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1 **Combined effects of environmental xeno-estrogens within multi-component mixtures:**
2 **comparison of *in vitro* human- and zebrafish-based estrogenicity bioassays**

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14 **ABSTRACT**

15 Some recent studies showed that *in vitro* bioassays based on fish or human estrogen
16 receptor (ER) activation may have distinct responses to environmental samples, highlighting
17 the need to better understand bioassay-specific ER response to environmental mixtures. For
18 this purpose, we investigated a 12-compound mixture in two mixture ratios (M1 and M2) on
19 zebrafish (zf) liver cells stably expressing zfER α (ZELH α cells) or zfER β 2 (ZELH β 2 cells)
20 and on human ER-reporter gene (MELN) cells. The mixture included well-known ER ligands
21 bisphenol A (BPA) and genistein (GEN), and other compounds representatives of a
22 freshwater background contamination. In this context, the study aimed at assessing the
23 robustness of concentration addition (CA) model and the potential confounding influence of
24 other chemicals by testing subgroups of ER activators, ER inhibitors or ER activators and
25 inhibitors combined. Individual chemical testing showed a higher prevalence of ER inhibitors
26 in zebrafish than human cells (e.g. propiconazole), and some chemicals inhibited zfER but
27 activated hER response (e.g. benzo(a)pyrene, triphenylphosphate). The estrogenic activity of
28 M1 and M2 was well predicted by CA in MELN cells, whereas it was significantly lower than
29 predicted in ZELH β 2 cells, contrasting with the additive effects observed for BPA and GEN
30 binary mixtures. When testing the subgroups of ER activators and inhibitors combined, a
31 deviation from additivity was caused by zebrafish-specific inhibiting chemicals. This study
32 provides novel information on the ability of environmental pollutants to interfere with zfER
33 signaling and shows that non-estrogenic chemicals can influence the response to a mixture of
34 xeno-estrogens in a bioassay-specific manner.

35 **KEY WORDS:** estrogenicity, anti-estrogen, mixture, *in vitro* reporter gene, human, zebrafish

36

37 **1. Introduction**

38 The occurrence of numerous endocrine disrupting chemicals (EDC) in aquatic ecosystems
39 has raised concern over their potential adverse effects in aquatic organisms, such as fish (Sumpter,
40 2005). Many EDCs, such as natural and synthetic hormones, pesticides or industrial chemicals,
41 are xeno-estrogens, *i.e.* they bind the estrogen receptors (ERs) and subsequently alter the
42 transcription of target genes involved in key physiological functions (Sumpter, 2005). *In vitro*
43 bioassays based on ER transactivation have been used to assess the estrogenic activity of
44 chemicals, but also of environmental samples (Jarošová et al., 2014; Snyder et al., 2001;
45 Zacharewski, 1997). In case of environmental monitoring, they are expected to enable an
46 integrative detection of various ER-active contaminants within complex environmental mixtures
47 considering both known and unknown xeno-estrogens. They provide a unique quantitative
48 response which may be summarized as estradiol-equivalent (E2-Eq, Kase et al., 2018, Jarošová et
49 al., 2014).

50 To date, a large majority of *in vitro* bioassays used in environmental bio-monitoring are
51 based on mammalian or yeast cell systems that stably express a reporter gene which expression is
52 controlled by the human ER subtype α (hER α) (Könemann et al., 2018; Kunz et al., 2015; Leusch
53 et al., 2010). However, the relevance of using human-based assay to assess hazard and risk for
54 aquatic species is a question of concern in environmental assessment (Hotchkiss et al., 2008). For
55 instance, humans express two ER subtypes, ER α and ER β , but most teleost fish express at least
56 three ER subtypes, ER α , ER β 1 and ER β 2 (Menuet et al., 2002; Tohyama et al., 2015). Fish and
57 human ER have relatively low sequence homologies in their ligand binding domain (Menuet et
58 al., 2002; Tohyama et al., 2015). These structural differences are believed to contribute to the
59 distinct sensitivity to certain xeno-estrogens (Miyagawa et al., 2014), along with other factors
60 linked to the cell specificities, such as cell metabolic capacities (Le Fol et al., 2015),
61 presence/absence of transcriptional cofactors or cross-talks with other signalling pathways (Navas
62 and Segner, 2000; Ohtake et al., 2003).

63 In a recent study, we reported that some surface water samples were active on a zebrafish
64 liver cell line stably expressing zebrafish ER β 2 (zfER β 2), the ZELH β 2 cells, but not on human
65 breast cancer MELN cells that endogenously express hER α (Sonavane et al., 2016). Similarly,
66 some effluent extracts from sewage treatment plants produced very different *in vitro* responses in
67 cells expressing human or medaka ER α (Ihara et al., 2014). These differences were further
68 confirmed *in vivo* by measuring vitellogenin induction in exposed male medaka (Ihara et al.,
69 2015). In the latter study, the estrogenic chemicals identified were not sufficient to explain the
70 distinct response of fish bioassays. However, the authors showed that the anti-estrogenic activity
71 measured in the samples may contribute to the different responses of medaka and human ER.

72 Several studies have addressed the combined effect of ER ligands in reconstituted
73 mixtures, generally concluding on their additive effects based on concentration addition (CA)
74 predictions (Kortenkamp, 2007). However, xeno-estrogens occur in the aquatic ecosystem
75 together with other chemicals that have various and distinct modes of action (e.g. Escher et al.,
76 2014; Neale et al., 2015, Busch et al., 2016). To date, few studies have investigated additive
77 effects of xeno-estrogens in more diverse exposure scenarios, such as with non- or weak
78 estrogenic chemicals (Evans et al., 2012) or with anti-estrogenic chemicals (Yang et al., 2015).
79 Recently, a mixture of 12 selected environmental chemicals was tested in zebrafish and human-
80 based bioassays as part of a larger round-robin study. The aim was to investigate whether the
81 estrogenic activity of the ER ligands in this mixture (e.g. genistein and bisphenol A) was
82 detectable against the background of the other environmental pollutants (Altenburger et al., 2018).
83 This study concluded that in human MELN cells the overall estrogenic activity of the mixtures
84 was accurately predicted by an assumed additivity of the estrogenic chemicals. However, in
85 zebrafish ZELH β 2 cells the measured estrogenic response of the mixture was lower than
86 expected. The reasons of this discrepancy between human and zebrafish-based ER-reporter gene
87 assays were unknown, and therefore raised the question about potential limitations of a presumed
88 CA additivity.

89 In this context, the present study was designed to investigate the different responses of
90 zebrafish- and human-based *in vitro* reporter gene assays to chemical mixtures. We hypothesized
91 that estrogenic chemicals within environmental mixtures have additive effects following default
92 model of CA that are well detected by zebrafish and human-based bioassays. In such way, we
93 investigated (1) the additivity of xeno-estrogens in zebrafish and human-based bioassays and (2)
94 the influence of non-estrogenic chemicals of the mixtures. As in Altenburger et al. (2018), we
95 used the same 12-compound mixture in two different mixture ratios (M1 and M2), which included
96 xeno-estrogens (e.g. bisphenol A and genistein), and non-estrogenic chemicals representatives of
97 a freshwater contamination background. The general experimental set-up design is outlined in
98 Figure 1. Firstly, each chemical was tested for both estrogenic and anti-estrogenic activities in
99 zebrafish-and human-based bioassays. Secondly, combinations of chemicals that proved to be
100 active at M1 and M2 mixture ratios (either ER activating, ER inhibiting, or both) were tested and
101 then discussed in relation to the outcomes from the 12-component mixture response. The
102 concentration addition model was used to evaluate the additivity of active chemicals in each
103 mixture scenario.

