Synergistic toxicity of some sulfonamide mixtures on Daphnia magna

Synergistic toxicity of some sulfonamide mixtures on *Daphnia magna*.

Marco De Liguoro, Alessia Riga, Piero Fariselli

Dept. of Comparative Biomedicine and Food Science - BCA, University of Padua, Italy.

Abstract

In livestock farming, sulfonamides (SAs) are used prophylactically and simultaneously in large numbers of animals. Therefore, traces of these compounds, alone or in combination, have been repeatedly detected in the environment. Synergistic interactions among chemicals in such mixtures represent an area of concern for the regulatory authorities. In this study, the acute toxic effects of binary and ternary mixtures of SAs were evaluated in *Daphnia magna*, in order to verify whether, based on their individual toxicity, they jointly exert a larger effect than would be predicted by individual actions alone. First, following the Concentration Addition (CA) principle, some preliminary observations were made by testing a number of drug combinations with an expected 50% effect. Then, mixtures more recognised for their synergistic effect (four binary and two ternary) were assayed in a range of reducing concentrations. The data acquired were processed using CompuSyn software, which integrates the different shape of the curves obtained in calculating the Combination Index (CI) for the evaluation of synergistic effects. For binary mixtures, synergy was also evaluated using the curvilinear isobologram method for heterodynamic drugs. Results indicate that most of the selected mixtures exhibit a synergistic effect using the CI methodology. For binary mixtures, these findings were also confirmed by isobologram analysis. Detected synergies indicate

that the CA is not always precautionary as a reference model for the evaluation of the aquatic toxicity of SAs mixtures.

Keywords: *Veterinary Sulfonamides, Drug-Mixtures, Daphnids, Synergy, CompuSyn.*

Introduction

In livestock farming, antibacterial drugs are used not only for therapeutic treatment of infected animals, but also for the so-called ‘mass treatments’ involving, simultaneously, a large number of animals. The following are carried out: growth promoting treatments, characterized by small doses of antibacterial added daily to the food during much of the production cycle and aimed at increasing the productivity of the animals; prophylactic treatments, routinely scheduled at critical times of the breeding cycle (weaning, change of housing, transport, etc.); and metaphylactic treatments, implemented promptly at the onset of disease in one or more subject of the group and aimed at treating infection in those already sick and prevent it in the still healthy ones. In the EU, despite the ban of the use of antibiotics as growth promoters, there seems to be no significant decrease in the consumption of antibiotics in the veterinary sector, as they continue to be used systematically for "prophylactic" purposes, due to unsustainable agricultural practices (Bond and Jewel, 2014).

Sulfonamides (SAs) are the oldest antibacterial agents and remain among the most widely used active pharmaceutical ingredients in veterinary medicine (EMA, 2015), mainly because of low cost and relative efficacy in some common bacterial and protozoan diseases.

Many of the available Veterinary Medicinal Products (VMPs) containing SAs are marketed as a premix, to be added to feed, or as an oral solution to be added to water. Using these formulations, animals may be treated simultaneously for preventive purposes and this can result in a substantial environmental load of the drugs. SAs are

subject to weak metabolism in the body of livestock and thus are eliminated mainly as such, or in the form of active metabolites in the excreta (Białk-Bielińska et al., 2013). As manure and slurry from farms are usually employed for the fertilization of agricultural land, a contamination of soil with SAs residues is clearly expected. During rainy days these residues can, partially at least, be transferred from soil to surface water by runoff (Boxall et al., 2002). Furthermore, SAs may also be directly released to watercourses as their use is extended to aquaculture in various countries. It is a matter of fact that residues of SAs have been repeatedly detected in the aquatic environment (Boxall *et al*., 2005; Perret *et al*., 2006; Xu *et al*., 2007; García-Galán *et al*., 2009; Santos et al., 2010; Guedes-Alonso et al., 2013; Giang et al., 2015).

For many years, the small crustacean *Daphnia magna* has been recognised as a keystone species in the food webs of many continental water bodies, and has served as an important model for ecotoxicological research (Seda and Petrusek, 2011). Impacts on daphnid populations may reverberate across the entire aquatic ecosystem as they are principal grazers of algae and primary forage for fish in lentic in-land ecosystems (Colbourne et al., 2011). The acute toxicity of SAs to *D. magna* is usually low (EC_{50s} >100 mg L^{-1}) with the notable exception of Sulfaguanidine (EC_{50} 6.2 mg L^{-1}) (Dalla Bona *et al*., 2014). However, as SAs occur in natural environment not just as a single entity, but usually together with other compounds of the same family or the same type (Managaki *et al*., 2007; Baran *et* al., 2011; García-Galán *et al*., 2011), it is of interest to evaluate the toxicity of their mixtures.

Here we adopted the concept of Concentration Addition (CA) to express the contribution of each chemical to the final mixture toxicity (Loewe and Muischnek, 1926). The concept is based on the assumption that all chemicals in a mixture act on the same biological target site and therefore could be viewed as being dilutions of each other, each having a different chemical potency (Cedergreen, 2014). Each chemical contribution to the overall toxicity of a mixture can be expressed as the quotient of its dose in the mixture and the dose of the same chemical alone that would be required to elicit the effect of the whole mixture. However, experimental data have often shown deviation

from this rule (Cedergreen, 2014), indicating more than additive interaction (an effect higher than expected, based on CA) or less than additive interaction (an effect lower than expected, based on CA).

To evaluate the CA deviations we prepared different binary and ternary mixtures containing SAs, with concentrations of each compound that, based on the CA concept, would be expected to result in a 50% immobilisation of *D. magna* after incubation for 48h. The scope of this experiment was to provide only a preliminary assessment of the synergistic tendencies of the molecules studied to allow later selection of the most appropriate mixtures for further evaluation. Mixtures that showed a strong indication of interactions that were more than additive, were then tested using a range of reducing concentrations of the components, in an equi-toxicity concentration ratio design. These latter data were processed using CompuSyn software (Chou and Martin, 2005) to identify the EC_{50} of each mixture, and to evaluate, more precisely, the interactions of its components (antagonism/synergy) at all effect levels. For binary mixtures, synergy was also evaluated using the curvilinear isobologram method proposed by Tallarida (2006) for heterodynamic drugs.

Materials and Methods

Culture conditions

Ephippia of *D. magna* were originally provided by ECOTOX (Milano, Italy). A single clone culture was selected based on the correct level of sensitivity to potassium dichromate (ISO, 1996) which was then rechecked periodically (every four months).

The subject organisms were maintained in Aachener Daphnien Medium (ADaM: hardness 193 mg $CaCO_3L^{-1}$; Klüttgen *et al*., 1994a,b) at 20±1°C, with a photoperiod of 16 h light (2.6 µE m^{-2} s^{-1}): 8 h dark. Their health status was optimal, and they did not show any sign of stress: mortality rate was ≤ 2% per week; reproduction rate was around 10 neonates per day per individual; *ephippia* and/or males never appeared in the culture.

116 They were fed three times per week with *Scenedesmus dimorphus* (8 x 10^5 cells mL^{-1}).
117 The alga was cultured in 2L BBM (Bold Basal Medium) enriched with 3 g of sterilised
118 poultry dung and suspended by bubbling filtered air. Before it was fed to the *Daphnia*
119 culture, the chlorophyte was filtered through a 50 µm laboratory test sieve (Endecotts
120 LTD, London, England), centrifuged at 3000 *g* for 10 min, resuspended in 25% BBM
121 medium at a concentration of 2 x 10^8 cells mL^{-1} and stored at 4 ± 1 °C.
122
123 *Chemicals*
124
125 Analytical grade compounds were purchased from Sigma–Aldrich (Milano, Italy) and
126 were of the following minimum purity: Sulfadiazine [68-35-9] (SDZ) 99%,
127 Sulfaguanidine [57-67-0] (SGD) 99%, Sulfamerazine [127-79-7] (SMA) 99%,
128 Sulfadimethoxine [122-11-2] (SDM) 98%, Sulfamethazine[57-68-1] (SMZ) 99%,
129 Sulfaquinoxaline [59-40-5] (SQO) 95%. The majority of these compounds have good
130 water solubility at a slight alkaline pH (O'Neil, 2006; Białk-Bielińska et al., 2012),
131 therefore for these compounds the preparation of their solutions in ADaM at
132 concentrations equal to their individual EC_{50} could be achieved by simple stirring at
133 room temperature. In the cases of SQO and SDZ, complete solubilisation in ADaM was
134 achieved by returning the pH of the medium to its original value (8.0) using 1 M NaOH
135 (De Liguoro *et al.,* 2009, 2010)
136
137 *Assayed mixtures*
138
139 Drug mixtures for the immobilisation test were prepared taking into account the EC_{50} of
140 individual compounds. All the possible binary mixtures (15) of the 6 compounds were
141 assayed. Ternary mixtures to be assayed were chosen on the basis of the results already
142 obtained with binary mixtures. After preparing solutions of each single compound in
143 ADaM medium, corresponding to its individual EC_{50}; equal volumes of two or three of
144 these solutions were mixed to generate the binary and ternary mixtures (Table 1). In this

