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Simulating synergism or antagonism in binary mixtures with different modeling approaches – A case study focused on the effect of disinfection by-products on algal growth

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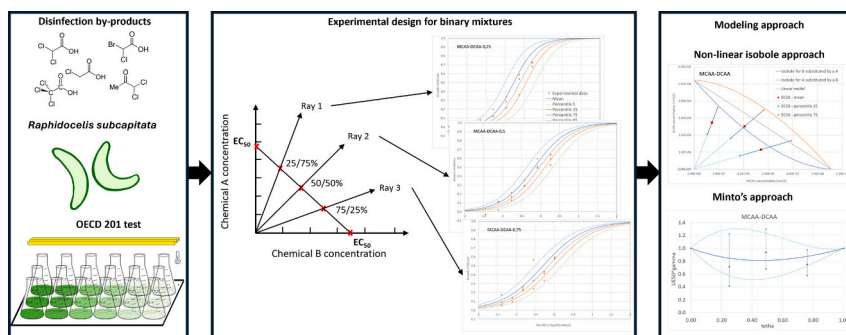
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HIGHLIGHTS

- Growth inhibition of *Raphidocelis subcapitata* was measured on DBPs binary mixtures.
- Chemical interactions in binary mixtures were modeled with two approaches.
- Non-linear isobole approach was tested to predetermined effect levels.
- Minto's approach was tested over the entire Hill concentration-response curve.
- Minto's model discriminated additive, synergistic and antagonist interactions.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper aims to test several modeling approaches for predicting toxicity of binary mixtures with potential synergy and antagonism. The approach based on the construction of isoboles was first tested and criticized. In contrast to conventional approaches, and in order to be mathematically consistent with the additivity assumptions, non-linear isoboles have been constructed. This approach was compared with that proposed by Minto et al. (2000), which measures deviations from additivity by considering standardized variables and which considers the entire Hill concentration-response curves. The selected models were tested on a case study related to chlorine-based disinfectant by-products (DBPs), using experimental data describing the effect of five DBPs (monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, bromochloroacetic acid and 1,1-dichloropropan-2-one) on a unicellular green algae *Raphidocelis subcapitata*. The approach based on the construction of isoboles has shown its limitations. Indeed, in cases where the individual substances involved have different slopes in terms of their Hill concentration-effect relationships, the so-called zone of indetermination can be large. Furthermore, conclusions drawn from isoboles based on EC_{50} s or EC_{20} s may not be consistent. Minto's approach

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makes it possible to construct interaction indicators that consider the entire Hill concentration-response curve. Response surfaces can be constructed to visualize the areas of concentration of the two substances involved that maximize the interaction effects.

1. Introduction

So far, ecotoxicological effects of chemicals are generally evaluated according to 'single' compound approaches, assuming that biota is exposed to only one chemical at the same time. Chemical substances are therefore evaluated one by one, independently of possible co-exposures with other compounds. Nevertheless, the most frequent scenarios involve simultaneous exposures to a mixture of chemicals. Only conservative approaches, like the Concentration Addition (CA) or Independent Action (IA) models, are usually applied for predicting mixture toxicity, but they do not account for potential synergism (i.e. supra-additive effects) or antagonism (i.e. sub-additive effects). CA approach assumes that mixture components have the same or similar mode of action (MOA), whereas IA approach assumes they have different or dissimilar MOA (Backhaus et al., 2004). However, Cedergreen et al. (2008) tested the CA and IA models on a set of 98 different mixtures and concluded that approximately 20 % of the mixtures were adequately predicted by IA, 10 % were adequately predicted by CA. On the other hand, the probability of synergy highly depends on the chemical class of substances in mixture: Cedergreen (2014) conducted a review on a large number of mixtures and observed that synergy occurred in 7 %, 3 % and 26 % of the binary mixtures of pesticides, metals and antifoulants respectively. In some cases, predicting potential deviations to CA or IA assumptions, i.e. to additivity, can then be of high concern for subsequent risk assessment.

Nevertheless, it is an insurmountable task to assess ecotoxicological effects of all possible combinations of chemical mixtures through purely experimental approaches. To overcome this obstacle, models for predicting mixture toxicity with potential synergy and antagonism were developed (Bliss, 1939; Loewe and Muischnek, 1926). However, Geary (2013) pointed out that, despite the apparent simplicity of the concept of supra- or sub-additivity, the quantitative models used so far are often based on mathematical inconsistencies between the basic assumptions of additivity and their applications. In particular, the method based on linear isoboles is typically used to estimate a potential deviation from additivity, and to estimate synergy or antagonism (Sørensen et al., 2007). Many who apply the isobole approach assume that it is applicable to any combination of substances, regardless of the shape of the concentration-response curves of the individual substances. From a practical point of view, it is generally considered that a Model Deviation Ratio (MDR, i.e. the ratio between a predicted and an observed endpoint respectively) calculated from a linear isobole of <0.5 indicates antagonism and that a MDR of >2 indicates synergy (Belden et al., 2007). Nevertheless, these thresholds are not justified from a mathematical or mechanistic point of view and are purely arbitrary. Bosgra et al. (2009), Geary (2013), Lederer et al. (2019, 2018), Ezechiáš and Cajthaml (2018) and Schindler (2022, 2017) (these works come from the field of pharmacology rather than ecotoxicology) criticized the validity of such an approach because they showed mathematically that 'linear isoboles are a rarity' and not the general rule.

Besides, most models developed for detecting antagonism and/or synergy only use a part of the concentration-response curves for single substances and mixtures. In particular, linear isoboles and subsequent MDRs are generally built from EC_{50} s (i.e. the individual or mixture concentration provoking 50 % effect). Numerous mathematical alternatives to the linear isobole model have been proposed in the literature to overcome the conceptual limitations of this approach (Lee, 2010). Considering this background, one challenge is to develop modeling approaches able to exploit the entire concentration-response relationships and to build a unified model for additivity, synergism and antagonism

limiting arbitrary and inconsistent choices. The objective of this paper is then to compare and discuss several potential modeling approaches simulating mixture effects with a quantification of synergy or antagonism strengths.

The selected models were tested on a case study related to chlorine-based disinfectants and their by-products. Chlorine-based disinfectants are used in water systems and their reaction with natural organic matter (NOM) leads to the formation of disinfection by-products (DBPs), with varying chemical composition and concentrations depending on the NOM and physico-chemical properties of the environment (Bougeard et al., 2010; Zhang et al., 2013). The most prevalent groups of DBP identified are trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs) and halo ketones (HKs) (Cowman and Singer, 1996; Richardson and Postigo, 2016). Thus, by definition, DBPs are generated in mixtures. DBPs can display toxicity towards organisms belonging to every trophic level in aquatic ecosystems (Cui et al., 2021). Microalgae are one the potential organisms impacted by the release of DBPs in the environment and are of interest when assessing the effects of a substance because of their belonging to the group of primary producers. Most of the toxicity data available in the literature relate only to individual DBPs but a few studies have focused on mixtures. Melo et al. (2019) showed that binary and ternary mixtures of bromoacetic acid (BAA), monochloroacetic acid (MCAA) and trichloroacetic acid (TCAA) were well correlated with CA and had a synergistic tendency when compared to IA. Chen et al. (2019) observed that binary mixtures of seven aromatic halogenated DBPs towards the freshwater bacteria *Vibrio qinghaiensis* sp.-Q67 were found to be mainly synergistic (>50 % of mixtures at EC_{50}). In contrast, toxic effects on the marine bacteria *Aliivibrio fischeri* were mainly additive. More recently, Qin et al. (2021) studied the effects of six haloacetic acids in binary mixtures on *Raphidocelis subcapitata* and observed that in 72 % of the mixtures tested, CA and IA models didn't predict well enough the EC_{50} , with almost 50 % of the mixtures being synergistic. Considering this background, experiments were conducted in the present study to determine effects of binary mixtures built from five DBPs (monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromochloroacetic acid (BCAA) and 1,1-dichloropropan-2-one (1,1-DCP)) on a microalgae species, *R. subcapitata*. Different alternative modeling approaches were tested on the dataset thus generated.

