TOXICITY OF INDIVIDUAL PHARMACEUTICALS AND THEIR MIXTURES TO *ALIIVIBRIO FISCHERI*: EVIDENCE OF TOXICOLOGICAL INTERACTIONS IN BINARY COMBINATIONS

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Abstract: The combined toxicities of binary mixtures of veterinary pharmaceutical active compounds were examined using the bioluminescent bacterium *Aliivibrio fischeri* as a test organism (Microtox® test). Mixtures were prepared at an equitoxic ratio that corresponded to the inhibitory concentration, 10% (IC10) of individual pharmaceutical active compounds. In addition, the toxicity was determined of a multicomponent mixture that contained all of the investigated pharmaceutical active compounds mixed at a ratio corresponding to their individual predicted no-effect concentration (PNEC) values. The experimental results were successively compared with those obtained by applying the 2 most widely used models for predicting mixture toxicity, the concentration addition (CA) and independent action (IA) models. Although the toxicity of the multicomponent mixture tested was well predicted by the CA and IA models, deviations from the model predictions were found for almost all of the binary mixtures. The deviations from the CA and IA models were greater at lower concentrations, particularly when diclofenac sodium and amoxicillin were present in the mixture. Based on these results, another hypothesis was tested, that of toxicological interactions occurring in binary mixtures (in the direction of synergistic or antagonistic effects), by applying the combination index method, which allowed for computerized quantification of synergism, the additive effect and antagonism. The application of this method confirmed, for at least half of the binary combinations, the clear presence of synergistic deviations at the lowest tested concentrations, with a tendency toward antagonism at the higher ones. In 1 case, a relevant antagonistic interaction was observed. *Environ Toxicol Chem* 2017;36:815–822. © 2016 SETAC

Keywords: *Aliivibrio fischeri*   Mixture toxicity   Veterinary pharmaceuticals   Combination index   Synergism

INTRODUCTION

Organisms are typically exposed to a cocktail of chemicals rather than 1 individual substance. This is particularly true in surface water systems, where monitoring studies have generally detected a multitude of heterogeneous and potentially toxic substances [1–4]. Hence, the risk posed by the presence of mixtures in environmental compartments should be evaluated. During the last decade, the European Commission became aware of the problem of chemical mixtures in the environment [5] and defined the challenges that require scientific support in its communication on the combination effects of chemicals [6]. However, presently, environmental risk assessment procedures are generally developed for single substances, which may lead to a severe underestimation of the real risk facing the environment [7,8].

Our understanding of the ecotoxicology of mixtures is in the first stages, because of the complexity of the variables that need to be taken into consideration [9]. The experimental assessment of the ecotoxicity of every potentially occurring mixture is not feasible and is still limited because of the wide temporal and spatial variability of compounds in a given area. In addition, obtaining a full prediction of mixture effects by using models is not easy. Two simple component-based predictive models for assessing the responses of mixtures are commonly used: the concentration addition (CA) or dose addition [10] and independent action (IA) [11] models. The CA approach is thought to be applicable to mixtures of chemicals that share a similar or common mode of action. Conversely IA, which is also known as response addition, is widely held to be appropriate for mixtures of agents that show a dissimilar mode of action. These concepts are both based on knowledge of the concentration–response relationships of the single components. In many cases mixtures in which components had similar or dissimilar modes of action were correctly predicted with the concepts of CA and IA, respectively [12–14]. The results from the IA model usually lead to slightly lower responses than those predicted by CA, and thus CA is often used as the acceptable worst case. However, in some cases, the so-called prediction window [15,16] is very small, which indicates that both models are able to predict the response of mixtures independently based on the mode of action of single components [7,17,18].

By definition, the CA and IA concepts ignore synergistic and antagonistic effects, and both models assume that the components of a mixture do not interact chemically or in toxicokinetic/toxicodynamic phases [9]. For the quantification of both phenomena, Chou and Talalay introduced the combination index method [19]. This approach was historically applied in pharmacology to understand the nature of the interactions among pharmaceutical active compounds [20], and it was more recently applied in the environmental field to study the interactions among chemical contaminants present in environmental mixtures [21–23]. In the first part of our study, the toxicity of 10 different pharmaceutical active compounds (for both veterinary and human use) to *Aliivibrio fischeri* (formerly *Vibrio fischeri* or *Photobacterium phosphoreum*) was determined using the Microtox® test system [24]. In this second part, we focused on pharmaceutical active compounds registered for veterinary use. In particular, we investigated the effects of a series of their binary or multicomponent mixtures on *A. fischeri*. In the previous study [24], we demonstrated that almost all the selected pharmaceutical active compounds act as polar-narcotic compounds toward *A. fischeri*. The only exception was the antibiotic chlortetracycline, which had a specific mode of action. Based on these findings, the mixture

