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Original Citation:

Availability:

This version is available at: 11577/3281325 since: 2020-01-23T11:14:20Z

Publisher:

Elsevier

Published version:

DOI: 10.1016/j.ecoenv.2018.08.011

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(Article begins on next page)

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

2

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5

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8

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

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

31

32

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

34

35 **Introduction**

36

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

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

54 Many of the available Veterinary Medicinal Products (VMPs) containing SAs are
55 marketed as a premix, to be added to feed, or as an oral solution to be added to water.
56 Using these formulations, animals may be treated simultaneously for preventive
57 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 $>100 \text{ mg L}^{-1}$) 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

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

90 To evaluate the CA deviations we prepared different binary and ternary mixtures
91 containing SAs, with concentrations of each compound that, based on the CA concept,
92 would be expected to result in a 50% immobilisation of *D. magna* after incubation for
93 48h. The scope of this experiment was to provide only a preliminary assessment of the
94 synergistic tendencies of the molecules studied to allow later selection of the most
95 appropriate mixtures for further evaluation. Mixtures that showed a strong indication of
96 interactions that were more than additive, were then tested using a range of reducing
97 concentrations of the components, in an equi-toxicity concentration ratio design. These
98 latter data were processed using CompuSyn software (Chou and Martin, 2005) to
99 identify the EC₅₀ of each mixture, and to evaluate, more precisely, the interactions of its
100 components (antagonism/synergy) at all effect levels. For binary mixtures, synergy was
101 also evaluated using the curvilinear isobologram method proposed by Tallarida (2006)
102 for heterodynamic drugs.

103

104 **Materials and Methods**

105

106 *Culture conditions*

107

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

111 The subject organisms were maintained in Aachener Daphnien Medium (ADaM:
112 hardness 193 mg CaCO₃L⁻¹; Klüttgen *et al.*, 1994a,b) at 20±1°C, with a photoperiod of
113 16 h light (2.6 µE m⁻² s⁻¹): 8 h dark. Their health status was optimal, and they did not
114 show any sign of stress: mortality rate was ≤ 2% per week; reproduction rate was around
115 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.

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 EC₅₀ 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 EC₅₀ 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 EC₅₀; equal volumes of two or three of
144 these solutions were mixed to generate the binary and ternary mixtures (Table 1). In this

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

151

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

153

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

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

171

172 *Toxicity tests*

173

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.

186

187 *Data Analysis*

188

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 EC₅₀ 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

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

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

218

219
$$b = B_{50} \left(1 - \frac{a}{A_{50}} \right)^{q/p} ; \quad b = B_{50} \left(1 - \left(\frac{a}{A_{50}} \right)^{q/p} \right)$$

220

221 where q and p are the exponents of the Hill curves (m in the previous equation) for the
222 chemicals A and B, respectively (Tallarida, 2006). A_{50} and B_{50} refer to the EC_{50} of each
223 of the two chemicals, while a and b are the doses (or concentrations) of each chemical
224 A and B. When $p = q$, the dose equivalent for B collapses to a single straight line, as can
225 be seen from the equation above. However, in the general case, the greater the difference
226 between q and p , the farther from this diagonal the two isoboles are (Tallarida, 2006).
227 Since we evaluated the Hill shape exponent with CompuSyn, and the program reports
228 the associated error for the computed exponent, we highlighted in the isobologram the
229 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_{50} s, 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 ($20 \pm 1^\circ\text{C}$) 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_{50} s 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 EC₅₀ 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 EC₅₀ of each component): their concentration-effect
265 curves and EC₅₀s 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 EC₅₀s (CVMP/VICH/790/03),
269 were always > 40 µg L⁻¹, with the exception of SGD (> 1 µg L⁻¹).

270

271 Discussion

272

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

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

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

342

343 **Conclusions**

344

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

354

355 **Acknowledgements**

356

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

360

361

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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, sulfadimethoxine; 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 sulfadimethoxine; 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 sulfadimethoxine; 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 sulfadimethoxine; 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

Sulphonamide mixtures

[illegible]