Dose–response modelling with two agents: Application to the bioassay of oil and shoreline cleaning agents

Miguel A. Murado [a,*], José A. Vázquez [a], Diego Rial [b], Ricardo Beiras [b]

[a] Grupo de Reciclado e Valorización de Materiais Residuais (REVAL), Instituto de Investigacións Mariñas (CSIC)., r/Eduardo Cabello, 6. Vigo-36208, Galicia, Spain
[b] Laboratorio de Ecoloxía Mariña (LEM), Faculdade de Ciencias do Mar (Universidade de Vigo)., Campus Universitario Lagoas-Marcosende. Vigo-36200, Galicia, Spain

ABSTRACT

Single and joint effects of hydrocarbons and a shoreline cleaning agent (SCA) were studied by measuring the inhibition of the larval growth of sea urchin. Different dosage methods of hydrophobic compounds were compared. The results obtained in the evaluation of CytoSol toxicity revealed that the method of variable dilution of water accommodated fraction (WAF) led to the more conservative toxicological approach. Regarding to Libyan oil, the use of DMSO as carrier allowed us the evaluation of its potential toxicity in comparison with the limitations imposed to the use of WAF method. A reparametrised form of the Weibull equation was slightly modified to be useful for dose–response analysis. This was the basis for modelling single sigmoid responses, which were used to simulate biphasic profiles with addition of effects and to describe both the concentration addition (CA) and independent action (IA) hypotheses. In all cases, its descriptive ability was graphically and statistically satisfactory. The IA model was the best option to explain the combined experimental responses obtained.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Seven years after the Prestige oil spill, tons of fuel has remained on supra-tidal rocks of the Galician coast (NW Spain). Nowadays, the only option to collect it is by means of shoreline cleaning agents (SCA) combined with high-pressure water washing. Recently, the SCA CytoSol has been studied and selected for this purpose due to its efficacy in dealing with weathered fuel oil [1]. This chemical does not contain surfactants and is mainly composed of fatty acid methyl esters with a small proportion of bioremediation enhancers [2].

The runoff produced after a cleaning treatment with a SCA will contain the chemical and the removed oil. Therefore, it should be noted that the combined toxic effects of chemicals must be taken into account when estimating ecological risk. In this sense, the toxicity of a compound on marine invertebrates is usually assessed in early-life stages using embryogenesis, early larval growth, survival, and morphological abnormalities as the endpoints [3]. Early-life stages are more sensitive than adult stages and are a critical period in the life cycle of an organism. Sea urchins [4] are among the organisms most frequently used in embryo-larval bioassays.

Toxicological evaluation of mixtures of two or more substances in aqueous media requires a proper dosage. Since hydrophobic substances present two basic problems, maintaining a constant and bioavailable concentration [5], it is necessary to choose a method appropriate to the objectives of the study and the nature of the substances. Different methods for media preparation have been applied for these substances [5]: direct addition and mixing, use of solvents and systems able to produce dispersion and emulsion. While the use of solvents and their concentrations in the media test is a much-discussed methodology [6]; preparation of water-accommodated fraction (WAF), defined as medium containing only the fraction of petroleum that remains in the aqueous phase once any source of mixing energy has been removed and after a period sufficient for phase separation, is recommended for multi-component substances not fully soluble in water, such as oil or CytoSol. Thus, the suitability of two different methods has been suggested for preparing a WAF: (1) variable loading, in which each WAF is prepared individually [5–7] and (2) variable dilution obtained by serial dilution of a single stock of WAF [8].

An important consideration to keep in mind to assess environmental risks associated with CytoSol application is to formalise the mathematical resources from dose–response (DR) analyses when a SCA and oil are combined. Several concepts concerning joint toxic effects have been applied to describe responses to chemical mixtures. The reference models are concentration addition (CA) [9,10] and independent action (IA) [11,12]. Concentration addition

---

is based on the expectation that the compounds of a mixture act on similar physiological systems in the tested organism [11,13,14]. Conversely, IA is based on the idea that chemicals present different modes of action inside the organism but generate a common global effect [15–18]. There is no consensus on whether the CA or IA model is superior, and the validity of the models seems to be case-specific.

In this sense, CA and IA models have been modified to allow the description of antagonistic or synergistic responses [19,20]. For the CA model, the shape of isoboles corresponding to 50% of the maximal effect is often used to characterise these combined effects [19,21]. The half-maximal effective concentration (EC_{50}) is a robust parameter that can be estimated with greater reliability than other levels of effect. This is the main reason why it is used in these approaches. If the analysis of the whole response surface for two agents is considered, the number of cases and the power of the statistical tests would be increased [22]. Furthermore, the ray design provides satisfactory results and is the most common experimental design for the study of binary mixtures [22,23]. A 3D plot of the dose-effect surface predicted by a model with the two agents as the X- and Y-axes and response as the Z-axis is useful to infer the shape of the isoboles [23].

However, the conventional CA and IA models descriptions do not take into account the biphasic response surfaces that can be obtained when binary mixtures are evaluated [19,20,24–27] as well as when hormetic phenomena are present [28,29]. Southam and Ehrlich [30] defined hormesis as "*a stimulatory effect of subinhibitory concentrations of any toxic substance on any organism*". This phenomenon, almost forgotten for a half century, has generated an abundant number of reports in the last few years [31–33]. These reports blame classic toxicological analysis for blocking the importance and the generality of hormesis [34–38]. They suggest that this generality could lead to a revision of the environmental protection policies, which may be unnecessarily expensive [33,39,40].

In this work, we propose a toxicological assessment of single agents, CytoSol and light Libyan crude oil, using different dosage methods. We applied the proposed mathematical resources to real cases related with the joint effects of CytoSol and light Libyan crude oil as well as CytoSol and fluoranthene on the larval growth of the sea urchin. These mathematical proposals were based on reparametrised Weibull equation for non-linear modelling and CA and IA hypothesis for effect of agents' mixture.

2. Materials and methods

2.1. Agents and dosages

Agents assayed were light Libyan crude petroleum, obtained from Repsol YPF SA; the shoreline cleaning agent CytoSol (CytoCulture International), a mixture of methyl esters of fatty acids from vegetable origin, and the polyaromatic hydrocarbon fluoranthene (Sigma). We utilised two basic procedures:

B1) Dilutions of a saturated aqueous extract (or water-accommodated fraction, WAF) that were obtained by (1) orbital shaking (150 rpm/48 h/20 °C), in a 2 l screw-capped separatory funnel, of a mixture (0.5:9.5, v/v) of agent and filtered seawater, (2) separation of phases after a rest period of 2 h, and drainage of aqueous one through a slight plug of glass wool saturated in the same extract.
B2) Acetone or dimethyl sulfoxide (DMSO) as solvents.

For comparative purposes or specific necessities of some tests, these basic methods were applied in the concrete forms which are detailed below.

2.1.1. CytoSol

P1. Dilutions of a WAF stock prepared by method B1. The experimental concentrations that were tested (0, 10, 20, 50, 100, 200 and 500 ml l^{-1}) were obtained by dilution of the stock in 0.22 μm filtered sea water (FSW).
P2. Dilutions of an emulsion were obtained by direct injection of 100 μl of CytoSol in 500 ml of seawater with a micro-syringe (200 ppm, v/v, $\rho_{CytoSol} = 0.887$ g cm^{-3}). We treated the mixture three times, 20 s each, with an Ultraturrax homogeniser. The experimental concentrations that were tested (in the interval 0.5–200 μl l^{-1}) were obtained by dilution of the stock in FSW.
P3. Variable loading, in which each WAF is individually prepared by addition of CytoSol with microsyringe to FSW and orbital shaking (150 rpm/48 h/20 °C), in a 2 l screw-capped separatory funnel. The nominal loadings tested were 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 μl l^{-1}.
P4. Dosage using acetone as carrier, at a constant concentration of 500 ppm (v/v) in the medium. The experimental concentrations tested were 0, 4, 10, 25, 75, 150 and 250 μl l^{-1}.

2.1.2. Light Libyan crude petroleum

P5. We followed procedure P1, and doses were expressed in ml of saturated aqueous extract per litre. The experimental concentrations tested (0, 10, 20, 50, 100, 200, 500 and 1000 ml l^{-1}) were obtained by dilution of the stock in FSW.
P6. A mixture of crude:DMSO (1:4, v/v) was produced after shaking for 48 h. Solutions of the extract of crude were obtained by dilution in DMSO and 12.5 μl of each solution was added to vials. The aqueous dilutions of the extract of crude were 5, 10, 25, 50, 100, 250, 500 and 1250 μl l^{-1} and constant concentration of the carrier was 1250 ppm (v/v).