responses containing narcotic-type pharmaceutical active compounds should be well predicted by the CA model. In contrast, the IA approach should be more suitable for the prediction of mixture toxicities containing chlortetracycline. The binary mixtures were prepared at an equitoxic ratio that corresponded to the individual inhibitory concentration, 10% (IC10) obtained in our previous work [24]. The multicomponent mixture was prepared at a ratio corresponding to the individual predicted no-effect concentration (PNEC) value. The experimental results were compared with those obtained by the application of the CA and IA models, to assess the additive behavior of chemicals in a mixture or the existence of deviations from conceptual expectations. Finally, the nature of the pharmaceutical active compound interactions was also investigated by applying the combination index method to verify the existence of possible synergistic and/or antagonistic effects.

MATERIALS AND METHODS

Chemicals and testing procedures

The tested veterinary pharmaceutical active compound compounds were the same as those reported by Di Nica et al. [24]: amoxicillin, chlortetracycline, sulfamethizole, diclofenac sodium, and acetylsalicylic acid. The CAS numbers, main physicochemical properties, and individual acute toxicity data (IC50 and IC10) for *A. fischeri* have been reported by Di Nica et al. [24]. The stock solutions were prepared using the method described in Vighi et al. [25]. Acute toxicity (15 min) to the bioluminescent bacterium *A. fischeri* (purchased from Ecotox) was measured using the Microtox model 500 analyzer (Ecotox). The testing procedure was described by Di Nica et al. [24]. For binary mixtures, the tested concentrations (from 9 to 22 experimental concentrations) ranged from 0.02 to 35 toxic units, where the toxic units of the mixture correspond to the sum of the ratio between the actual concentration of the chemicals present in the mixture and their relative IC10. Solutions were buffered with 0.1 M sodium phosphate and pH was adjusted to a range of 6 to 8.50 (DIN EN ISO 11358-3) using a pH meter model 250 (Denver Instruments). All tests are referred to nominal (= initial) concentrations of pharmaceutical active compounds. Tests were performed in 4 replicates. Stock solutions of sulfamethizole, amoxicillin, and acetylsalicylic acid were prepared by using dimethyl sulfoxide (DMSO). For mixtures containing these pharmaceutical active compounds, the final test samples contained a concentration of DMSO that was always below 1.5% (v/v). As reported in our previous study [24], at this level of concentration, the influence of DMSO for low-water-solubility compounds is negligible.

Experimental design for selected mixtures

Eleven mixtures were prepared by mixing individual chemicals at equitoxic concentrations in the saline solution (2% NaCl) that was used for the toxicity test. This concentration ratio was kept constant, and, using a serial dilution, different concentrations of stock solutions were tested (fixed ratio design).

The tested mixtures were as follows. First, 10 combinations of binary mixtures of compounds mixed in a ratio corresponding to their individual IC10 value (acetylsalicylic acid–amoxicillin, acetylsalicylic acid–chlortetracycline, acetylsalicylic acid–diclofenac sodium, acetylsalicylic acid–sulfamethizole, chlortetracycline–amoxicillin, chlortetracycline–diclofenac sodium, chlortetracycline–sulfamethizole, diclofenac sodium–amoxicillin, diclofenac sodium–sulfamethizole, sulfamethizole–amoxicillin, with individual IC10 values of the selected pharmaceutical active compounds shown in the Supplemental Data, Table S1). Second, 1 multicomponent mixture of the 5

compounds mixed in a ratio corresponding to their individual PNEC value (mix–PNEC: chlortetracycline–diclofenac sodium–acetylsalicylic acid–sulfamethizole–amoxicillin).

In line with the current guidelines on environmental risk assessment of pharmaceuticals [26,27], PNECs were extrapolated by applying an appropriate assessment factor to the lowest relevant observed value within the toxicity dataset available in the literature. The calculated PNEC values are reported in the Supplemental Data, Table S2.