2. Materials and methods

2.1. Experimental design and toxicity test

2.1.1. Chemicals

Monochloroacetic acid (MCAA, CAS: 79-11-8; purity >99 %) and trichloroacetic acid (TCAA, CAS: 76-03-9; purity >99 %) were obtained from Thermo Scientific™. Dichloroacetic acid (DCAA, CAS: 79-43-6; purity >99 %) was obtained from Alfa Aesar. Bromochloroacetic acid (BCAA, CAS: 5589-96-8; purity = 97 %) was obtained from Sigma-Aldrich. Halo ketone 1,1-dichloropropan-2-one (1,1-DCP, CAS: 513-88-2), also known as 1,1-dichloroacetone (purity = 95 %), was obtained from Novachemistry. The OECD TG 201 growth medium was used to prepare the stock solutions of DBPs (OECD, 2011).

2.1.2. Chemical analyses

Previous experiments demonstrated the stability of the exposure concentrations for the four HAA and the volatility of 1,1-DCP individually (Ciccia et al., 2023). In order to check DBPs exposure during the algal growth inhibition tests in mixtures, two mixtures rays were

selected for chemical analyses, one with two HAAs (MCAA and TCAA) and one with 1,1-DCP and MCAA. In both cases, one replicate of three out of the six tested concentrations (low, medium and high) were sampled after inoculation of the microalgae (0 h) and at the end of the test (72 h). Chemical analyses were carried out by liquid-liquid extraction-gas chromatography-mass spectrometry (LLE-GC-MS). By examining the results of the chemical analyses, it was established that, for HAA mixtures, measured exposure concentrations were stable and close to nominal exposure concentrations (<27 % deviation in the worst case). Thus, nominal concentrations were considered for HAAs. However, 1,1-DCP exposure concentrations were shown to decrease over time (>80 % deviation compared to nominal in the worst case). Therefore, the time-weighted mean concentrations have been used in the following work when dealing with 1,1-DCP (OECD, 2012).

2.1.3. Microalgal cultures

The freshwater microalgae *Raphidocelis subcapitata* (previously known as *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*) was employed in the present study. The original strain was purchased from Culture Collection of Algae and Protozoa (CCAP 278/4; Dunbeg, UK). The stock culture used in this study was maintained in 250 mL glass flasks containing 100 mL Lefevre-Czarda medium at 23 ± 2 °C, on an orbital shaker (100–150 rpm) and subjected to a dark:light cycle of 8:16 h, while being renewed once a week. Pre-cultures were inoculated (0.6–1 mL of stock culture) in 100 mL OECD 201 medium (OECD, 2011) for 96 h in 250 mL Erlenmeyer flasks on an orbital shaker (125 rpm), maintaining a temperature between 21.3 and 23.3 °C and under continuous illumination ($60\text{--}120 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). These conditions and exposure duration ensured that algae were in the exponential growth phase when used for the assays. Microalgal cultures, pre-cultures, and experimental media were systematically manipulated in a sterile environment. Algal cell concentrations used for growth-rate determination were measured with a Coulter Z2 particle counter (Beckman Coulter France SAS, Villepinte, France).

2.1.4. Exposure of *R. subcapitata* to disinfection by-products

Algal growth inhibition tests were conducted according to OECD 201 test guideline (OECD, 2011). Prior to the experiments on mixtures, the five DBPs were tested individually under similar conditions to establish concentration-response curves and derive EC_x . The latter values have been used to set up the experimental design for testing mixtures. Experiments on individual DBPs are described in detail in Ciccia et al. (2023). Briefly, for each experiment, unicellular algae (initial concentration of $10^4 \text{ cell}\cdot\text{mL}^{-1}$) were exposed to an increasing concentration gradient of DBP (6 to 7 concentrations) for 72 h (without renewal) in Erlenmeyer glass flasks filled with a total volume of 100 mL. Incubation conditions were identical to those described above for pre-cultures. Three replicates with algae and one replicate without algae (negative control) were carried out for each concentration. For the control condition (algae without treatment), six replicates were prepared. The concentration ranges for each individual and binary mixture were selected to establish concentration-response curves based on growth inhibition (with the maximum concentration leading to effect close to EC_{70} or EC_{80} if possible). Cell concentration measurements at 72 h were performed using a Coulter Z2 particle counter.

2.1.5. Experimental design for binary mixtures

The experimental design selected for the study of the binary mixtures is called isobolographic or direct equipartition ray design (EquRay) (Dou et al., 2011; Gessner, 1995). After determining the concentration-response curve of each individual DBP and their respective EC_{50} , 3 different mixture rays (R1, R2 and R3) corresponding to 3 different equivalent concentration ratios (25 %:75 %, 50 %:50 %, 75 %:25 % for R1, R2 and R3, respectively) were designed for each binary combination of DBP (Fig. 1). 72 h-experiments were then carried out for each ray. Using such experimental design allowed us to limit the number of

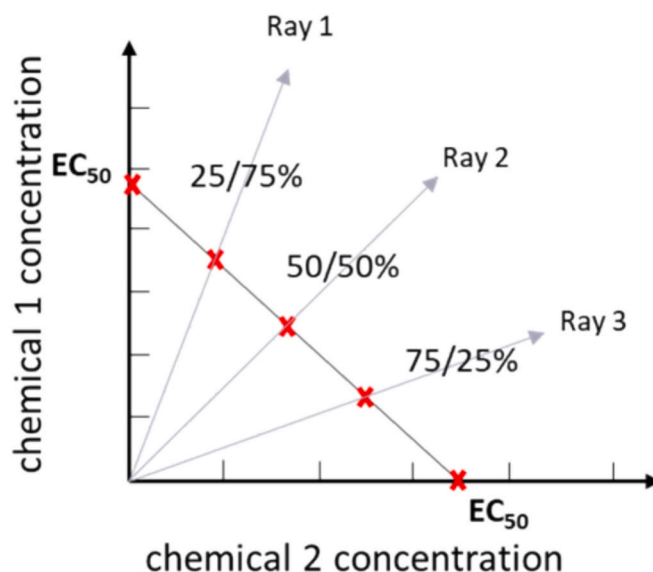


Fig. 1. Illustration of an isobolographic/direct equipartition ray design. On x and y axis are represented the concentrations of each mixture component. The three rays (25 %:75 %, 50 %:50 %, 75 %:25 %) are represented by the red crosses for which concentration-response curves are then established.

experiments necessary to give an overview of the mixture effects, which is not the case when using a full factorial or orthogonal design (Liu et al., 2016). Also, testing different ratios for the same combination of DBPs allows better representing the natural variations of these chemicals in the environment. By taking into account all the possible combinations for the five studied DBPs, ten binary combinations were selected with the previously mentioned design (MCAA:DCAA, MCAA:TCAA, MCAA:TCAA, MCAA:BCAA, MCAA:1,1-DCP, DCAA:TCAA, DCAA:BCAA, DCAA:1,1-DCP, TCAA:BCAA and TCAA:1,1-DCP) for a total of 30 mixture rays. Since the mixture MCAA-BCAA showed synergistic interactions (see Results), the experiment for this mixture was repeated for confirmation; the two tested experiments were noted MCAA:BCAA-1 and MCAA:BCAA-2.

