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1 **Development of a new approach using mathematical modeling to predict**  
2 **cocktail effects of micropollutants of diverse origins.**

3

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5

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9

10 **Highlights**

- 11 • To reduce the number of cytotoxicity experiments, an orthogonal array composite design was used.  
12 • Cocktail effects were looked for by using a response surface model.  
13 • A neutralizing effect was observed between bisphenol A and tramadol.

14

15 **Abstract**

16 A wide variety of micropollutants (MP) of diverse origins is present in waste and surface waters without  
17 knowing the effect of their combination on ecosystems and human. The impact of chemical mixtures  
18 is poorly documented and often limited to binary mixtures using MP of the same category. Knowing  
19 that it is not realistic to test every possible combination found in mixtures, we aimed to develop a new  
20 method helping to predict cocktail effects. Six chemicals of agriculture, industry or pharmaceutical  
21 origin were selected: cyproconazole, diuron, terbutryn, bisphenol A, diclofenac and tramadol.  
22 Individual MP were first used in vitro to determine the concentration at which 10 % (Effective  
23 Concentration EC10) or 25% (EC25) of their maximal effect on human cytotoxicity was observed. Using  
24 an Orthogonal Array Composite Design (OACD), relevant complex mixtures were then tested. Multiple  
25 linear regression was applied for response surface modeling in order to evaluate and visualize the  
26 influence of the different MP in mixtures and their potential interactions. The comparison of the  
27 predicted values obtained using the response surface model with those obtained with the model of  
28 independent effects, evidenced that the hypothesis of independence was unjustified. The cocktail  
29 effect was further investigated by considering micropollutant response surfaces pairwise. It was  
30 deduced that there was a neutralizing effect between bisphenol A and tramadol. In conclusion, we  
31 propose a new method to predict within a complex mixture of MP the combinations likely involved in  
32 cocktail effects. The proposed methodology coupling experimental data acquisition and mathematical  
33 modeling can be applied to all kind of relevant bioassays using lower concentrations of MP. Situations

34 at high ecological risk and potentially hazardous for humans will then be identified, which will allow to  
35 improve legislation and policies.

36

37 284 words

38

39 **Keywords:** micropollutant, surface water, cocktail effect, orthogonal array composite design,  
40 response surface model

41

42 **Abbreviations:** MP: micropollutant; EC: Effective Concentration; EDC: endocrine disrupting compound;  
43 HUVEC: Human Umbilical Vein Endothelial Cells; OACD: Orthogonal Array Composite Design; REACH:  
44 Registration, Evaluation, Authorisation and restriction of Chemicals; RSM: Response Surface Model

45

## 46 **Introduction**

47

48 Complex contamination of water by organic chemicals threatens ecosystems and human  
49 health as micropollutants (MP) exhibit potential harmful effects on non-target organisms. The  
50 numerous chemical components are generally found in all matrices and also accumulate in wild-life  
51 organisms. Pico et al. (2019) recently showed that contaminants of emerging concern are present as  
52 mixtures in most freshwater fish species at low levels in European rivers. MP are predominantly  
53 aromatic compounds used as pesticides, pharmaceuticals or industrial chemicals (41%, 30% and 16%  
54 of total contaminating organic compounds, respectively; Busch et al., 2016). Pesticides widely used for  
55 agriculture, are constantly present in the environment and consequently, included in environmental  
56 monitoring programs. Poorly reduced by municipal wastewater treatment processes (Stamatis et al.,  
57 2010), significant levels enter river streams. Co-occurrence of multiple pesticides in a given water  
58 sample is often observed (2 or more pesticides in 90% urban and agricultural streams). Cyproconazole,  
59 one of the most frequent detected fungicide in influent and effluent samples with 93% and 79%  
60 detection percentages and also present in river samples (Stamatis et al., 2010), was chosen in our  
61 study, associated with diuron and terbutryn.

62

63 Pharmaceuticals are a class of emerging contaminants found in the aquatic environment with  
64 poor degradation and growing utilization rates. Like pesticides, pharmaceuticals are not completely  
65 removed from wastewater treatment plants, leading to their potential presence in drinking water  
66 (Jones et al., 2001). Among them, diclofenac, a prevalent non-steroidal anti-inflammatory drug  
67 (veterinary and human use) is often detected. Anti-inflammatories and analgesic drugs such as

68 tramadol belong to the therapeutic group the most commonly detected in soils and sediments (Biel-  
69 Maeso et al., 2017). A recent study (Schmitz et al., 2018) evidenced that combined occurrence of  
70 common pharmaceutical drugs at concentrations present in surface water environments may act as  
71 endocrine-disrupting compounds (EDC).

72  
73 EDC are defined as exogenous substances (individual or mixture) belonging to a broad family  
74 of compounds that alter hormone secretion, potentially affecting different functions of the organism  
75 (individual, offspring or subpopulations): metabolism, reproductive functions, nervous system...The  
76 sources of exposure are numerous and difficult to control. Among them, bisphenol A, classified as  
77 category 1B reprotoxic substance from REACH legislation (Registration, Evaluation, Authorisation and  
78 restriction of CHemicals), was included in our study. Bisphenol A is ubiquitous in the environment as a  
79 result of its high production, consumption, and subsequent environmental introduction (Corrales et  
80 al., 2015). It is well documented that bisphenol A affects aquatic organism reproduction (Luo et al.,  
81 2017, Wang et al., 2019).