2.1.3. CytoSol:Libyan crude mixtures

P7. Dilutions of a saturated aqueous extract were obtained by method B1 starting from a seawater:crude:CytoSol (9.0:0.5:0.5) mixture. The aqueous phase was used to obtain the FSW dilutions to be tested (in the range 1–1000 ml l^{-1}).
P8. A ray design (see later on) was applied to combinations of separate extracts of CytoSol and crude independently prepared by method B1. Four mixture ratios: 100:0% (0°), 36.6:63.4 (30°), 63.4:36.6 (60°) and 100:0% (90°); six chemical dilutions and a point corresponding to the maximum concentration tested (500 ml l^{-1} CytoSol, 500 ml l^{-1} Lybian crude, 50:50) were considered.

2.1.4. CytoSol:fluoranthene mixtures

P9. A ray design using acetone as a carrier at a constant concentration of 500 ppm (v/v). Four mixture ratios: 100:0 (0°), 36.6:63.4 (30°), 63.4:36.6 (60°) and 100:0% (90°); six chemical dilutions and the maximum concentration tested for both agents (250 μl l^{-1} CytoSol, 250 μg l^{-1} fluoranthene, 50:50) were considered.

2.2. Analytical methods

Serial dilutions of CytoSol were performed in hexane and determined by chromatography-mass spectrometry (GC-MS) methods [41]. A litre of an aqueous extract of CytoSol was extracted two times each with hexane (1:5, v/v) and dichloromethane (1:10, v/v). The organic extracts were dried over anhydrous Na$_2$SO$_4$ and concentrated by vacuum evaporation before determination by GC–MS. The equipment was an HP 5850 GC with a selective mass detector HP 5971 (series J) in mode scan 40–450 and a column HP-5MS of 60 m × 0.25 mm. Temperatures were 300 °C (injector), 280 °C (detector), and a column programming from 40 °C (1 min) up to 300 °C (20 min) with a gradient of 6 °C/min.

2.3. Bioassays

Tests were performed by conventional methods [42,43] in larvae of sea urchin (*Paracentrotus lividus*). Larvae were exposed to the test agents for 48 h at 20 °C without shaking. All the assays were performed in the dark except for those in which fluoranthene was involved (photoperiod with 14 h of light and irradiance of 20 μE m^{-2} s^{-1}). Light conditions were chosen for the fluoranthene assay because fluorescence light exposure enhances its toxicity for sea urchin embryo-larval test [44].

Gametes were obtained by dissection from a single pair of adults and checked for optimum quality (round eggs and motile sperm) under the microscope. Eggs were delivered into experimental vials in quadruplicate (the control was performed in quintuplicate), at a density of 40/ml, within 30 min after fertilisation in a gently stirred measuring cylinder filled with filtered sea water. After the exposure time, the material was fixed with 0.1 ml of 40% formaldehyde, and the lengths (maximum dimension) of at least 45 organisms, either larvae or earlier life stages, were measured in each replicate to evaluate the increments with the average ovum diameter. Response (R_i) was quantified in terms of growth inhibition as $R_i = 1 - (\Delta L_i / \Delta L_0)$, where ΔL_0 and ΔL_i are the increments corresponding to the control and the ith dose, respectively.

2.4. Mathematical models

2.4.1. Simple sigmoid response for single agent effects

In previous works [24,28,45–47], we have compared different mathematical equations commonly applied to the DR analysis by means of numerical simulations and experimental methods. The results demonstrated the special validity of the cumulative function of Weibull's distribution. In the context of DR modelling, the original form of this function should be modified and reparametrised so that the asymptote can take values different from 1 (e.g., subpopulations resistant to the toxic effect), and the dose for semi-maximum response can be explicit [45]. The definitive form, which we will denote as ^mW, is as follows:

$$R = K \left\{ 1 - \exp\left[-\ln 2 \left(\frac{D}{m} \right)^a \right] \right\}; \quad \text{briefly:} \quad R = {}^mW(D; K, m, a) \tag{1}$$

R is the response (with K as maximum value), D is the dose, m is the dose for semi-maximum response, and a is a form parameter related to the maximum slope of the function. The inverse of Eq. (1), which was necessary to apply the CA hypothesis (see later on), is as follows:

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

2.4.2. Response surfaces describing the joint action of two agents

The analysis of the joint response to two agents is commonly developed by the contrast of the experimental results with two hypotheses [9,13,17]: independent action (IA) and concentration addition (CA).

The IA hypothesis admits the statistical independence of the phenomena that underlie the individual responses. The probability theory allows us to define the joint response as the sum of the probabilities of the individual phenomena minus the probability of their joint occurrence. The response herein studied was the inhibition of the sea urchin larval growth as it is described in the Bioassays section. Thus, if R_d is the response to the joint action of the doses d_1 and d_2, and R_{d_1} and R_{d_2} are the responses to the same doses considered individually, this hypothesis proposes the following model [12]:

$$R_d = R_{d_1} + R_{d_2} - R_{d_1}R_{d_2} \tag{3}$$

This model is usually written in the following form:

$$R_d = 1 - (1 - R_{d_1})(1 - R_{d_2}) \tag{4}$$

The CA hypothesis used in the present work is given by Berenbaum [10] and is derived from the classical isobologram representation for the joint effect of two chemicals [48]. This CA model is initially based on the concept of null interaction between chemicals with the assumption that agents act via a similar mechanism to elicit an effect. In this sense, if D_1 and D_2 are the equivalent effect doses of two agents 1 and 2 that produce the individual response R_a, and d_1 and d_2 are the individual concentrations of the agents 1 and 2 that produces the same joint response R_a, it can be established that the isobole for the effect R_a, under the hypothesis of null interaction, satisfies the following equation of additivity [9]:

$$\frac{d_1}{D_1} + \frac{d_2}{D_2} = 1 \tag{5}$$

When the left-hand side of Eq. (5) is less than 1 then a positive interaction can be shown (synergy) and when it is higher than 1 a negative interaction can be claimed (antagonism). In a similar way, straight isoboles indicate null interactions, and concave up and down isoboles indicate synergy or antagonism, respectively. Under these conditions, assuming that the expression of DR individual models is $R_i = f_i(D_i)$ and their inverse function $D_i = g_i(R_i)$ have an explicit mathematical expression, the applicable form of Eq. (5) in practice is as follows [9]:

$$\frac{d_1}{g_1(R_a)} + \frac{d_2}{g_2(R_a)} = 1 \tag{6}$$

This equation can be used with diverse modifications which improve its applicability or establish quantitative indices for non-null interactions [9]. A consequence of the CA hypothesis is that a dose of an agent is replaceable for the equieffective dose of the other, which is equivalent to saying that the equation $R = f(D)$ has the same parametric values for any agent if we express the doses in the same coded values (one chemical acts as a dilution of the other and can be substituted at a constant proportion for the other).

Both hypotheses can be summarised in terms of the models that they propose for the joint response. Thus, if we assume that the DR individual models obey Eq. (2), the model for the joint response can be briefly written as the following:

IA hypothesis:

$$R = 1 - [1 - {}^mW(D_1; K_1, m_1, a_1)][1 - {}^mW(D_2; K_2, m_2, a_2)] \tag{7}$$

CA hypothesis: $\quad R = {}^mW[(D_1 + D_2); K, m, a] \tag{8}$

2.4.3. Biphasic responses model

The biphasic responses occasionally detected with several agent mixtures can be explained by admitting an addition or subtraction of effects (not of concentrations). In fact, we have proposed the following equation to model these responses [24,28]:

$$R = K_1 \left\{ 1 - \exp\left[-\ln 2 \left(\frac{D}{m_1} \right)^{a_1} \right] \right\} \pm K_2 \left\{ 1 - \exp\left[-\ln 2 \left(\frac{D}{m_2} \right)^{a_2} \right] \right\} \tag{9}$$

Biphasic profiles with a stimulatory section at low doses and an inhibitory section at high doses are often considered to be an indication of a hormetic response, according to the aforementioned definition of Southam and Ehrlich [30]. The subtractive form of model (9), with $K_2 < K_1$ and $m_2 < m_1$, describes appropriately these cases. Hormesis, however, is not the only phenomenon when biphasic profiles are obtained in the assessment of complex solutions [25].
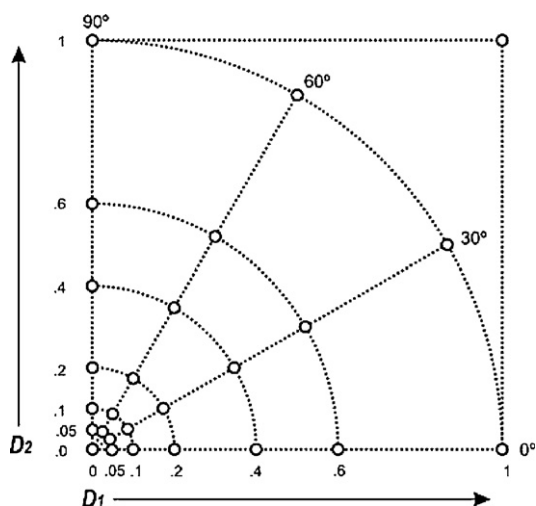
Fig. 1. Example of ray experimental design applicable to the modeling of the joint response to two agents (D1 and D2, both in normalized values) with different rays and combinations of both agents.