2.2. Modeling of the concentration-response relationships

Concentration-response relationships for each experimental condition (i.e. individual chemicals and mixtures with different proportions of binary components) were modeled by a sigmoid function, i.e. the Hill function, relating growth inhibition (I) and chemical concentration (C):

$$I(C) = \frac{C^\gamma}{C_{50}^\gamma + C^\gamma} = \frac{\left(\frac{C}{C_{50}}\right)^\gamma}{1 + \left(\frac{C}{C_{50}}\right)^\gamma} \quad (1)$$

where I is the growth inhibition on algae; C_{50} is the concentration associated with 50 % inhibition effect; γ is the 'sigmoidicity factor' that determines the steepness of the relation. The 95 % and 50 % observation confidence intervals of each concentration-response curve were calculated.

2.3. Construction of non-linear isoboles and rules for detecting antagonism and/or synergy

Contrary to many publications in the field of ecotoxicology, we do not consider a priori here that the isoboles are linear and we do not consider arbitrary thresholds for MDRs (e.g. <0.5 and >2 as in Belden et al., 2007) in order to detect antagonisms and/or synergies. The model described below is based on the mathematics described in Geary (2013),

Lederer et al. (2018) and Schindler (2022, 2017). The aim of the demonstration described below is to determine the equation for the two isoboles, considered as potentially non-linear, i.e. the set of combinations $(C_A; C_B)$ leading to EC_{50} when B is substituted by A, and when A is substituted by B respectively. For this purpose, we assume that we can exchange A by B, and vice-versa, to reach the same effect, i.e. EC_{50} .

If we consider a binary mixture of two substances A and B, additivity (according to CA, or Loewe, principles) is defined as:

$$I_{mix}(C_A + C_B) = I_A(C_A) + I_B(C_B) \quad (2)$$

where $I_A(C_A)$ is the growth inhibition on algae of substance A at concentration C_A ; $I_B(C_B)$ is the growth inhibition on algae of substance B at concentration C_B ; $I_{mix}(C_A + C_B)$ is the growth inhibition on algae of substances A and B in mixture at concentrations C_A and C_B respectively.

This relationship also means that we can exchange A by B, and vice-versa, to reach the same effect, i.e. if B is substituted by A:

$$I_{mix,B \rightarrow A}(C_A + C_B) = I_A(C_A + C_A^{equ}(C_B)) \quad (3)$$

where $I_{mix,B \rightarrow A}$ is the effect of the mixture when B is substituted by A; I_A refers to Eq. (1) with parameters calibrated from the concentration-response relationship with only A, i.e.

$$I_A(C) = \frac{C^{\gamma_A}}{C_{50,A}^{\gamma_A} + C^{\gamma_A}} = \frac{\left(\frac{C}{C_{50,A}}\right)^{\gamma_A}}{1 + \left(\frac{C}{C_{50,A}}\right)^{\gamma_A}} \quad (4)$$

and $C_A^{equ}(C_B)$ is the equivalent concentration C_A^{equ} of A having the same effect of concentration C_B of B. The definition of $C_A^{equ}(C_B)$ (i.e. concentration C_A^{equ} of A has the same effect of concentration C_B of B) can be mathematically translated by:

$$I_A(C_A^{equ}) = I_B(C_B), \text{ or} \quad (5)$$

$$C_A^{equ} = I_A^{-1}(I_B(C_B)) \quad (6)$$

where I_A^{-1} is the inverse function of the function $I_A(C)$ (Eq. (4)), i.e.

$$I_A^{-1}(C) = C_{50,A} \cdot \left(\frac{C}{1-C}\right)^{1/\gamma_A} \quad (7)$$

Combining Eqs. (6) and (7), we obtain:

$$C_A^{equ}(C_B) = C_B^{\gamma_B/\gamma_A} \cdot \frac{C_{50,A}}{C_{50,B}^{\gamma_B/\gamma_A}} \quad (8)$$

Combining Eqs. (3) and (8), we obtain:

$$\begin{aligned} I_{mix,B \rightarrow A}(C_A + C_B) &= I_A(C_A + C_A^{equ}(C_B)) = I_A\left(C_A + C_B^{\gamma_B/\gamma_A} \cdot \frac{C_{50,A}}{C_{50,B}^{\gamma_B/\gamma_A}}\right) \\ &= \frac{\left(C_A + C_B^{\gamma_B/\gamma_A} \cdot \frac{C_{50,A}}{C_{50,B}^{\gamma_B/\gamma_A}}\right)^{\gamma_A}}{C_{50,A}^{\gamma_A} + \left(C_A + C_B^{\gamma_B/\gamma_A} \cdot \frac{C_{50,A}}{C_{50,B}^{\gamma_B/\gamma_A}}\right)^{\gamma_A}} \end{aligned} \quad (9)$$

If we want to determine the isobole corresponding to EC_{50} , we can write:

$$I_{mix,B \rightarrow A}(C_A + C_B) = 0.5 = \frac{\left(C_A + C_B^{\gamma_B/\gamma_A} \cdot \frac{C_{50,A}}{C_{50,B}^{\gamma_B/\gamma_A}}\right)^{\gamma_A}}{C_{50,A}^{\gamma_A} + \left(C_A + C_B^{\gamma_B/\gamma_A} \cdot \frac{C_{50,A}}{C_{50,B}^{\gamma_B/\gamma_A}}\right)^{\gamma_A}} \quad (10)$$

And we obtain the corresponding isobole, i.e. the set of combinations

$(C_A; C_B)$ leading to EC_{50} when B is substituted by A. It can be seen that this isobole is linear only if $\gamma_A = \gamma_B$.

Similarly, when A is substituted by B, we obtain:

$$I_{mix,A \rightarrow B}(C_A + C_B) = 0.5 = \frac{\left(C_B^{\gamma_B/\gamma_B} \cdot \frac{C_{50,B}}{C_{50,A}^{\gamma_B/\gamma_B}}\right)^{\gamma_B}}{C_{50,B}^{\gamma_B} + \left(C_B^{\gamma_B/\gamma_B} \cdot \frac{C_{50,B}}{C_{50,A}^{\gamma_B/\gamma_B}}\right)^{\gamma_B}} \quad (11)$$

We can observe that the isoboles for B substituted by A and A substituted by B respectively are not the same if $\gamma_A \neq \gamma_B$. This is illustrated in Fig. 2. The interpretation of such different isoboles is problematic. Tallarida (2007) considers that each isobole represents additivity (according to two different basic assumptions, i.e. A substituting for B and vice versa) and that the area contained between them is a region of additivity. On the contrary, Geary (2013) prefers to interpret the area between the two isoboles as indeterminate solutions (i.e. where deviation against additivity is not demonstrated). Synergy is restricted to the area below both isoboles and antagonism to the area above them.

2.4. An alternative model for detecting antagonism and/or synergy: Minto's approach

Minto et al. (2000) proposed an interaction model for drugs and chemicals whose concentration-effect curves fit the Hill equation. The interaction model is an extension of the model for a single chemical to a binary mixture that considers each ratio of two chemicals as a virtual chemical. For this purpose, concentrations of A and B in the mixture are normalized to their respective potency $C_{50,A}$ and $C_{50,B}$, and dimensionless units of potency U_A and U_B are defined as:

$$U_A = \frac{C_A}{C_{50,A}} \quad (12)$$

$$U_B = \frac{C_B}{C_{50,B}} \quad (13)$$

Note that the normalization could be done with any $C_{x,A}$ and $C_{x,B}$ levels, but the classical 50 % level was chosen here.