82  
83 Current risk assessment studies mostly focus on exposure to single chemicals. Studies involving  
84 mixtures of MP mainly deal with chemicals of the same group and are used as binary mixtures.  
85 Pesticides and antifouling chemicals are used in more than 90 % and 75 % of the publications,  
86 respectively (reviewed by Cedergreen, 2014), without considering the large variety of chemicals  
87 present in a same location at a same time. Different mathematical models have been developed to  
88 predict the effects of mixtures. Hence, using Concentration Addition (CA) or Independent Action (IA)  
89 models, the overall toxicity of a mixture of known composition can be determined to some extent  
90 (Kienzler et al., 2016). Synergistic or antagonistic effects are also evidenced when compounds interact  
91 together (Laetz et al., 2009). The effect resulting from the interaction between chemicals is called  
92 cocktail effect. Other studies use environmental samples where the mixture is tested as a whole. A  
93 screening approach consisting of the analysis of a maximum of chemicals is then compared with the  
94 individual chemical known effects. The monitoring of water quality is mostly based on chemical  
95 analyses of surface water using liquid chromatography coupled to tandem mass spectrometry. This  
96 approach gives accurate information regarding the structure and the number of numerous  
97 contaminants but is complex and cost-effective.

98  
99 In this context, the objective of our study is to propose a new methodology using mathematical  
100 modeling to easily determine cocktail effects within complex mixtures containing diverse categories of  
101 MP, whatever their mode of action. We use a statistical design termed Orthogonal Array Composite  
102 Design (OACD) based on a two-level factorial design (two concentrations of MP) and a three-level

103 orthogonal array (three concentrations of MP). This new class of composite design recently developed  
104 by Ding et al. (2013) and Xu et al. (2014) is effective for response surface modeling. A second-degree  
105 polynomial model is then applied to estimate the potential interactions between MP. Briefly, the  
106 experimental procedures were carried out with Human Umbilical Vein Endothelial Cells (HUVEC).  
107 These cells are directly exposed to chemicals circulating in the blood and may consequently be a site  
108 for adverse effects of xenobiotics, metabolically activated by cytochrome P450 enzymes and/or other  
109 Ah receptor-regulated enzymes (Annas et al., 2000; Dauchy et al., 2009). Six MP were selected:  
110 bisphenol A used in the chemical industry, diclofenac and tramadol from the pharmaceutical industry  
111 and cyproconazole, diuron and terbutryn, three pesticides from agriculture activities. The cytotoxicity  
112 of the six MP was first evaluated individually. Then different mixtures were tested at the concentration  
113 at which 10 % (Effective Concentration EC10) or 25% (EC25) of their maximal effect on human  
114 cytotoxicity was observed. The OACD allowed to reduce the number of the subsequent in vitro  
115 experiments using MP mixtures. A response surface model was then applied to look for cocktail effects.

116

## 117 **Materials and Methods**

118

### 119 **Chemicals**

120

121 Six chemicals corresponding to micropollutants of different categories and origins were used  
122 for the in vitro tests. 75 mM stock solutions were aliquoted in 100% methanol and stored at -20°C.  
123 100X solutions were prepared in 100% methanol for the treatments. Bisphenol A (#15824) was  
124 purchased from Acros Organics. Other chemicals: cyproconazole (#46068), diclofenac (#93484), diuron  
125 (#45463), terbutryn (#45677) and tramadol (#42965) were purchased from Sigma.

126

### 127 **Cell culture and single compound treatment**

128

129 HUVEC obtained from Lonza, were maintained in Endothelial Growth medium (EGM-2, Lonza)  
130 at 37°C and 5% CO<sub>2</sub>. The medium was changed every 2 days, and the cells were passaged when they  
131 reached 80% confluence. They were used at passage 6 for all the treatments. HUVEC cells were seeded  
132 into 96-well plates (10<sup>4</sup> cells/well) and were allowed to adhere overnight in phenol red-free M200  
133 medium (Gibco), supplemented with LSGS (Low Serum Growth Supplement kit), 10% (v/v) charcoal-  
134 stripped-fetal bovine serum and 1% (v/v) penicillin–streptomycin before starting the experiments. The  
135 primary cell line was first treated with increasing concentrations of individual MP (0, 100, 200, 300,  
136 400 and 500 µM) to determine their cytotoxicity and in particular the effective concentration for which

137 10% (EC10) and 25% (EC25) between the minimum and the maximum cytotoxic effect was obtained.  
138 The untreated condition (0 μM) consisting of M200 contained 1% methanol (reference) as this  
139 concentration of methanol was used when performing all the treatments. Each treatment was  
140 performed in triplicate and three independent experiments were used for EC determination. Mixed  
141 pollutants were then tested as described in the OACD design section.

142

### 143 **Crystal violet staining**

144

145 HUVEC cells seeded into 96-well plates were treated with individual or mixed MP for 24 h. The  
146 medium with 1% methanol was used as a blank. At the end of the treatment, the medium in each well  
147 was discarded, wells were rinsed with Phosphate Buffer Saline, then 100 μl of formaldehyde 3.7% was  
148 added to fix the cells (10 min, room temperature). The plate was washed three times with deionized  
149 water before adding 0.1 % crystal violet (30 min incubation.) After 3 additional rinses, acetic acid 10%  
150 was added for 30 min prior to absorbance reading at 590 nm using FLUOstar® Omega Microplate  
151 Reader (BMG LABTECH, Ortenberg, Germany). % cell survival evaluated by the percentage of adherent  
152 cells was calculated as follows:

$$153 \quad \% \text{ survival cells} = \frac{Abs - \bar{X} \text{ blank}}{\bar{X} \text{ reference} - \bar{X} \text{ blank}} \times 100 \quad (1)$$

154

155 where Abs is the measured absorbance in each well;  $\bar{X}$  blank, the mean absorbance in blank (no cell in  
156 the well);  $\bar{X}$  reference, the mean absorbance for the reference (untreated cells). % mortality was then  
157 deduced (100 - % survival cells) to evaluate the cytotoxic effect.