104 2. Material and methods

105 2.1 Chemical selection, mixtures design and experimental approach

106 Twelve environmentally relevant chemicals were selected following (1) a prioritization
107 exercise based on occurrence, hazard and available environmental quality standard (Busch et al.,
108 2016), and (2) a screening of prioritized contaminants through multiple bioassays (Neale et al.,
109 2017a). As a result, two fixed-ratio mixtures of 12 chemicals with dissimilar mode of actions were
110 designed (Table SI-1) and tested as part of a benchmarking exercise (Altenburger et al., 2018).
111 The first mixture ratio (M1) was composed in such way that the diverse bioactivities of the
112 individual chemicals had a chance to be detected experimentally by an array of 19 bioassays. The
113 second mixture ratio (M2) was chosen to mimic a realistic freshwater contamination scenario. In

114 the current study, all 12 chemicals were tested individually for their capacity to induce or inhibit
115 ER-mediated luciferase response in different cellular assays. Based on the information on the
116 activity of individual chemicals in each bioassay, chemicals predicted to contribute to M1 and M2
117 responses based on CA prediction were identified. Based on toxic unit distribution (as defined in
118 Scholtz et al., 2017), only chemicals expected to contribute to at least 1% of the total response at
119 non-cytotoxic concentrations were selected. Subgroup mixtures were then designed containing
120 either only ER activators or only ER inhibitors, or both ER activators and inhibitors (Figure 1,
121 Table 1). These mixtures were designed such that their relative concentration ratios agreed to that
122 from the original M1 and M2 mixtures (i.e. real sub-mixtures), to allow the best possible
123 comparison to the outcomes from the 12 compound mixtures.

124 ***2.2 Chemicals and reagents***

125 17 β -estradiol (E2, CAS#50-28-2, purity of >98%), triclosan (TCS, CAS#3380-34-5, purity
126 of 97% - 103%), bisphenol A (BPA, CAS#80-05-7, purity of 97%), genistein (GEN, CAS#446-
127 72-0, purity of > 98%), propiconazole (CAS#60207-90-1, purity of >98%), diclofenac
128 (CAS#15307-79-6), diazinon (CAS#333-41-5, purity of >98%), diuron (CAS#330-54-1, purity
129 >98%), cyprodinil (CAS#121552-61-2, purity of >98%), triphenylphosphate (TPP, CAS#115-86-
130 6, purity >99%), benzo(a)pyrene (BaP, CAS#50-32-8, purity >96%), benzo(b)fluoranthene (BbF,
131 CAS#205-99-2, purity of 98%), chlorophene (CAS#120-32-1, purity of 95%), hydroxy-tamoxifen
132 (OH-TAM, CAS#68392-35-8, purity of >98%) and dimethylsulfoxide (DMSO) were purchased
133 from Sigma-Aldrich (France). The cell culture medium and reagents Leibovitz 15 culture medium
134 (L-15), fetal calf serum (FCS), 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES),
135 epidermal growth factor (EGF), G418, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-
136 tetrazoliumbromide (MTT) and D-luciferin were purchased from Sigma Aldrich (St-Quentin
137 Fallavier, France); Dulbecco's Modified Eagle Medium (DMEM), DMEM High Glucose (DMEM
138 HG) powder, F-12 nutrient mixture (Ham's F12) powder, penicillin and streptomycin were

139 purchased from Gibco (France); insulin, hygromycin B and sodium bicarbonate were purchased
140 from Dominique Dutscher (France).

141 ***2.3 In vitro bioassays: cell lines, luciferase and cell viability assays***

142 The zebrafish *in vitro* assays have been derived from the zebrafish liver (ZFL) cell line
143 (Cosnefroy et al., 2012). ZFL were stably transfected, first, with an ERE-driven firefly luciferase
144 gene, yielding the ZELH cell line, and then either with zfER α subtype, yielding the ZELH α cell
145 line, or with zfER β 2 subtype yielding the ZELH β 2 cell line (Cosnefroy et al., 2012).
146 Establishment of these cell models and their response to different classes of well-known xeno-
147 estrogens have been previously described (Cosnefroy et al., 2012; Sonavane et al., 2016). The
148 human-derived MELN cell line (Balaguer et al., 1999) was kindly provided by Dr Patrick
149 Balaguer (INSERM Montpellier, France). It is derived from the breast cancer MCF-7 cells, which
150 endogenously express the hER α , but no functional hER β (P. Balaguer, *personal communication*).
151 MELN cells were stably transfected with an ERE-driven firefly luciferase reporter gene.

152 Conditions for routine cell culture have been detailed previously (Balaguer et al., 1999;
153 Cosnefroy et al., 2012). The cells used were pathogen-free and controlled on a regular basis. For
154 exposure experiments, ZELH-derived cells were seeded in 96-well white opaque culture plates
155 (Greiner CellStar™, Dutscher, France) at 25,000 cells per well in phenol red-free LDF-DCC
156 medium (containing L-15 50%, DMEM HG 35%, Ham's F12 15%, HEPES 15 mM, 0.15 g/L
157 sodium bicarbonate, 0.01 mg/mL insulin, 50 ng/mL EGF, 50 U/mL penicillin and streptomycin
158 antibiotics, 5% v/v stripped serum). MELN were seeded at 80,000 cells per well in phenol red-free
159 DMEM medium containing 5% v/v stripped serum. Cells were left to adhere for 24h. Then, they
160 were exposed in triplicates to serial dilutions of test compound for either 72h at 28°C for zebrafish
161 cells or 16h at 37°C for MELN cells. Each plate included both solvent and positive controls (in
162 two triplicates each). E2 was used as a positive quality control for ER activation, and hydroxy-
163 tamoxifen (OH-TAM) for ER inhibition. In addition, a serial dilution of 7 to 8 concentrations of

164 E2 was tested in each experiment. At the end of exposure, the culture medium was removed and
165 replaced by 50 μ L per well of medium containing 0.3 mM luciferin. The luminescence signal was
166 measured in living cells using a microtiter plate luminometer (Synergy H4, BioTek).

167 The cell viability was assessed by using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5diphenyl
168 tetrazolium bromide (MTT) assay (Mosmann, 1983). After cell exposure, the culture medium was
169 removed and replaced by 100 μ L of medium containing 0.5 mg/mL MTT. Cells were incubated
170 for 3h. In metabolically active cells, MTT is reduced onto a blue formazan precipitate, which is
171 dissolved by adding 100 μ L of DMSO after removal of MTT-containing medium. Plates were
172 read at 570 nm against a 640 nm reference wavelength on a microplate reader (KC-4, BioTek
173 Instruments, France) and results are expressed as absorbance units relative to control cells.