The ratio between A and B is described by the parameter θ defined by:

$$\theta = \frac{U_B}{U_A + U_B} \quad (14)$$

By definition, θ ranges from 0 (chemical A only) to 1 (chemical B only).

The extension of the Hill model (Eq. 1) to describe the concentration-response relation for any ratio θ of the binary mixture is given by:

$$I(U_A, U_B) = \frac{(U_A + U_B)^{\gamma(\theta)}}{U_{50}^{\gamma(\theta)} + (U_A + U_B)^{\gamma(\theta)}} = \frac{\left(\frac{U_A + U_B}{U_{50}(\theta)}\right)^{\gamma(\theta)}}{1 + \left(\frac{U_A + U_B}{U_{50}(\theta)}\right)^{\gamma(\theta)}} \quad (15)$$

where $U_A + U_B$ is the 'virtual chemical' normalized concentration (unitless); $U_{50}(\theta)$ is the number of units associated with 50 % of growth inhibition, depending on θ ; $\gamma(\theta)$ is the steepness of the concentration-response relation, depending on θ .

Note that for the substance A alone (and similarly for the substance B alone), Eq. (15) is equivalent to Eq. (1), i.e.

$$I(U_A, 0) = \frac{U_A^{\gamma_A}}{1 + U_A^{\gamma_A}} = \frac{\left(\frac{C_A}{C_{50}}\right)^{\gamma_A}}{1 + \left(\frac{C_A}{C_{50}}\right)^{\gamma_A}} \quad (16)$$

In other words, the values of $U_{50}(\theta = 0)$ (i.e. when only chemical A is

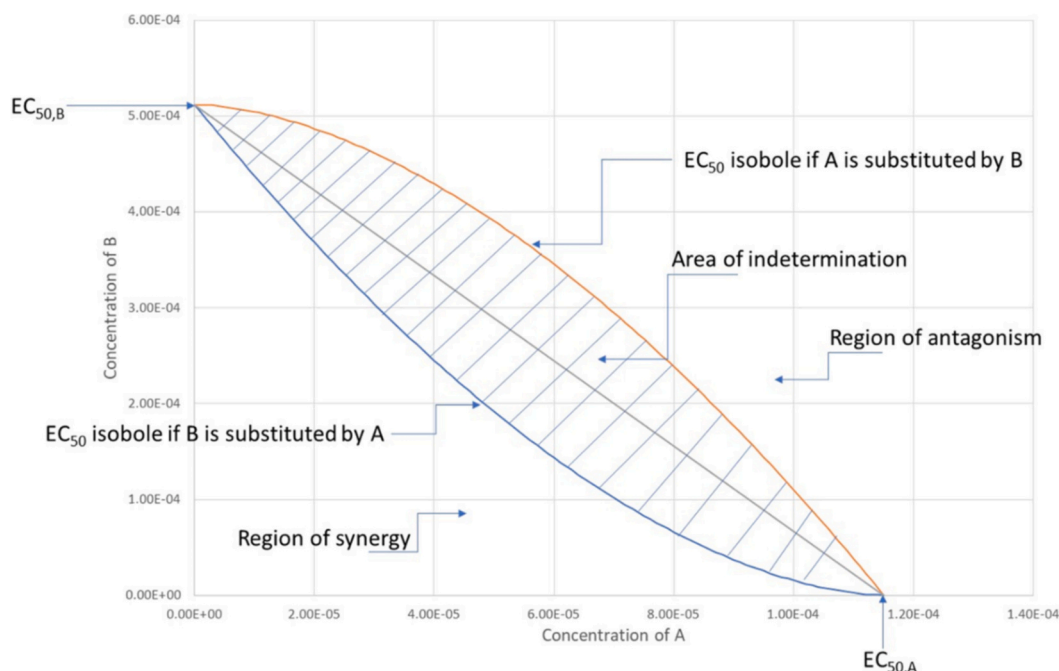


Fig. 2. EC₅₀ isoboles for two alternative assumptions, i.e. A is substituted by B or B is substituted by A.

present) and $U_{50}(\theta = 1)$ (i.e. when only chemical B is present) are:

$$U_{50}(\theta = 0) = 1 \tag{17}$$

$$U_{50}(\theta = 1) = 1 \tag{18}$$

Between these two limits (i.e. for $0 < \theta < 1$), several situations can be met:

- if $U_{50}(\theta) < 1$ (and a fortiori $U_{50}^\gamma(\theta) < 1$) for $0 < \theta < 1$, the effect is to decrease the denominator $(U_{50}(\theta))^{\gamma(\theta)} + (U_A + U_B)^{\gamma(\theta)}$ compared to individual compound in Eq. (15), making it appear that there is more chemical present. This will produce a greater than additive effect, i.e. synergy;
- if $U_{50}(\theta) > 1$ (and a fortiori $U_{50}(\theta)^{\gamma(\theta)} > 1$) for $0 < \theta < 1$, the effect is to increase the denominator $(U_{50}(\theta))^{\gamma(\theta)} + (U_A + U_B)^{\gamma(\theta)}$ compared to individual compound in Eq. (15), making it appear that there is less chemical present. This will produce a less than additive effect, i.e. antagonism;
- if $U_{50}(\theta) = 1$ for $0 < \theta < 1$, the effect is additive.

It should be noted that Geary (2013) criticized the terminology of ‘synergy’ and ‘antagonism’ in the case of the Minto model because the mathematical validity with regard to Loewe’s axioms has not been demonstrated. In this case, he prefers to speak of ‘cooperative effect synergy’. However, in the remainder of this paper, we will maintain the classic terminology of additivity, synergy and antagonism, even if this discussion would be relevant.

The advantage of using $U_{50}^\gamma(\theta)$ rather than $CE_{50,mix}$ is that U_{50}^γ is a normalized indicator, enabling different mixtures to be compared with each other, based on the deviation from unity. Minto et al. (2000) suggest describing the relationship between U_{50} and θ by a polynomial function. In our case (after analysis of experimental data), three-order polynomial functions were used to fit the $U_{50}^\gamma(\theta)$ relationships for each binary mixture, i.e.:

$$U_{50}^\gamma(\theta) = a_0 + a_1 \cdot \theta + a_2 \cdot \theta^2 + a_3 \cdot \theta^3 \tag{19}$$

Note that unlike Minto et al. (2000) and others (Lee, 2010), who establish separate relationships $U_{50}(\theta)$ and $\gamma(\theta)$, in this paper we

establish a single relationship $U_{50}^\gamma(\theta)$ (i.e. merging the parameters U_{50} and γ in a common relationship) because this term indicates the deviation from additivity in the Eq. (15).

Conditions at limits can provide information for the reduction of parameters. Since $U_{50}(\theta = 0) = 1$ (Eq. 7), it gives $a_0 = 1$. Since $U_{50}(\theta = 1) = 1$, $a_1 = -a_2 - a_3$. Eq. (9) can then be written with two parameters a_2 and a_3 , i.e.

$$U_{50}^\gamma(\theta) = 1 - (a_2 + a_3) \cdot \theta + a_2 \cdot \theta^2 + a_3 \cdot \theta^3 \tag{20}$$

Additivity, synergism and antagonism can be analyzed from Eq. (20), which establishes the position of $U_{50}^\gamma(\theta)$ in relation to unity. In this paper, we propose to use a global indicator to measure the ‘strength’ of deviation from additivity, i.e. the Area Under the Reference Curve ($U_{50} = 1$) (noted here AUC – Note that $AUC < 0$ of the function is negative and $AUC > 0$ of the function is positive) (see illustration on Fig. 3). Three cases may be encountered when calculating the AUC , as described below.