158

### 159 **Single micropollutant EC determination**

160

161 Dose response curves were obtained for the six micropollutants: bisphenol A (BIS), cyproconazole  
162 (CYP), diclofenac (DIC), diuron (DIU), terbutryn (TER) and tramadol (TRA). Each dose response curve  
163 was used to determine the cytotoxic effective concentration EC50 using the following formula:

164

$$165 \quad y = f(x) + \varepsilon \quad (2)$$

166 with

$$167 \quad f(x) = a + \frac{b - a}{1 + (x/EC50)^h} \quad (3)$$

168

169 where  $y$  is 100 times the percentage of surviving cells,  $x$  the concentration of micropollutant,  $a$  the  
170 minimal survival rate,  $b$  the maximal survival rate,  $EC50$  the micropollutant concentration for which  
171  $y=(a + b)/2$ .  $\epsilon$  is the Gaussian observation error. An adjustment was done using the maximum likelihood  
172 method i.e. by minimizing the least square criterion:

$$173 \quad C(a, b, EC50, h) = \sum_{i=1}^n (y_i - f(x_i))^2 \quad (4)$$

174 with  $a > 0$ ,  $b > a$ ,  $EC50 > 0$ ,  $h > 0$  and where  $n$  is the number of observations collected.  $y_i$  is the observed  
175 percentage of surviving cells at concentration  $x_i$ .

176

177 In order to obtain the  $EC10$  (10% cytotoxicity) and  $EC25$  (25% cytotoxicity) values, the adjusted  
178 function was reversed, that is to say  $EC_y$  was calculated using the following formula:

$$179 \quad EC_y = EC50 \times \left( \frac{b - 1 + y}{1 - y - a} \right)^{1/h} \quad (5)$$

180 The R software was used for all calculations.

181

## 182 **OACD design**

183

184 Since there were six micropollutants, it was not feasible to test in vitro all the combinations  
185 using three conditions corresponding to 0,  $EC10$  and  $EC25$  values (respectively coded by -1, 0 and +1).  
186 Hence, the complete factorial design plan  $3^k$  (three levels and 6 factors, making  $3^6 = 729$  tests without  
187 any replicate) was replaced by a reduced factorial design using FrF2 and DoE.base packages (R  
188 software). The proposed OACD was composed of a subset of the experimental conditions carefully  
189 chosen in order to maintain the desired level of statistical power. This fractional factorial design of  
190 resolution V combining a two-level fractional design and a three-level orthogonal array comprised a  
191 total of 55 conditions (supplementary data). These 55 tests, for  $k=6$  factors, denoted by  $x_1, \dots, x_k$ ,  
192 consisted of 34 cube points with all  $x_i = -1$  or  $+1$  (2 levels), 6 center points with all  $x_i = 0$  and 15  
193 additional points with  $x_i = -1, 0$  or  $+1$  (3 levels). We performed four replicates, to which 45 central tests  
194 (center points) were added. Eventually, 265 tests were performed in vitro. Experiments were carried  
195 out in a randomized order (shuffling program).

196

## 197 **Mathematical approach for cocktail effect analysis**

198

199 The biological data obtained with MP mixtures were collected to perform the mathematical  
200 analysis, which was based on the adjustment of a second-order Response Surface Model (RSM) to the  
201 experimental data. The RSM is a second-order polynomial approximation for the unknown true

202 functional relationship between the percentage of surviving cells and the concentration of  
203 micropollutants. This methodology was previously described in Cheng and Wu (2001), Ding et al.  
204 (2013), Xu et al. (2014) and Weiss et al. (2015). The second-order RSM of degree two is given by the  
205 following equation:

$$206 \quad y = \beta_0 + \sum_{i=1}^6 \beta_i x_i + \sum_{i=1}^6 \beta_{ii} x_i^2 + \sum_{i=1}^5 \sum_{j=i+1}^6 \beta_{ij} x_i x_j + \epsilon \quad (6)$$

207 where  $y$  is 100 times the percentage of surviving cells,  $x_i$  ( $i = 1$  to  $6$ ) represents the concentration of the  
208 micropollutants,  $\epsilon$  is the observation error term. The adjustment was made using the least square  
209 method and the  $\beta$ 's are the parameters of the model.

210

### 211 **Physicochemical characteristics of micropollutants**

212

213 SMILES (Simplified Molecular-Input Line-Entry System) strings using short ASCII (American  
214 Standard Code for Information Interchange) format were imported from PubChem database  
215 (Weininger, 1988). The six SMILES strings were compared using Hierarchical Clustering Analysis (HCA)  
216 with Ward's method in the R core environment. n-octanol/water partition coefficient or  $K_{ow}$  is an  
217 important parameter to estimate environmental exposure risk as it is assumed that the uptake of an  
218 organic substance depends on its hydrophobicity. Attempts were made to correlate  $\text{Log}(K_{ow})$  values  
219 with the observed EC10 and EC25 values (linear regression).