2.5. Experiments with two agents. The ray design

An experimental design able to model the joint response to two agents should contain a dose series of each agent in the absence of the other one as well as a certain number of combinations of both agents. An intuitive alternative consists of combining all the doses of an agent with all the doses of the other one. However, more economical experimentation can be achieved by means of a ray design (Fig. 1). Moreover, in order to avoid biases in the parametric estimates due to the different weight (concentration) of the independent variables, the doses should be coded (normalized) in the (0,1) domain. Finally, the proposed ray design demands that the normalized doses of both agents have the same values (Fig. 1). Generally, the nominal domains of both dose series will be different, and the following is an appropriate way to proceed:

1. Establish the maximum doses in nominal values, $^{nom}D_{1m}$ and $^{nom}D_{2m}$ of both agents as well as the (primary) dose series $^{nom}D_{1i}$ of the first agent in the absence of the second one.

2. Obtain the coded values D_{1i} in the [0,1] domain with the following equation:

$$D_{1i} = \frac{^{nom}D_{1i}}{^{nom}D_{1m}} \tag{10}$$

Because the values of the primary coded series D_{2i} of the second agent are the same as D_{1i}, the nominal series $^{nom}D_{2i}$ is obtained by means of the following decoding expression:

$$^{nom}D_{2i} = D_{1i} \times {}^{nom}D_{2m} \tag{11}$$

3. Supposing (see Fig. 1) radial beam at angles of $0°$, $30°$, $60°$ and $90°$ with a variable that represents the $^{n}D_{1i}$ series, the coded series of mixed doses (both agents) located on a given radius α_i is defined by the coordinates $(d_1[\alpha_i]_i, d_2[\alpha_i]_i)$ as follows:

$$d_1[\alpha_i]_i = D_{1i} \times \cos\alpha_i; \quad d_2[\alpha_i]_i = D_{2i} \times \sin\alpha_i \tag{12}$$

where corresponding nominal series can be obtained by means of Eq. (11).

4. Finally, it is convenient to include an additional point in the design defined by the maxima of both variables. Of course $^{nom}D_{1m}$ and $^{nom}D_{2m}$ values, as well as the number of radii and the α_i angles are able to change in accordance with the requirements of each specific case.

2.6. Numerical methods

Homoscedasticity of the experimental data was verified by means of the Levene's test ($\alpha = 0.05$). Fitting procedures and initial parametric estimations were performed by minimisation of the sum of quadratic differences between experimental and model-predicted values using the non-linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel spreadsheet. Parametric estimates were confirmed in the non-linear section of DataFit 9 software (Oakdale Engineering), which was also used for the significance of the parameters and the calculation of the parametric confidence intervals and model consistency (Student's t and Fisher's F tests, respectively, in both cases $\alpha = 0.05$).

The Akaike's information criterion (AIC) was also used to model comparison [49]. This statistical tool based on entropy concept, produces a relative quantification of the information lost when a given model is used to describe experimental data in comparison with another equation. The below AIC-equation is a measure of the
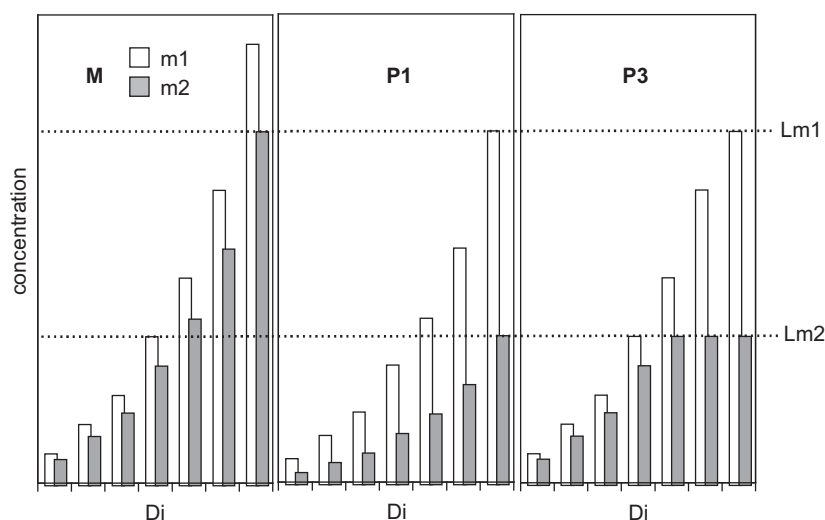

Fig. 2. Left: Representation of the nominal dose series of a mixture M with two hydrophobic components (m1 and m2 with solubility limits Lm1 and Lm2, respectively). Centre: Effect of the dosage method P1 on the nominal doses. Right: Effect of the dosage method P3 on the nominal doses.
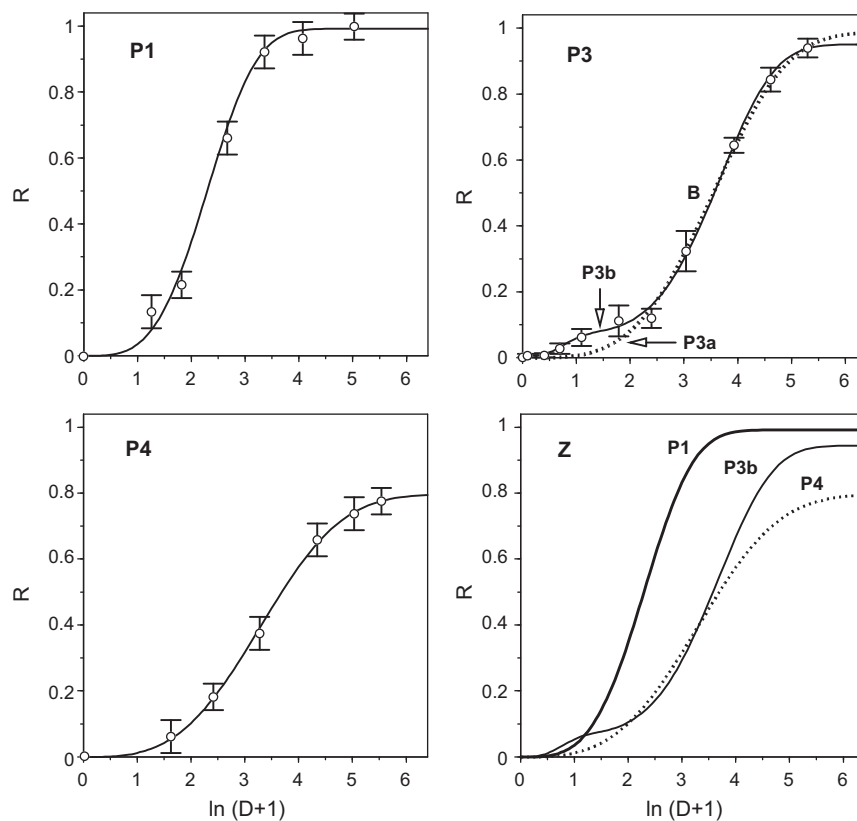
Fig. 3. Comparison among the responses of the sea urchin to *Cytosol* dosed according to the three methods (P1, P3, P4; jointly presented in Z) specified in the text. Experimental values (points) fitted (lines) to the models (1: P1, P3a, P4) and (9: P3b). D: real dose in ppm; R: response as inhibition of the sea urchin larval growth. Statistical analysis is summarised in Table 1. Error bars are confidence intervals ($n = 181$–190, $\alpha = 0.05$).

lack-of-fit of the chosen model (taking into account both, bias and variance) and the increased unreliability of the selected model to the increased number of model parameters (in terms of accuracy and complexity of the model):

$$\text{AIC} = n \ln\left(\frac{\text{SSR}}{n}\right) + 2(p+1) + \left[\frac{2(p+1)(p+2)}{n-p-2}\right] \quad (13)$$

The difference in the AIC of two models for the same set of data (n) balances the residual sum of squares (SSR) against the change in the number of parameters (p) to fit. The model with the lowest AIC is the one with the highest likelihood of being correct. The probability (Pr) of the chosen model being correct between two equations A and

B can be calculated as indicated now:

$$\Pr = \frac{\exp(-0.5\Delta\text{AIC}_{B-A})}{1 + \exp(-0.5\Delta\text{AIC}_{B-A})} \quad (14)$$

