L) is:

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

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

R response, with K as maximum value.

m dose for semi-maximum response.

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

Modified Weibull's function (from now on ^{m}W) is:

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m}\right)^{a}\right]\right\} \quad ; \text{ where:} \qquad [5A]$$

R response, with K as maximum value.

m dose for semi-maximum response.

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

With slight differences, both equations translate satisfactorily the basic facts of the DR phenomenology, and their parameters have precise biological meanings (although a in ^{m}W is more ambiguous than μ in ^{m}L). Both allow the direct calculation of the confidence intervals of the parametric estimates. Finally, both are suitable to describe the response also as a function of the time: it is sufficient to change D for t in [4A] and [5A], making the respective conceptual transferences in the parameters. Thus, in both equations m changes into $t_{0.5}$, or time for semimaximum response; in ^{m}L μ means the maximum increment of R per unit of R and unit of D, and in ^{m}W the a parameter changes to get involved in the temporal slope of the response.

In this way, a specific form of the equation [3A] could be the product of two ^{m}W equations, one of them with the time and the other with the dose as independent variable:

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{t}{t_{0.5}}\right)^{a_1}\right]\right\}\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m}\right)^{a_2}\right]\right\} \qquad [6A]$$

But either of the four products $^{m}W_t \times {}^{m}W_D$; $^{m}L_t \times {}^{m}L_D$; $^{m}W_t \times {}^{m}L_D$ y $^{m}L_t \times {}^{m}W_D$ is in principle a feasible model.

However, it must be pointed out that this approximation assumes the statistical independence of the equations that describe the response as a simultaneous function of the dose and the time. This involves that the m value is the same regardless of the considered time, an assumption which is

as extreme as the coincidence of both responses. In front of this alternative, it is much more realistic to accept that only some of the elements that respond at lower doses responds too at shorter times. It implies to accept that the $t_{0.5}$ parameter is not independent of the dose; that is, in [6] it must be changed $t_{0.5}$ to a function of the type:

$$t_D = j(D) \qquad [7A]$$

where t_D is now a variable. Undoubtedly, a function that relates the response time with the dose would be very useful. Regrettably, however, to establish its form does not exist general criteria so clear like those that lead to [4A] and [5A]. In this way, if the response time is considered as the response to an increasing series of doses, it must be resorted to models the unavoidable empiricism of which can only be accepted if they are included in other (*e.g.* equation [6A]) with a bigger theoretical base.

2. The moving averages method and its problems

A procedure that has been widely applied in the last decades is based on tables created over 50 years ago by Thompson and Weil (Thompson 1947, Thompson & Weil 1952, Weil 1952). The tables of Thompson and Weil are set up assuming 4 doses in geometric progression with factor q, and organised into sections according to the number of animals treated per dose (n, that can be 2, 3, 4, 5, 6, or 10, but always the same for all doses). When starting, the vector (r_1, r_2, r_3, r_4) of dead animals at each dose must be specified (the order between r_2 and r_3 can be interchanged), providing as the output two magnitudes (f and σ_f) that allow us to calculate LD_{50} and its confidence interval CI (with α=0.05) using the expressions:

$$\log LD_{50} = \log D_a + d \cdot (f + 1) \qquad [8A]$$

$$\log CI = 2 \cdot d \cdot \sigma_f \quad ; \quad \text{where:} \qquad [9A]$$

D_a lowest of the dosage levels used (ng/kg).

d logarithm of the constant ratio q between dosage levels (dimensionless).

f, σ_f numeric values from the table for the vector of dead animals (r_1, r_2, r_3, r_4).

In this way, the limits of the confidence interval (α=0.05) for the LD_{50} are:

upper limit = $10^{\log LD_{50} + \log CI}$; lower limit = $10^{\log LD_{50} - \log CI}$

The advantages that the authors attribute to this method are its simplicity and the absence of a link to a specific DR model, which avoids the «fitting of complex mathematical curves» (Weil 1952). Certainly, avoiding the implied calculation in the non-linear fittings was an important factor half century ago. This advantage, however, is practically irrelevant with the informatics resources available today, and advises that we should examine the possible cost in precision, in particular if we are dealing with highly active toxins.