way, based on the principle of CA, a 50% immobilisation effect would have been expected from each mixture after 48h incubation. Therefore, any detected effect >50% would have been considered as an indication of more than additive interaction. Similarly, any detected effect <50% would have been considered as an indication of less than additive interaction. In other words, for any number of additive agents the following equation holds:

$$\sum_{i=1}^{N} \frac{dA_i}{DA_i} = 1$$

where dA_i is the dose/concentration of A_i in a mixture that produces a specified effect, and DA_i is the dose/concentration of the single agent which on its own elicits the same effect as the mixture (Kortenkamp and Altenburger, 1998).

Given that the CA principle is rooted in the assumption of the constant relative potency of the drugs being combined (Tallarida, 2006) and that with the six SAs studied (Figure 1), this was not the case; as already indicated (see Introduction section), the preliminary tests were introduced in order to obtain an indication of which mixtures showed synergistic tendencies. Mixtures with a strong effect (> 90%) were then further assayed in a range of reducing concentrations (Table 2). This in order to plot their concentration-response curves, derive the EC_{50}s, and proceed to a reliable analysis of the interactions between their components, using CompuSyn software (Chou and Martin, 2005). The CompuSyn program integrates the different shapes of the curves in the calculation of the Combination Index for the evaluation of synergy; in this way, the constant relative potency of the combined drugs is not a prerequisite. For combinations of two drugs (not for three drugs), we also addressed the question using the equations proposed by Tallarida (2006) for heterodynamic drugs, which allow the isobole of additivity to be represented as a region bounded by two well-defined curves.

Toxicity tests

Acute toxicity tests were performed according to the Guideline 202 '*Daphnia sp*., Acute Immobilisation Test' (OECD, 2004). The ADaM medium was used for Controls and the dilution of test compounds. Eight groups of 5 young daphnids (third brood neonates; <24 h) were exposed to each of the assayed mixtures (Table 1 and Table 2) or used as controls. The organisms were fed for about 1 h with 100% pure, dried Spirulina powder (15 mg in 100 mL ADaM) just before the start of the experiment, and then each group was incubated in a 20 mL glass vessel loosely covered with parafilm, and containing 10 mL of the test solution, under the same conditions (light, temperature) used for culturing. Pre-feeding of the organisms is not deemed necessary by the test guideline, however in our experience is strongly advisable as it helps to sustain 100% survival in the control groups. The number of immobile daphnids recorded after 48h was the endpoint for effect calculation.

Data Analysis

Data were processed using CompuSyn software for Drug Combinations and General Dose-Effect Analysis (Chou and Martin, 2005). Raw data for the effects of both single drugs and mixtures were entered. CompuSyn fitted the data and provided the model parameters and the concentration-effect plots. The model parameters were the EC_{50} and the shape value "*m*" of the Hill curve *f* as a function of the chemical concentration *x,* as given by:

$$f(x) = \frac{1}{1 + \left(\frac{EC_{50}}{x}\right)^{m}}$$

If the exponent *m* is greater than 1, the curve is sigmoidal, when it is equal to 1 the curve is hyperbolic (Chou and Martin, 2005). The most relevant aspect of CompuSyn is to provide the evaluation and the plots that report Combination Indices. The Combination

Index (CI) quantifies the dose-effect relationship on the basis of "mass-action law" to evaluate the effect of combination of chemicals (Chou and Martin, 2010). The CI index furnishes a value that quantitatively indicates synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1). Here we used this tool to evaluate possible synergy among the different compounds.

To further evaluate the possible synergy between pairs of chemicals, we completed an isobologram analysis at EC_{50}. Since there is no obvious basis upon which to distinguish whether chemical A is contributing to chemical B or vice versa, the use of dose equivalence leads to not one but to two possible isoboles of additivity, depending on how the concept of dose equivalence is applied (Tallarida 2006). This means that rather than being a single straight line, the isobole becomes an area bordered by two curved lines. In particular, according to previous indications (Tallarida, 2006), when two compounds have two different shapes (or exponents) of the dose-effect curve, the signature of the synergy/antagonism of their mixture must be found outside the region bounded by two curves. In the EC_{50} isobologram estimation, the equivalent doses were computed using two equations that describe the upper and lower bounds of the additivity area and are expressed as:

$$b = B_{50}\left(1 - \frac{a}{A_{50}}\right)^{q/p} \quad ; \quad b = B_{50}\left(1 - \left(\frac{a}{A_{50}}\right)^{q/p}\right)$$

where q and p are the exponents of the Hill curves (m in the previous equation) for the chemicals A and B, respectively (Tallarida, 2006). A_{50} and B_{50} refer to the EC_{50} of each of the two chemicals, while a and b are the doses (or concentrations) of each chemical A and B. When $p = q$, the dose equivalent for B collapses to a single straight line, as can be seen from the equation above. However, in the general case, the greater the difference between q and p, the farther from this diagonal the two isoboles are (Tallarida, 2006). Since we evaluated the Hill shape exponent with CompuSyn, and the program reports the associated error for the computed exponent, we highlighted in the isobologram the uncertainty of the computed exponents. Thus, in the isobologram we also included the

230 "worst-case isobole", in which the larger exponent is increased by summing its error,
231 and the smaller exponent is decreased by subtracting its error. In practice, if $q>p$ and ε_q
232 and ε_p are the corresponding estimated errors, the worst-case isobole is computed with
233 the highest possible ratio $r=(q + \varepsilon_q)/(p - \varepsilon_p)$. In this way, if the measured point of the
234 combination dose exceeds this "fatter" isobole the indication of synergy (or antagonism)
235 gains greater confidence.
236
237
238 **Results**
239
240 Raw data for acute toxicity of single compounds were already available from previous
241 experiments run in our lab (Dalla Bona *et al*., 2014) under the same conditions (T, light
242 cycle, length of exposure, age of daphnids, feeding) used for mixture assays. The relative
243 concentration-response curves and EC_{50s}, generated using CompuSyn software, are
244 presented in Figure 1.
245 In all tests, validity criteria were fulfilled as control survival (mobility) was 100%, and
246 the recorded values of water quality parameters, measured at the beginning and at the
247 end of the test, were always within the following ranges: pH 7.9–8.1, dissolved oxygen
248 7.70–8.40 mg L^{-1}. Temperature stability (20±1°C) of the medium was guaranteed by the
249 use of a refrigerated incubator. Six binary mixtures of the 15 assayed, gave indications
250 of more than additive interaction (Figure 2). The following had more than 90% effect:
251 SMA+SDZ (97.5%); SQO+SDM (92.5%); SGD+SDZ (92.5%); SDM+SGD (100%).
252 These were re-assayed in a range of reducing concentrations (from 0.5 to 0.25 EC_{50} of
253 each component) under the same conditions used in the previous tests: their
254 concentration-effect curves and EC_{50s} are shown in Figure 3 and compared to the effect
255 curves predicted by CA. In general, their effects were confirmed to be synergic (Figure
256 4); at high effect levels - in three cases out of four, the synergy was strong (Combination
257 Index < 0.3; Chou and Martin, 2005). At the 50% effect level, synergy was also
258 confirmed by applying the equations proposed by Tallarida (2006); however, with

SGD+SDM and SMA+SDZ the EC_{50} fell just below the confidence limit of the additivity area (Figure 5).