220

### 221 **Results**

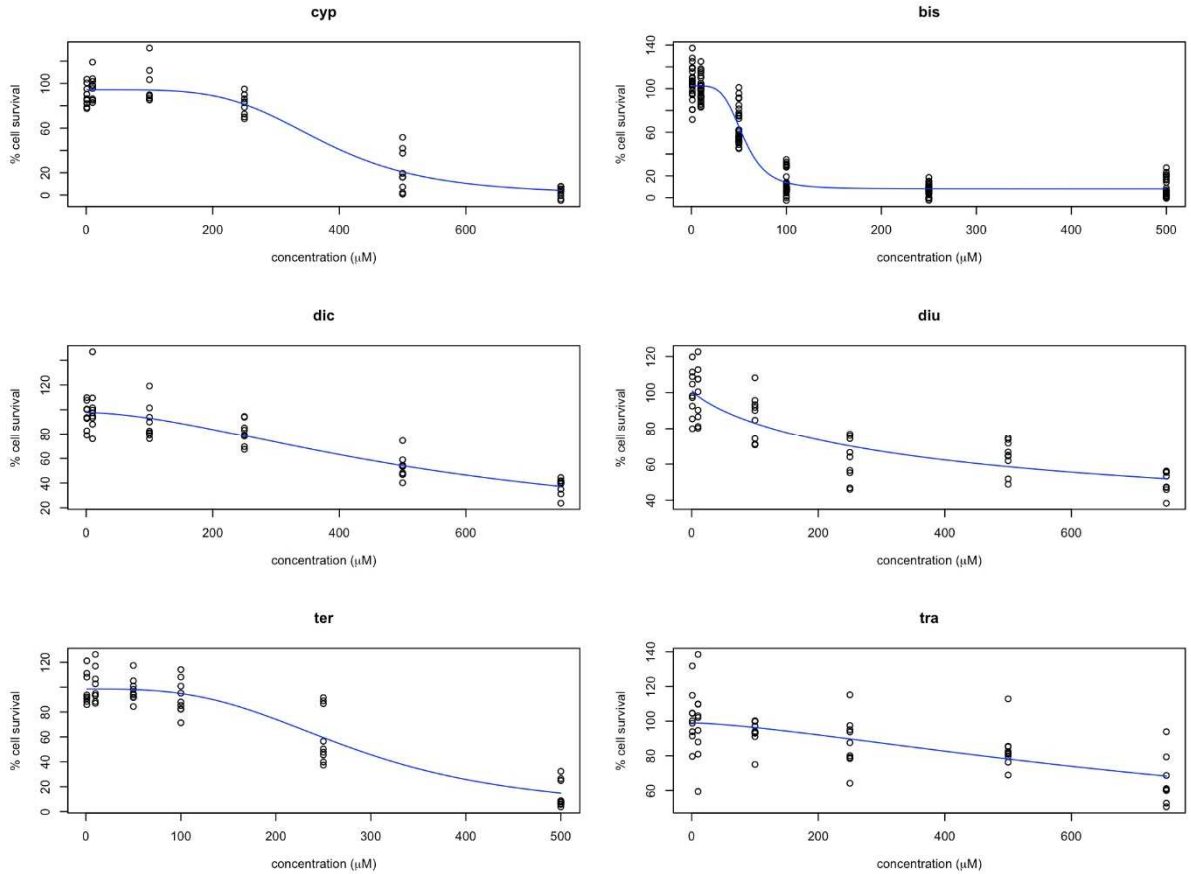
222

223 HUVEC cells were first treated with increasing concentrations of each micropollutant for 24h  
224 to establish dose response curves. Figure 1 shows the dose response curves we obtained for the six  
225 individual MP. Three independent experiments testing each dose in triplicate were performed to  
226 consolidate the concentration response pattern. The Hill equation was selected for the best-fitting  
227 regression model. The survival data collected using crystal violet staining were used to calculate  
228 cytotoxic effects. 100% mortality was obtained at higher doses for three MP: bisphenol A,  
229 cyproconazole and terbutryn. Tramadol used for human pain management was the less cytotoxic. The  
230 effective concentration (EC) values obtained for the different micropollutants are indicated in Table 1.  
231 Distinct EC10 and EC25 values were observed among the different MP, which highlights the need of  
232 concentration-effect analysis instead of using fixed and identical concentrations.

233

234





235  
236  
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238  
239

**Figure 1:** Dose response curves following single MP treatment. After 24h treatment of HUVECs with increasing concentrations of each micropollutant, a dose-response curve was obtained (BIS: bisphenol A; CYP: cyproconazole; DIC: diclofenac; DIU: diuron; TER: terbutryn; TRA: tramadol).