3. Results and discussion

3.1. Simple sigmoid responses: influence of the dosage method in multicomponent substances

3.1.1. CytoSol

Procedures P1 and P3 (see methods) are common in the dosage of hydrophobic mixtures, but both bias the original mixture compo-
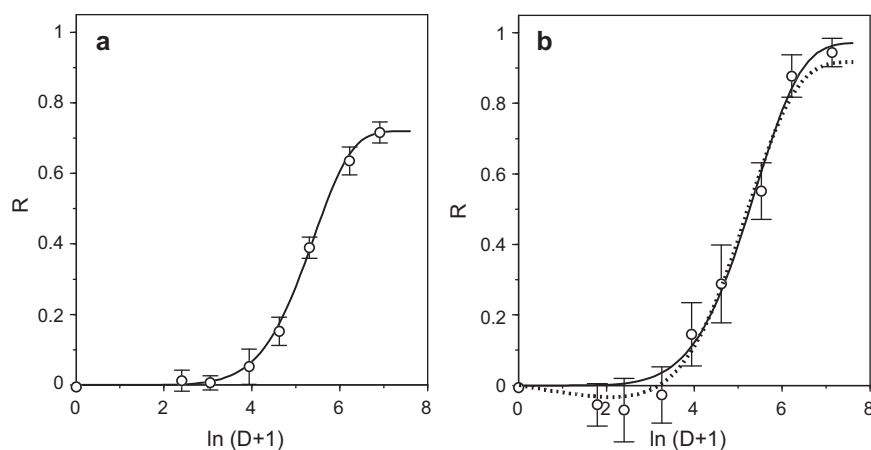

Fig. 4. Inhibition of the sea urchin larval growth by aqueous (a: dose in $\text{ml}\,\text{l}^{-1}$) and DMSO (b: dose in $\mu\text{l}\,\text{l}^{-1}$) extracts of light Libyan crude oil (R). Experimental values (points) fitted to the models (1, continuous line) and (9, dotted line in b). Error bars are confidence intervals ($n = 41$–189, $\alpha = 0.05$).

Table 1
Growth inhibition of sea urchin larvae by *Cytosol*. Parametric estimates (model ^mW) and confidence intervals (Student's t, $(= 0.05)$) corresponding to the dosage methods P1, P2, P3, and P4 described in the text. In all cases, the models were statistically consistent and robust (p-value from Fisher's F-test, $\alpha = 0.05$); r: correlation coefficient between observations and predictions. $D_{0.01K}$ and $D_{0.99K}$: doses corresponding to responses of the 1% and 99% of the maximum, respectively (confidence intervals for predicted response are also specified). Dose in ppm. See also Fig. 3.

P1	K	0.984 ± 0.076
	m (ED$_{50}$)	9.00 ± 1.88
	a	1.35 ± 0.42
	r (obs-pred)	0.999
	$D_{R=0.01}$	$0.40\ [0.0018 < R < 0.0489]$
	$D_{0.99K}$	$36.59\ [0.801 < R < 1.060]$
	p-value	<0.001
P2	K	0.953 ± 0.052
	m	11.40 ± 1.097
	a	1.53 ± 0.232
	r (obs-pred)	0.999
	$D_{R=0.01}$	$0.74\ [0.0043 < R < 0.0225]$
	$D_{0.99K}$	$39.22\ [0.859 < R < 1.004]$
	p-value	<0.001
P3	K	0.941 ± 0.058
	m	32.43 ± 4.78
	a	1.087 ± 0.158
	r (obs-pred)	0.998
	$D_{R=0.01}$	$0.70\ [0.0043 < R < 0.0224]$
	$D_{0.99K}$	$185.15\ [0.842 < R < 0.999]$
	p-value	<0.001
P4	K	0.763 ± 0.029
	m	26.33 ± 3.106
	a	1.005 ± 0.125
	r (obs-pred)	0.998
	$D_{R=0.01}$	$0.51\ [0.0052 < R < 0.0189]$
	$D_{0.99K}$	$173.27\ [0.707 < R < 0.791]$
	p-value	<0.001

$D_{R=0.01}$ represents the dose corresponding to a response of 1% of the whole population, without taking into account the existence of a fraction resistant to the effector. However, $D_{0.99K}$ represents the dose corresponding to a response equivalent to 99% of K.

sition in an aqueous medium. As shown in Fig. 2 with an example of two components at concentrations cm_1 and cm_2, P1 produces dose series with a constant ratio between components; however, this ratio involves the solubility limits Lm_1/Lm_2, instead of cm_1/cm_2. P3 does not maintain this constant ratio. Indeed, the low doses can reproduce the original value of cm_1/cm_2, but because the least hydrosoluble component reaches its solubility limit, its concentration remains constant in the subsequent doses, whereas the levels of the more hydrosoluble component continued increasing. The possible interactions among solutes, or the micellar character of their solutions can alter the values theoretically expected, but the basic situation is the one described. It is difficult to attribute more environmental realism to one method or another because it depends on the mixture components, their absolute concentrations and the environmental factors. However, P3 violates a basic condition of the DR analysis because it does not produce a simple increasing dose series.

These considerations help to interpret the differences among the responses shown in Fig. 3 and quantified in Table 1. It should also be kept in mind that, on the basis of oleic and linoleic methyl esters, the concentration of the saturated extract used in P1 was 250 ± 14 ppm at $20\,^\circ$C. This was in good agreement with the results of Walker et al. [2] for the solubility of CytoSol in water: 43 ppm at $12\,^\circ$C and 230 ppm at $18\,^\circ$C.

Thus, responses in P1 and P2 indicated the essential equivalence of both dosage methods when the initial concentration in P2 (Ultraturrax) did not exceed the solubility limit. The slight underestimation of toxicity in P3 can be attributed to minor losses of CytoSol on the recipient and homogeniser surfaces. This could be
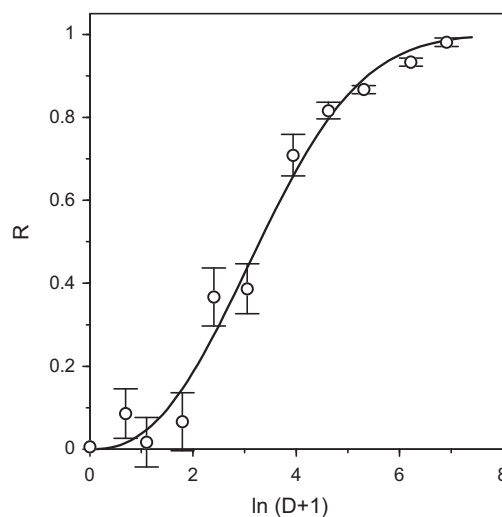

Fig. 5. Inhibition of the sea urchin larval growth by dilutions of an aqueous mixed extract of light Libyan crude oil and *Cytosol* (R). Dose is expressed as ml of the mixed saturated extract per litre and it was prepared following the procedure P7 (materials and methods). Experimental values (points) fitted (line) to the model (1). Error bars are confidence intervals ($n = 156$–200, $\alpha = 0.05$).

due to border effects by hydrophobic repulsion, which is a problem that is difficult to avoid at the low concentrations used here [7].

In P3, the underestimation of the toxicities of P1 and P2 was statistically significant. At the low CytoSol doses used, the border effect associated with the gentle orbital shaking excluded contact with water and a part of the agent, which produced lower concentrations than expected. This problem was avoided in P1. The profile of the response here was slightly biphasic, which requires the Eq. (9) for its modelling. We will discuss the interpretation of this type of fitting later, but the biphasic response could be due to that the CytoSol components with lower ED$_{50}$ values are more hydrophobic, and their levels do not increase correlatively with the rest when increasing the dose (Fig. 3. P3). The biphasic profile was not very marked; in fact, the parameter of shape $-a_2-$ of the low-dose sigmoid curve from model (9) is not statistically significant (Student's t test, $\alpha = 0.05$). However, the equation was found to be consistent by means of Fisher's F test ($\alpha = 0.05$) and the comparison between Eqs. (9) and (1), using Akaike's information criterion, revealed that with a probability of 100% the chosen model (9) is most likely to be correct for fitting experimental data than model (1). In addition, this biphasic curve took place in the only case in which it could be

Table 2
Parametric estimates and confidence intervals ($\alpha = 0.05$) corresponding to the fittings of the specified joint responses to IA and CA hypotheses. NS: non significant; r^2: adjusted coefficient of multiple determination.