In reality the method postulates a concrete DR model. The work performed by the tables is equivalent to smoothing a profile which is supposed sigmoidal by the moving averages method and to calculate the LD_{50} (m) after linearization of the smoothed values through the probitic transformation (the use of the dosage in a geometrical progression is simply a resource that facilitates the linearization). This way, the use of the probitic transformation postulates a normal distribution for the populational sensitivity to the effector, and the corresponding normal accumulative function for the DR profile. Although the distributions with domain $(-\infty;\infty)$ create some inconvenient in the DR context (Murado et al. 2002), this approach is clearly preferable to the empiric relationships as those mentioned in Table 3, its problems being of a more practical character.

Firstly, the vectors of death are of 4^{th} order, what –in tables performed with window=3 for moving averages– supposes to work with the minimum admissible number of doses (3+1), too low for a sigmoidal function. Secondly, it seems excessive to tolerate the permutation of the central values of the vector of death. Naturally, when the series (r_1, r_2, r_3, r_4) and (r_1, r_3, r_2, r_4) are smoothed by moving averages with window=3 the numerical result is the same; but often one of the series suggests the repetition of the assay. Finally, while it is true that it is always convenient to use doses with increasing spacing, the geometric progression is a very rigid and in general excessive criterion.

Appendix B. Dose-response and survival models used.

1. Modified logistic equation (^mL)

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:

$$R = \frac{K}{1+\exp\left(c-\mu D\right)} \;;\; \text{with} \;\; c = \ln\left(\frac{K}{R_0}-1\right), \;\text{where:} \qquad \text{[B1]}$$

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 [B1] 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:

$$R = \frac{K}{1+\exp\left[\mu\left(m-D\right)\right]} \qquad \text{[B2]}$$

Now, since the intercept (R for D=0) of [B2] is:

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

the logistic equation without intercept is:

$$R = K\left(\frac{1}{1+\exp\left[\mu\left(m-D\right)\right]}-\frac{1}{1+\exp\left(\mu m\right)}\right) \qquad [B3]$$

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 [B3]:

$$K_r = \lim_{D\to\infty} R = K\left[\frac{\exp\left(\mu m\right)}{1+\exp\left(\mu m\right)}\right] \qquad [B4]$$

$$m_r = \frac{1}{\mu}\ln\left[2+\exp\left(\mu m\right)\right] \qquad [B5]$$

So that the model includes such real values, K and m could be isolated from [B4] and [B5], and the resulting expressions to be introduced in [B3]. A simpler resource, however, is to reorder [B5] in the form:

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

and to substitute, in [B3] and [B4], the term $\exp(\mu m)$ for its equivalent one, what leads to the form:

$$R = \frac{K_r\left[\exp\left(\mu m_r\right)-1\right]}{\exp\left(\mu m_r\right)-2}\left\{\frac{1}{1+\exp\left(-\mu D\right)\left[\exp\left(\mu m_r\right)-2\right]}-\frac{1}{\exp\left(\mu m_r\right)-1}\right\} \qquad [B6]$$

For simplifying the notation, it can be made:

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

$$R = \frac{KA}{B}\left[\frac{1}{1+B\exp\left(-\mu D\right)}-\frac{1}{A}\right] \qquad [B7]$$

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):

$$R = 1 - \exp\left[-\left(\frac{D}{\beta} \right)^{a} \right] \qquad \text{[B8]}$$

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:

$$R = K\left\{ 1 - \exp\left[-\left(\frac{D}{\beta} \right)^{a} \right] \right\} \qquad \text{[B9]}$$

2. Reparametrization of the equation, to make explicit the dose (*m*) for semi-maximum response. This way, if we make *R*=*K*/2 in [B9], we have:

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

what leads to the definitive form:

$$R = K\left\{ 1 - \exp\left[-\ln 2 \left(\frac{D}{m} \right)^{a} \right] \right\} \;;\; \text{where:} \qquad \text{[B10]}$$

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.