174 ***2.4 Testing of multi-component mixtures***

175 The mixture compositions are given in Table SI-1, SI-2 and SI-3. The two 12-component
176 mixtures were prepared in methanol (as part of a round robin study on bioassays, Altenburger et
177 al., 2018). Stocks solutions and serial dilutions of single chemicals and 2-, 3-, 4- and 5-component
178 mixtures were prepared in DMSO. The response of MELN cells to TPP and BPA using either
179 DMSO or methanol as vehicle were similar (data not shown), thus, no significant effect of the
180 solvent was to expect. To investigate the anti-estrogenic activity of the chemicals or mixtures, the
181 cells were exposed in the presence of E2 at a concentration leading to 80% of maximal response,
182 i.e. 0.1 nM in MELN and ZELH β 2 and 1 nM in ZELH α assays. The ZELH cells, that correspond
183 to the parent cell line of ZELH α and ZELH β 2 cells but lack functional ER, were used additionally
184 as a control for non-specific luciferase modulation. As for the other cell lines, cytotoxicity was
185 measured in parallel in the way previously described. Final solvent concentrations in culture
186 medium were 0.1% v/v (agonist assay) or 0.15% v/v (in case of co-exposure with E2), which do
187 not affect luciferase expression or cell viability. Stock solutions of chemicals in DMSO and
188 methanol were maintained at -20°C for up to three months.

189 **2.5 Data analysis**

190 **2.5.1 Data treatment and analysis**

191 Luciferase activity (LUC) was normalized to a response range between 0 and 1 on an
192 experiment-to-experiment basis as follows:

$$193 \quad \text{Response} = \frac{LUC_{\text{chemical}} - LUC_{\text{control}}}{LUC_{E2} - LUC_{\text{control}}} \quad (1)$$

194 where LUC_{chemical} is the luminescent signal induced by the tested chemical, LUC_{control} is the
195 average luminescent signal of the solvent controls and LUC_{E2} is the average luminescent signal of
196 the E2 positive controls. Only non-cytotoxic concentrations (i.e. more than 80 % of cell viability
197 in the MTT assay) were considered for data analysis. Concentration-effect data analysis was
198 performed in the same way for individual compounds and mixtures. In short, a nonlinear
199 regression model best-fit approach was used to describe pooled data sets in the best possible way
200 (Scholze et al., 2001). If different regression functions led to similar goodness-of-fits, the logit
201 model (which is a re-parameterised form of the Hill equation) was given preference. To account
202 for inter-study variations we included experiments as random factor in the best-fit data analysis
203 (nonlinear mixed effect model). A detailed description can be found in Altenburger et al. (2018).

204 **2.5.2 Mixture prediction and uncertainty assessment**

205 The combined response from individual substances was assumed to follow the concept of
206 concentration addition (CA). Here we used the standard form of non-interaction, i.e.:

$$207 \quad \sum_{i=1}^n \left(\frac{C_i}{EC_{Xi}} \right) = 1 \quad (2)$$

208 where C_i is the concentration of the i^{th} substance in the mixture expected to produce a mixture
209 response X , and EC_{Xi} the concentration of the i^{th} substance leading to the same response X as
210 expected for the mixture.

211 To account for the statistical uncertainty in the CA prediction, a combination of Monte-Carlo
212 (MC) simulations and bootstrapping nonlinear regression functions (Tibshirani and Efron, 1993)
213 was conducted to simulate approximate 95% confidence limits around the predicted mean
214 response of the mixture. Here the MC step is responsible for linking the data input from the single
215 compounds (i.e. estimates about ECs or individual effects) to the mixture prediction, and the
216 bootstrapping step is responsible for generating data information relevant for input variables (i.e.
217 uncertainty distributions around the single substance EC's or effects). We followed a parametric
218 bootstrap with resamples drawn from the fitted nonlinear mixed effect model. Differences
219 between predicted and observed mixture effects (concentration) were deemed statistically
220 significant when the 95% confidence belts of the prediction did not overlap with those of the
221 experimentally observed mixture effects (Altenburger et al., 2018). The comparative assessment
222 was performed on mixture concentrations leading to 20% ER activation (EC20) or inhibition
223 (IC20).

224 **3. Results**

225 ***3.1. Activation and inhibition of ER response by single chemicals***

226 The results of ER activation and inhibition by all 12 chemicals and the reference compounds (E2
227 and OH-TAM) on MELN, ZELH α and ZELH β 2 cells are presented in Table 2, and the
228 concentration-response data are provided in supplementary information (Figure SI-1 for ER
229 activation and SI-2 for ER inhibition).

230 As expected, genistein and BPA were active in all cell lines, but at different sensitivity and
231 efficacy levels. MELN cells responded to BPA with an EC20 of 0.12 μ M and a maximal
232 induction of 86% of the positive E2 control response, while ZELH α and ZELH β 2 cells showed a
233 lower sensitivity with an EC20 of 2.1 μ M and 5.0 μ M, respectively, and a maximum luciferase
234 induction around 30 % (Table 2). In case of genistein, MELN (EC20 of 0.0121 μ M) and ZELH β 2

235 cells (EC20 of 0.015 μ M) were more responsive than ZELH α cells (EC20 of 1.4 μ M). BaP, TPP
236 and diazinon weakly induced luciferase activity in MELN cells with an EC20 of 0.57 μ M, 4.1 μ M
237 and 15 μ M, respectively, whereas no activity was recorded at non-cytotoxic concentrations in
238 zebrafish cells. No other chemicals showed any estrogenic response up to 30 μ M in any
239 bioassays.

240 The inhibition of ER response by the 12 chemicals revealed distinct response between the
241 bioassays (Table 2). Overall, several chemicals were identified as new ER inhibitors, mainly in
242 ZELH-zfERs cells. TPP and BaP decreased ER response in ZELH α and ZELH β 2 cells at
243 concentrations where they did not affect cell viability or the luciferase activity in the ER-negative
244 ZELH cells. Conversely, benzo(b)fluoranthene and propiconazole decreased E2-induced
245 luciferase activity up to 90% in ZELH α and ZELH β 2 and in ER-negative ZELH cells. Cyprodinil
246 decreased E2-induced luciferase activity across all the cell lines with similar sensitivity,
247 suggesting a likely non-specific effect of this chemical on luciferase activity (Table 2, Figure SI-
248 3).

249 ***3.2. Combined effects of xeno-estrogens in multi-component mixtures***

250 The concentration-response curves estimated for the single chemicals were used to predict
251 the ER activation and ER inhibition of M1 and M2 mixtures using the CA model. Since CA can
252 describe only ER activation or ER inhibition, but not their co-occurrence, the additive response of
253 a mixture containing both ER activators and inhibitors is predicted solely from the ER activators
254 in case of ER activation or from the ER inhibitors in case of ER inhibition. Therefore, the
255 chemicals expected to induce ER activation or ER inhibition in M1 and M2 mixtures were
256 identified for each cell line based on CA prediction. They were then tested as subgroup mixtures
257 containing either ER activating (M1_A, M2_A), ER inhibiting (M1_I, M2_I), or both ER
258 activating and inhibiting chemicals (M1_A+I, M2_A+I) (Table 1). The relative concentration
259 ratios were always kept in accordance to the 12-compound mixtures M1 and M2. All subgroup

260 mixture results are presented in Figure 2 (mixture composition according to M1) and Figure 3
261 (mixture composition according to M2), together with the outcomes for M1 and M2 (Altenburger
262 et al., 2018). Details about the mixture composition are given in Tables SI-1 (12-component
263 mixtures) and in SI-2 and SI-3 (subgroup mixtures).