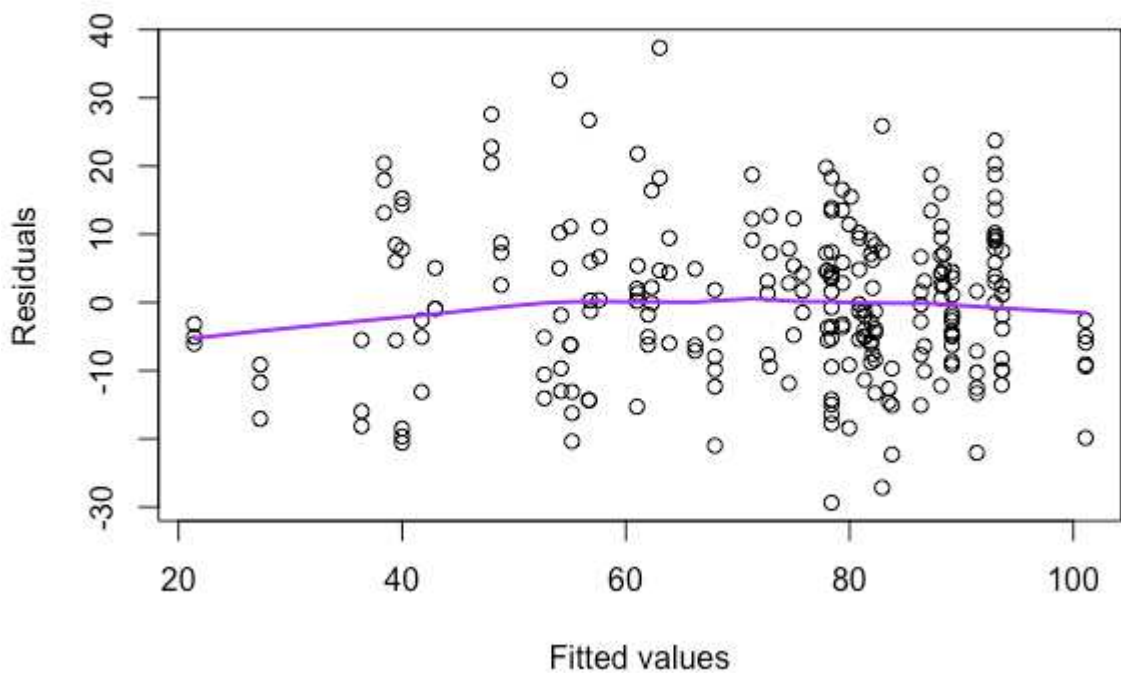
Micropollutant	EC <sub>10</sub> (µM)	EC <sub>25</sub> (µM)
Bisphenol A	36	45
Cyproconazole	191	278
Diclofenac	136	284
Diuron	49	182
Terbutryn	134	197
Tramadol	244	575

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246

**Table 1:** Comparison of EC10 and EC25 values. The effect of MP was individually tested on HUVECs. EC10 and EC25 values are the doses where 10 % and 25 %, respectively, of the maximal cytotoxic effect was observed for each MP.

MP mixtures were then tested using EC10 and EC25 values and an OACD design. The concentration 0 was coded by -1, the EC10 value by 0 and EC25 value by +1. The OACD plan composed

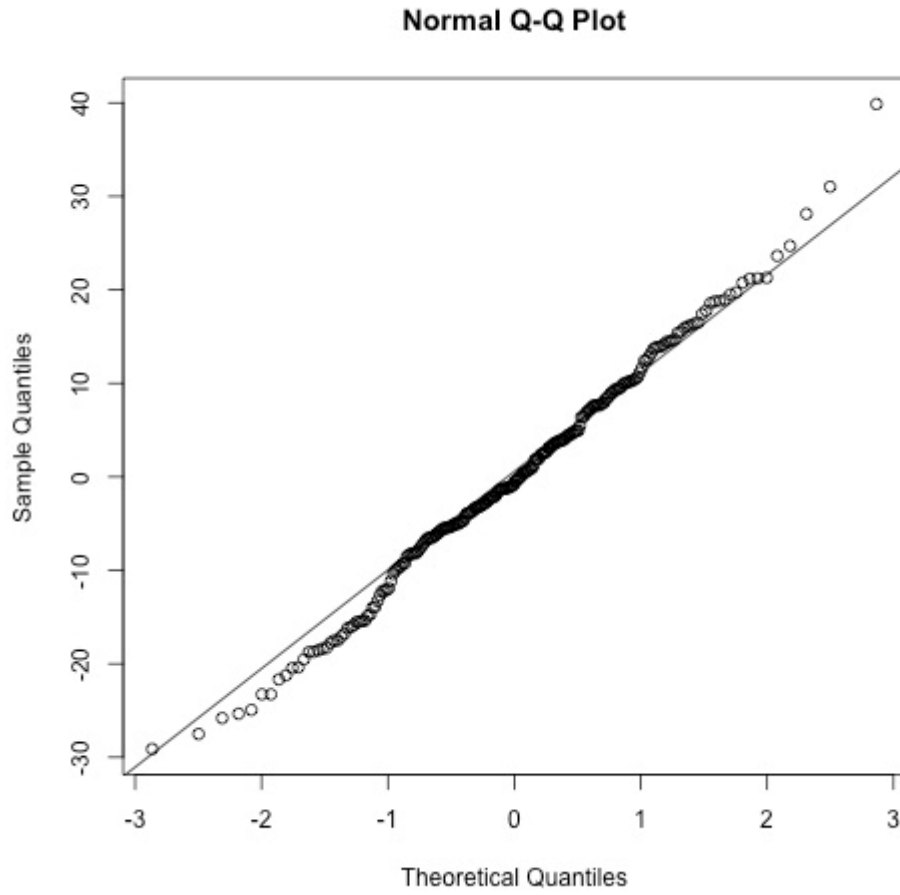
247 of 55 tests with replicates eventually consisted in 265 randomly distributed tests. The survival data  
248 were collected, and a second-order linear regression equation corresponding to the response surface  
249 model was applied. The pure error residuals, that are the observed values minus the predicted  
250 adjusted values, collected from all treatment groups, were used to assess whether the proposed  
251 statistical model was consistent with the data (Figure 2).  
252



253  
254 **Figure 2:** Comparison of residuals and predicted adjusted values. The plot of residuals against predicted adjusted  
255 values allowed to assess the response surface model.