Note that mathematically $U_{50}^\gamma(\theta) = 1$ for three θ values (since it is a three-order polynomial function). Two of them are 0 and 1. The third one is:

$$\theta_{U_{50}^\gamma=1} = -1 - \frac{a_2}{a_3} \tag{21}$$

For the sake of simplicity, we will note this root $\theta_{U_{50}^\gamma=1} = \theta_{lim}$. AUC s were calculated for the intervals $[0; \theta_{lim}]$ and $[\theta_{lim}; 1]$ if $\theta_{lim} \in [0; 1]$ and on $[0; 1]$ instead. Depending on the values of AUC_{inf} and AUC_{sup} , there are four possible situations:

1. $\theta_{lim} \notin [0; 1]$ and $AUC > 0$ (picture A in Fig. 2), the mixture is antagonistic whatever the proportions of A and B in the mixture;
2. $\theta_{lim} \notin [0; 1]$ and $AUC < 0$ (picture B in Fig. 2), the mixture is synergistic whatever the proportions of A and B in the mixture;
3. $\theta_{lim} \in [0; 1]$ and $AUC_{inf} > 0$ on $[0; \theta_{lim}]$ (picture C in Fig. 2), the mixture is antagonistic for $\theta \in [0; \theta_{lim}]$ and synergistic for $\theta \in [\theta_{lim}; 1]$;
4. $\theta_{lim} \in [0; 1]$ and $AUC_{inf} > 0$ on $[0; \theta_{lim}]$ (picture D in Fig. 2), the mixture is synergistic for $\theta \in [0; \theta_{lim}]$ and antagonistic for $\theta \in [\theta_{lim}; 1]$.

To facilitate the interpretation of the results for all pairs of concentrations ($C_A; C_B$), an interaction index can be proposed to ‘measure’ the

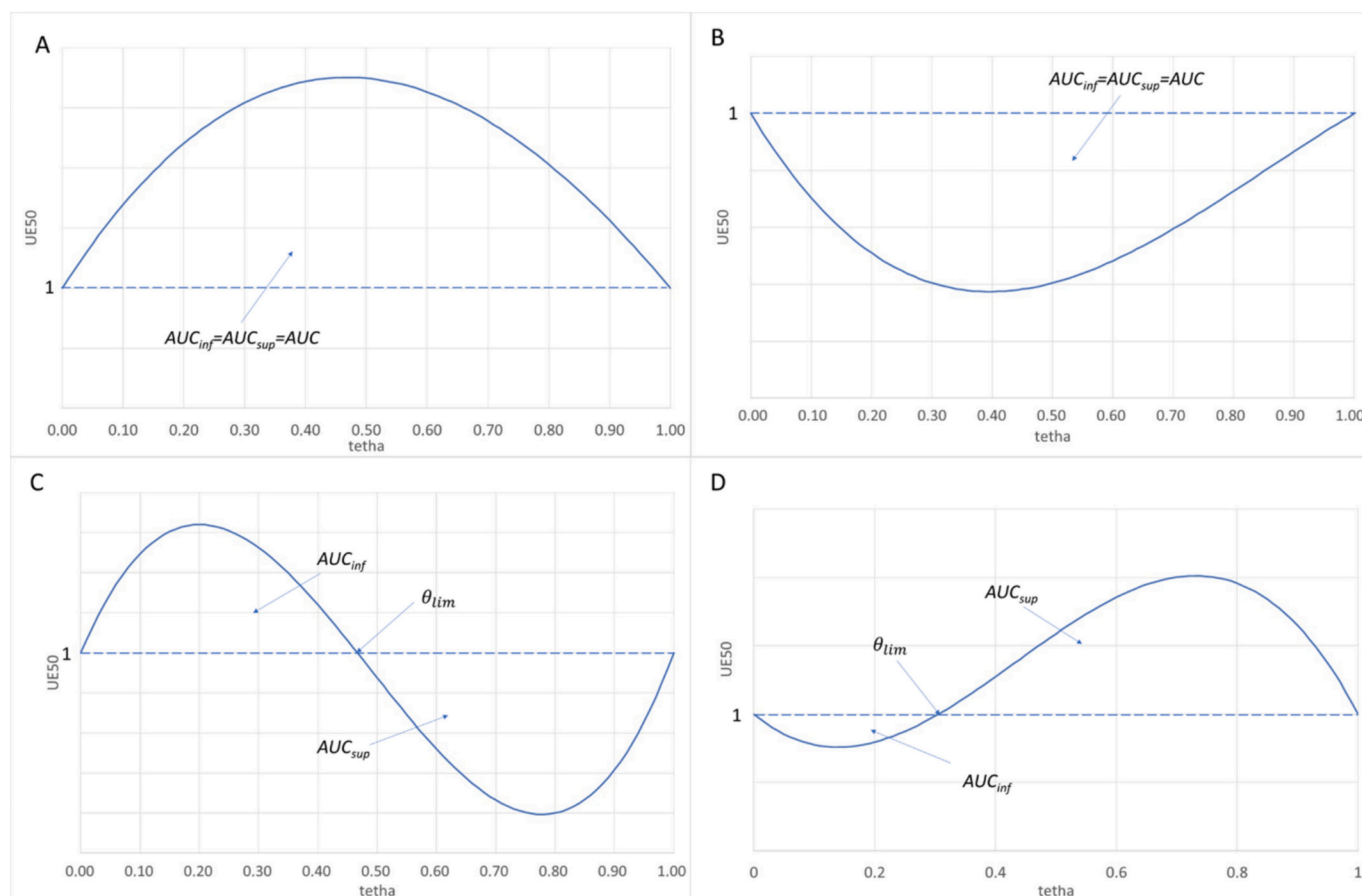


Fig. 3. Typical functions $UE_{50}^{\gamma} = f(\theta)$.

strength of deviation in relation to additivity:

$$\begin{aligned}
 \text{Interaction Index}(C_A; C_B) &= \frac{I(U_A, U_B)_{\text{simulated}}}{I(U_A, U_B)_{\text{expected under additivity assumption}}} \\
 &= \frac{U_{50}^{\gamma}(\theta) + (U_A + U_B)^{\gamma(\theta)}}{1 + (U_A + U_B)^{\gamma(\theta)}} \quad (22)
 \end{aligned}$$

3. Results and discussion

3.1. Concentration-effects relationships

The Hill concentration-effects relationships are presented in Supplementary Information for all the individual substances and binary mixtures (Figs. S1, S2 and Table S1). Specifically, parameter values of each individual (EC_{50} , γ) and binary mixture (EC_{50} , U_{50} , γ , θ) concentration-effects curve are described in Table S1. We can observe a general good reproducibility of the experimental replicates and a good fit between experimental points and the Hill functions. We can also observe that the slope of the Hill functions may be quite different according to the substances involved (e.g. the slopes of the DCAA and 1,1-DCP individual substances are quite different). We can conclude that the theory based on the Hill model described in the Material and methods is relevant and consistent in the frame of this work.

3.2. Isoboles for EC_{50} s

Results related to isoboles for EC_{50} s are presented in Fig. 4. The 50% confidence intervals of each binary mixture were considered for data analysis. First of all, we can observe that the zone of additivity (according to the interpretation of Tallarida, 2007) or indeterminacy

(according to the interpretation of Geary, 2013) varies greatly according to the pairs of chemicals (A;B) involved. According to Eqs. (10) and (11), these differences can be explained by the different slopes γ_A and γ_B of the Hill functions of the individual substances A and B. For example, TCAA and BCAA have almost the same γ value (1.29 and 1.34 respectively) and the curved isoboles are then merged, and the zone of indeterminacy is almost null. On the contrary, DCAA and 1,1-DCP show significant differences in γ values (0.51 and 2.06 respectively) and the isoboles built from the Hill functions $I_{mix,B \rightarrow A}$ and $I_{mix,A \rightarrow B}$ are distant. These two examples show that experimental data cannot be interpreted solely in terms of arbitrary thresholds (such as $MDR < 0.5$ or $MDR > 2$ as in Belden et al., 2007), but that the zone of indeterminacy (or additivity) depends on the Hill function parameters of the two substances involved.