Concentration–response curve fitting

To quantitatively describe the concentration–response curves, the observed concentration–effect data were fitted to the nonlinear regression Weibull model (Equation 1):

$$I = (c, \beta) = 1 - \exp\{-\exp\{\ln(\beta_2(\ln(c) - \ln(\beta_1)))\}\} \quad (1)$$

where I is the fractional response ($0 \leq I \leq 1$) in terms of the inhibition of luminescence, c is the chemicals concentration and β_1 and β_2 are the parameters of the model. The I_x ($100 \times I_x\%$) and the confidence intervals were obtained using the R$^{\circledR}$ software package [28,29].

Prediction of mixture toxicities by CA and IA models

Based on the fixed ratio of the mixture components, it was possible to express the concentration of single chemicals as a fraction of the total concentration. Therefore, it was possible to apply the CA model using Equation 2 [15]:

$$ECx_{\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}}\right)^{-1} \quad (2)$$

where ECx_{mix} is the total concentration of the mixture that produces an $x\%$ of the effect, p_i (C_i/C_{mix}) is the fraction of the component i in the mixture, and $EC_{x,i}$ is the concentration of the ith component when present individually and elicits the same effect ($x\%$) as the mixture. It was possible to derive the predicted concentration-response curve using Equation 2.

The alternative IA model is commonly used to predict the mixture toxicity of substances that have different or dissimilar mechanisms of action. It may be applied using Equation 3:

$$E(C_{\mathrm{mix}}) = 1 - \Pi_{1=1}^{n}(1 - E(C_i)) \text{ with } C_{\mathrm{mix}}$$
$$= c_l.. \ \ldots \ \ldots + c_n \quad (3)$$

where C_i denotes the concentrations of the ith mixture component, $E(c_i)$ is its corresponding effect, and $E(C_{\mathrm{mix}})$ is the overall effect as a result of the mixture [11,15].

Application of the combination index-isobologram equation

To highlight possible synergistic or antagonistic effects, a further analysis of the response of *A. fischeri* to pharmaceutical active compound mixtures was carried out using the combination index approach [20,30,31] (Equation 4):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{\frac{[D]_j}{\sum_1^n [D]}\right\}}{(D_m)_j\left\{\frac{(fa_x)_j}{[1-(fa_x)_j]}\right\}^{1/m_j}} \quad (4)$$

where $^{n}(CI)_x$ is the combination index for n chemicals at $x\%$ inhibition, $(D_x)_{1-n}$ is the sum of the dose of n chemicals that

exerts $x\%$ inhibition in combination, $\{([D]_j/\sum[D]\}$ is the proportionality of the dose of each of n drugs that exerts $x\%$ inhibition in combination, and $(D_m)_j \{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts $x\%$ inhibition. D_m is the median-effect dose, f_{ax} is the fractional inhibition at $x\%$ inhibition, and m is the slope of the median-effect plot.

From Equation 4, combination index < 1, combination index = 1, and combination index > 1 indicate synergism, the additive effect, and antagonism, respectively.

The computer program CompuSyn was used to calculate the combination index values for the different mixtures at different ranges of effect levels (10%, 25%, 50%, 75%, and 90% of inhibition of bioluminescence) [32].

RESULTS AND DISCUSSION

Experimental mixture toxicities

Binary mixtures of pharmaceutical active compounds. Values of IC10 and IC50 were derived from the concentration–response curves of the tested binary pharmaceutical active compound combinations (Table 1). As shown in Table 1, the IC50 values obtained spanned over 2 orders of magnitude.

In particular, the binary mixture chlortetracycline–diclofenac sodium showed the highest toxicity (IC10 = 0.68 mg L^{-1} and IC50 = 10.1 mg L^{-1}), followed by the diclofenac sodium–acetylsalicylic acid combination (IC10 = 18.2 mg L^{-1} and IC50 = 111.6 mg L^{-1}). In contrast, the binary combinations containing amoxicillin showed the lowest IC50.

These results were not surprising considering that, in our previous study [24], chlortetracycline and diclofenac sodium tested singly were the most toxic pharmaceutical active compounds to *A. fischeri* (IC10 = 2.0 mg L^{-1} and IC50 = 12.1 mg L^{-1} for chlortetracycline; IC10 = 5.9 mg L^{-1} and IC50 = 15.9 mg L^{-1} for diclofenac sodium) whereas the amoxicillin was the least toxic 1 (IC50 = >1702 mg L^{-1}).