		Cytosol-Libyan oil	Cytosol-fluoranthene
IA hypothesis. Eq. (7)	K_1	0.975 ± 0.022	0.726 ± 0.064
	K_2	0.709 ± 0.094	0.377 ± 0.059
	m_1	0.074 ± 0.004	0.101 ± 0.025
	m_2	0.372 ± 0.077	0.465 ± 0.079
	a_1	1.401 ± 0.103	0.743 ± 0.122
	a_2	1.429 ± 0.333	3.846 ± 2.225
	adj r^2	0.996	0.982
CA hypothesis. Eq. (13)	K	0.985 ± 0.025	0.750 ± 0.055
	m	0.062 ± 0.019^a	0.058 ± 0.082^aNS
	a	1.303 ± 0.113	0.867 ± 0.149
	b_1	7.912 ± 1.006	8.683 ± 2.931
	adj r^2	0.994	0.978

[a] Values of m in Eq. (13) are corrected taking into account the coefficient of relative power b_1. The result forces to reject this hypothesis in the case of *Cytosol*-fluoranthene.
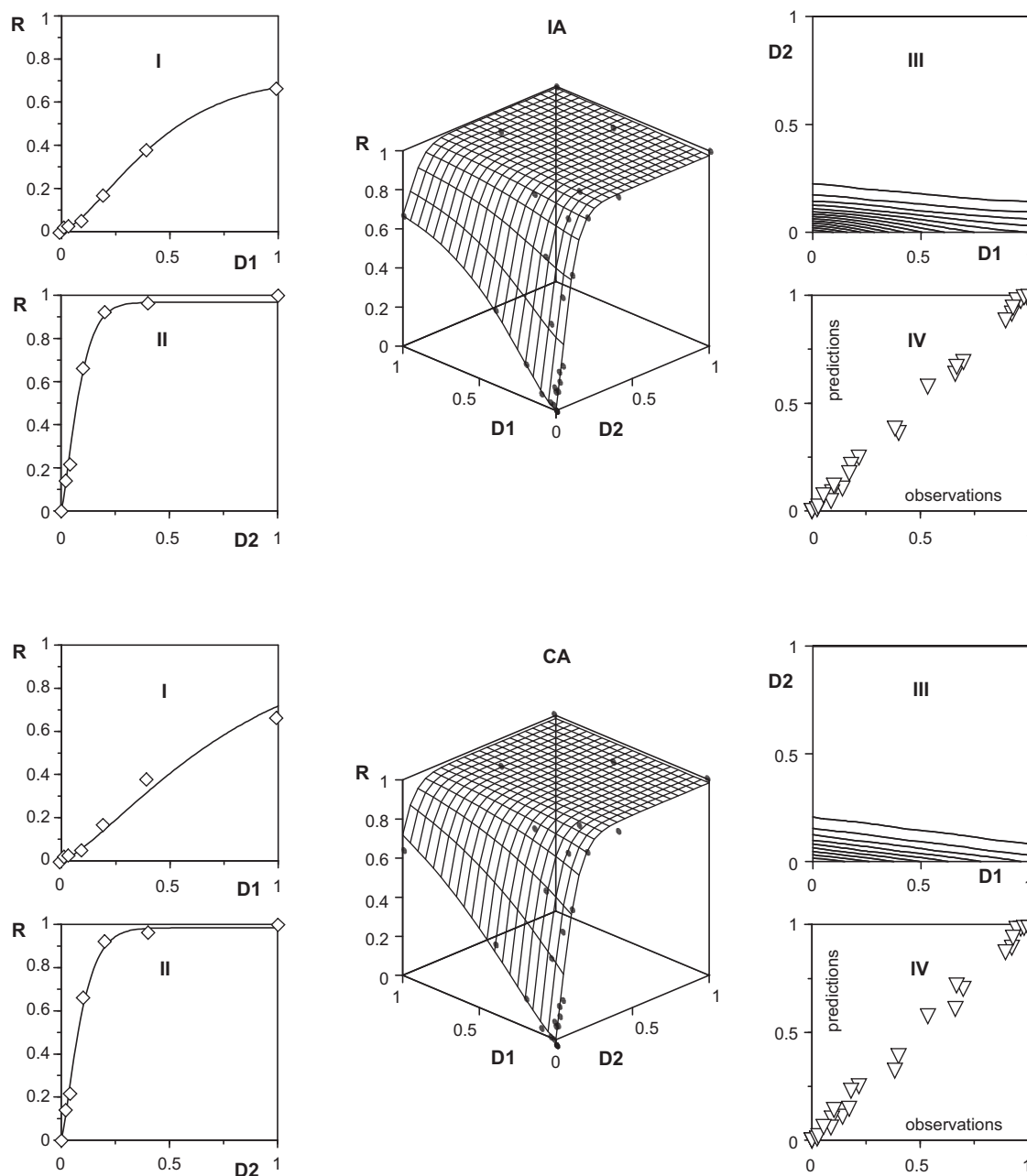
Fig. 6. Inhibition of the sea urchin larval growth by joint action of Libyan crude oil and *Cytosol* (*R*), according to IA and CA hypotheses. I and II: individual responses to Libyan oil and *Cytosol*, respectively; III: isobolograms; IV: plots of experimental versus model-predicted values. See also Table 2.

expected as a consequence of the dosage method but it might be attributed to experimental error. The underestimation of the toxicity in P4 was not only shown by a significantly higher ED_{50} than in P1 and P3 but also by a lower asymptote, which was significantly lower than 1.

3.1.2. Light Libyan crude oil

In view of the preceding results, the crude oil dosage was initially carried out by method P5 with the results shown in Fig. 4a. The doses are expressed in ml of saturated aqueous extract per litre. Eq. (1) provided a consistent fitting and statistically significant parametric estimates, but the asymptote ($K=0.719\pm0.029$) can only be interpreted as the limit that the hydrosolubility of the oil components imposes to the response (notice that the maximum dose assayed, $1000\,\mathrm{ml}\,\mathrm{l}^{-1}$, was equivalent to the use of the undiluted saturated aqueous extract).

This result demonstrates that the method of the aqueous extract, which tends to simulate the environmental conditions, imposes a restriction on the bioassay that it is no due to the nature of the toxic action of the compound studied but to the lack of hydrosolubility of the oil components. In this situation the underestimation in the toxicity of these components is obtained. In the beaten sea of a coastal environment, for example, non-polar compounds tend to produce emulsions and micelles to migrate towards the interfaces and concentrate on the particles in suspension by means of partition, absorption, adsorption and hydrophobic repulsion phenomena. Although they do not exist as molecular solutes, they can easily enter into the metabolism (e.g., of a filter feeder). In a complementary way, the use of a carrier changes the natural conditions of the assay and allows us to model the response to an agent without imposing restrictions on the mode in which the agent reaches the target.
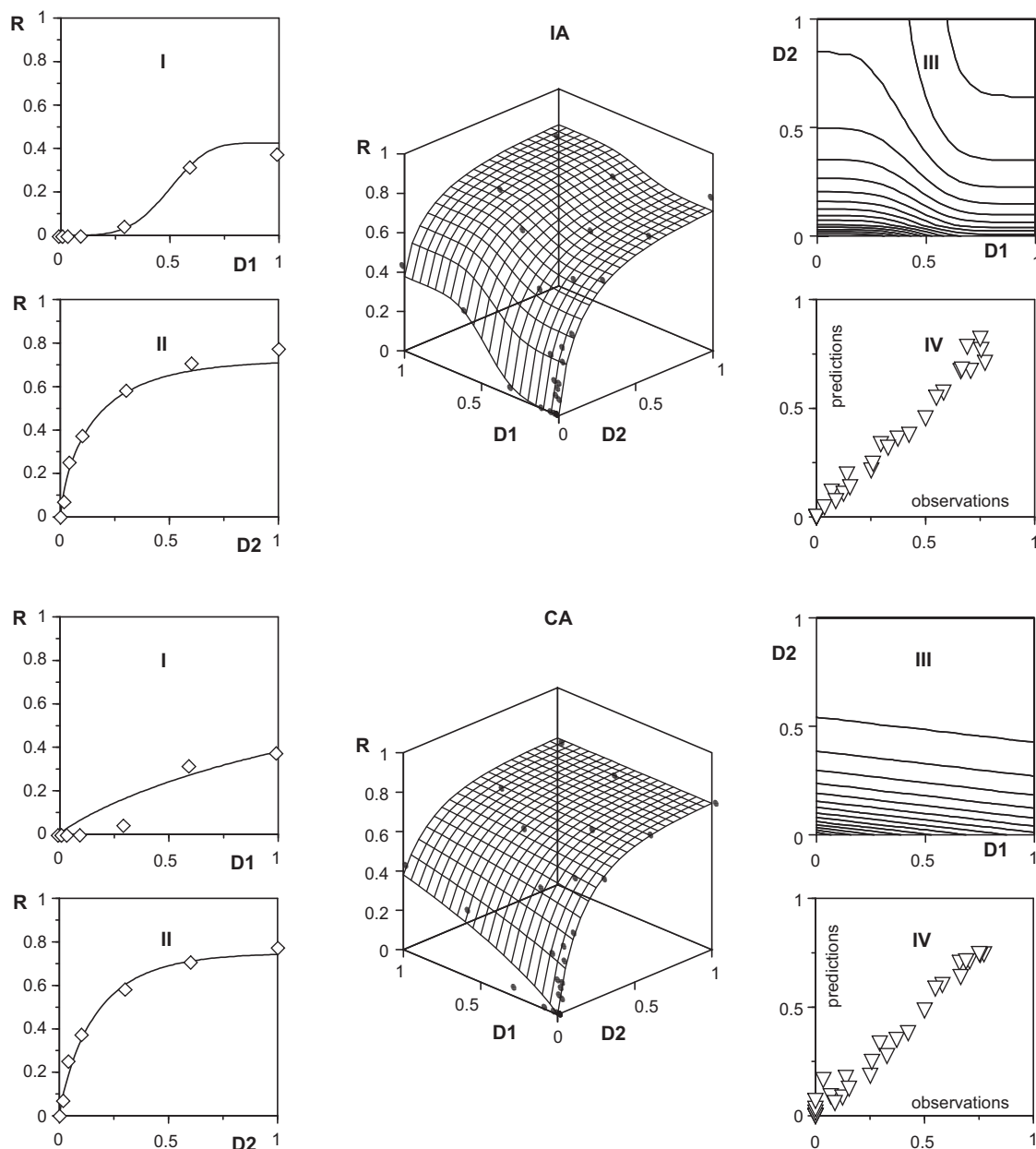
Fig. 7. Inhibition of the sea urchin larval growth by joint action of fluoranthene and *Cytosol* (R), according to IA and CA hypotheses. I and II: individual responses to fluoranthene and *Cytosol*, respectively; III: isobolograms; IV: plots of experimental versus model-predicted values. See also Table 2.