Considering the zones of indeterminacy and the confidence interval of each experiment, we can see that most of the experimental points are located in this zone, or at its borders, with the exception of a few cases. For the MCAA-BCAA combination (MCAA-BCAA-1 and MCAA-BCAA-2), the experimental points lie below the isoboles (except for one point for MCAA-BCAA-1); we can therefore conclude that there is synergy. Some combinations are difficult to interpret. For the MCAA-DCAA, BCAA-DCAA and BCAA-1,1-DCP combinations, two of the three experimental points are in the zone of indeterminacy and one lie below the isobole. On the contrary, MCAA-TCAA and TCAA-BCAA combinations are in the zone of indeterminacy for two of the ratios and one point is above this zone, indicating antagonism. For the combination TCAA-1,1-DCP, one point is in the zone of indeterminacy, one below (indicating synergism) and one above the zone (indicating antagonism). In those cases, the interaction is depending on the combination of concentrations ($C_A; C_B$) which clearly show the complexity of the ecotoxicological assessment of even the simplest mixture.

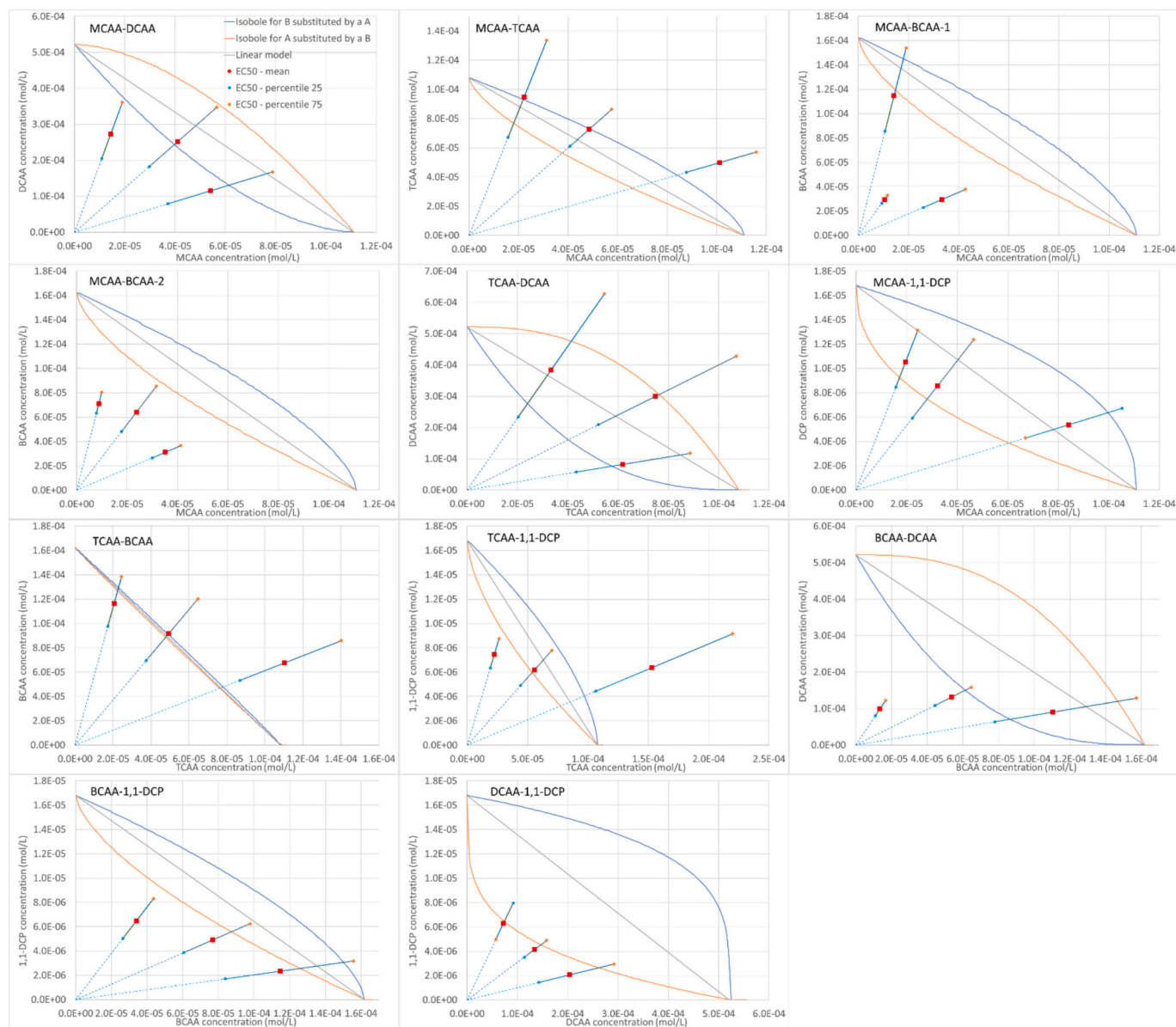


Fig. 4. Curved isoboles and experimental data for the tested mixtures for EC_{50} s.

3.3. Comparison of isoboles for EC_{20} s and EC_{50} s

Results related to isoboles for EC_{50} s and EC_{20} s are presented in Figs. 4 and 5, respectively.

The comparison between two different effect thresholds (i.e. EC_{50} s and EC_{20} s) shows two contrasted situations. For some combinations of chemicals, i.e. MCAA-BCAA-2, MCAA-1,1-DCP, TCAA-BCAA, TCAA-1,1-DCP and BCAA-1,1-DCP, the isoboles for EC_{50} and EC_{20} are globally consistent. In the other cases, there were significant differences, or even reversals of trends (from synergy to antagonism and vice versa) for one or more experimental points. For example, for the DCAA-1,1-DCP combination, the experimental points are at the lower limit of the EC_{50} isoboles, indicating a slight synergy, while two experimental points are in the antagonistic zone for EC_{20} . These examples show the difficulty of interpreting isoboles, which give only a partial picture of potential interactions between chemical substances.

3.4. Minto's model

Results of the functions $U_{50}^{\gamma}(\theta)$ are presented in Fig. 6 and

parameters of this functions, i.e. θ_{lim} and AUC (mean value and interquartile), are presented in Table 1. For AUC_{tot} , not only are the best estimate values presented, but also the interquartile ranges, calculated from the interquartile ranges of each U_{50}^{γ} . When the null value is included in the interquartile range, no conclusion can be drawn as to the synergistic or antagonistic nature of the mixture over the whole range $\theta \in [0; 1]$.