264 ***3.2.1 Additivity of ER activating or inhibiting chemicals***

265 Regarding subgroup mixtures of ER activating chemicals, there was overall a good
266 agreement between observed and predicted EC20 across all cell lines and for both mixtures M1
267 and M2 compositions. In MELN cells, TPP, BPA and genistein at M1 mixture ratio had additive
268 effects very well predicted by CA model with a ratio between observed and predicted EC20 of 1.3
269 (M1_A_{MELN}, Figure 2A, Table 3). In comparison, the measured estrogenic activity of BPA and
270 genistein in M2_A_{MELN} was below the predicted response, although not statistically significant
271 (M2_A_{MELN}, Figure 3A, Table 4). BPA and genistein were the only two identified estrogenic
272 chemicals in ZELH α and ZELH β 2 cells. Their binary mixture induced an estrogenic response in a
273 good agreement with CA prediction at M1 and M2 concentration ratios in ZELH α (Figure 2E and
274 3E) and ZELH β 2 cells (Figure 2I and 3I). The ratio of observed against predicted EC20 was of
275 0.40 and 0.55 in ZELH α cells, and 0.71 and 0.73 in ZELH β 2 cells for M1 and M2, respectively.

276 As observed for single chemicals, ER inhibiting chemicals were more prevalent in ZELH α
277 and ZELH β 2 cells than in MELN cells. In MELN cells, cyprodinil was predicted to inhibit E2
278 response in M1, but only at high concentrations (M1_I_{MELN}, Figure 2B), and no inhibiting
279 chemical was identified for M2. In contrast, TPP, chlorophene and propiconazole were identified
280 as ER inhibiting chemicals of M1 in ZELH α and ZELH β 2 cells. In subgroup mixtures, they
281 induced a strong ER inhibition in ZELH α (M1_I_{ZELH α} , Figure 2F) and ZELH β 2 cells (M1_I_{ZELH β 2},
282 Figure 2J), well predicted by the CA model (EC20 ratio of 0.87 and 0.83, respectively). Similarly,
283 the subgroup mixtures of ER inhibitors based on M2 mixture ratio induced a strong inhibition,
284 well predicted by CA model (M2_I_{ZELH α} , figure 3F and M2_I_{ZELH β 2}, Figure 3J, respectively).

285 Overall, the combined effects of ER activating or ER inhibiting chemicals were in good
286 agreement with CA predictions for both M1 and M2 mixture ratios and across all cell lines.

287 *3.2.2 Estrogenic response to the 12-component mixtures: influence of inhibiting* 288 *chemicals*

289 For each cell line, the combined effects of activator and inhibitor subgroup mixtures
290 (M1_A+I and M2_A+I) were determined and compared to the results of the 12 component
291 mixtures M1 and M2 (Figures 2 and 3, right part). The observed and predicted EC20 or IC20 of
292 each mixture are presented in Tables 3 (M1) and 4 (M2).

293 In MELN cells, the estrogenic activity of M1_A+I_{MELN} (Figure 2C) was well predicted by
294 CA, and this accuracy was not impacted negatively by the presence of 9 other environmental
295 substances (M1, Figure 2D). No active ER inhibitors were present at non-cytotoxic concentration
296 in the mixture M2, and therefore a mixture of activators and inhibitors was not tested.
297 Nevertheless, the mixture effect of all 12 substances was well explained by the additivity of the
298 only two estrogenic chemicals identified, BPA and genistein (M2, Figure 3D).

299 In zebrafish ZELH α cells, M1 was not expected to induce any estrogenic response in the
300 range of tested concentrations, and indeed no estrogenic response was observed neither with the
301 5-component mixture (M1_A+I_{ZELH α} , Figure 2G) nor with the 12-component mixture M1 (Figure
302 2H). Conversely, a strong ER inhibiting response was measured (up to 80% inhibition) for both
303 the 5- and 12-component mixtures, which was well predicted by the CA model (IC20 ratio of 0.74
304 and 0.95, respectively). Thus, the ER inhibition measured remained unaffected by addition of
305 estrogenic and inactive chemicals, including ER inhibiting chemicals present at non-effective
306 concentrations (e.g. cyprodinil). In case of M2, the estrogenic activity of ER activating and
307 inhibiting chemicals was correctly predicted by CA model (Figures 3G and 3H). However, the
308 estrogenic activity measured was lower than that of BPA and genistein binary mixture results
309 (Figure 3E), suggesting an influence of ER inhibiting compounds.

310 In zebrafish ZELH β 2 cells, an estrogenic response was expected according to CA for the
311 mixture of activators and inhibitors, as supported by the additive outcomes from the binary
312 mixture of BPA and genistein (M1_A_{ZELH β 2}, Figure 2I). However, M1_A+I_{ZELH β 2} did not induce
313 any estrogenic response at test concentrations (Figure 2K). Instead, a strong inhibition of ER
314 response was measured, which was in line with the M1_I_{ZELH β 2} results and CA prediction (Figure
315 2J). As observed for the subgroup mixture of ER activating and inhibiting chemicals
316 (M1_A+I_{ZELH β 2}), M1 mixture did not induce any estrogenic activity but inhibited E2-induced
317 response (Figure 2H). Hence, these results indicate that inhibiting chemicals in M1 indeed
318 influenced ER response in ZELH β 2 cells. Compared with M1, the estrogenic activity measured
319 for the subgroup mixture of ER activators and inhibitors corresponding to M2 mixture ratio was
320 well predicted by CA model (M2_A+ I_{ZELH β 2}, Figure 3K), although the maximal efficacy
321 observed was well below the one of the BPA and genistein binary mixture (M2_A_{ZELH β 2}, Figure
322 3I). When ER activating and inhibiting chemicals were grouped with inactive chemicals in M2,
323 the estrogenic activity was well predicted by CA up to 20% (Figure 3L), but the maximal
324 estrogenic response remained lower than expected based on the M2_A_{ZELH β 2} mixture results
325 (Figure 3I). In comparison, the inhibition of ER response was well predicted by CA for both
326 M2_A+I_{ZELH β 2} (Figure 3K) and M2 (figure 3L). The results of the 4-component mixture
327 M2_A+I_{ZELH β 2} on ZELH β 2 cells are very similar to M2 results, considering both ER activation
328 and inhibition (Figure 3K and 3L).

329 **4. DISCUSSION**

330 The current study investigated the distinct responses of zebrafish ZELH α and ZELH β 2 and human
331 MELN cells ER reporter gene bioassays to 12-component mixtures composed of xeno-estrogens
332 and other environmental relevant chemicals (Altenburger et al., 2018). By using a stepwise
333 experimental approach from individual chemicals to subgroup mixture testing, we were able to

334 explain the distinct response of human and zebrafish bioassays to the same 12-component
335 mixtures.

336 *4.1. Distinct responses of human and zebrafish cell lines to individual chemicals*

337 BPA and genistein are well-known ER agonist ligands and were indeed active in all ER-
338 based bioassays, in agreement with previous studies using the same cellular models (Balaguer et
339 al., 1999; Cosnefroy et al., 2012; Le Fol et al., 2017; Sonavane et al., 2016). Apart from these two
340 compounds, the screening of individual chemicals highlighted some marked differences between
341 cell assays for some of the 10 chemicals.