Three ternary mixtures were tested, and all gave indications of greater than additive interaction (Figure 2). The following had greater than 90% effect: SDM+SGD+SDZ (100%); SMA+SGD+SDZ (100%). These were re-assayed in a range of reducing concentrations (from 0.33 to 0.165 EC_{50} of each component): their concentration-effect curves and EC_{50}s are shown in Figure 3 and compared to the effect curves predicted by CA. Their effects were confirmed to be synergic at all effect levels (Figure 4).

Predicted No-Effect Concentrations (PNECs), for individual compounds in mixtures, obtained by applying an Assessment Factor of 1000 to the EC_{50s} (CVMP/VICH/790/03), were always > 40 $\mu g\, L^{-1}$, with the exception of SGD (> 1 $\mu g\, L^{-1}$).

Discussion

Following an in-depth study on the hydrolysis of SAs in aqueous solutions, Białk-Bielińska *et* al. (2012) concluded that under typical environmental conditions (pH and temperature) SAs are hydrolytically stable with a long half-life, and that all could be assumed to be hydrolytically stable at pH 9 and 25°C for least 1 year. Moreover, in previous experiments with *D. magna* (De Liguoro et al., 2009, 2010) it was verified, using HPLC analysis, that the 48 h level of decline of the above mentioned compounds under the conditions used in the tests (pH 8.0; 20°C) was between 0 and 13%. Based on the CRED (Moermond *et al*., 2016), in acute toxicity tests with stable substances, nominal concentrations without further measurements are acceptable. Furthermore, Guideline 202 '*Daphnia sp*., Acute Immobilisation Test' (OECD, 2004) states that if the concentration of the test substance has been maintained throughout the test within ± 20 per cent of the nominal initial concentration, the results can be based on the nominal values. Thus, in the present study, the use of HPLC analysis was rendered redundant and consequent undesirable excess use of solvents was avoided, with test results being based on nominal concentrations.

The various SAs evaluated in this study share the same mechanism of action and cellular target (Eguchi *et al*., 2004); consequently, their combinations should follow the CA principle (Cedergreen, 2014). The 15 (preliminary) binary tests, where all possible pairs were tested, showed 9 cases of less than additive interaction (Combination Index >1), and 6 cases of more than additive interaction (Combination Index <1). The 3 (preliminary) ternary tests, chosen, based on binary test results, all showed more than additive interaction. In some cases the deviation from the rule of CA, in one way or another, was strong (Figure 2). The CA principle, however, is rooted in the assumption of a constant relative potency of the drugs being combined. In other words, the Hill coefficients (Faust *et al*., 2003), which respectively describe their concentration-effect relations, should be equal (Tallarida, 2006). With the studied SAs this assumption did not hold true (Figure 1). Therefore, the positive results of the preliminary tests were taken only as a possible indication of synergy, rather than definitive proof. The more promising mixtures were then re-assayed in a range of five concentrations, spreading experimental data points below and above the EC_{50} value, as suggested by Chou for the use of the CompuSyn application (Chou and Martin, 2005). At the 50% effect level, synergy was confirmed for all four binary mixtures by applying the equations of Tallarida. To further check the synergetic effects, worst-case isoboles were also included by adding the computed error to the larger exponent and subtracting the computed error from the lowest exponent. Figure 5 shows that all the binary mixtures, passed this more restrictive test, indicating significant synergy around the EC_{50} combined dose.

For a more comprehensive evaluation of the drug interactions, we used the CompuSyn program, which allows the evaluation of synergy at all effect levels, both for binary and ternary mixtures. Detected synergies (Figure 4) indicate that the concept of CA is not always precautionary as a reference model for the evaluation of the aquatic toxicity of SA mixtures. Interestingly, SDZ that is the only SA licensed for aquaculture in EU, and therefore more prone than other compounds to the contamination of the aquatic environment, was frequently involved in synergic interactions. It should be noted, however, that synergies were generally stronger when immobilisation percentages were

very high (Figure 4), i.e. when relatively high concentrations of SAs were mixed. This means that at the very low concentrations usually encountered in the natural environment, SA synergies may be of more limited relevance to *D. magna*. More generally, calculated PNECs for single components of each mixture indicate that the currently reported level of SA contamination (<1 μg L^{-1}) should have no impact on the freshwater environment. Nevertheless, it would be of interest to assess the effects of the selected SA mixtures in the chronic *D. magna* Reproduction Test, which is indeed far more sensitive than the acute immobilisation test and allows the estimation of NOEC for PNEC calculation.

The authors think that some experiments with similar SAs mixtures, on more sensitive species, such as cyanobacteria, would complement the present work. Indeed, cyanobacteria are generally considered to be the most sensitive aquatic organism to antibacterials; however, it has also been shown that green algae are more sensitive than daphnids to the toxicity of some selected SAs, with NOEC values in the range 0.02-1 mg L^{-1} (Eguchi *et* al., 2004; De Liguoro *et al*., 2010). In the natural environment, some cascade effect on daphnids may also be expected, as green algae are their basic food resource. Such an effect could not be highlighted by the acute immobilisation test where, in order to avoid any nutritional variation among the experiments, daphnids were fed only with a calibrated quantity of dried spirulina. Possible synergic interactions with Trimethoprim (TMP) or Pyrimethamine (PMT), two SA potentiators frequently included in VMPs, should be taken into consideration in addition. For instance, Eguchi *et al*. (2004) showed that pairing SMZ, SDZ and SDM with TMP or PMT strongly enhanced their algal growth inhibition effects. Overall, the indications of synergy between SAs observed during these tests open the way for a range of new experiments to further deepen our understanding of this phenomenon.

Conclusions

A range of methods is available for the evaluation of the synergistic interactions of drug mixtures. As suggested by Foucquier and Guedj (2015), in the absence of a reference methodology appropriate for all situations, the evaluation of the impacts of various drug combinations may be facilitated by the collective use of different approaches. Here, binary mixtures of veterinary SAs were evaluated using two different models, and both generally confirmed the possibility of synergistic interactions among these compounds. Whilst their combined acute toxicity to *D. magna* still seems too low to represent a real threat in the natural environment, future studies with SAs mixtures should focus on the possible chronic harm to daphnids and to other, more sensitive, aquatic organisms.

Acknowledgements

This investigation was funded by the DOR grants (2016) of the University of Padua. The authors are grateful to Jonathan Dancer MRPharmS for contributing to the language used in the manuscript.

References

Baran, W., Adamek, E., Ziemiańska, J., Sobczak, A., 2011. Effects of the presence of sulfonamides in the environment and their influence on human health. Journal of Hazardous Materials 196, 1–15.

Białk-Bielińska, A., Stolte, S., Matzke, M., Fabiańska, A., Maszkowska, J., Kołodziejska, M., Liberek, B., Stepnowski P., Kumirska J., 2012. Hydrolysis of sulphonamides in aqueous solutions. Journal of Hazardous Materials 221-222, 264-274.Bond V. and Jewell J., 2014. Business Benchmark on Farm Animal Welfare -The impacts of antibiotic use in animals on human health and animal welfare. http://www.bbfaw.com

Boxall, A.B., Blackwell, P., Cavallo R., Kay,P., Tolls J., 2002. The sorption and transport of a sulphonamide antibiotic in soil systems. Toxicology Letters 131 (1,2), 19–28.

Boxall, A.B., Fogg, L.A., Baird, D.J., Lewis, C., Telfer, T.C., Kolpin, D., Gravell, A., 2005. Targeted monitoring study for veterinary medicines in the UK environment. Final Report to the UK Environment Agency.

Cedergreen N., 2014. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. PLoS ONE 9(5): e96580.

Chou, TC., 2010. Drug Combination Studies and their Synergism Quantification Using Chou-Talalay Method. Cancer Research 70: 440-446.

Chou TC., Martin N., 2005. CompuSyn for drug combinations: pc software and user's guide. ComboSyn Inc Paramus NJ.

CVMP/VICH/790/03-FINAL, 2003. VICH Topic GL38 (Ecotoxicity Phase II) for Implementation at Step 7 Guideline on Environmental Impact Assessment (EIAS) for Veterinary Medicinal Products – Phase II.

De Liguoro, M., Fioretto, B., Poltronieri, C., Gallina, G., 2009. The toxicity of sulfamethazine to *Daphnia magna* and its additivity to other veterinary sulfonamides and trimethoprim. Chemosphere 75, 1519–1524.