Mixtures can be classified into three major groups: (i) some mixtures, i.e. MCAA-DCAA, MCAA-BCAA-1, MCAA-BCAA-2, BCAA-DCAA, and DCAA-1,1-DCP, meet the conditions $\theta_{lim} \notin [0; 1]$ and $AUC < 0$, meaning that they are, on average, synergistic over the whole range of proportions in the mixture. If we consider the interquartile ranges, we observe that the null value is included in the range of the MCAA-DCAA mixture, indicating that this mixture cannot be considered synergistic if uncertainties are taken into account. On the contrary, the null value is not included in the interquartile range of the other mixtures, indicating their synergistic potential, even when uncertainties are taken into account. The strength of the synergy can be quantified by the value of AUC , and the mixtures considered have similar values (from -0.32 to -0.41) indicating similar synergetic potential; (ii) several mixtures

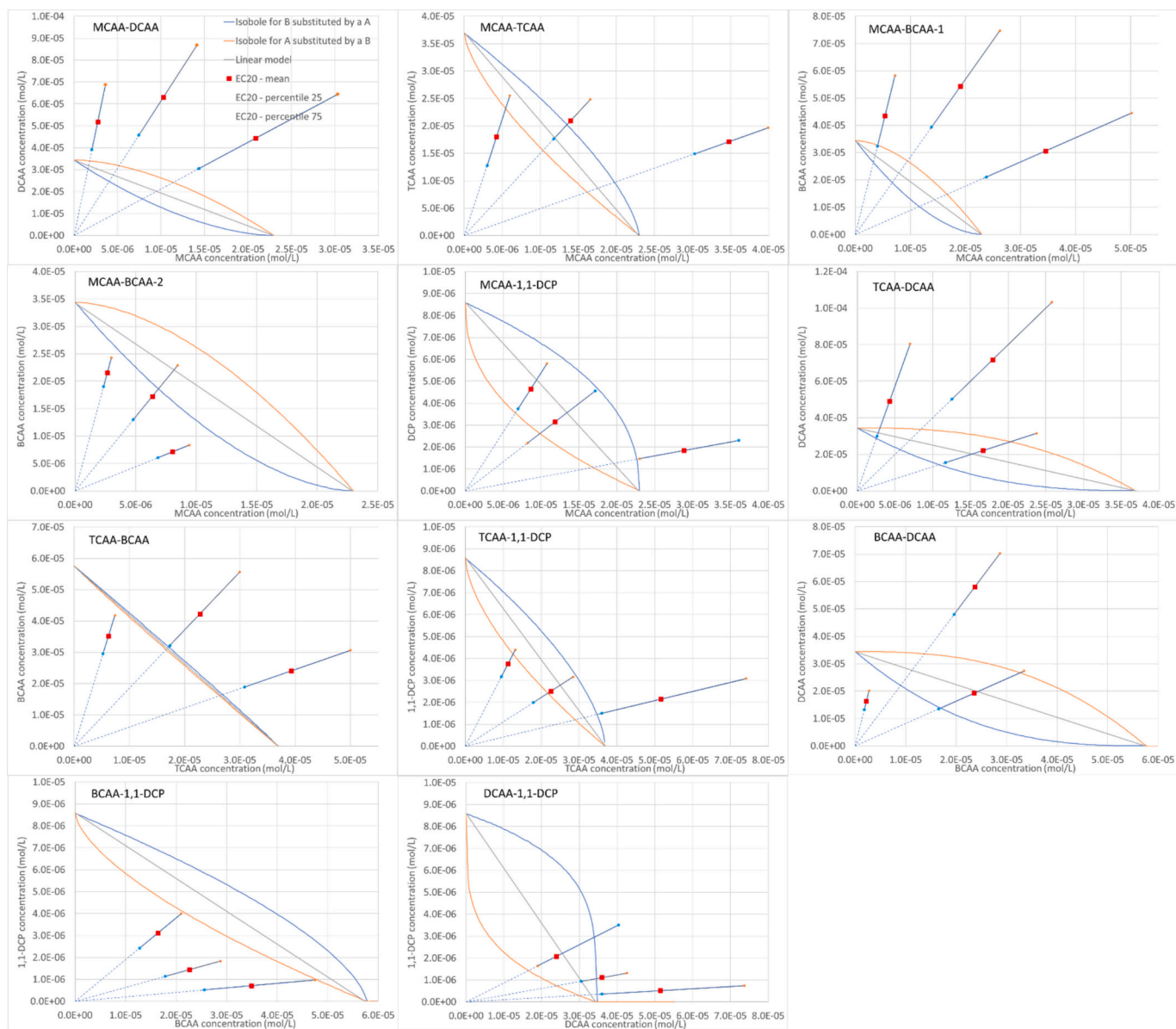


Fig. 5. Curved isoboles and experimental data for the tested mixtures for EC₂₀s.

show values of θ_{lim} in the range $[0; 1]$ (with θ_{lim} close to 0.5), and significant deviation from unity, which means that they are synergistic for certain proportions of the substances involved, and antagonistic for other proportions. This is the case for MCAA-TCAA, TCAA-BCAA and TCAA-1,1-DCP (with $|AUC|$ values in the same order, even more for TCAA-1,1-DCP, as those observed for interaction effects identified in item (i)); (iii) three chemical combinations, i.e. MCAA-1,1-DCP, BCAA-1,1-DCP and TCAA-DCAA show a low deviation from unity, indicating additivity.

It can be observed that BCAA is rather associated to synergetic interactions (i.e. negative AUC values for MCAA-BCAA, BCAA-DCAA and in the mixture TCAA-BCAA in the zone where BCAA is the major component). On the contrary, TCAA is rather associated to antagonist interactions (i.e. positive AUC values for the majority fraction of TCAA in the mixtures TCAA-BCAA and TCAA-1,1-DCP).

3.5. Isobole vs Minto models

These examples show the advantages of the Minto's approach over the isoboles approach. Indeed, Minto's approach makes it possible to

derive an overall indicator representing the interaction effects over the entire Hill concentration-response curve, as a function of the relative proportions of the two substances involved in the binary mixture. The analysis of the $U_{50}^{\gamma}(\theta)$ function can also be supplemented by the interaction indicator proposed in Eq. (22). Two examples of surface response obtained from this Interaction index are provided in Fig. 7 for BCAA-DCAA and BCAA-1,1-DCP. It can be observed that interaction is maximized for some specific $(C_A; C_B)$ combinations. It can be explained because at extreme concentrations, interactions are expected to be low: when C_A and/or C_B are high or quite different, the effect of the majority substance is much greater than the potential interaction effect. The interaction therefore only occurs if the two substances are 'balanced', i.e. if their respective concentrations cause similar individual effects. The comparison between two couples of chemicals (e.g. here BCAA-DCAA on one part and BCAA-1,1-DCP on the other part) also allows to visualize the differences in term of interaction according to the surface response curvature.

Compared to conventional approaches such as CA and IA, Minto's model and the non-linear isobole approach offer the advantage of not interpreting the data solely using arbitrary thresholds (such as MDR <