342 One major outcome relates to the higher prevalence of chemicals inhibiting E2-induced
343 luciferase activity in ZELH-zfERs cells than in MELN cells (Table 2). Some chemicals had
344 opposite responses in zebrafish and human cells. For instance, BaP -a known AhR-ligand- and
345 TPP were estrogenic in MELN cells but decreased E2-induced response in ZELH α and ZELH β 2
346 cells. The mechanistic interaction between AhR and ER signalling pathways has been
347 documented in human (Matthews and Gustafsson, 2006; Ohtake et al., 2003) and in fish (e.g.
348 Navas and Segner, 2000). The prototypical AhR ligand TCDD was shown to induce a weak
349 estrogenic response in MELN cells (Balaguer et al., 1999) while it decreased E2 response in all
350 ZELH-zfER cells (Sonavane, 2015). The distinct responses to BaP in ZELH-zfERs and MELN
351 cells might thus be explained, at least partially, by AhR-ER interactions. In comparison, less
352 information is available on the ability of TPP to interact with ER signalling. Previous studies have
353 reported a weak agonist effect on hER α transactivation (Kojima et al., 2013), as observed in the
354 current study in MELN cells, while some TPP metabolites are reported to have an anti-estrogenic
355 activity on hER β transactivation (Kojima et al., 2016). However, TPP was unable to induce the
356 ER-regulated brain aromatase expression gene in transgenic cyp19a1b-GFP zebrafish embryos
357 (Neale et al., 2017a). Considering the anti-estrogenic activity of TPP evidenced in zebrafish liver

358 cells, further research would be warranted to assess whether TPP (or metabolites) either binds
359 directly zfERs or alters zfER transactivation through cross-talk(s) with other signaling pathways.

360 Other chemicals, such as propiconazole and cyprodinil, decreased E2-induced estrogenic
361 activity in an ER non-specific manner, i.e. they decreased firefly luciferase also in the parent cell
362 line ZELH that does not express functional zfER (Table 2, Figure SI-5). Such inhibition may
363 reflect either a direct effect on luciferase enzyme or an indirect effect on baseline transcriptional
364 machinery in the promoter region of the reporter gene, irrespectively of ER activity. Despite a
365 weak estrogenic activity on hER α reported *in vitro* (Medjakovic et al., 2014; Schlotz et al., 2017),
366 cyprodinil decreased firefly luciferase activity in all cells, irrespectively of E2 addition. The
367 structural similarities of cyprodinil with known firefly luciferase inhibitor (Auld and Inglese,
368 2004) and its capacity to interfere with ATP production (Coleman et al., 2012) suggest a possible
369 effect on the reporter gene system. In case of propiconazole, a weak hER α agonist activity was
370 reported in the high μ M range in MVLN cells (Kjeldsen et al., 2013) and anti-proliferative effects
371 measured in MCF-7 cells (Kjaerstad et al., 2010). In fish, interference of propiconazole with
372 estrogen signalling pathway has been reported *in vivo* (Skolness et al., 2013) but no information
373 on ER agonist or antagonist activity is available. Thus, additional assays would be warranted to
374 assess the specific activity of propiconazole and cyprodinil on ER-signalling pathway in
375 zebrafish.

376 ***4.2. Deciphering cell-specific response to xeno-estrogen mixtures***

377 BPA and genistein were the main drivers for ER agonistic response in M1 and M2. When
378 combined as binary mixture, they induced in all zebrafish and human-based bioassays responses
379 that were in good agreement with CA predictions. This additivity is consistent with several
380 previous studies which reported additive effects of selected estrogens on different biological
381 models such as mammalian cells (Ghisari and Bonefeld-Jorgensen, 2009; Heneweer et al., 2005)
382 or *in vitro* fish cells (Le Page et al., 2006; Petersen and Tollefsen, 2011) and *in vivo* in fish (Brian

383 et al., 2005; Brion et al., 2012). Furthermore, our results demonstrate for the first time the
384 suitability of the ZELH-zfER cell line to investigate mixture effects of ER agonists at the receptor
385 level in a zebrafish cell context.

386 The screening for anti-estrogenic activity showed that some inhibiting chemicals active on
387 ZELH-zfER cells were present at effective concentrations in M1 and M2, e.g. TPP and
388 propiconazole. Although the underlying mechanism of ER inhibition remains unclear, the
389 subgroup mixtures of inhibiting chemicals had additive effects in ZELH α and ZELH β 2 cells, in
390 all co-exposure scenario, i.e. with inactive and/or estrogenic chemicals. In case of M1, a
391 decreased luciferase activity was also observed in ZELH cells, well predicted by the additive
392 effects of TPP and propiconazole (Figure SI-4). These results indicate that the inhibition observed
393 in ZELH-zfERs cells for M1 may involve non-ER specific luciferase inhibition.

394 Interestingly, we observed in ZELH β 2 cells that the addition of the inhibiting chemicals to
395 the binary mixture of BPA and genistein resulted in a decrease in the expected estrogenic
396 response to a similar level as observed in the 12-component mixtures M1 and M2. In case of M1,
397 the presence of inhibiting chemicals silenced entirely the estrogenic activity expected, whereas in
398 M2, only the efficacy of the response was decreased. To a lesser extent, a similar trend was
399 observed for M2 in ZELH α cells. The experimental approach consisting of testing ER activating
400 and inhibiting chemicals separately and then together allowed us to evidence the role of inhibiting
401 chemicals in the deviation from expected additivity of genistein and BPA in ZELH β 2 cells. The
402 experimental results from the stepwise testing approach demonstrate that the response to the 12-
403 chemical mixtures in each bioassay can be explained knowing the individual responses of the 12
404 chemicals.

405 *4.3. Differences between zebrafish and human-based bioassay responses*

406 Our results highlight marked differences between human and zebrafish cells responses.
407 Each cell line displays cell-specific features, such as co-activator recruitment or metabolic
408 capacities. For instance, ZELH cells originate from zebrafish liver cells and have retained some
409 metabolic capacities qualitatively similar to zebrafish hepatocytes but distinct from MELN cells
410 (Le Fol et al., 2015), which may have played a role in the specific response to inhibiting
411 chemicals in our study. Indeed, metabolism has been previously suggested to negatively influence
412 the response to xeno-estrogen mixtures in rainbow trout hepatocytes (Petersen and Tollefsen,
413 2011) and in the E-SCREEN assay (Evans et al., 2012). The characterization of internal
414 concentrations of chemicals in MELN and ZELH-zfER cells would be needed to estimate the
415 influence of metabolism on the xeno-estrogen response.

416 To further investigate the relevance of the estrogenic mixture response in fish, both M1
417 and M2 were tested on transgenic zebrafish embryos expressing GFP under control of *cyp19a1b*
418 promoter in radial glial cells in the EASZY assay (Brion et al., 2012). Indeed, in previous studies,
419 we showed that ZELH-zfER response profile to individual chemicals or environmental samples
420 was better correlated than the MELN assay with *in vivo* estrogenic activity measured in the
421 EASZY assay (Neale et al., 2017b; Sonavane et al., 2016). As a result, no estrogenic activity was
422 measured for both M1 and M2 mixtures because of a high embryo mortality, especially for M1
423 (Altenburger et al., 2018). Thus, we could not confirm *in vitro* combined effects in zebrafish *in*
424 *vivo*.