De Liguoro, M., Di Leva, V., Gallina, G., Faccio, E., Pinto, G., Pollio, A., 2010. Evaluation of the aquatic toxicity of two veterinary sulfonamides using five test organisms. Chemosphere, 81, 788–793.

391 Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y.S., Goto, K., Hirata, K., Miyamoto, K.,
392 Yoshimura, H., 2004. Evaluation of antimicrobial agents for veterinary use in the
393 ecotoxicity test using microalgae. Chemosphere, 57, 1733–1738.
394 EMA (2015) European Surveillance of Veterinary Antimicrobial Consumption 'Sales of
395 veterinary antimicrobial agents in 26 EU/EEA countries in 2013'. (EMA/387934/2015).
396 Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P.,
397 Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2003. Joint algal toxicity of 16
398 dissimilarly acting chemicals is predictable by the concept of independent action. Aquat
399 Toxicol, 63, 43–63.
400 Foucquier, J. and Guedj M., 2015. Analysis of drug combinations: current methodological
401 landscape. Pharmacology Research & Perspectives, 3 (3), 1-11.
402 García-Galán, M.J., Garrido, T., Fraile, J., Ginebreda, A., Díaz-Cruz, M.S., Barceló, D.,
403 2009. Simultaneous occurrence of nitrates and sulfonamide antibiotics in two ground
404 water bodies of Catalonia (Spain). J Hydrol, 383 (1–2), 93–101.
405 García-Galán M.J., Díaz-Cruz M.S., Barcélo D., 2011. Occurrence of sulfonamide
406 residues along the Ebro river basin. Removal in wastewater treatment plants and
407 environmental impact assessment. Environment International, 37(2), 462–473.
408 Giang C.N., Sebesvari Z., Renaud F., Rosendahl I., Minh Q.H., Amelung W., 2015.
409 Occurrence and Dissipation of the Antibiotics Sulfamethoxazole, Sulfadiazine,
410 Trimethoprim, and Enrofloxacin in the Mekong Delta, Vietnam. PlosOne DOI:
411 10.1371/journal.pone.0131855.
412 Guedes-Alonso, R., Afonso-Olivares, C., Montesdeoca-Esponda, S., Sosa-Ferrera, Z.,
413 Santana-Rodríguez, J.J., 2013. An assessment of the concentrations of pharmaceutical
414 compounds in wastewater treatment plants on the island of Gran Canaria (Spain).
415 Springerplus 2, 24. doi:10.1186/2193-1801-2-24
416 ISO, 1996. ISO 6341. Water quality – Determination of the inhibition of the mobility of
417 *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test. International
418 Organization for Standardization, Switzerland.
419 Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994a. ADaM, an artificial freshwater

420 for the culture of zooplankton. Water Res. 28, 743–746.
421 Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994b. Corrigendum. Water Res 28, I.
422 Loewe S, Muischnek H., 1926. Über kombinationswirkungen. 1. Mitteilung: Hilfsmittel
423 der Fragestellung. Naunyn-Schmiedebergs Arch Exp Pathol Pharmakol 114: 313–326.
424 Managaki, S., Murata, A., Takada, H., Tuyen, B.C., Chiem, N.H., 2007. Distribution of
425 macrolides, sulfonamides, and trimethoprim in tropical waters: ubiquitous occurrence
426 of veterinary antibiotics in the Mekong Delta. Environ Sci Technol. 41:8004–10.
427 Moermond, C.T.A., Kase, R., Korkaric, M., Ågerstrand, M., 2016. CRED: Criteria for
428 reporting and evaluating ecotoxicity data. Environ Toxicol Chem, 35 (5), 1297-1309.
429 OECD, 2004. Guidelines for the Testing of Chemicals. Guideline 202: *Daphnia sp*.,
430 Acute immobilisation Test. Organization for Economic Cooperation and Development,
431 Paris.
432 O'Neil, M.J., (Ed.). (2006). The Merck Index: An Encyclopedia of Chemicals, Drugs, and
433 Biologicals (14th ed.). NJ: Merck.
434 Perret D., Gentili A., Marchese S., Greco A., Curini R., 2006. Sulphonamide residues in
435 Italian surface and drinking waters: a small scale reconnaissance, Chromatographia 63,
436 225–232.
437 Santos L.H., Araújo A.N., Fachini A., Pena A., Delerue-Matos C., Montenegro M.C.,
438 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic
439 environment. J. Hazard. Mater. 175, 45–95.
440 Tallarida, R.J., 2006. An overview of drug combination analysis with isobolograms.
441 J.Pharmacol. Exp. Ther. 319, 1–7.
442 Xu W.H., Zhang G., Zou S.C., Li X.D., Liu Y.C., 2007. Determination of selected
443 antibiotics in the Victoria Harbour and the Pearl River, South China using high
444 performance liquid chromatography-electrospray ionization tandem mass spectrometry.
445 Environ. Pollut. 145, 672–679.
446
447 **Figure Captions**
448

Figure 1. Concentration-effect curves of single SAs in *D. magna* immobilisation test (48h). *r*=correlation coefficient; *m*=exponent of the Hill-curve that defines the curve slope. Vertical error bars show standard deviation (4 vessels, each with 5 daphnids). SDM, sufadimethoxine; SGD, sulfaguanidine; SDZ, sulfadiazine; SMA, sulfamerazine; SQO, sulfaquinoxaline; SMZ, sulfamethazine.

Figure 2. Effect percentage of binary and ternary mixtures of SAs in *D. magna* immobilisation test (48h); based on the CA principle a 50% effect was to be expected. However, deviations from this rule may also be the consequence of the inconstant relative potency of the drugs being combined (Tallarida, 2006) Horizontal error bars show standard deviation (8 vessels, each with 5 daphnids). SMA, sulfamerazine; SGD, sulfaguanidine; SDZ, sulfadiazine; SDM, sulfadimethoxine; SQO, sulfaquinoxaline; SMZ, sulfamethazine.

Figure 3. Concentration-effect curves of binary (a,b,c,d) and ternary (e,f) mixtures of SAs in *D. magna* immobilisation test (48h). Vertical error bars show standard deviation (8 vessels, each with 5 daphnids). Dashed lines are the concentration-effect curves predicted by Concentration Addition principle. SDM, sufadimethoxine; SGD, sulfaguanidine; SDZ, sulfadiazine; SMA, sulfamerazine; SQO, sulfaquinoxaline.

Figure 4. Graphic representations obtained from the CompuSyn Report for SAs binary (a,b,c,d) and ternary mixtures (e,f) assayed on *D. magna*: Combination Index <1 indicates synergic interaction. SDM, sufadimethoxine; SGD, sulfaguanidine; SDZ, sulfadiazine; SMA, sulfamerazine; SQO, sulfaquinoxaline.

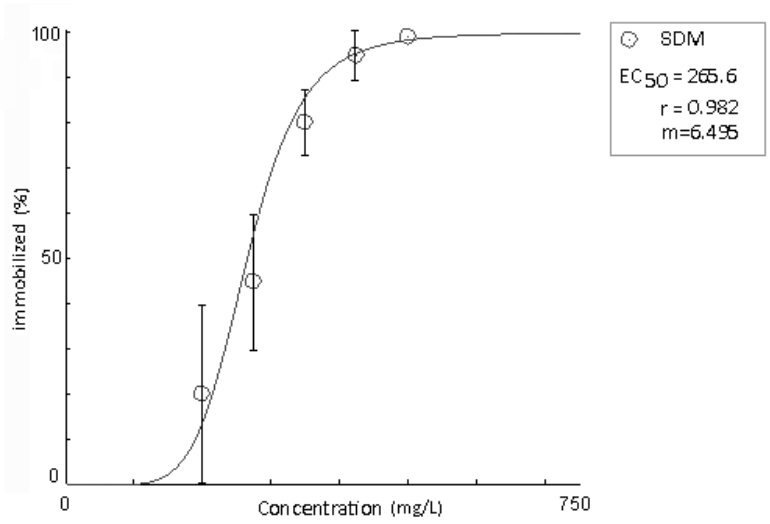
Figure 5. Isobolograms of SAs binary mixtures assayed on *D. magna*. For compounds with a variable potency ratio, synergy is detected only if the EC_{50} of the mixture lies below the region of the plane bounded by the two curves of additivity for a 50% effect (Tallarida, 2006). Dotted lines represent curves of additivity. Dashed lines are their confidence limits based on Hill coefficient variability. SGD, sulfaguanidine; SDM, sufadimethoxine; SDZ, sulfadiazine; SMA, sulfamerazine; SQO, sulfaquinoxaline.