Fig. 4b shows the response to the specified dilutions of DMSO extract of crude oil. The direct comparison between Fig. 4a and b demonstrates that the toxic potential of crude oil (if the barrier of its hydrophobia is reduced for any one of the above mentioned mechanisms) is higher than the aqueous extracts method can show.

Additionally, it can be pointed out that slight stimulatory effects at low doses were detected in this last case. Because it is not attributable to DMSO, it could be due to a stimulatory action (with low K and ED_{50}) of some oil components or to a case of hormesis (see Section 3.3). Determining the cause of the stimulatory effects would require additional experimentation that exceeds the objectives of this work. In any case, both suppositions could be described with model (9) in its subtractive form (Fig. 4b). Although two parameters (K_2 and a_2) were not statistically significant the robustness of this equation was high and, with a probability of 72% (AIC test), the model (9) seems to be the correct choice instead of model (1).

3.2. Joint response to two agents. CytoSol-Libyan crude and CytoSol-fluoranthene

In a first approach, the dosage was carried out with dilutions of a saturated aqueous extract prepared by method P7 with doses expressed as dilutions ($ml\,l^{-1}$) of this extract and results (Fig. 5) satisfactorily described by means of Eq. (1). If we express the ED_{50} corresponding to this case and the previous single assays of CytoSol and crude oil in the same units (ml of the saturated aqueous extract per litre), we obtain the following values:

$$\text{crude oil: } ED_{50} = 190.4 \pm 16.4 \text{ ml } l^{-1}$$
$$\text{CytoSol: } ED_{50} = 36.0 \pm 7.5 \text{ ml } l^{-1}$$
$$\text{crude oil + CytoSol: } ED_{50} = 26.2 \pm 7.6 \text{ ml } l^{-1}$$

These values only demonstrate that a mixed aqueous extract of crude oil and CytoSol was more toxic than any of the two sep-
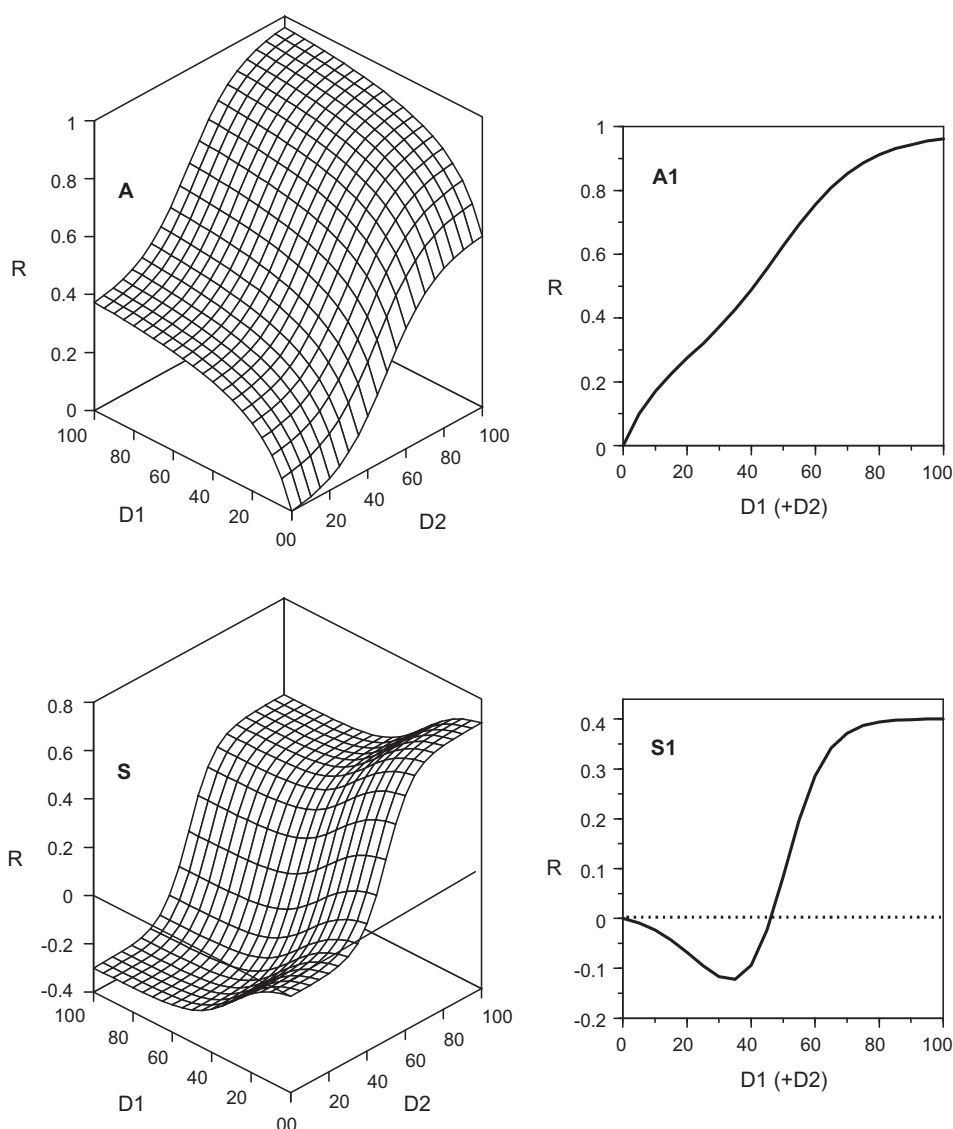
Fig. 8. Simulation of additive (A) and subtractive (S) responses to joint action of two agents. Right: non-conventional profiles (A1 and S1) show the simulations when the independent variable is a dilution series of a mixed solution. This result is equivalent to select the responses along a line bisecting the plane of the doses on the surfaces A and S. Note that S1 could be interpreted like a case of hormetic response.

arate extracts under the same conditions. Nevertheless, since the concentrations of both agents vary in strictly correlative form, they can only be treated as a single variable, and not as two true independent variables, which prevents us from knowing the nature of the joint response. To describe such a joint response, a ray design was applied using aqueous extracts prepared by method P8. For comparative purposes, a similar design (P9) was applied to the joint action of CytoSol and fluoranthene.

In both cases (Figs. 6 and 7 and Table 2), the description of the single responses by means of model (1) produced statistically significant fittings ($\alpha = 0.05$). When these parametric estimates were used as initial values for fitting each group of observations to model (7), which corresponded to the IA hypothesis, the description led to statistically significant new estimates ($\alpha = 0.05$; Table 2). The values of these estimates were very close to the initial estimates, and there were strong correlations between observations and predictions with residuals randomly distributed.

