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

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9

10 **Abstract**

11

12 In livestock farming, sulfonamides (SAs) are used prophylactically and simultaneously

13 in large numbers of animals. Therefore, traces of these compounds, alone or in

14 combination, have been repeatedly detected in the environment. Synergistic interactions

15 among chemicals in such mixtures represent an area of concern for the regulatory

16 authorities. In this study, the acute toxic effects of binary and ternary mixtures of SAs

17 were evaluated in *Daphnia magna*, in order to verify whether, based on their individual

18 toxicity, they jointly exert a larger effect than would be predicted by individual actions

19 alone. First, following the Concentration Addition (CA) principle, some preliminary

20 observations were made by testing a number of drug combinations with an expected

21 50% effect. Then, mixtures more recognised for their synergistic effect (four binary and

22 two ternary) were assayed in a range of reducing concentrations. The data acquired were

23 processed using CompuSyn software, which integrates the different shape of the curves

24 obtained in calculating the Combination Index (CI) for the evaluation of synergistic

25 effects. For binary mixtures, synergy was also evaluated using the curvilinear

26 isobologram method for heterodynamic drugs. Results indicate that most of the selected

27 mixtures exhibit a synergistic effect using the CI methodology. For binary mixtures,

28 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.

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

58 subject to weak metabolism in the body of livestock and thus are eliminated mainly as
59 such, or in the form of active metabolites in the excreta (Białk-Bielińska et al., 2013).
60 As manure and slurry from farms are usually employed for the fertilization of
61 agricultural land, a contamination of soil with SAs residues is clearly expected. During
62 rainy days these residues can, partially at least, be transferred from soil to surface water
63 by runoff (Boxall et al., 2002). Furthermore, SAs may also be directly released to
64 watercourses as their use is extended to aquaculture in various countries. It is a matter
65 of fact that residues of SAs have been repeatedly detected in the aquatic environment
66 (Boxall *et al.*, 2005; Perret *et al.*, 2006; Xu *et al.*, 2007; García-Galán *et al.*, 2009; Santos
67 et al., 2010; Guedes-Alonso et al., 2013; Giang et al., 2015).
68 For many years, the small crustacean *Daphnia magna* has been recognised as a keystone
69 species in the food webs of many continental water bodies, and has served as an
70 important model for ecotoxicological research (Seda and Petrusek, 2011). Impacts on
71 daphnid populations may reverberate across the entire aquatic ecosystem as they are
72 principal grazers of algae and primary forage for fish in lentic in-land ecosystems
73 (Colbourne et al., 2011). The acute toxicity of SAs to *D. magna* is usually low (EC$_{50s}$
74 >100 mg L^{-1}) with the notable exception of Sulfaguanidine (EC$_{50}$ 6.2 mg L^{-1}) (Dalla
75 Bona *et al.*, 2014). However, as SAs occur in natural environment not just as a single
76 entity, but usually together with other compounds of the same family or the same type
77 (Managaki *et al.*, 2007; Baran *et* al., 2011; García-Galán *et al.*, 2011), it is of interest to
78 evaluate the toxicity of their mixtures.
79 Here we adopted the concept of Concentration Addition (CA) to express the contribution
80 of each chemical to the final mixture toxicity (Loewe and Muischnek, 1926). The
81 concept is based on the assumption that all chemicals in a mixture act on the same
82 biological target site and therefore could be viewed as being dilutions of each other, each
83 having a different chemical potency (Cedergreen, 2014). Each chemical contribution to
84 the overall toxicity of a mixture can be expressed as the quotient of its dose in the
85 mixture and the dose of the same chemical alone that would be required to elicit the
86 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* (8×10^5 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 2×10^8 cells mL^{-1} and stored at 4 ± 1 °C.

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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*

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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

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174 Acute toxicity tests were performed according to the Guideline 202 '*Daphnia sp.*, Acute
175 Immobilisation Test' (OECD, 2004). The ADaM medium was used for Controls and the
176 dilution of test compounds. Eight groups of 5 young daphnids (third brood neonates;
177 <24 h) were exposed to each of the assayed mixtures (Table 1 and Table 2) or used as
178 controls. The organisms were fed for about 1 h with 100% pure, dried Spirulina powder
179 (15 mg in 100 mL ADaM) just before the start of the experiment, and then each group
180 was incubated in a 20 mL glass vessel loosely covered with parafilm, and containing 10
181 mL of the test solution, under the same conditions (light, temperature) used for culturing.
182 Pre-feeding of the organisms is not deemed necessary by the test guideline, however in
183 our experience is strongly advisable as it helps to sustain 100% survival in the control
184 groups. The number of immobile daphnids recorded after 48h was the endpoint for effect
185 calculation.