425 ***4.4. Implication for quantifying the estrogenic activity of samples***

426 A consistent body of literature exist regarding the assessment of additivity of xeno-
427 estrogens according to CA. However, very few studies investigated the robustness and validity of
428 CA model in more complex and realistic mixture scenarios. In the current study, the main factors
429 differentiating zebrafish and human ER response to M1 and M2 was the presence of inhibiting
430 chemicals that had higher influence on zfER activation in zebrafish cells. This agrees well with

431 the findings of Ihara et al. (2014) that evidenced that anti-estrogenic activity in wastewater
432 treatment plant extracts was a key factor to explain the different estrogenic activity measured in
433 human and medaka ER α transactivation *in vitro*.

434 The 12-component mixtures were designed to mimic a simplified scenario of
435 environmental surface water contamination. To assess whether the mixture context would have
436 influenced the quantification of estrogenic activity mediated by xeno-estrogens, the mixture
437 results were used to quantify estradiol-equivalents (E2-Eq) in each bioassay (Table SI-4). Overall,
438 M2 was predicted to be more estrogenic (mean E2-Eq > 10 μ M) than M1 (mean E2-Eq < 1 μ M).
439 In MELN cells, the estrogenicity of M1 and M2 was almost not affected by the mixture context:
440 the ratio of observed to predicted E2-Eq was close to 1 for both mixtures. In contrast, ZELH α and
441 ZELH β 2 responses to xeno-estrogens in this specific mixture scenario were more susceptible to
442 co-occurrence of inhibiting chemicals: the estrogenic activity was underestimated in M1 and M2,
443 whenever quantified. In case of ZELH β 2 cells, similar IC20 were derived for both M1 and M2,
444 however, the inhibiting chemicals abolished the estrogenic response in case of M1, while they
445 only partially decreased the maximal efficacy level in case of M2, without altering significantly
446 the EC20 measured. These results suggest the presence of a balance between estrogenic and ER
447 inhibiting chemicals which can influence the detection, and thus the quantification, of xeno-
448 estrogens in ZELH β 2 cells.

449 5. CONCLUSION

450 In summary, this study demonstrates that BPA and genistein had additive effects *in vitro* in
451 zebrafish bioassays, comforting their use to assess combined effects of xeno-estrogens. In
452 addition, we show that the distinct responses of zebrafish and human-based bioassays to a 12-
453 component mixture were due to newly identified ER inhibiting chemicals selectively active in
454 ZELH α and ZELH β 2 cells (e.g. TPP, propiconazole) and altering zfER response to xeno-
455 estrogens. In the context of water bio-monitoring, this study illustrates the need for a mindful

456 consideration of the bioassay specificities (e.g. fish vs human ER, cell context) to ensure a proper
457 interpretation of results, as environmental chemicals may interfere with ER response, positively or
458 negatively, in a cell-specific manner. In future works, comparative assessment of real water
459 samples using human and zebrafish bioassays will help further documenting the environmental
460 relevance of such cross-species differential effects to complex mixtures.

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466 **DECLARATIONS OF INTEREST**

467 The authors declare that no conflict of interest regarding the publication of this paper.

468

469 **AUTHOR CONTRIBUTIONS:**

470 H.S., M.S., R.A., W.B., H.B., F.B and S.A conceived and designed the experiments; H.S. has
471 performed the experiments; H.S. and M.S. analysed the data; H.S., M.S. and S.A. have written the
472 manuscript; all authors have read and approved the final manuscript.

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673 **TABLES AND FIGURES**

674

675 **Table 1:** Overview of mixtures and their abbreviations tested on four different cell lines.
 676 More details about the composition of the mixtures are provided in the Supplementary
 677 Information (Tables SI 1-3). ⁽¹⁾ published in Altenburger et al; ⁽²⁾ corresponds to cyprodinil
 678 which was the only ER inhibitor.

679

mixture	ER activation		ER inhibition	
	M1	M2	M1	M2
MELN				
activators	M1_A _{MELN}	M2_A _{MELN}	-	-
inhibitors	-	-	M1_I _{MELN} ⁽²⁾	-
activators + inhibitors	M1_A+I _{MELN}	-	M1_A+I _{MELN}	-
activators + inhibitors + inactives	M1 ⁽¹⁾	M2 ⁽¹⁾	M1	-
ZELHα				
activators	M1_A _{ZELHα}	M2_A _{ZELHα}	-	-
inhibitors	-	-	M1_I _{ZELHα}	M2_I _{ZELHα}
activators + inhibitors	M1_A+I _{ZELHα}	M2_A+I _{ZELHα}	M1_A+I _{ZELHα}	M2_A+I _{ZELHα}
activators + inhibitors + inactives	M1 ⁽¹⁾	M2 ⁽¹⁾	M1	M2
ZELHβ2				
activators	M1_A _{ZELHβ2}	M2_A _{ZELHβ2}	-	-
inhibitors	-	-	M1_I _{ZELHβ2}	M2_I _{ZELHβ2}
activators + inhibitors	M1_A+I _{ZELHβ2}	M2_A+I _{ZELHβ2}	M1_A+I _{ZELHβ2}	M2_A+I _{ZELHβ2}
activators + inhibitors + inactives	M1 ⁽¹⁾	M2 ⁽¹⁾	M1	M2
ZELH				
inhibitors	-	-	M1_I _{ZELH}	M2_I _{ZELH}
inhibitors + inactives	-	-	M1	M2

Table 2: ER activation (EC20) and inhibition (IC20) of 12 test substances in MELN, ZELH α , ZELH β 2 and ZELH cells. Results are expressed in EC20 (activation) or IC20 (inhibition) are expressed in M concentration. E2 and OH-TAM were the positive control substances for ER activation and inhibition, respectively. Data originate from at least 2 independent experiments done in triplicates. Chemicals were tested in the 0.01 – 30 $\times 10^{-6}$ M range, except for genistein (from 10 $^{-9}$ M). All concentration-response data are presented in SI-1 and SI-2.