The differences among the individual parametric estimates in each couple suggested non replaceable equieffective doses and, therefore, a joint response that did not obey the CA hypothesis. In fact, Eq. (8) did not produce a statistically significant description

in either of the two cases. However, if we include a coefficient, b_1, in order to balance the toxic potential of both agents, we obtain:

$$R = {}^m W[(b_1 D_1 + D_2); K, m, a] \tag{15}$$

This equation produced statistically significant descriptions ($\alpha = 0.05$) in both couples, and response surfaces whose linear isoboles indicated the absence of interaction between the agents involved in each couple. In the case of CytoSol-fluoranthene the value of m calculated from (15) was not statistically significant when it was corrected taking into account the coefficient b_1 (Table 2). In the case of CytoSol-Libyan oil, the difference between both hypotheses was small, but the better fittings obtained with the IA hypothesis (F-Fisher value and r coefficient) suggested that this option was preferred over the CA hypothesis. Comparison of IA and CA models by AIC test also suggested that the IA model was superior in describing the experimental data of dose–response obtained with both mixtures (CytoSol-fluoranthene and Cytosol-Libyan oil).

Isobole analysis is usually restricted to the context of the CA hypothesis, where the expression (6) allows us to decide between null interaction, synergy or antagonism. However, the meaning of the isobole's curvature can be generalized to any system in which

interaction exists among the variables, that is, in which the effect of one of them on the response depends on the values of the other one. In any system – interactive or not – if the isobole of a given response, R_a, intersects the axes of the doses in the points d_{1a} and d_{2a}, it is obvious that when this isobole is concave up, the response R_a requires lower doses than those corresponding to the straight line between d_{1a} and d_{2a}, and the opposite occurs when the isobole is convex up. In a system with interaction, concave and convex isoboles mean synergy and antagonism, respectively. When this approach is applied to a surface that follows the IA hypothesis – which involves an interactive response, in accordance with the Eq. (3) – it reveals that synergy and antagonism notions cannot be used to describe the interaction between agents without making reference to a specific dose domain. Indeed, it can clearly be observed in Fig. 7 that surface response not only includes sections with concave and convex isoboles but also isoboles with concave and convex sections in the same profile. It allows us to define a low-dose subdomain in which the response corresponds to a null interaction or an antagonistic effect, and another high-dose subdomain where synergistic effects were observed.

3.3. Biphasic profiles, hormesis and degenerate responses

The biphasic responses obtained in the bioassay of CytoSol measured out by the P2 method and assessment of Libyan crude oil using DMSO as the solvent (Fig. 4b) raises a problem of interest in relation with the hormesis concept. A biphasic profile reveals a sum or subtraction of effects due to two different phenomena. It does not correspond with the "sum minus multiplication" of responses that the IA hypothesis needs (7). It also does not correspond to the sum of dose that the CA hypothesis demands (8), but it does correspond to the sum of response that Eq. (9) shows. The specific feature of the hormesis phenomenon is the presence of a single agent. The double effect of this agent can be explained if we assume that it operates on two different mechanisms of the target organism, is metabolically transformed into more than one chemical, or even by means of other hypotheses. Regardless, the possibility that the effects of a toxic agent are stimulatory to sub-inhibitory dose, together with the supposed generality of this phenomenon, is what induced several authors to suggest a relaxation of environmental protection policies in certain cases [33,39,40].

Some of these authors also pointed out that the presence of biphasic profiles in dose–response assessments are not always derived from the hormesis phenomena [28]. In this sense, an appropriate description of the joint action of two agents demands a design with a series of non-correlative doses of both agents and a mathematical function of two independent variables for its modelling. This equation produces surfaces as A or S (Fig. 8) according to that the individual responses have similar or opposite sign, respectively. However, when the response of a bioassay with two agents is defined as a function of the dilutions from a one solution of both chemicals, using the dilution as the only independent variable, the 2D profile generated is a degenerate response of the real 3D surface that should be obtained. This result can be described by Eq. (9), and it will be equivalent to the results obtained throughout a straight line in the A or S surfaces from the plane defined by the two independent variables (A1 or S1 profiles in Fig. 8).

It is likely that nobody would design an experiment with two agents in this way. However, in the bioassay of complex solutions (extracts of tissues, biological fluids, microbial cultures, polluted waters, lixiviates) it is common to express the response as a function of the dilution or to pay attention to a specific compound. If these solutions contain more than one active agent, observation of a biphasic response could be a consequence of this degenerate design.

It is surprising that multiphasic responses are not more frequent in assays of complex products (e.g., petroleum, CytoSol and many others). In principle such responses could be described, for a group of h-agents, by means of Eq. (9) modified with series of h-sigmoid sums. However, it should be kept in mind that the first condition of a biphasic or multiphasic response is the addition of the effects, different from IA and the addition of concentrations that are more common. Although the difference between mono and biphasic curves can be very large, the profile waves tend to be overlapped when h increases and, in practice, they are absorbed by experimental error.

4. Conclusions

The use of SCA to eliminate petroleum that remains in the rocky substrates after mechanical cleaning of oil spills demands an evaluation of the toxicity of both agents in terms of single and combined action. The present work studied the inhibitory response of larval growth of sea urchin to CytoSol, Libyan crude oil and fluoranthene individually and in two binary combinations. The results led to a discussion of the problem of the dosage of hydrophobic mixtures, the anomalous biphasic responses (similar to the hormesis phenomena) obtained in some cases and the notions of synergy and antagonism in the framework of the mathematical models that can be applied to the joint response to two agents.

In relation to the dosage methods, it could be concluded that the dilution of a saturated aqueous extract was a better option than using CytoSol separately accommodated in different volumetric relationships. The first method produced equivalent responses to those obtained by diluting an emulsion vigorously mixed with Ultraturrax without exceeding the limit of solubility. Individual accommodation underestimated the toxicity with regard to the other two methods and, according with what we expected if two compounds from a mixture have solubility limits, it generated anomalous responses due to changes in the initial composition with an increasing series of doses. The use of a solvent without significant effect as carrier (DMSO) led to higher estimates of the toxicity. It is not clear whether the accommodation method underestimated the biodisponibility of hydrophobic compounds under environmental conditions of beaten sea and particulate material that could behave as a carrier for filter feeders.

Experimental data were, in all cases, statistically well-described by using a simple modification of the Weibull equation as the modelling basis. Biphasic responses obtained in two bioassays were able to fit a model including addition of effects (not of concentrations), which only involved a sum or subtraction of two Weibull equations. Biphasic profiles with a stimulatory section at low-dose and an inhibitory section at high-dose are often considered as an indication of the hormesis phenomenon (characterised by duplicity of opposite effects due to a single agent). However, based on toxicodynamic results, hormesis cannot be differed from those obtained by a degenerate design.

The Weibull equation in the framework of the mathematical models corresponding to the IA and CA hypotheses was also utilised in the descriptions of two combined responses (CytoSol-light Libyan crude oil and CytoSol-fluoranthene), which were studied by means of a ray design. Modelling results were significant in both the IA hypothesis and the CA hypothesis with null interactions (Student's t and Fisher's F tests with $\alpha = 0.05$). However, the lowest confidence intervals of numerical parameters, the highest correlation coefficient between observed and predicted values as well as the results of probability obtained with AIC test favoured the IA hypothesis. In spite of what this name suggests, the isoboles exam, whose meaning is independent of the used hypothesis, showed surface responses with cases of synergy and antagonism (specially

clear in the CytoSol-fluoranthene test). As other authors have previously pointed out [22,50,51], these concepts cannot be applied to the interaction among two agents without specifications because synergy and antagonism not only depend on the nature of such agents but also on the subdomain of doses considered.

Acknowledgements

References

[1] D. Rial, R. Beiras, J.A. Vázquez, M.A. Murado, Acute toxicity of a shoreline cleaner Cytosol and oil, and ecological risk assessment of its use on the Galician Coast, Arch. Environ. Contam. Toxicol. 59 (2010) 407–416.

[2] A.H. Walker, J.H. Kucklick, J. Michel, Effectiveness and environmental considerations for non-dispersant countermeasures, Pure Appl. Chem. 71 (1999) 67–81.

[3] E. His, R. Beiras, M.N.L. Seaman, The assessment of marine pollution-bioassays with bivalve embryos and larvae, in: P.A. Tyler, C.M. Young, A.J. Southward (Eds.), Advances in Marine Biology, Academic Press, 1999, pp. 1–178.

[4] R. Beiras, L. Saco-Alvarez, Toxicity of seawater and sand affected by the *Prestige* fuel-oil spill using bivalve and sea urchin embryogenesis bioassays, Water Air Soil Pollut. 177 (2006) 457–466.

[5] OECD, Series on Testing and Assessment, Number 23, Guidance Document on Aquatic Tocicity Testing of Difficult Substances and mixtures, 2000.

[6] T.H. Hutchinson, N. Shillabeer, M.J. Winter, D.B. Pickford, Acute and chronic effects of carrier solvents in aquatic organisms: a critical review, Aquat. Toxicol. 76 (2006) 69–92.

[7] M.M. Singer, D. Aurand, G.E. Bragin, J.R. Clark, G.M. Coelho, M.L. Sowby, R.S. Tjerdema, Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing, Mar. Pollut. Bull. 40 (2000) 1007–1016.