256  
257 Additionally, the Q-Q plot analyzing the sample quantile versus theoretical quantile  
258 appears linear, which means that the residuals come from the expected normal distribution (Figure 3).  
259 There is a slight deviation at each extremity, suggesting slightly more extreme values than expected.  
260 This analysis of residues confirmed that the fit was correct.

261



262

263 **Figure 3:** Analysis of the residuals' distribution. Data distribution was checked by Q-Q Plot analysis.

264

265 The proposed model contains regression parameters that include coefficients for main  
 266 effects regarding one MP, coefficients for quadratic main effects regarding one MP and coefficients  
 267 for two MP interaction effects. A first exploratory adjustment of the response surface, including a  
 268 screening of the highest cell survival values, highlighted that relatively few quadratic terms had  
 269 significant coefficients different from 0, at the level of 10 % statistical significance. The model was  
 270 readjusted by keeping only the terms whose coefficient was significantly different from 0. We get an  
 271  $R^2$  of 0.7037 and an adjusted  $R^2$  of 0.6907. The coefficients obtained are shown in Table 2. The Fisher  
 272 statistic value is 54.38 with 10 and 229 degrees of freedom, with a p-value lower than  $2.2e-16$ , which  
 273 confirms that the response surface model is globally significant.

274

275

276

277

Coefficient	Estimation	p-value
C <sup>st</sup>	78.1339	***
CYP	-7.2737	***
DIU	-1.9785	*
BIS	-4.3555	***
TER	-15.6562	***
TRA	3.1778	***
CYP <sup>2</sup>	-14.1550	***
CYP*DIC	2.9985	**
CYP*BIS	-1.7606	.
CYP*TER	-2.2637	*
BIS*TRA	3.0041	**

278

279 **Table 2:** Estimates of the parameters for the response surface model. All coefficients are significantly  
 280 different from 0 to 10% level. "." corresponds to a p-value less than 0.1 and "\*" to a p-value less than  
 281 0.05. "\*\*\*" and "\*\*\*\*" correspond to lower levels.

282

283 Regarding the linear effects, all micropollutants except diclofenac are identified as significant.  
 284 Concerning the other parameters, the quadratic term CYP<sup>2</sup> and the bilinear effects CYP × DIC and BIS ×  
 285 TRA are the most significant terms (p-value < 0.05).

286

287 Next, the response surfaces corresponding to individual micropollutants were deduced from  
 288 the adjusted response surface, replacing the level of all others by -1. Each response surface is noted as  
 289 a function  $f_1(x_1), \dots, f_6(x_6)$  where  $x_1$  is the concentration of BIS, ...,  $x_6$  the concentration of TRA. When  
 290 assuming independent effects, the cell survival rate is given by the formula:

$$291 \quad 100 \times \frac{1}{100^5} \prod_{i=1}^6 f_i(x_i) \quad (7)$$

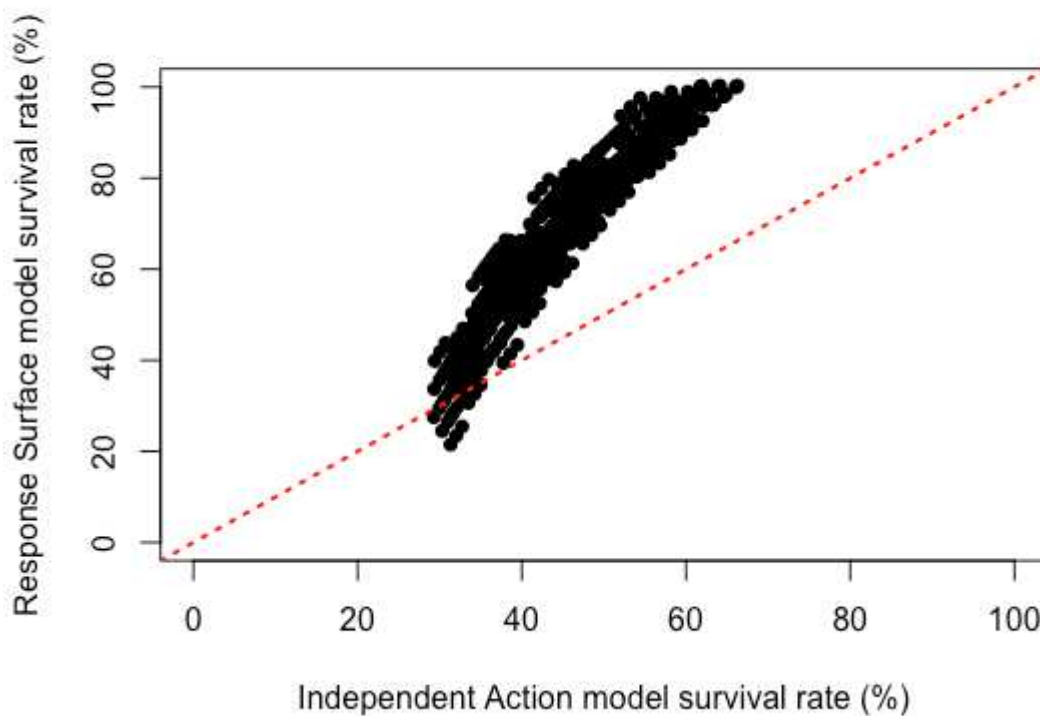
292

293 Thus, we can compare the survival rate obtained under the assumption of independence with  
 294 the survival rate obtained from the global response surface, for all combinations of levels (-1, 0, +1) of  
 295 the micropollutants. This is done graphically in Figure 4. Under the assumption of independence of the  
 296 effects, these must be placed along the first bisector of the Cartesian plane. As shown in Figure 4, these  
 297 are well above. Hence the hypothesis of independence seems unjustified from the graph.