For the majority of the tested binary mixtures, a complete concentration–response relationship was obtained (Figure 1). In 4 cases the maximum percentage of inhibition reached was near 50% (acetylsalicylic acid–amoxicillin and sulfamethizole–amoxicillin 43%; acetylsalicylic acid–sulfamethizole and chlortetracycline–amoxicillin 48%).

Multicomponent mixtures of pharmaceutical active compounds. A complete concentration–response curve was obtained for the tested multicomponent mixture of veterinary
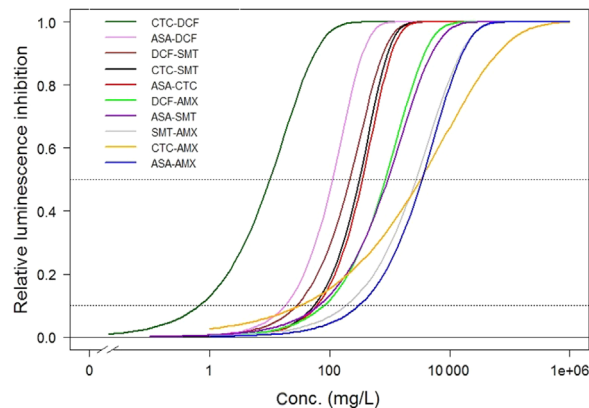

Figure 1. Concentration response curves (Weibull model) of the tested binary mixtures of veterinary pharmaceutical active compounds (dashed lines = intersections at values of individual acute toxicity data [IC50 and IC10, respectively]). AMX = amoxicillin; ASA = acetylsalicylic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

pharmaceutical active compounds (mixed at their PNEC ratio). The resulting IC10 and IC50 values were 25.0 mg L^{-1} and 220.7 mg L^{-1}, respectively. The results obtained showed that at concentrations corresponding to the sum of individual PNECs (0.16 mg L^{-1}), a very negligible effect was measured on *A. fischeri*; in addition, the IC10 was 2 orders of magnitude greater than the sum of the single-compound safety threshold for aquatic organisms, and at least 4 orders of magnitude above their joint measured environmental concentrations in European water bodies that are in the range of ng L^{-1} [24].

These results are not surprising considering that PNECs have been calculated by applying an assessment factor to acute or chronic toxicity data for the most sensitive species and that *A. fischeri* is not the most sensitive for all the tested compounds, as demonstrated in our previous study [24].

Predicted mixture toxicity by CA and IA models and comparison with experimental results

The experimental concentration–response curves obtained were compared with the classical models of CA and IA. Some of the results are depicted in Figure 2, with the confidence intervals also reported for the experimental concentration–responses curves. A more complete picture of the results can

Table 1. Inhibitory concentration (IC10 and IC50; 15-min acute Microtox test; mg L^{-1}) values with the corresponding 95% confidence intervals (in parentheses) for the 10 tested binary combinations of pharmaceutical active compounds

		CTC	DCF	ASA	SMT	AMX
	mg L^{-1}					
CTC	IC10	—				
	IC50	—				
DCF	IC10	0.68 (0.41–0.96)	—			
	IC50	10.1 (8.72–11.5)	—			
ASA	IC10	56.0 (50.3–61.8)	18.2 (15.6–20.8)	—		
	IC50	378.6 (340.7–406.4)	111.6 (105.9–117.2)	—		
SMT	IC10	55.6 (46.8–64.3)	28.3 (25.8–30.9)	66.1 (49.1–83.2)	—	
	IC50	311.1 (294.6–327.6)	215.4 (208.4–222.3)	927.6 (774.8–1080.5)	—	
AMX	IC10	32.7 (20.9–45.2)	80.7 (63.3–98.0)	308.7 (187.5–430.0)	187.4 (150.5–224.3)	—
	IC50	3270.4[a] (2471.9–4068.9)	846.2 (783.8–908.6)	3549.2[a] (2397.0–4501.4)	2738.2[a] (2352.2–3135.2)	—