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

Aune T, Larsen S, Aasen JAB, Rehmann N, Satake M, Hess P (2007) Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. Toxicon 49:1–7

Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Magno GS, Tartaglione L, Grillo C, Melchiorre N (2006) The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean Ostreopsis ovata by a new liquid chromatography tandem mass spectrometry method. Analytical Chemistry 78:6153-6159

EC (2007) Commission recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes. (2007/526/EC). Official Journal of the European Union. L 197, 50:1-89

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

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

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

Kodama AM, Hokama Y, Yasumoto T, Fukui M, Manea SJ, Sutherland N (1989) Clinical and laboratory findings implicating palytoxin as cause of ciguatera poisoning due to Decapterus macrosoma (mackerel). Toxicon 27:1051-1053

Lehane L, Lewis RJ (2000) Ciguatera: recent advances but the risk remains. International Journal of Food Microbiology 61:91–125

Lenoir S, Ten-Hage L, Turquet J, Quod JP, Bernard C, Hennion MC (2004) First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in southwestern Indian Ocean. Journal of Phycology 40:1042-1051

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

Penna A, Vila M, Fraga S, Giacobbe MG, Andreoni F, Riobó P, Vernesi C (2005) Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the Western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8s rDNA sequences. Journal of Phycology 41:212-245

Riobó P, Paz B, Fernández ML, Fraga S, Franco JM (2004) Lipophylic toxins of different strains of Ostreopsidaceae and Gonyaulaceae. In: Steidinger KA, Landsberg JH, Thomas CR, Vargo GA (eds) Harmful Algae 2002. Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commission and Intergovernamental Oceanographic Commission of UNESCO., Florida, p 119-121

Riobó P, Paz B, Franco JM, Vázquez J, Murado MA (2007) Proposal for a simple and sensitive haemolytic assay for palytoxin. Toxicological dynamics, kinetics, ouabain inhibition and thermal stability. Harmful Algae doi:10.1016/j.hal.2007.09.001

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

Teh YF, Gardiner JE (1974) Partial purification of *Lophozozymus pictor* toxin. Toxicon 12:603-610

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

Usami M, Satake M, Ishida S, Inoue A, Kan Y, Yasumoto T (1995) Palytoxin analogs from the dinoflagellate Ostreopsis siamensis. Journal of the American Chemical Society 117:5389-5390

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

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

Yasumoto T, Oshima Y, Yamaguchi M (1978) Ocurrence of a new type of shellfish poisoning in the Tohoku district. Bulletin of the Japanese Society of Scientific Fisheries 44:1249-1255

Yasumoto T, Yasumura D, Ohizumi Y, Takahashi M, Alcala AC, Alcala LC (1986) Palytoxin in two species of xanthid crab from the Philippines. Agric. Biol. Chem. 50:163-167