	ER activation (EC20)			ER inhibition (IC20)			
	MELN mean (95% CI)	ZELH α mean (95% CI)	ZELH β 2 mean (95% CI)	MELN mean (95% CI)	ZELH α mean (95% CI)	ZELH β 2 mean (95% CI)	ZELH mean (95% CI)
E2	3.4 $\times 10^{-12}$ (2.6 $\times 10^{-12}$ - 4.3 $\times 10^{-12}$)	1.3 $\times 10^{-10}$ (1.1 $\times 10^{-10}$ - 1.6 $\times 10^{-10}$)	6.0 $\times 10^{-12}$ (4.74 $\times 10^{-12}$ - 7.7 $\times 10^{-12}$)	-	-	-	-
OH-TAM	-	-	-	5.2 $\times 10^{-9}$ (4.5 $\times 10^{-9}$ - 6.0 $\times 10^{-9}$)	1.8 $\times 10^{-9}$ (9.4 $\times 10^{-10}$ - 3.4 $\times 10^{-9}$)	1.9 $\times 10^{-9}$ (1.4 $\times 10^{-9}$ - 2.8 $\times 10^{-9}$)	> 3 $\times 10^{-5}$
Bisphenol A	1.2 $\times 10^{-7}$ (8.2 $\times 10^{-8}$ - 1.7 $\times 10^{-7}$)	2.1 $\times 10^{-6}$ (1.3 $\times 10^{-6}$ - 3.6 $\times 10^{-6}$)	5.0 $\times 10^{-6}$ (2.4 $\times 10^{-6}$ - 6.1 $\times 10^{-6}$)	> 3 $\times 10^{-5}$	2.02 $\times 10^{-5}$ (1.1 $\times 10^{-5}$ - 3.6 $\times 10^{-5}$)	8.8 $\times 10^{-6}$ (8.7 $\times 10^{-7}$ - 1.3 $\times 10^{-5}$)	> 3 $\times 10^{-5}$
Genistein	1.21 $\times 10^{-8}$ (6.0 $\times 10^{-9}$ - 2.9 $\times 10^{-8}$)	1.4 $\times 10^{-6}$ (9.5 $\times 10^{-7}$ - 1.9 $\times 10^{-6}$)	1.5 $\times 10^{-8}$ (6.9 $\times 10^{-9}$ - 3.1 $\times 10^{-8}$)	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$
Diazinon	1.5 $\times 10^{-5}$ (1.2 $\times 10^{-5}$ - 1.9 $\times 10^{-5}$)	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 1 $\times 10^{-5}$	> 3 $\times 10^{-5}$
Triphenylphosphate	4.1 $\times 10^{-6}$ (2.9 $\times 10^{-6}$ - 5.7 $\times 10^{-6}$)	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	8.0 $\times 10^{-6}$ (3.2 $\times 10^{-7}$ - 1.3 $\times 10^{-5}$)	1.7 $\times 10^{-6}$ (8.3 $\times 10^{-7}$ - 3.5 $\times 10^{-6}$)	1.1 $\times 10^{-5}$ (3.0 $\times 10^{-7}$ - 1.3 $\times 10^{-5}$)
Benzo(a)pyrene	5.7 $\times 10^{-7}$ (4.6 $\times 10^{-7}$ - 7.2 $\times 10^{-7}$)	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	4.2 $\times 10^{-6}$ (2.5 $\times 10^{-6}$ - 7.3 $\times 10^{-6}$)	1.4 $\times 10^{-6}$ (7.7 $\times 10^{-7}$ - 2.4 $\times 10^{-6}$)	> 3 $\times 10^{-5}$
Benzo(b)fluorantene	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	1.95 $\times 10^{-6}$ (1.1 $\times 10^{-6}$ - 3.4 $\times 10^{-6}$)	1.5 $\times 10^{-6}$ (5.4 $\times 10^{-7}$ - 4.1 $\times 10^{-6}$)	1.8 $\times 10^{-6}$ (7.2 $\times 10^{-7}$ - 4.4 $\times 10^{-6}$)
Chlorophene	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	1.0 $\times 10^{-5}$ (2.6 $\times 10^{-6}$ - 1.7 $\times 10^{-5}$)	6.2 $\times 10^{-6}$ (3.4 $\times 10^{-6}$ - 9.8 $\times 10^{-6}$)	> 1 $\times 10^{-5}$
Propiconazole	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	8.1 $\times 10^{-6}$ (3.1 $\times 10^{-6}$ - 1.9 $\times 10^{-5}$)	4.4 $\times 10^{-6}$ (2.6 $\times 10^{-6}$ - 7.7 $\times 10^{-6}$)	2.4 $\times 10^{-6}$ (3.7 $\times 10^{-7}$ - 1.4 $\times 10^{-5}$)
Cyprodinil	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	4.9 $\times 10^{-6}$ (3.0 $\times 10^{-6}$ - 8.1 $\times 10^{-6}$)	2.0 $\times 10^{-6}$ (1.2 $\times 10^{-6}$ - 3.4 $\times 10^{-6}$)	4.2 $\times 10^{-6}$ (1.4 $\times 10^{-6}$ - 1.3 $\times 10^{-5}$)	4.1 $\times 10^{-6}$ (2.6 $\times 10^{-6}$ - 1.6 $\times 10^{-5}$)
Triclosan	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$
Diuron	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$
Diclofenac	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$	> 3 $\times 10^{-5}$

Table 3: Observed and predicted ER activation and inhibition for mixture M1 and its subgroups in MELN, ZELH α and ZELH β 2 cells. All concentrations are in M. (-) not tested as none of the individual compounds showed activity below its cytotoxic concentration range. (n.a.): the calculation is not applicable. Star indicates statistical significance (p<0.05). ^(a) re-calculated from Altenburger et al., 2018; ^(b) corresponds to cyprodinil which was the only ER inhibitor; ^(c) above cytotoxic concentration range.

Cell line	Mixture (name)	ER activation (EC20)			ER inhibition (IC20)		
		Observed Mean (95% CI)	Predicted Mean (95% CI)	Ratio obs/pred	Observed Mean (95% CI)	Predicted Mean (95% CI)	Ratio obs/pred
MELN	M1_A _{MELN}	1.2×10^{-6} (9.3×10^{-7} - 1.6×10^{-6})	8.9×10^{-7} (5.9×10^{-7} - 1.3×10^{-6})	1.3	-	-	-
	M1_I _{MELN}	-	-	-	4.9×10^{-6} ^(b) (3.0×10^{-6} - 8.2×10^{-6})	4.9×10^{-6} ^(b) (3.0×10^{-6} - 8.2×10^{-6})	1
	M1_A+I _{MELN}	2.1×10^{-6} (1.5×10^{-6} - 2.9×10^{-6})	2.6×10^{-6} (1.7×10^{-6} - 3.8×10^{-6})	0.81	$> 2 \times 10^{-5}$ ⁽⁴⁾	8.3×10^{-5} (5.0×10^{-5} - 1.4×10^{-4})	n.a.
	M1	6.1×10^{-6} ^(a) (3.9×10^{-6} - 9.2×10^{-6})	6.7×10^{-6} ^(a) (4.4×10^{-6} - 9.5×10^{-6})	0.91	3.4×10^{-5} (1.1×10^{-5} - 1.0×10^{-4})	5.9×10^{-4} ^(c) (3.6×10^{-4} - 9.8×10^{-4})	0.058*
ZELH α	M1_A _{ZELHα}	8.2×10^{-7} (6.5×10^{-7} - 1.6×10^{-6})	2.0×10^{-6} (1.0×10^{-6} - 3.0×10^{-6})	0.41	-	-	-
	M1_I _{ZELHα}	-	-	-	2.7×10^{-6} (1.9×10^{-6} - 3.6×10^{-6})	3.1×10^{-6} (1.2×10^{-6} - 1.2×10^{-5})	0.87
	M1_A+I _{ZELHα}	$> 4 \times 10^{-5}$ ^(c)	2.1×10^{-4} (1.3×10^{-4} - 3.2×10^{-4})	n.a.	4.2×10^{-6} (1.9×10^{-6} - 9.5×10^{-6})	5.7×10^{-6} (2.4×10^{-6} - 2.3×10^{-5})	0.74
	M1	$> 10^{-5}$ ^(c)	3.0×10^{-4} (1.8×10^{-4} - 4.6×10^{-4})	n.a.	4.2×10^{-6} (2.0×10^{-6} - 8.7×10^{-6})	4.4×10^{-6} (1.7×10^{-6} - 1.7×10^{-5})	0.95
ZELH β 2	M1_A _{ZELHβ2}	8.6×10^{-8} (3.7×10^{-8} - 1.8×10^{-7})	1.2×10^{-7} (5.5×10^{-8} - 2.4×10^{-7})	0.71	-	-	-
	M1_I _{ZELHβ2}	-	-	-	2.9×10^{-6} (2.0×10^{-6} - 4.0×10^{-6})	3.5×10^{-6} (2.1×10^{-6} - 5.1×10^{-6})	0.83
	M1_A+I _{ZELHβ2}	$> 2 \times 10^{-5}$ ^(c)	1.3×10^{-5} (5.8×10^{-6} - 2.5×10^{-5})	n.a.	4.4×10^{-6} (3.0×10^{-6} - 6.3×10^{-6})	6.4×10^{-6} (4.0×10^{-6} - 9.0×10^{-6})	0.69
	M1	$> 3 \times 10^{-5}$ ^(c)	1.8×10^{-5} (8.1×10^{-6} - 3.5×10^{-5})	n.a.	3.7×10^{-6} (2.1×10^{-6} - 6.3×10^{-6})	5.0×10^{-6} (3.0×10^{-6} - 7.1×10^{-6})	0.74