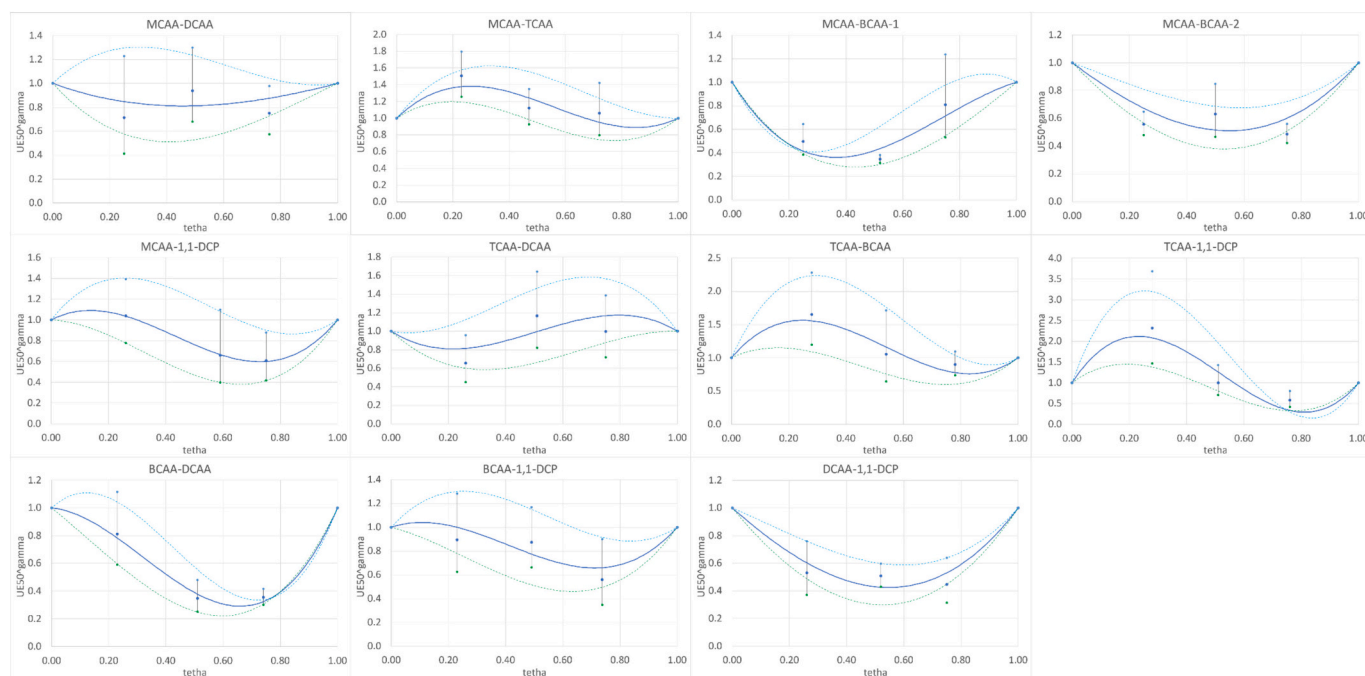


Fig. 6. $UE_{50}^\gamma = f(\theta)$ relationships for the different tested mixtures.

Table 1
Parameters of the $U_{50}^\gamma(\theta)$ functions.

Mixture	θ_{lim}	AUC_{inf}	AUC_{sup}	AUC_{tot}	Interquartile of AUC_{tot}
MCAA-DCAA	>1	-0.125		-0.125	[-0.31;0.15]
MCAA-TCAA	0.67	0.24	-0.10	0.14	[-0.04;0.35]
MCAA-BCAA1	>1	-0.39		-0.39	[-0.47;-0.27]
MCAA-BCAA2	<1	-0.32		-0.32	[-0.41;-0.21]
MCAA-1,1-DCP	0.30	0.06	-0.22	-0.16	[-0.35;0.14]
TCAA-DCAA	0.51	-0.12	0.11	-0.01	[-0.23;0.30]
TCAA-BCAA	0.62	0.36	-0.20	0.16	[-0.13;0.59]
TCAA-1,1-DCP	0.57	0.72	-0.51	0.20	[-0.11;0.69]
BCAA-DCAA	<1	-0.41		-0.41	[-0.28;-0.50]
BCAA-1,1-DCP	0.23	0.02	-0.18	-0.16	[-0.32;0.09]
DCAA-1,1-DCP	<1	-0.38		-0.38	[-0.47;-0.26]

0.5 or $MDR > 2$), but rather using the confidence intervals determined using the experimental variability. Furthermore, the non-linear isobole approach also factors in the specific parameters of the concentration-response curves of the DBPs making up the mixture, resulting in the

zone of indetermination. Therefore, these approaches allow interaction to be categorized via a mechanistic threshold, which is not the case for CA and IA conventional use.

In a regulatory context, the use of reference values such as CEX is common. The non-linear isobole approach would therefore be more relevant than Minto's, and would add an advantage in terms of categorising the interaction compared with CA and IA.

4. Conclusions

In this paper, two modeling approaches were compared to assess the potential interactions (synergy or antagonism) involved in binary mixtures of chemical substances. The approach based on the construction of isoboles, classically used in ecotoxicology, has shown its limitations. Indeed, in cases where the individual substances involved have different slopes in terms of their Hill concentration-effect relationships, the so-called zone of indetermination can be large. Non-linear isoboles would be relevant in a regulatory context because classification of the interaction around reference values such as EC_{50} would no longer rely on

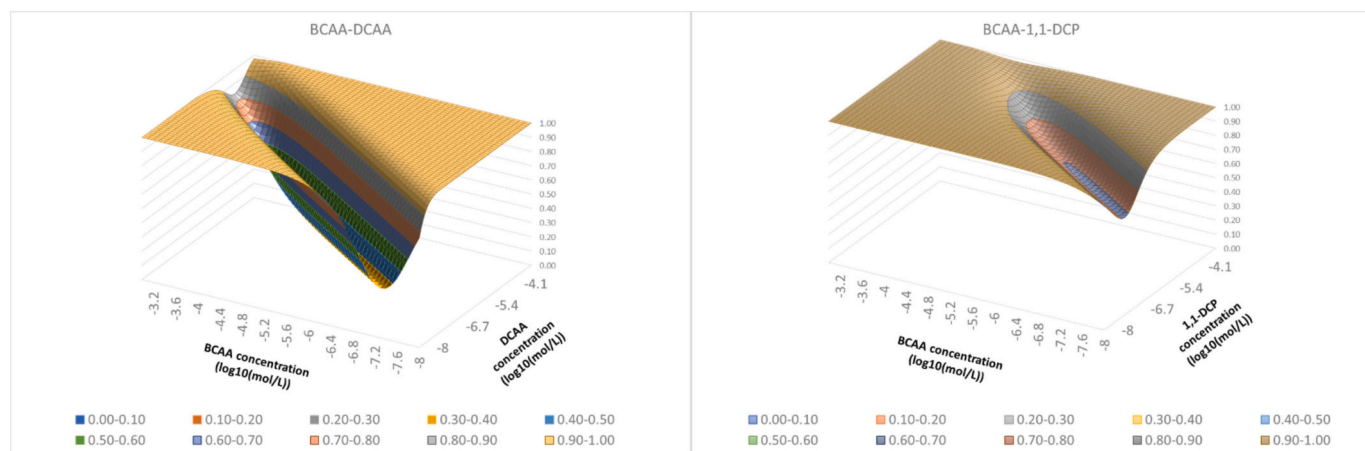


Fig. 7. Response surfaces of Interaction index for BCAA-DCAA (left) and BCAA-1,1-DCP (right) built from the Minto's model.

arbitrary criteria. Yet, conclusions drawn from isoboles based on EC₅₀ or EC₂₀ may not be consistent. In some cases, it is therefore difficult to obtain an overall view of the effects of interactions in binary mixtures using this approach. Minto's approach, on the other hand, makes it possible to construct interaction indicators that consider the entire Hill concentration-response curve (including its curvature characteristics), as a function of the relative proportions of the two substances involved in the binary mixture. Response surfaces can be constructed to visualize the areas of concentration of the two substances involved that maximize the interaction effects. The use of these modeling approaches was illustrated using original experimental data relating to the inhibition of algal growth caused by chlorinated and/or brominated DBPs. The extension of the Hill formalism to mixtures of *n* agents is possible (Minto et al., 2000; Schindler, 2022, 2017) and its application is a perspective for future works.

CRedit authorship contribution statement

Théo Ciccia: Writing – original draft, Methodology, Investigation, Formal analysis. **Anne Bado-Nilles:** Writing – review & editing, Supervision, Resources. **Pascal Pandard:** Writing – review & editing, Supervision, Resources, Methodology. **Nastassia Urien:** Writing – review & editing, Supervision. **Léo Lafay:** Writing – review & editing, Supervision. **Philippe Ciffroy:** Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2025.178437>.

Data availability

Data will be made available on request.

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