FIGURES

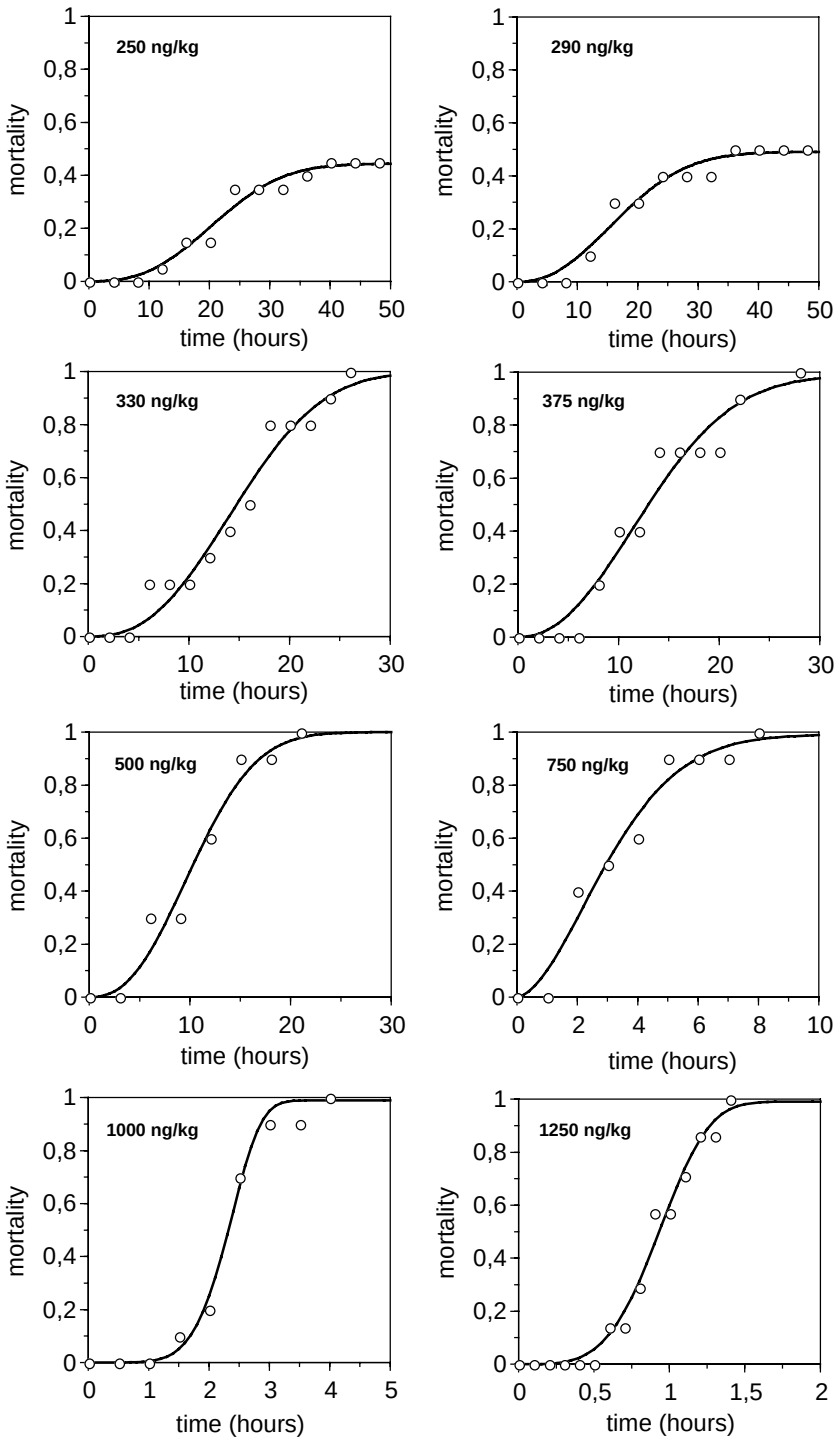

Figure 1: Mortality curves of mice treated with the specified doses of palytoxin. Normalized experimental values (points) fitted to the model ^{m}W (lines). Note the different time scales.

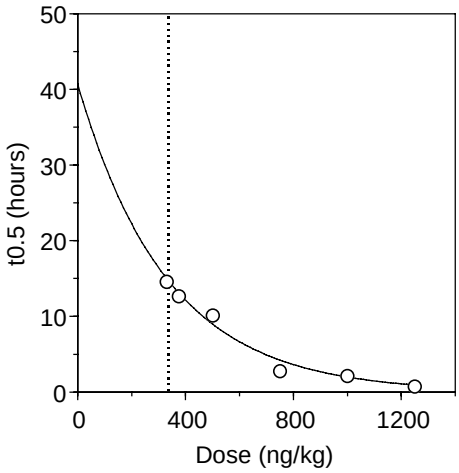

Figure 2: Effect of palytoxin dose (D) on survival half time ($t_{0.5}$) calculated from the model ^{m}W. Experimental data (points) and fitting (continuous line) to the exponential negative model [1].

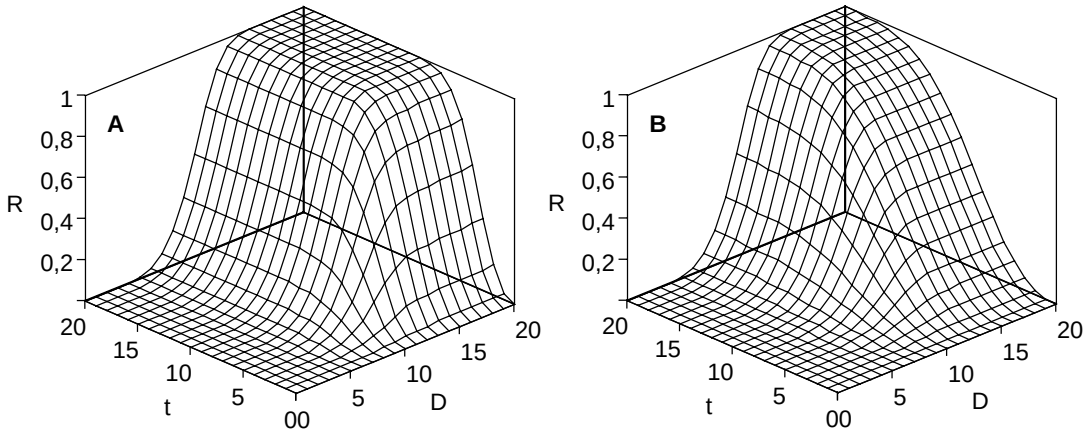

Figure 3: Simulation of two response surfaces (R) to an effector as a simultaneous function of the time (t) and the dose (D), under the hypothesis of dependence (A: model [2]) and independence (B: model [6A]) between both variables.