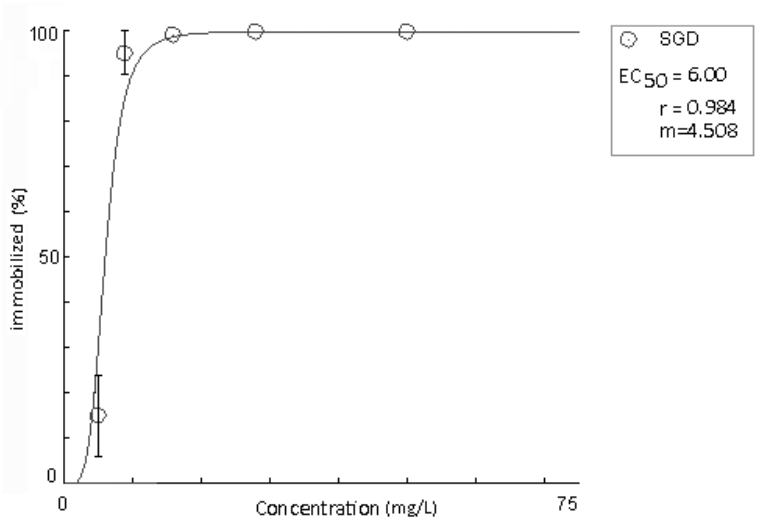
Table 1. Preliminary assays of binary (B) and ternary (T) mixtures of SAs in *D. magna* immobilisation test.

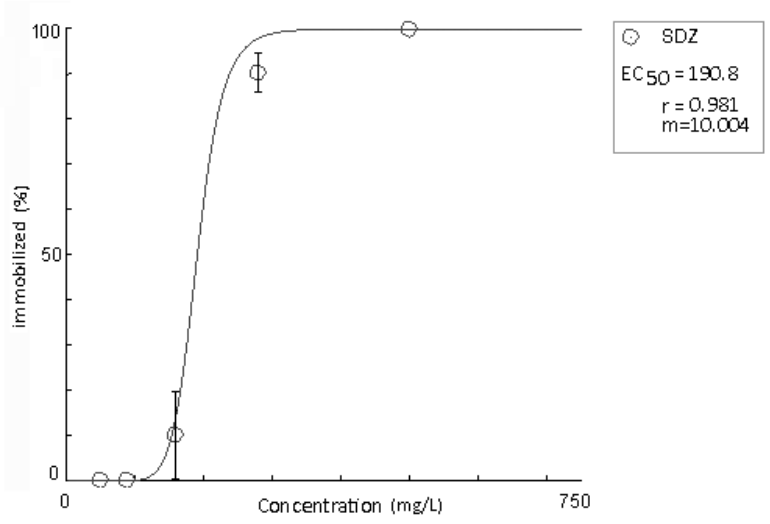
Mixture	Sulfadimethoxine (mg/L)	Sulfaguanidine (mg/L)	Sulfadiazine (mg/L)	Sulfaquinoxaline (mg/L)	Sulfamethazine (mg/L)	Sulfamerazine (mg/L)
B1	132.8	3				
B2	132.8		95.4			
B3	132.8			69		
B4	132.8				104.4	
B5	132.8					102.5
B6		3	95.4			
B7		3		69		
B8		3			104.4	
B9		3				102.5
B10			95.4	69		
B11			95.4		104.4	
B12			95.4			102.5
B13				69	104.4	
B14				69		102.5
B15					104.4	102.5
T1	88.5	2	63.6			
T2	88.5	2		46		
T3		2	63.6			68.3

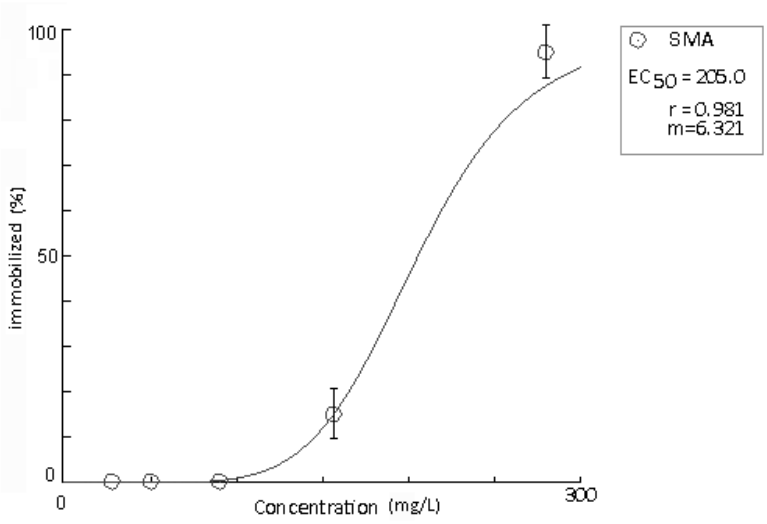
Table 2. Assays in a range of reducing concentrations of selected binary and ternary mixtures of SAs in *D. magna* immobilisation test.

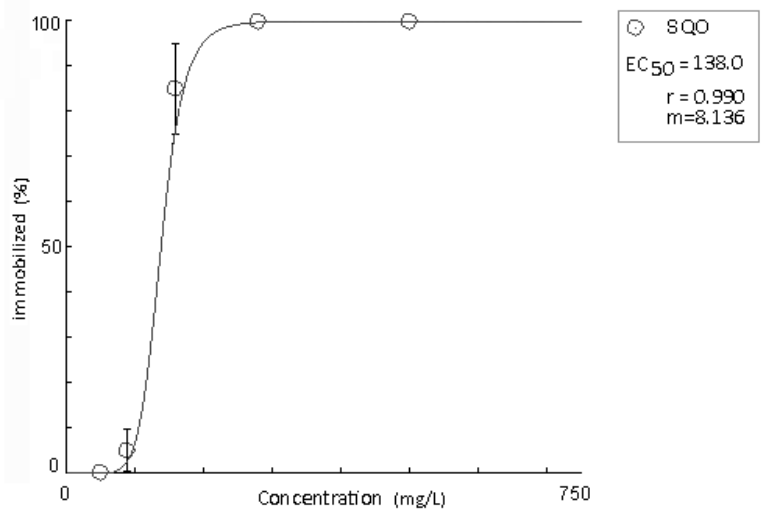
Combination	**SAs**	**Assayed concentrations (mg/L)**				
Binary	Sulfadimethoxine	132.8	116.2	99.6	83.0	66.4
	Sulfaguanidine	3.0	2.6	2.3	1.9	1.5
Binary	Sulfamerazine	102.5	89.7	76.9	64.1	51.3
	Sulfadiazine	95.4	83.5	71.6	59.6	47.7
Binary	Sulfaguanidine	3.0	2.6	2.3	1.9	1.5
	Sulfadiazine	95.4	83.5	71.6	59.6	47.7
Binary	Sulfaquinoxaline	69.0	60.4	51.8	43.1	34.5
	Sulfadimethoxine	132.8	116.2	99.6	83.0	66.4
Ternary	Sulfadimethoxine	88.5	77.5	66.4	55.4	44.3
	Sulfaguanidine	2.0	1.8	1.5	1.3	1.0
	Sulfadiazine	63.6	55.7	47.7	39.8	31.8
Ternary	Sulfaguanidine	2.0	1.8	1.5	1.3	1.0
	Sulfadiazine	63.6	55.7	47.7	39.8	31.8
	Sulfamerazine	68.3	59.8	51.2	42.7	34.2