[a]Values estimated with the Weibull model.
AMX = amoxicillin; ASA = acetylsalicylic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.
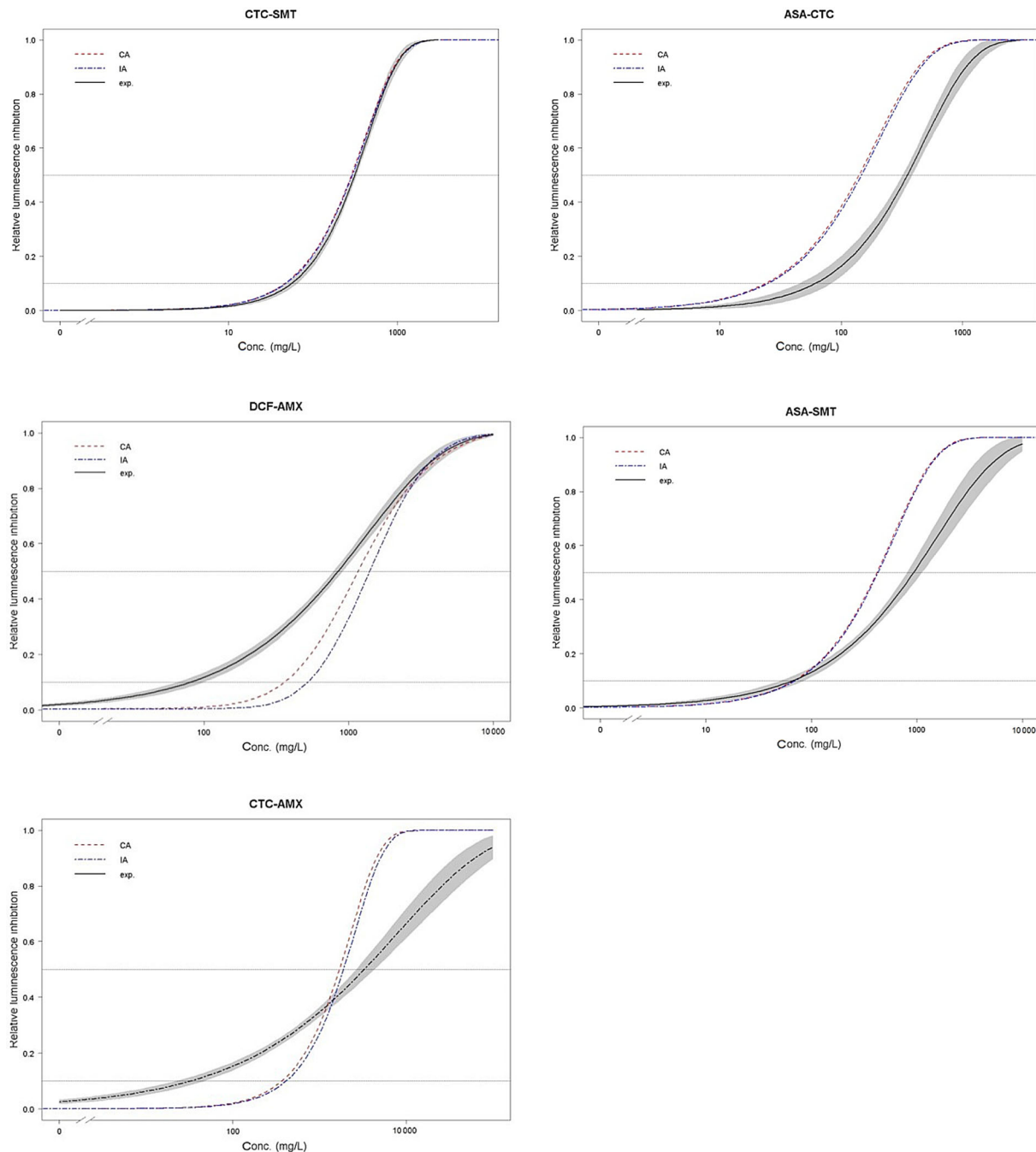
Figure 2. Predicted (concentration addition = red line; independent action = blue line) and observed toxicity values (black line) of binary mixtures (at inhibition concentration [IC10] concentration ratio) of veterinary pharmaceutical active compounds (gray shading = 95% confidence intervals). AMX = amoxicillin; ASA = acetylsalicylic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