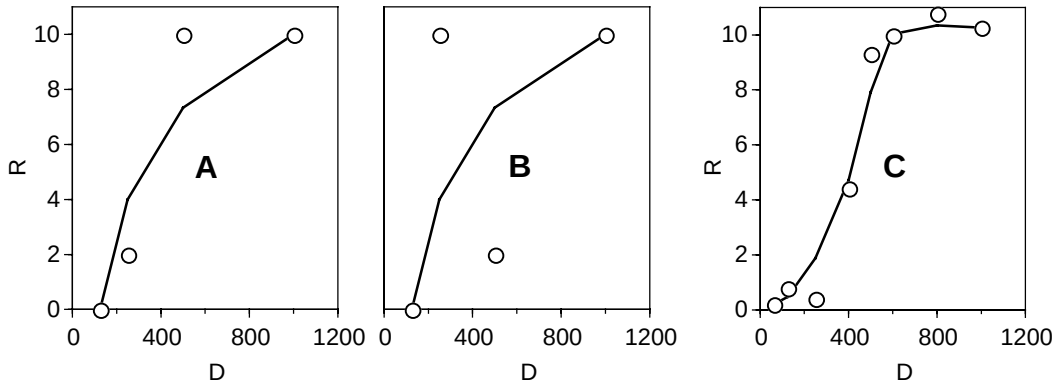

Figure 4: DR relationships, smoothed by means of the moving averages method described in Appendix A, section 2 (A), together with those corresponding to the permutation of the central values for the death vector (B) which will lead to the same LD_{50}. In (C) we can see the effect of increasing the number of points used for smoothing (moving averages, window=3) of a sigmoid profile with an arbitrary error of normal distribution (μ=0; σ=1).

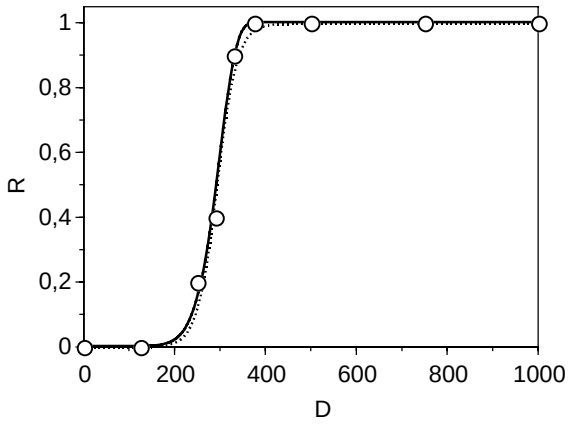

Figure 5: Effect of palytoxin dose (D in ng/kg) on the normalized mortality of mice after 24 hours (response: R). Experimental values (points) fitted to the ^{m}W (solid line) and ^{m}L (dotted line) models. See also Table 3.

TABLES

Toxins	Symptoms after ip. injection
PSP	Jumping in the early stages, ataxia, ophthalmia, paralysis, gasping and death (usually in <15 min) by respiratory arrest
Domoic acid	Spasms, scratching ears
AO, DTX1,DTX2	Deep depression, weak of limbs, convulsion (40´-24h)
DTX3	Deep depression, weak of limbs, convulsion (1h-48h)
Pectenotoxins	Similar to PSP, survival time over 20´(30´-24h)
Yessotoxins	Similar to PSP, survival time over 20´(40´-5h)
Brevetoxins B1, B2	Similar to PSP, survival time over 20´(40´-48h)
Azaspiracid	Similar to PSP, survival time over 20´(40´-36h) (with low doses, creeping paralysis)
Gymnodimine	Similar to PSP
Espirolids	Similar to PSP
Ciguatoxins	Diarrhea, dysnea, paralysis, convulsion
Palytoxins	Creeping paralysis, cyanosis, deep depression

Table 1: Symptoms of detectable toxins by mouse bioassay (Yasumoto et al. 1978). PSP (Paralytic Shellfish Poisoning), AO (Okadaic Acid), DTX (Dinophysitoxins)

Table 2: Data regarding response to palytoxin (mouse assay, intraperitoneal injection) according to different authors.