[8] M.G. Barron, L. Kaĭaihue, Critical evaluation of CROSERF test methods for oil dispersant toxicity testing under subarctic conditions, Mar. Poll. Bull. 46 (2003) 1191–1199.

[9] M.C. Berenbaum, The expected effect of a combination of agents: the general solution, J. Theor. Biol. 114 (1985) 413–431.

[10] M.C. Berenbaum, What is synergy? Pharmacol. Rev. 41 (1989) 93–141.

[11] C. Gennings, W.H. Carter, J.A. Campain, D.S. Bae, R.S.H. Yang, Statistical analysis of interactive cytotoxicity in human epidermal keratinocytes following exposure to a mixture of four metals, J. Agric. Biol. Environ. Stat. 7 (2002) 58–73.

[12] C.I. Bliss, The toxicity of poisons applied jointly, Ann. Appl. Biol. 26 (1939) 585–615.

[13] M. Junghans, T. Backhaus, M. Faust, M. Scholze, L.H. Grimme, Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures, Aquat. Toxicol. 76 (2006) 93–110.

[14] M. Goldoni, C. Johansson, A mathematical approach to study combined effects of toxicants *in vitro*: evaluation of the Bliss independence criterion and the Loewe additivity model, Toxicol. In Vitro 21 (2007) 759–769.

[15] S.Y. Euling, C. Gennings, E.M. Wilson, J.A. Kemppainen, W.R. Kelce, C.A. Kimmel, Response-surface modeling of the effect of 5α-dihydrotestosterone and androgen receptor levels on the response to the androgen antagonist vinclozolin, Toxicol. Sci. 69 (2002) 332–343.

[16] T. Backhaus, Å. Arrhenius, H. Blanck, Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition, Environ. Sci. Technol. 38 (2004) 6363–6370.

[17] C. Gennings, W.H. Carter Jr., R.A. Carchman, L.K. Teuschler, J.E. Simmons, E.W. Carney, A unifying concept for assessing toxicological interactions: changes in slope, Toxicol. Sci. 88 (2005) 287–297.

[18] A. Loureiro, M.J.B. Amorin, B. Campos, S.M.G. Rodrigues, A.M.V.M. Soares, Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint, Environ. Poll. 157 (2009) 625–636.

[19] H. Sørensen, N. Cedergreen, I.M. Skovgaard, J.C. Streibig, An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments, Environ. Ecol. Stat. 14 (2007) 383–397.

[20] M.J. Jonker, C. Svendsen, J.J.M. Bedaux, M. Bongers, J.E. Kammenga, Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose–response analysis, Environ. Toxicol. Chem. 24 (2005) 2701–2713.

[21] W.R. Greco, G. Bravo, J.C. Parsons, The search for synergy: a critical review from a response surface perspective, Pharmacol. Rev. 47 (1995) 331–385.

[22] D.B. White, H.M. Faessel, H.K. Slocum, L. Khinkis, W.R. Greco, Nonlinear response surface and mixture experiment methodologies applied to the study of synergy, Biometr. J. 46 (2004) 56–71.

[23] P.K. Gessner, Isobolographic analysis of interactions: an update on applications and utility, Toxicology 105 (1995) 161–179.

[24] J.A. Vázquez, M.ª.P. González, M.A. Murado, Effects of lactic acid bacteria cultures on pathogenic microbiota from fish, Aquaculture 245 (2005) 149–161.

[25] M.J. Jonker, A.M. Piskiewicz, N. Ivorra i Castellá, J.E. Kammenga, Toxicity of binary mixtures of cadmium-copper and carbendazimcopper to the nematode Caenorhabditis elegans, Environ. Toxicol. Chem. 23 (2004) 1529–1537.

[26] M.J. Jonker, R.A.J.C. Sweijen, J.E. Kammenga, Toxicity of simple mixtures to the nematode *Caenorhabditis elegans* in relation to soil sorption, Environ. Toxicol. Chem. 23 (2004) 480–488.

[27] S. Loureiro, C. Svendsen, A.L.G. Ferreira, C. Pinheuro, F. Ribeiro, A.M.V.M. Soares, Toxicity of three binary mixtures to Daphnia Magna: comparing chemical modes of action and deviations from conceptual models, Environ. Toxicol. Chem. 29 (2010) 1716–1726.

[28] M.A. Murado, J.A. Vázquez, The notion of hormesis and the dose–response theory: a unified approach, J. Theor. Biol. 244 (2007) 489–499.

[29] R.G. Belz, N. Cedergreen, H. Sørensen, Hormesis in mixtures-can it be predicted? Sci. Total. Environ. 404 (2008) 77–87.

[30] C.M. Southam, J. Ehrlich, Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture, Phytopathology 33 (1943) 517–524.

[31] E.J. Calabrese, L.A. Baldwin, The frequency of U-shaped dose responses in the toxicological literature, Toxicol. Sci. 62 (2001) 330–338.

[32] E.J. Calabrese, L.A. Baldwin, Defining hormesis, Human Exp. Toxicol. 21 (2002) 91–97.

[33] J.G. Teeguarden, Y. Dragan, H.C. Pitot, Hazard assessment of chemical carcinogens: the impact of hormesis, J. Appl. Toxicol. 20 (2000) 113–120.

[34] E.J. Calabrese, L.A. Baldwin, The marginalization of hormesis, Human Exp. Toxicol. 19 (2000) 32–40.

[35] E.J. Calabrese, L.A. Baldwin, The hormetic dose–response model is more common than the treshold model in toxicology, Toxicol. Sci. 71 (2003) 246–250.

[36] E.J. Calabrese, L.A. Baldwin, Toxicology rethinks its central belief, Nature 421 (2003) 691–692.

[37] E.J. Calabrese, R. Blain, The occurrence of hormetic dose responses in the toxicological literature, the hormetic database: an overview, Toxicol. Appl. Pharmacol. 202 (2005) 289–301.

[38] E.J. Calabrese, Toxicological awakenings: the rebirth of hormesis as a central pillar of toxicology, Toxicol. Appl. Pharmacol. 204 (2005) 1–8.

[39] E.J. Calabrese, Hormesis: a revolution in toxicology, risk assessment and medicine, EMBO Rep. (5) (2004) 537–540 (special issue).

[40] L.M. Ellman, C.R. Sunstein, Hormesis, the precautionary principle, and legal regulation, Human Exp. Toxicol. 23 (2004) 601–611.

[41] S. Daling, L.G. Faksness, Laboratory and reporting instructions for the CEN/BT/TF 120 oil spill identification Round Robin test, SINTEF, Nordtest Technical Report no. 499 (2002).

[42] N. Fernández, R. Beiras, Combined toxicity of dissolved mercury with copper, lead and cadmium on embrypgenesis and early larval growth of the *Paracentrotus lividus* seaurchin, Ecotoxicology 10 (2001) 263–271.

[43] J Bellas, R. Beiras, J.C. Mariño-Balsa, N. Fernández, Toxicity of organic compounds to marine invertebrate embryos and larvae: a comparison between the sea-urchin embryogenesis bioassay and alternative test species, Ecotoxicology 14 (2005) 335–351.

[44] J. Bellas, L. Saco-Álvarez, O. Nieto, R. Beiras, Ecotoxicological evaluation of polycyclic aromatic hydrocarbons using marine invertebrate embryo-larval bioassays, Mar. Pollut. Bull. 57 (2008) 493–502.

[45] P. Riobó, B. Paz, J.M. Franco, J.A. Vázquez, M.A. Murado, Proposal for a simple and sensitive haemolytic assay for palytoxin. Toxicological dynamics, kinetics, ouabain inhibition and thermal stability, Harmful Algae 7 (2008) 415–429.

[46] M.A. Murado, M.ª.P. González, J.A. Vázquez, Dose–response relationships. An overview, a generative model and its application to the verification of descriptive models, Enzyme Microb. Technol. 31 (2002) 439–455.

[47] P. Riobó, B. Paz, J.M. Franco, J.A. Vázquez, M.A. Murado, E. Cacho, Mouse bioassay for palytoxin. Specific symptoms and dose–response against dose-death time relationships, Food Chem. Toxicol. 46 (2008) 2639–2647.

[48] S. Loewe, The problem of synergism and antagonism of combined drugs, Arzneimittle Forsch. 3 (1953) 285–290.

[49] K.P. Burnham, D.R. Anderson, Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach, 2nd ed., Springer-Verlag, 2002, ISBN:0-387-95364-7.

[50] S. Savelev, E. Okello, N.S.L. Perry, R.M. Wilkins, E.K. Perry, Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia lavandulaefolia* essential oil, Pharm. Biochem. Behav. 75 (2003) 661–668.

[51] M. Kong, J. Jack Lee, A generalized response surface model with varying relative potency for assessing drug interaction, Biometrics 62 (2006) 986–995.