be found in the Supplemental Data, Figure S1 and Table S3 (IC50 and IC10 predicted by CA and IA). The observed concentration responses are not always sufficiently described by the CA and IA models. In fact, with the exception of 3 binary combinations (chlortetracycline–sulfamethizole, diclofenac sodium–sulfamethizole, and acetylsalicylic acid–diclofenac sodium) and of the multicomponent mixture, the experimental toxicity data deviate from the conceptual expectations of the CA and IA. In 1 case (the binary mixture acetylsalicylic acid–chlortetracycline), both models clearly overestimate the bioluminescence inhibition response at all concentrations tested. In all the other cases, the extent of the

deviation is mainly dependent on the mixture components and the effect level considered. In particular, for binary combinations showing greater observed toxicities than those predicted by models (e.g., chlortetracycline–diclofenac sodium, diclofenac sodium–amoxicillin), the deviations are considerably higher at lower concentrations. Recently, Marx et al. [33], using literature data, analyzed the presence of synergistic and antagonistic effects in antibiotic mixtures and found a potential increase in synergistic effects with decreasing concentrations. However, the authors concluded that because of the low number of investigations into the concentration dependency of mixture interactions and because

of the dependency of this influence on the targeted organism and on the specific combination of chemicals, no general statement can be made at present.

In contrast, for acetylsalicylic acid–sulfamethizole (Figure 2) and acetylsalicylic acid–amoxicillin (Supplemental Data, Figure S1) combinations, the magnitude of deviation increased with an increase in concentration. However, in both cases, the deviations could be an artifact resulting from the lack of experimental data. (As reported above in the section *Binary mixtures of pharmaceutical active compounds*, the maximum percentage of inhibition reached was near 50%.) Finally, both models seem to underestimate the effects of chlortetracycline–amoxicillin (Figure 2) and sulfamethizole–amoxicillin (Supplemental Data, Figure S1) mixtures at lower tested concentrations.

Toxicological interactions of the tested pharmaceutical active compounds in mixtures

The results reported in the section *Predicted mixture toxicity by CA and IA models and comparison with experimental results* suggested possible interactions among the mixture components. The presence of synergism or antagonism in the mixtures of pharmaceutical active compounds (particularly in binary mixtures) or other pollutants has been reported in previous studies [14,21,23,34–37] and in the recent review by Backhaus [9]. Cleuvers [14] tested combinations of various pharmaceutical active compounds on algae and *Daphnia* and demonstrated that the evaluated compounds behaved as nonpolar narcotic compounds toward both organisms. In the algal test, the combination effect of a binary mixture of ibuprofen and diclofenac (2 nonsteroidal anti-inflammatory drugs [NSAIDs]) was well predicted by the CA model (as expected for substances with similar modes of action). However, in the *Daphnia* test, the effect of the mixture was stronger than that predicted by the CA model. In a further study, Cleuvers [34] evaluated a 4-component mixture of NSAIDs and confirmed that, in the algal test, the effect of increasing the number of NSAID components in the mixture was well predicted by the CA. In the *Daphnia* test, the results were quite different: at low doses (up to EC20/4), the mixture showed practically no effect (much lower than that predicted by the CA model), whereas at the higher concentrations, the effect was much higher than that predicted by the model. Rodea-Palomares et al. [21] found contrasting results when assessing the toxicological interactions of binary combinations of fibrates in *V. fischeri* and *Anabaena*. In fact, these authors reported that in the *Vibrio* test, the binary mixtures showed antagonism at low effect levels that turned into an additive effect or synergism at higher effect levels. However, in the *Anabaena* test, they found a strong synergism at the lowest effect levels that turned into a very strong antagonism at high effect levels. Gonzalez-Pleiter et al. [36] studied the toxicity of binary and multicomponent mixtures of 5 antibiotics to the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Anabaena* CPB4337. The study found a clear predominance of synergistic behavior for most of the antibiotic interactions in these organisms. In particular, the amoxicillin–tetracycline combination tested on cyanobacterium was strongly synergistic. Conversely, the same authors [36] found a dual behavior with rapid changes of interactions (from strong synergism to strong antagonism) when tetracycline in combination with erythromycin was tested in *Anabaena*. Brezovsek et al. [37] tested the toxicity of 3 binary mixtures of antineoplastic drugs to the green alga *P. subcapitata* and the cyanobacterium *Synechococcus leopolinensis* and compared their results with predictive models (CA and IA).