298

299

300

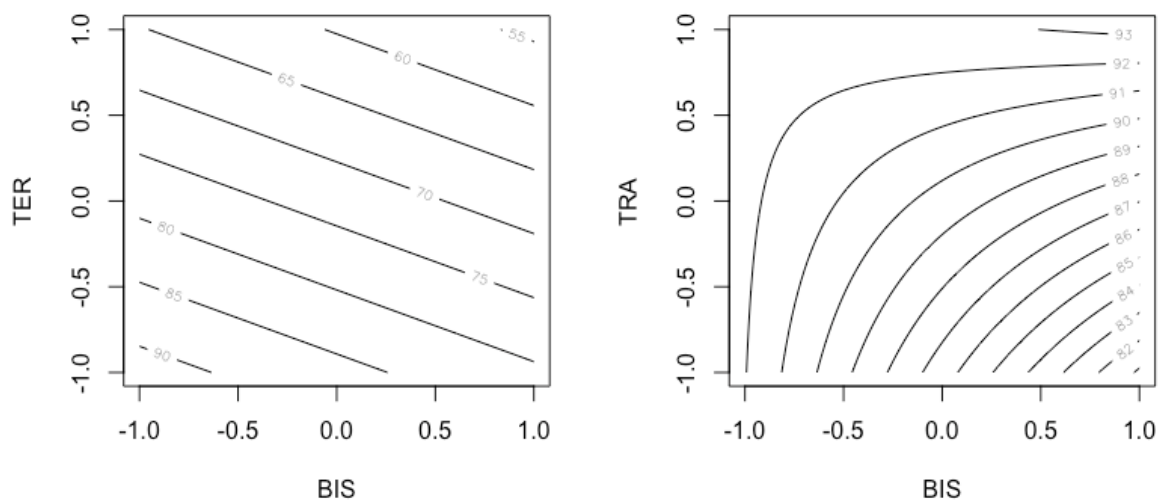


301  
 302 **Figure 4:** Comparison of cytotoxic effects estimated by the response surface model versus the independent  
 303 action model.

304  
 305 The values obtained using the response surface being mostly much higher than those  
 306 obtained under the hypothesis of independent effects, it was deduced that there is a strong global  
 307 cocktail effect between the micropollutants.

308  
 309 This effect can be investigated by considering micropollutant response surfaces taken  
 310 in pairs, the concentrations of the others being set at -1 in the overall response area. For example  
 311 (Figure 5), for BIS and TER, there is no interaction as the contour diagram reflects it and as it is reflected  
 312 in the response surface equation, where there is no significant interaction term corresponding to BIS  
 313 and TER. It is different for BIS and TRA: the interaction term in the response surface having a  
 314 significantly positive coefficient, it can be concluded that there is a neutralization between these two  
 315 micropollutants.

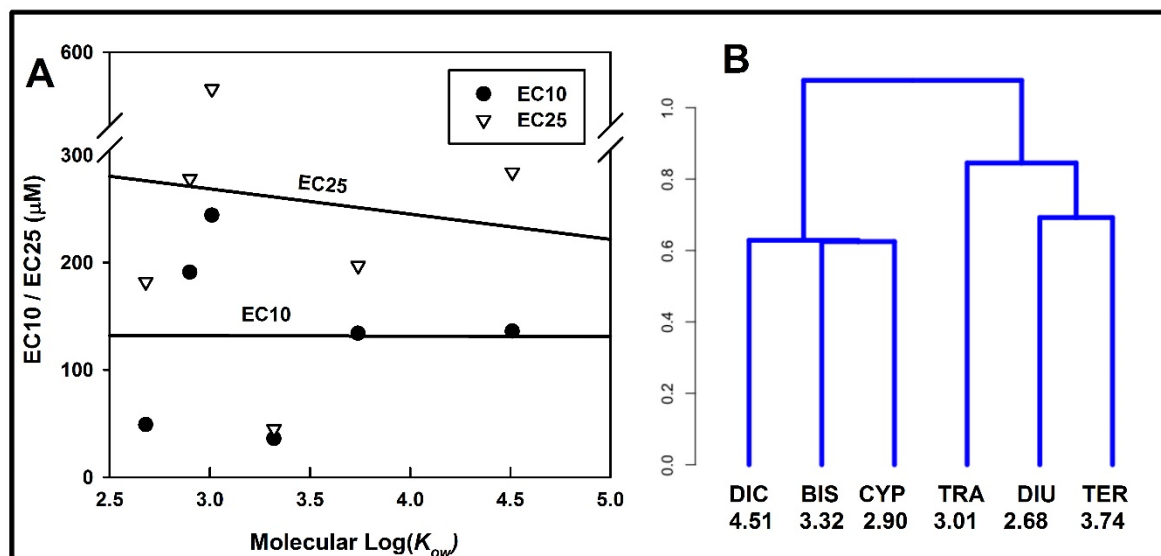
316  
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 320



321  
 322 **Figure 5:** Contour plot of response surface for BIS and TER (left panel) and for BIS and TRA (right panel) when the  
 323 concentration of the other MP is set at -1 in the overall response surface.