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187 *Data Analysis*

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

195

196
$$f(x) = \frac{1}{1 + \left(\frac{EC_{50}}{x}\right)^{m}}$$

197

198 If the exponent *m* is greater than 1, the curve is sigmoidal, when it is equal to 1 the curve
199 is hyperbolic (Chou and Martin, 2005). The most relevant aspect of CompuSyn is to
200 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:

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

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.

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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

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

261 Three ternary mixtures were tested, and all gave indications of greater than additive
262 interaction (Figure 2). The following had greater than 90% effect: SDM+SGD+SDZ
263 (100%); SMA+SGD+SDZ (100%). These were re-assayed in a range of reducing
264 concentrations (from 0.33 to 0.165 EC_{50} of each component): their concentration-effect
265 curves and EC_{50}s are shown in Figure 3 and compared to the effect curves predicted by
266 CA. Their effects were confirmed to be synergic at all effect levels (Figure 4).

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

270

271 **Discussion**

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

288 The various SAs evaluated in this study share the same mechanism of action and cellular
289 target (Eguchi *et al*., 2004); consequently, their combinations should follow the CA
290 principle (Cedergreen, 2014). The 15 (preliminary) binary tests, where all possible pairs
291 were tested, showed 9 cases of less than additive interaction (Combination Index >1),
292 and 6 cases of more than additive interaction (Combination Index <1). The 3
293 (preliminary) ternary tests, chosen, based on binary test results, all showed more than
294 additive interaction. In some cases the deviation from the rule of CA, in one way or
295 another, was strong (Figure 2). The CA principle, however, is rooted in the assumption
296 of a constant relative potency of the drugs being combined. In other words, the Hill
297 coefficients (Faust *et al*., 2003), which respectively describe their concentration-effect
298 relations, should be equal (Tallarida, 2006). With the studied SAs this assumption did
299 not hold true (Figure 1). Therefore, the positive results of the preliminary tests were
300 taken only as a possible indication of synergy, rather than definitive proof. The more
301 promising mixtures were then re-assayed in a range of five concentrations, spreading
302 experimental data points below and above the EC_{50} value, as suggested by Chou for the
303 use of the CompuSyn application (Chou and Martin, 2005). At the 50% effect level,
304 synergy was confirmed for all four binary mixtures by applying the equations of
305 Tallarida. To further check the synergetic effects, worst-case isoboles were also included
306 by adding the computed error to the larger exponent and subtracting the computed error
307 from the lowest exponent. Figure 5 shows that all the binary mixtures, passed this more
308 restrictive test, indicating significant synergy around the EC_{50} combined dose.
309 For a more comprehensive evaluation of the drug interactions, we used the CompuSyn
310 program, which allows the evaluation of synergy at all effect levels, both for binary and
311 ternary mixtures. Detected synergies (Figure 4) indicate that the concept of CA is not
312 always precautionary as a reference model for the evaluation of the aquatic toxicity of
313 SA mixtures. Interestingly, SDZ that is the only SA licensed for aquaculture in EU, and
314 therefore more prone than other compounds to the contamination of the aquatic
315 environment, was frequently involved in synergic interactions. It should be noted,
316 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

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446

447 **Figure Captions**

448

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

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

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

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

465   Figure 5. Isobolograms of SAs binary mixtures assayed on *D. magna*. For compounds with a variable potency
466   ratio, synergy is detected only if the EC_{50} of the mixture lies below the region of the plane bounded by the
467   two curves of additivity for a 50% effect (Tallarida, 2006). Dotted lines represent curves of additivity. Dashed
468   lines are their confidence limits based on Hill coefficient variability. SGD, sulfaguanidine; SDM,
469   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

Figure (six dose-response panels):

Panel 1 (top left): SDM, EC$_{50}$ = 265.6, r = 0.982, m = 6.495. Axes: immobilized (%) vs Concentration (mg/L), x-axis 0 to 750.

Panel 2 (top right): SGD, EC$_{50}$ = 6.00, r = 0.984, m = 4.508. Axes: immobilized (%) vs Concentration (mg/L), x-axis 0 to 75.

Panel 3 (middle left): SDZ, EC$_{50}$ = 190.8, r = 0.981, m = 10.004. Axes: immobilized (%) vs Concentration (mg/L), x-axis 0 to 750.

Panel 4 (middle right): SMA, EC$_{50}$ = 205.0, r = 0.981, m = 6.321. Axes: immobilized (%) vs Concentration (mg/L), x-axis 0 to 300.

Panel 5 (bottom left): SQO, EC$_{50}$ = 138.0, r = 0.990, m = 8.136. Axes: immobilized (%) vs Concentration (mg/L), x-axis 0 to 750.

Panel 6 (bottom right): SMZ, EC$_{50}$ = 208.7, r = 0.961, m = 5.176. Axes: immobilized (%) vs Concentration (mg/L), x-axis 0 to 500.

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

(b) SGD+SDZ

(c) SMA+SDZ

(d) SQO+SDM

(e) SDM+SGD+SDZ

(f) SMA+SGD+SDZ