The binary combination of 5-fluorouracil and imatinib had a synergistic effect for *P. subcapitata* and an antagonistic effect on *S. leopolinensis*. Based on these results, the authors noted the importance of species-dependent interactions between the pharmaceuticals in a mixture.

Backhaus [9] highlighted that, for pharmaceuticals, the synergisms and antagonisms were specific for the tested mixture and the toxicity test. In particular, synergistic mixtures seemed to be largely confined to mixtures of only a few compounds, usually not more than 2 or 3. In contrast, synergistic or antagonistic mixture effects were rarely observed in toxicity tests for multicomponent mixtures. Backhaus [9] argued that this phenomenon might be explained by the presence of a sort of buffering against synergistic or antagonistic interactions when the contributions of the latter are not predominant.

Warne and Hawker [38], in their work on the funnel hypothesis, explained the variation in the toxicity of multicomponent mixtures of nonspecific toxicants (narcotics) and concluded that mixtures of this type of toxicants tended to approach additivity (becoming less synergistic or antagonistic) as the number of components increased.

In the present study, to evaluate the presence and eventually quantify the nature of such interactions (e.g., synergism, antagonism) at any effect level, the combination index method was applied. Figure 3 shows a plot of the combination index values obtained as a function of effect levels (f_a) for all of the tested mixtures. The corresponding combination index values at the main representative f_a levels (effect level of fraction inhibited with respect to the control) are reported in the Supplemental Data, Table S3. The f_a-combination index plots allowed us to observe, at any f_a level, the trend of the nature of the interactions among pharmaceutical active compounds in mixtures. In Figure 3, combination index values up to the effect level (f_a) of 0.5 are reported, for those binary mixtures for which the entire concentration–response curves were not obtained.

From the analysis of Figure 3 and Supplemental Data, Table S4, the following inferences may be made. First, for almost all the tested mixtures, f_a-combination index plots and combination index values seems to confirm the presence of interactions among the components. The only exceptions are the chlortetracycline–sulfamethizole, diclofenac sodium–sulfamethizole, and acetylsalicylic acid–diclofenac sodium binary combinations, which are fairly additive (combination index ∼1) at all the effect levels considered. Second, synergism or antagonism seem to be independent of the mode of action toward the tested organism. As highlighted in our previous study [24], we demonstrated that, with the exception of chlortetracycline, all of the selected pharmaceutical active compounds act as polar narcotic compounds to *A. fischeri*. In agreement with the previously reported results of Cleuvers [14] on *Daphnia*, even if these substances share (broadly speaking) the same mechanism of action, we found that the effect of the binary mixtures deviates from CA predictions. In addition, binary combinations composed of substances with different mechanisms of action (i.e., those including chlortetracycline) show very different and unpredictable behaviors. Third, clear synergistic effects are present for the chlortetracycline–diclofenac sodium and diclofenac sodium–amoxicillin combinations up to the effect level (f_a) of 0.65 to 0.70. The results obtained for chlortetracycline–diclofenac sodium seem to be in line with those of previous studies. Olajuyigbe and Afolayan [39] tested combinations of tetracycline and amoxicillin in 8 different kinds of antibiotic-resistant bacteria (either gram-negative or gram-positive bacteria) and found that the combinations often had a synergistic effect. Interestingly the
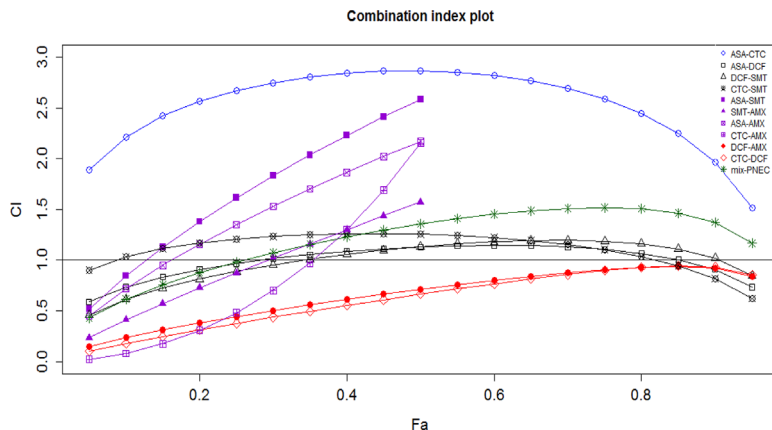
Figure 3. Combination index plot (f_a-combination index plot) for binary and multicomponent mixtures of tested pharmaceutical active compounds toward *Aliivibrio fischeri*. The combination index values are plotted as a function of the fractional inhibition of bioluminescence (f_a) by computer simulation (CompuSyn). Combination index <1, $=1$, and >1 indicate synergism, the additive effect, and antagonism, respectively [32]. AMX = amoxicillin; ASA = acetylsalicylic acid; CTC = chlortetracycline; DCF = diclofenac sodium; SMT = sulfamethizole.