324  
 325 Eventually,  $\text{Log}(K_{ow})$  values of the six MP, used to measure their hydrophobicity, were  
 326 compared to the observed EC10 and EC25 values (Figure 6A). The absence of correlation suggests the  
 327 absence of a link between the uptake of each MP and its biological effect. We then used SMILES strings  
 328 (Weininger, 1988) to classify the six MP according to their physicochemical characteristics (Figure 6B).  
 329 The dendrogram calculated with the Ward's Hierarchical Clustering Method shows two clusters: one  
 330 containing bisphenol A and another one containing tramadol (neutralization with bisphenol A) and  
 331 terbutryn (no interaction with bisphenol A). These results suggest that the cocktail effect can't be  
 332 predicted using structural analysis of MP.

333



334  
 335 **Figure 6:** Physicochemical characteristics of MP. (A) Log(K<sub>ow</sub>) values were compared with biological activity of  
 336 MP. (B) SMILES strings were used for Hierarchical Clustering Analysis of MP with Ward's method (Numbers  
 337 indicate Log(K<sub>ow</sub>) values).

338

### 339 Discussion

340

341 We present a study focusing on MP cocktail effects where a selection of micropollutants was  
 342 made to reflect the heterogeneity of water contamination. Hence, six organic compounds, namely  
 343 cyproconazole, diuron, terbuthryn, bisphenol A, diclofenac and tramadol were tested. Single MP dose–  
 344 response curves were first generated for experimental data collection. Three doses per MP were then  
 345 retained for complex mixture analysis: EC25, the highest tested concentration, the dose where 25 %  
 346 of the maximal response was observed, EC10, where 10 % of the maximal response was observed, and  
 347 dose 0, where no MP was present. An OACD plan lead to test 55 combinations in 4 replicates, instead  
 348 of the complete factorial design plan made of 3<sup>6</sup> combinations. Then a response surface model allowed  
 349 to generate significant regression coefficients corresponding to single-chemical linear and quadratic  
 350 effects as well as two-chemical pair-wise interaction effects. A neutralizing effect between  
 351 micropollutants was observed, in particular, between bisphenol A and tramadol.

352 The proposed methodology was inspired by clinical methodologies developed to test the effect  
 353 of drug combinations (Weiss et al., 2015). The advantage of using drug mixtures is that they often have  
 354 higher efficacy and lower drug dosages than individual drugs. From an ecotoxicological point of view,  
 355 with the increase of MP present in the aquatic environment, important questions are emerging: is the  
 356 predicted toxicity of single compounds realistic in presence of other micropollutants, with same or  
 357 different modes of action?

358

359           The mixture risks are a major concern as illustrated with the recent growing number of studies  
360 dealing with mixtures of MP and mathematical modeling for combined effect prediction (Altenburger  
361 et al., 2004, 2005, Ermler et al., 2014). Altenburger et al. (2018) compared predicted with observed  
362 combined effects using twelve micropollutants at the estimated low effect levels (10 or 20% maximal  
363 effect). The use of 21 bioassays show variable results: response most of the time in agreement with  
364 concentration additive model, or statistically higher or lower activity than expected. The independent  
365 action model was the best way to predict the observed mixture effects when looking at apical effects  
366 (observable outcome in a whole organism). Usually, concentration addition model is retained for the  
367 similarly acting components, and subsequently, the dissimilarly acting compound groups are analyzed  
368 using independent action model (Altenburger et al., 2004). In environmental mixtures, the information  
369 on modes of action for all compounds is, however, not always easily available.

370           Very few studies investigate the robustness and validity of mathematical models to assess the  
371 additive effect of micropollutants with the same mode of action in complex and more realistic  
372 mixtures. Using human and fish reporter gene assays, Serra et al (2019) tested the combined effects  
373 of environmental xenoestrogens, among them the binary mixture bisphenol A and genistein, within  
374 multicomponent mixtures, comprising diuron and diclofenac. They evidenced that non-estrogenic  
375 chemicals can interfere with the response to a mixture of xenoestrogens; for example, the triazole  
376 fungicide propiconazole decreased estradiol-induced estrogenic activity in a non-specific manner.  
377 Using the response surface model, we show for the first time that bisphenol A interacts with the  
378 painkiller tramadol. It is largely described that bisphenol A perturbs normal endocrine functions.  
379 Surprisingly, it can also act on the nervous system (Ogiue-Ikeda et al., 2008), modulate synaptic  
380 plasticity in the hippocampus, interfere with anxiety-related behavior in rats (Farabollini et al., 1999)  
381 or disrupt physiologic neurotransmitter function (Alyea and Watson, 2009). More recently, Mizuta et  
382 al. (2017) showed that bisphenol A could inhibit compound action potentials (nerve conduction block)  
383 in the frog sciatic nerve, like tramadol (Katsuki et al., 2006). Our results show that these two chemicals  
384 can interact within the cell, whatever their mode of action. This cocktail effect can't be predicted using  
385 structural analysis of MP.

386

387           In conclusion, there is an urgent need to prioritize the risk assessment of chemical mixtures by  
388 testing MP under REACH legislation. We propose a method that can be transposed to in situ studies  
389 and monitoring programs. Hence, once the most prominent micropollutants in a given site have been  
390 identified, our mathematical approach can be applied to predict situations at risk. Other bioassays such  
391 as Reactive Oxygen Species detection, nuclear receptor-based responses... can be used to evaluate



392 environmentally relevant MP concentrations. Hopefully, new legislation dealing with priority cocktail  
393 monitoring will appear to reinforce micropollutant monitoring policy.

394

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396

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