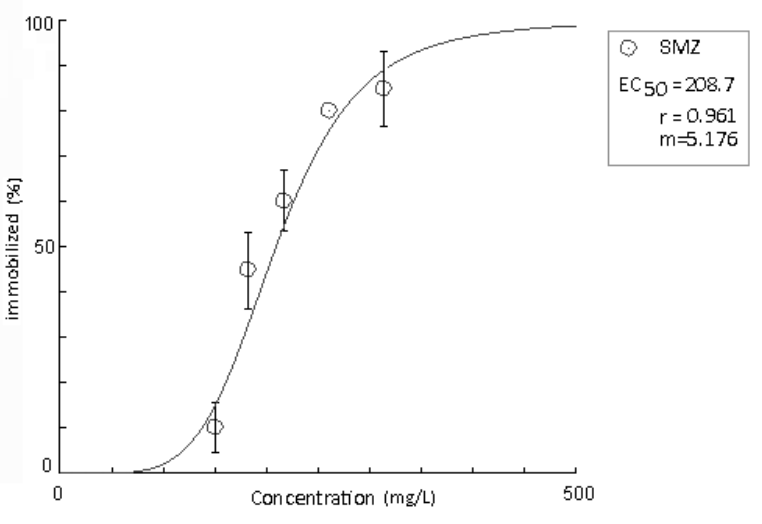

SDM
EC50 = 265.6
r = 0.982
m=6.495
immobilized (%)
100
50
0
0
750
Concentration (mg/L)

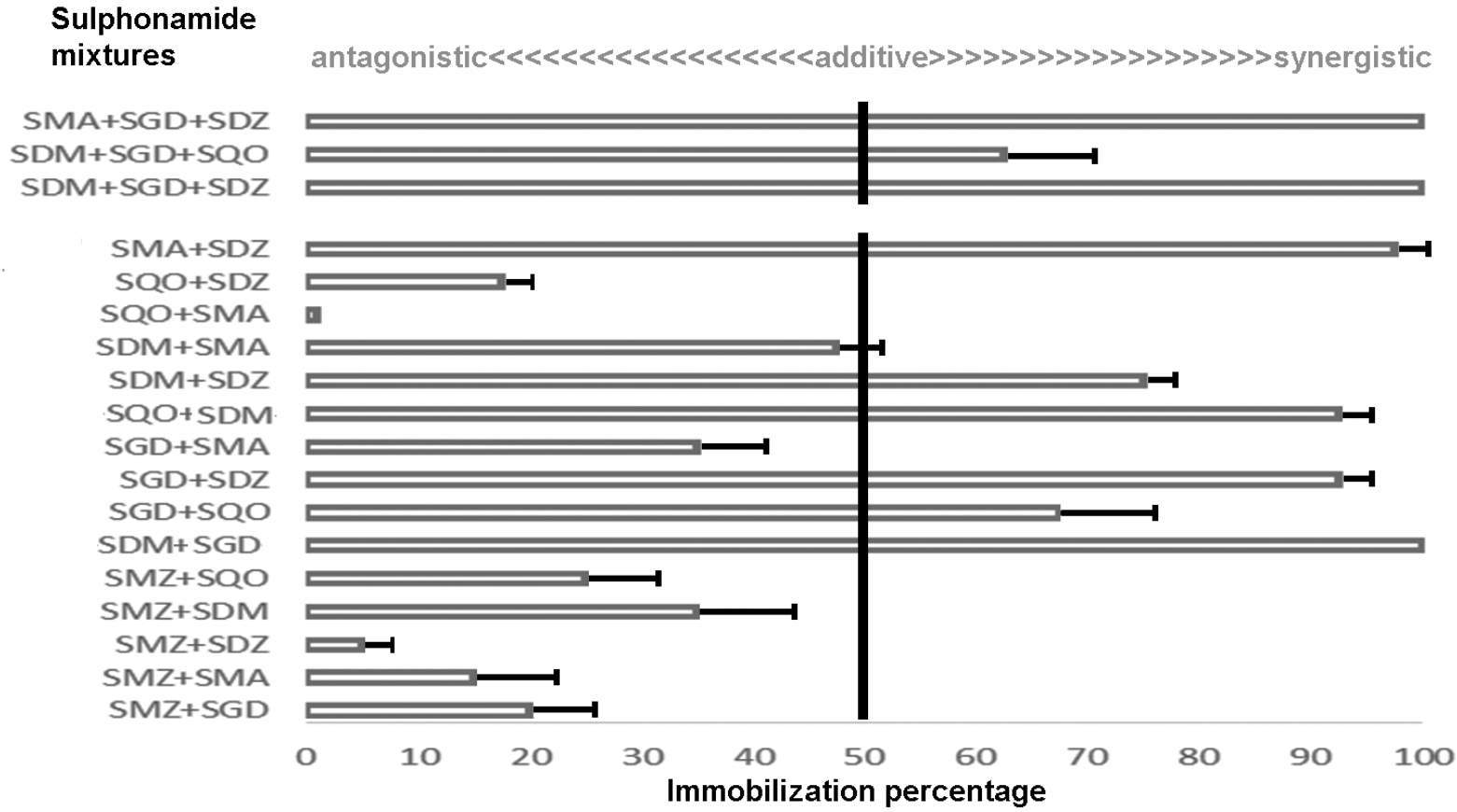

SGD
EC50 = 6.00
r = 0.984
m=4.508
immobilized (%)
100
50
0
0
75
Concentration (mg/L)

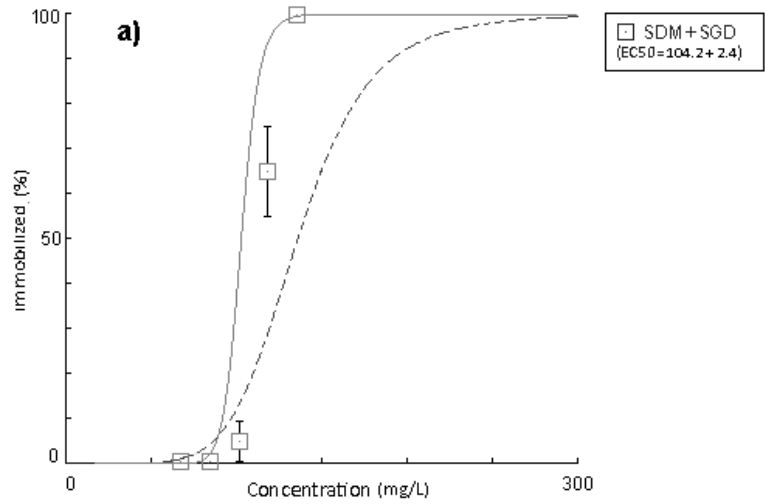

SDZ
EC50 = 190.8
r = 0.981
m=10.004
immobilized (%)
100
50
0
0
750
Concentration (mg/L)

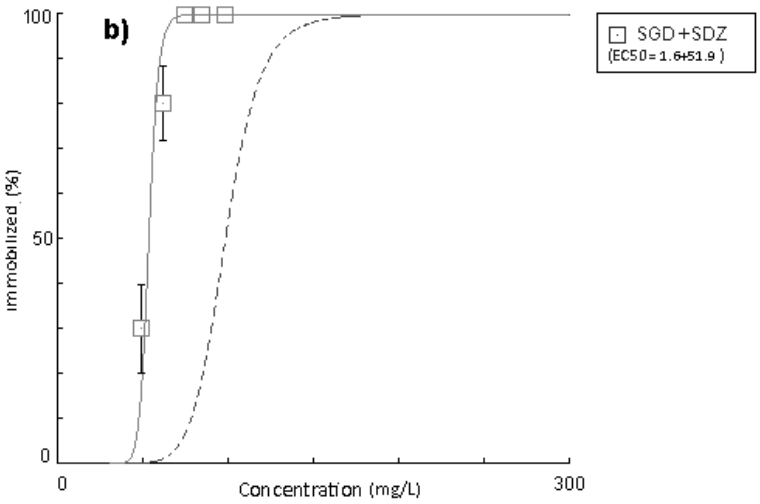

SMA
EC50 = 205.0
r = 0.981
m=6.321
immobilized (%)
100
50
0
0
300
Concentration (mg/L)