same authors [39] also found that the synergistic effects were concentration dependent between the 2 antibiotics. In contrast, acetylsalicylic acid–chlortetracycline clearly interacted in an antagonistic way over the whole range of effect levels. Fourth, for other binary combinations (chlortetracycline–amoxicillin, sulfamethizole–amoxicillin acetylsalicylic acid–sulfamethizole, acetylsalicylic acid–amoxicillin), a somewhat heterogeneous pattern is observed, with interactions changing from synergism at low f_a (0.1−0.3) to a clear antagonism within a narrow range of concentrations. The presence of synergism at lower concentrations and the tendency toward additivity, or antagonism, at the higher levels is not easy to explain, because the nature of the interactions are complex and mostly unknown. In any case, as discussed previously, similar results are found in the literature [21,35,36]. For instance, Magdaleno et al. [40] tested the effects on the growth of *P. subcapitata* of combinations of several antibiotics and found that at low concentrations ($<10\,\mathrm{mg\,L^{-1}}$), all binary combinations showed synergism, whereas in almost all cases, at the higher concentrations tested, the response became close to the predictions.

In some cases, the nature of the interactions seems to be related to the presence of certain compounds. For instance, at the lower tested concentrations, the presence of the anti-inflammatory diclofenac sodium seems to enhance the effects of the antibiotics amoxicillin, chlortetracycline, and (to a lesser extent) sulfamethizole). Several studies have noted that the efficiency of some antibiotics (streptomycin, gentamicin, tetracycline, and ampicillin) toward bacteria can be synergistically enhanced by the presence of diclofenac sodium [41–45]. These studies found that diclofenac sodium has the pability to damage the bacterial membrane [46], and to favor alteration in the permeability of the microorganism to antibiotics [45,47,48]. In contrast, in the present study, the presence of the anti-inflammatory acetylsalycilic acid seems to weaken their toxicity.

Finally, the multicomponent mixture showed no strong synergism or antagonism. In fact, the combination index values ranged from 0.61 (IC10) to 1.37 (IC90) until the tested effect level. The results we obtained in the toxicity test for the multicomponent mixtures seem to support the buffering hypothesis proposed by Backaus [9].

As reported by Kortenkamp et al. [49], synergistic cases are highly specific for specific compounds, their concentrations, and their mixture ratios. In the present study, the concentrations

of the tested mixtures giving synergistic responses are above the environmentally relevant concentrations and in addition, for our experimental purpose, substances were combined in equal ratios according to their individual IC10 value, which could be quite different from the real ratio at which toxicants might be found in the environment. Thus responses from naturally occurring combinations could be different.

CONCLUSIONS

The toxicity of binary and multicomponent mixtures of widely used veterinary pharmaceuticals was evaluated using the Microtox test system. For the multicomponent mixtures tested, the results indicated that at concentrations corresponding to the sum of individual PNECs, no effects on *A. fischeri* were present. This finding suggested that PNEC values were sufficiently protective when the investigated pharmaceutical active compounds were contemporaneously present in aquatic environments, at least for microorganisms and for short-term exposure. By comparing the experimental toxicity curves with those predicted by the CA and IA models, it was possible to hypothesize the presence, in binary mixtures, of toxicological interactions between the components, which, in several cases, leads to synergistic effects at low concentrations (particularly when either diclofenac sodium or amoxicillin was 1 of the components). The subsequent application of the combination index method confirmed the presence of synergistic and antagonistic interactions. Finally, synergism or antagonism seemed to be independent of the mode of action of the toxicants tested. This seems to confirm that for combinations of pharmaceutical active compounds, use of the predictive models may have some limitations in terms of assessment of the real mixture toxicity.

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