observation time (h)	LD_{50} (ng/kg)	reference
4		Tan and Lau 2000
24	450	Onuma *et al.* 1999
48		Ballantine *et al.* 1988
48	150	Taniyama *et al.* 2002, Taniyama *et al.* 2003
48	720	Rhodes *et al.*2002
24	295	this work

Table 3: Some usual models for toxicological evaluation of marine toxins, with mouse as assay animal.

toxin	model		reference
ciguatoxin	$\log D = 2.3 \log (1+1/t)$	[a]	Lehane and Lewis (2000)
saxitoxin	$\log D = 2.3 \log (1+1/t)$	[b]	Fernández et al. (2003)
saxitoxin	$1/t = a+b \log D$	[c]	Holtrop et al. (2006)
palytoxin	$D = 225.19 t^{-0.99}$	[d]	Teh and Gardiner (1974)
OA and DTX2	contingency table	[e]	Aune et al. (2007)

[a]: t=death time in hours. [b]: as [a], but restricted to the interval in which the relationship between the transformed variables is lineal. [c]: t=death time in hours. [d]: t=death time in minutes; D=dose in Mouse Units (1 MU defined as dose that kills a mouse of 20 g in 4 hours). [e]: based on the binomial distribution and resulting in a second degree polynomial.

Table 4: List of the initial time of symptoms (t_0) recorded in all the injected mice

Dose (ng/Kg)	**t_0 (min:seg)**	**Average**	**%SD**
1500	1:44, 1:13, 2:14, 3:00, 2:15	2:05	32
1250	2:40, 2:30, 1:57, 1:50, 2:36, 1:30, 1:30	2:05	24
1000	2:40, 3:00, 3:00, 2:00, 2:00, 2:00, 2:00, 3:00, 1:00, 1:00	2:01	35
750	2:59, 2:10, 1:38, 2:24, 3:08, 2:41, 3:06, 2:26, 1:46, 3:10	2:33	22
500	5:00, 2:00, 3:00, 2:30, 2:00, 3:00, 2:00, 3:00, 2:00, 3:00	2:45	33
375	3:30, 3:40, 4:12, 2:24, 3:36, 1:19, 2:38, 2:57, 2:29, 2:55	2:58	28
330	3:49, 8:48, 7:58, 7:07, 5:22, 3:05, 6:42, 3:32, 3:13, 2:00	5:37	43
290	6:48, 3:48, 4:21, 4:00, 2:40, 4:45, 3:29, 4:08, 4:06, 3:13	4:08	27
250	3:00, 10:00, 3:25, 5:3, 6:00, 3:00, 2:30, 7:00, 6:00, 3:00 4:10, 4:14, 3:14, 3:00, 4:56, 2:56, 2:47, 2:36, 4:26, 2:23	4:08	45
125	5:40, 4:50, 5:00, 5:20, 2:29, 4:00, 2:37, 4:00, 3:34, 4:01	4:09	26

Table 5: List of the death times recorded in all the injected mice

Dose (ng/Kg)	**number of mice**	**dead (48 h)**	**Death time (min)**
1500	5	5	46, 43, 42, 55, 52
1250	7	7	84, 54, 36,48,54,61,72
1000	10	10	135, 135, 135, 165, 115, 135, 135, 90,180,220
750	10	10	285, 255, 255, 140, 90, 100, 120, 90, 195, 430
500	10	10	360, 300, 360, 660, 660, 690, 800, 800, 900, 1250
375	10	10	450, 570, 1230, 840, 840, 510, 450, 840,1320, 1440
330	10	10	250, 250, 610, 700, 960, 970, 1080, 1080,1330,1450
290	10	5	840, 840, 1410, 720, 2160
250	20	9	1320, 1320, 1320, 1320, 840, 840, 720, 1930, 2400
125	10	0	

Table 6: Parametric estimations (α=0.05) and correlations between expected and observed results referred to the palytoxin activity (mouse bioassay, mortalities at 24 hours), calculated by fitting of experimental results to ^{m}L and ^{m}W models. LD_{50} (m) in ng / kg. See also figure 5.

eq. ^{m}L [B7]		eq. ^{m}W [B10]	
K =	1.008 ± 0.040	K =	1.003 ± 0.029
μ =	0.045 ± 0.014	a =	9.340 ± 1.943
m =	293.5 ± 7.299	m =	294.6 ± 5.384
r =	0.996	r =	0.998