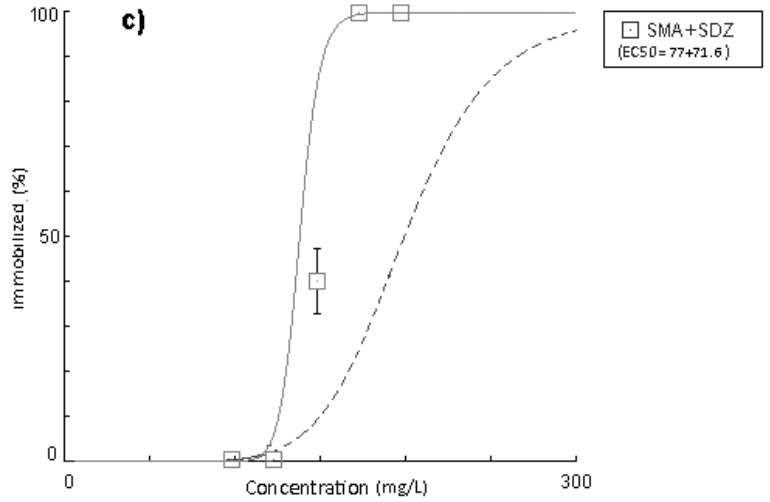

SQO
EC50 = 138.0
r = 0.990
m=8.136
immobilized (%)
100
50
0
0
750
Concentration (mg/L)

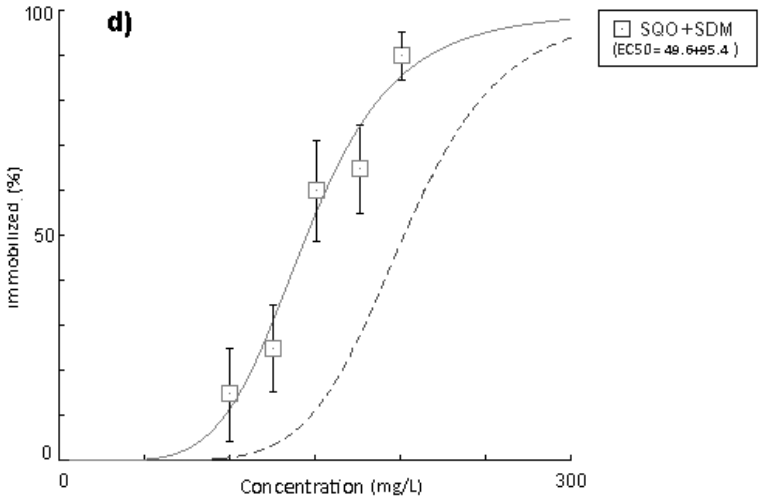

SMZ
EC50 = 208.7
r = 0.961
m=5.176
immobilized (%)
100
50
0
0
500
Concentration (mg/L)

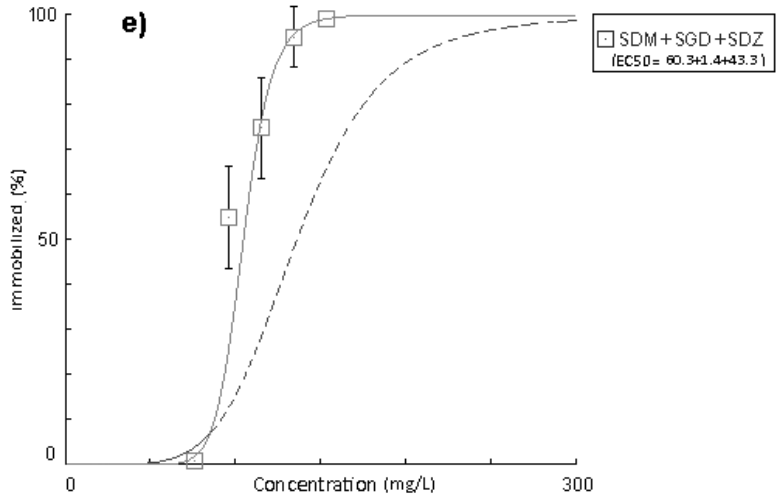

Sulphonamide mixtures
antagonistic<<<<<<<<<<<<<<<<<<additive>>>>>>>>>>>>>>>>>>synergistic
SMA+SGD+SDZ
SDM+SGD+SQO
SDM+SGD+SDZ
SMA+SDZ
SQO+SDZ
SQO+SMA
SDM+SMA
SDM+SDZ
SQO+SDM
SGD+SMA
SGD+SDZ
SGD+SQO
SDM+SGD
SMZ+SQO
SMZ+SDM
SMZ+SDZ
SMZ+SMA
SMZ+SGD
0
10
20
30
40
50
60
70
80
90
100
Immobilization percentage

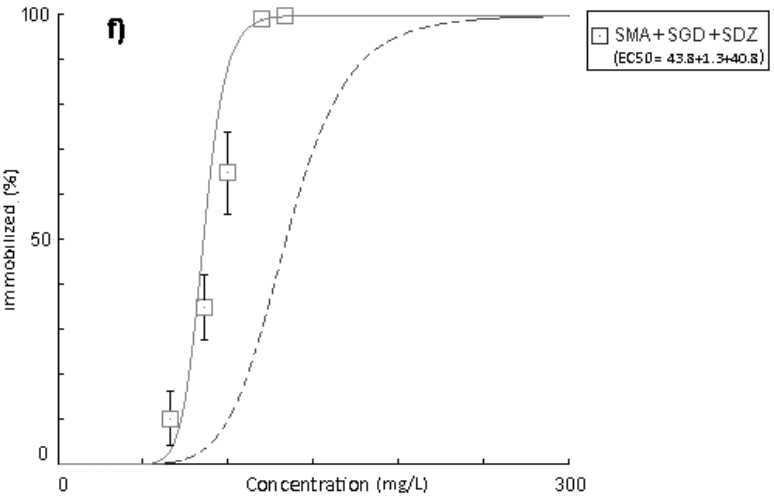

a)
SDM+SGD
(EC50=104.2+2.4)
immobilized (%)
Concentration (mg/L)
0
50
100
300

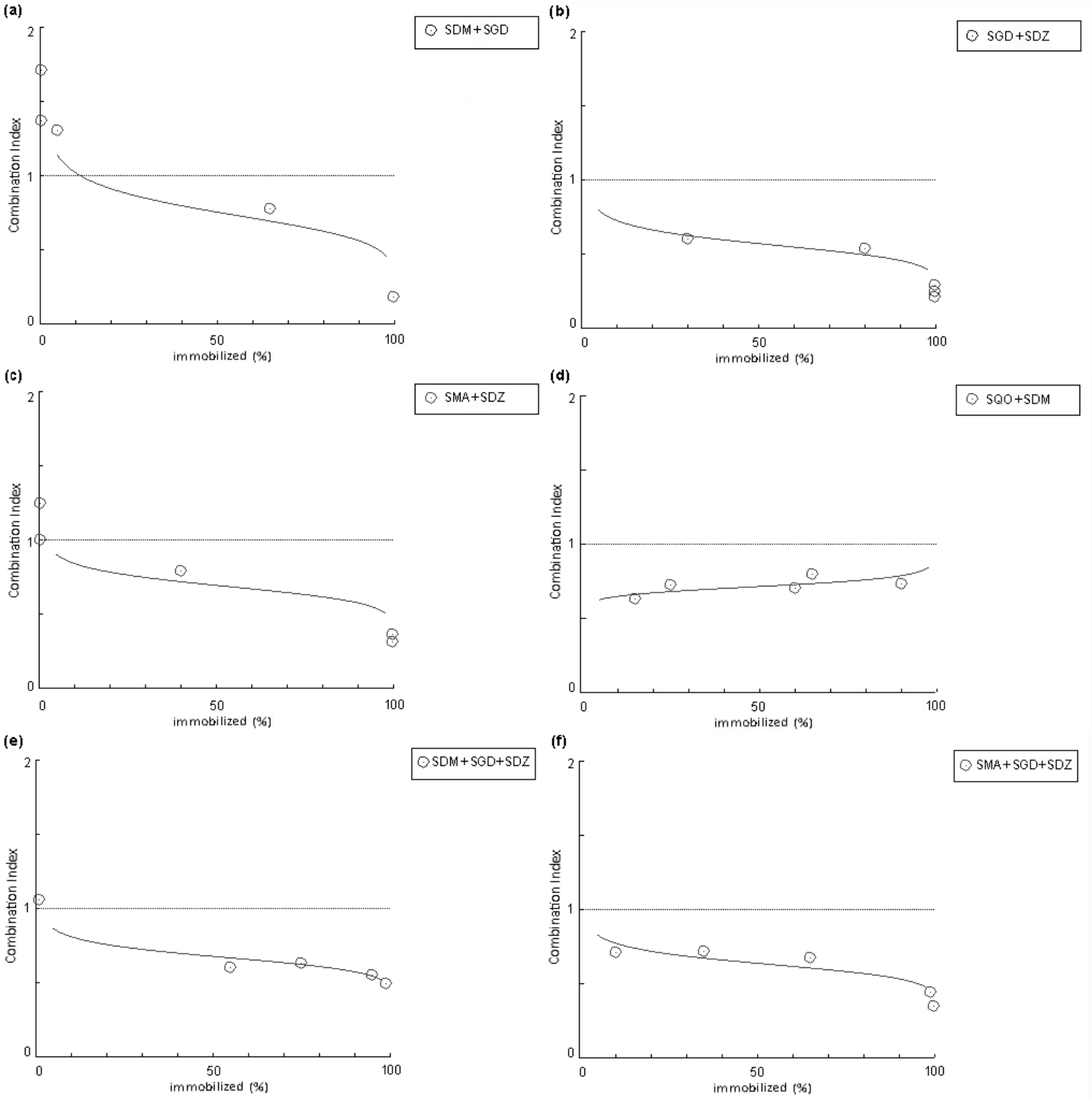

b)
SGD+SDZ
(EC50=1.6+51.9)
immobilized (%)
Concentration (mg/L)
0
50
100
300

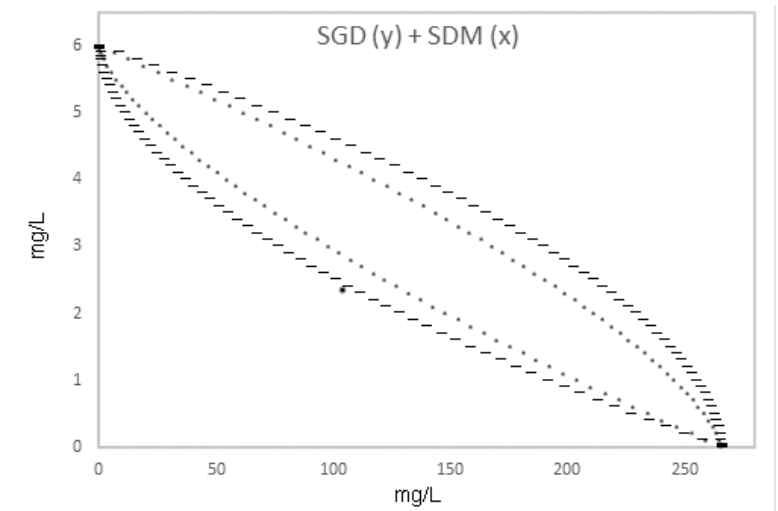

c)
SMA+SDZ
(EC50=77+71.6)
immobilized (%)
Concentration (mg/L)
0
50
100
300

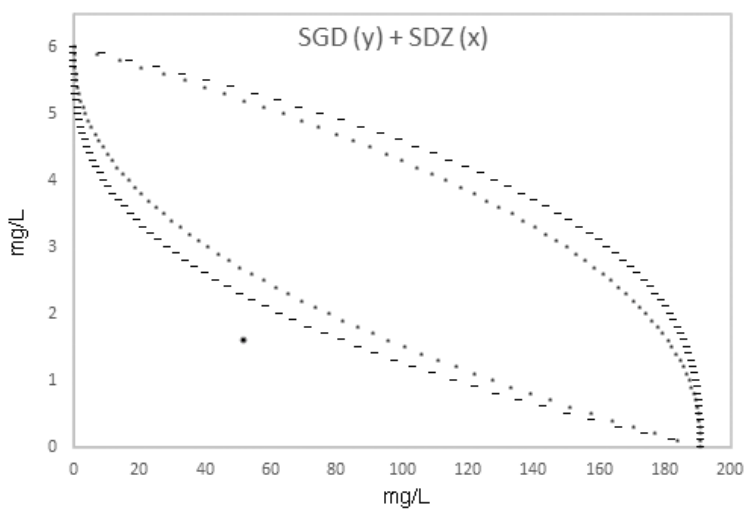

d)
SQO+SDM
(EC50=49.6+95.4)
immobilized (%)
Concentration (mg/L)
0
50
100
300

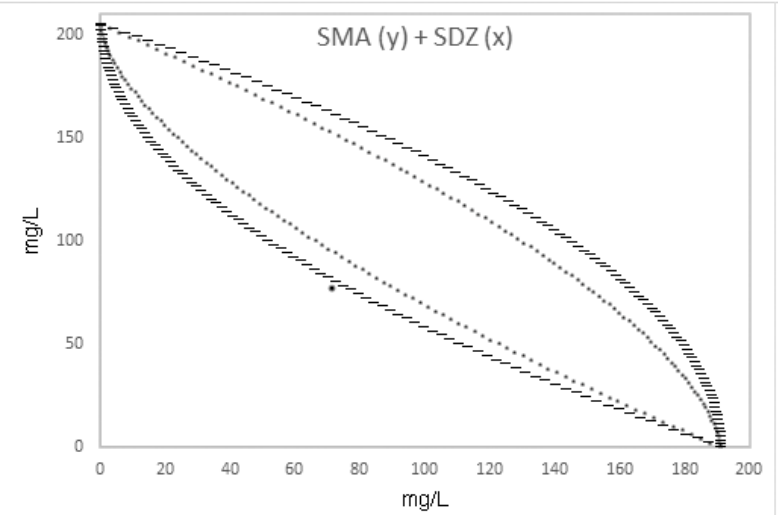

e)
SDM+SGD+SDZ
(EC50=60.3+1.4+43.3)
immobilized (%)
Concentration (mg/L)
0
50
100
300

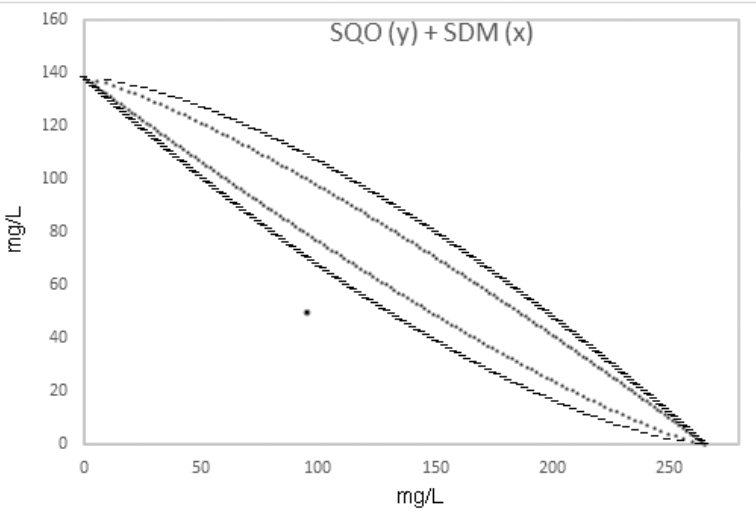

f)
SMA+SGD+SDZ
(EC50=43.8+1.3+40.8)
immobilized (%)
Concentration (mg/L)
0
50
100
300


(a)
SDM+SGD
Combination Index
immobilized (%)
(b)
SGD+SDZ
Combination Index
immobilized (%)
(c)
SMA+SDZ
Combination Index
immobilized (%)
(d)
SQO+SDM
Combination Index
immobilized (%)
(e)
SDM+SGD+SDZ
Combination Index
immobilized (%)
(f)
SMA+SGD+SDZ
Combination Index
immobilized (%)


SGD (y) + SDM (x)
mg/L
mg/L
0
1
2
3
4
5
6
0
50
100
150
200
250


SGD (y) + SDZ (x)
mg/L
mg/L
0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
160
180
200


SMA (y) + SDZ (x)
mg/L
mg/L
0
50
100
150
200
0
20
40
60
80
100
120
140
160
180
200


SQO (y) + SDM (x)
mg/L
mg/L
0
20
40
60
80
100
120
140
160
0
50
100
150
200
250