Table 4: Observed and predicted ER activation and inhibition for mixture M2 and its subgroups. All concentrations are in M. (-) not tested as none of the individual compounds showed activity below its cytotoxic concentration range. (n.a.): the calculation is not applicable. Star indicates statistical significance ($p < 0.05$). ^(a) re-calculated from Altenburger et al., 2018; ^(b) maximal induction measured below 20%.

Cell line	Mixture (name)	ER activation (EC20)			ER inhibition (IC20)		
		Observed Mean (95% CI)	Predicted Mean (95% CI)	Ratio obs/pred	Observed Mean (95% CI)	Predicted Mean (95% CI)	Ratio obs/pred
MELN	M2_A _{MELN}	1.6×10^{-7} (8.2×10^{-8} - 2.9×10^{-7})	6.4×10^{-8} (4.0×10^{-8} - 9.5×10^{-8})	2.5	-	-	-
	M2	1.5×10^{-7} ^(a) (6.8×10^{-8} - 2.8×10^{-7})	2.08×10^{-7} ^(a) (1.3×10^{-7} - 3.3×10^{-7})	0.72	-	-	-
ZELH α	M2_A _{ZELHα}	1.1×10^{-6} (7.4×10^{-7} - 1.7×10^{-6})	2.0×10^{-6} (1.2×10^{-6} - 3.1×10^{-6})	0.55	-	-	-
	M2_I _{ZELHα}	-	-	-	6.7×10^{-6} (2.9×10^{-6} - 1.3×10^{-5})	6.1×10^{-6} (2.2×10^{-6} - 1.1×10^{-5})	1.1
	M2_A+I _{ZELHα}	1.5×10^{-6} (7.8×10^{-7} - 2.8×10^{-6})	4.9×10^{-6} (3.0×10^{-6} - 7.5×10^{-6})	0.31*	7.6×10^{-6} (5.3×10^{-6} - 1.0×10^{-5})	1.0×10^{-5} (3.7×10^{-6} - 1.7×10^{-5})	0.76
	M2	$> 1.5 \times 10^{-7}$ ^(b)	6.6×10^{-6} (4.0×10^{-6} - 1.0×10^{-5})	n.a.	8.3×10^{-6} (6.0×10^{-6} - 1.1×10^{-5})	1.4×10^{-5} (5.3×10^{-6} - 2.4×10^{-5})	0.59
ZELH β 2	M2_A _{ZELHβ2}	1.1×10^{-7} (3.3×10^{-8} - 3.2×10^{-7})	1.5×10^{-7} (7.0×10^{-8} - 3.0×10^{-7})	0.73	-	-	-
	M2_I _{ZELHβ2}	-	-	-	7.5×10^{-6} (5.3×10^{-6} - 1.0×10^{-5})	6.6×10^{-6} (1.7×10^{-6} - 8.2×10^{-6})	1.1
	M2_A+I _{ZELHβ2}	1.2×10^{-6} (2.9×10^{-7} - 4.5×10^{-6})	3.7×10^{-7} (1.7×10^{-7} - 7.3×10^{-7})	3.2	7.7×10^{-6} (2.1×10^{-6} - 1.8×10^{-5})	6.8×10^{-6} (1.8×10^{-6} - 8.6×10^{-6})	1.1
	M2	1.8×10^{-6} (3.2×10^{-7} - 6.6×10^{-6})	5.0×10^{-7} (2.3×10^{-7} - 9.8×10^{-7})	3.6	4.1×10^{-6} (3.2×10^{-6} - 5.1×10^{-6})	9.2×10^{-6} (2.4×10^{-6} - 1.2×10^{-5})	0.44

Figure 1: Experimental approach selected to study the combined effects of ER activating and inhibiting chemicals within the 12-component mixtures M1 and M2. Subgroups of activators and inhibitors were designed with the chemicals predicted to contribute to the 12-component mixture responses at M1 and M2 mixture ratios.

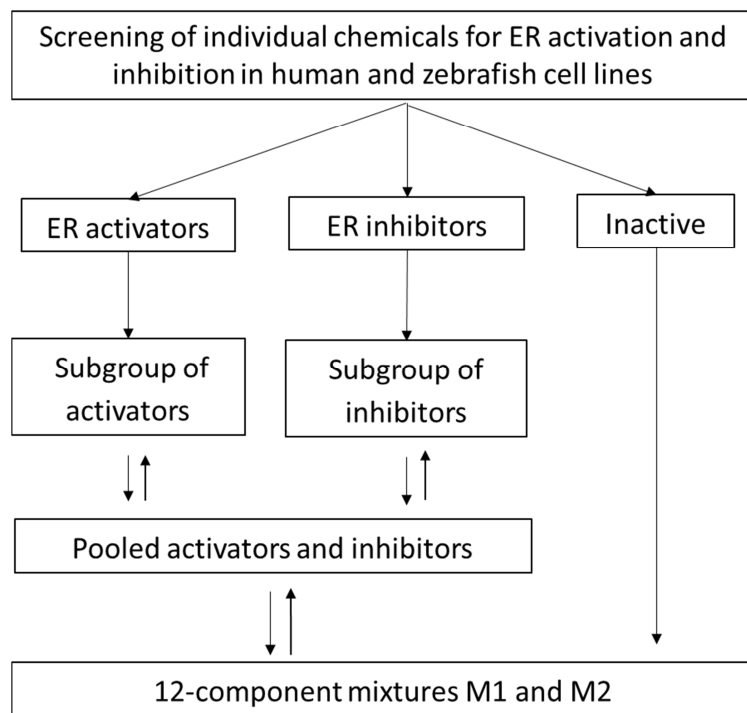


Figure 2: Predicted and measured effects of multi-component mixtures based on M1 concentration ratios. Data represent the mean (+/- SD) of a minimum of 3 independent experiments done in triplicates and pooled together. The green line represents CA prediction for ER activation and the orange line ER inhibition, and their respective dotted line represent the 95% CI belt. Cytotoxic concentrations (measured by MTT) were removed.

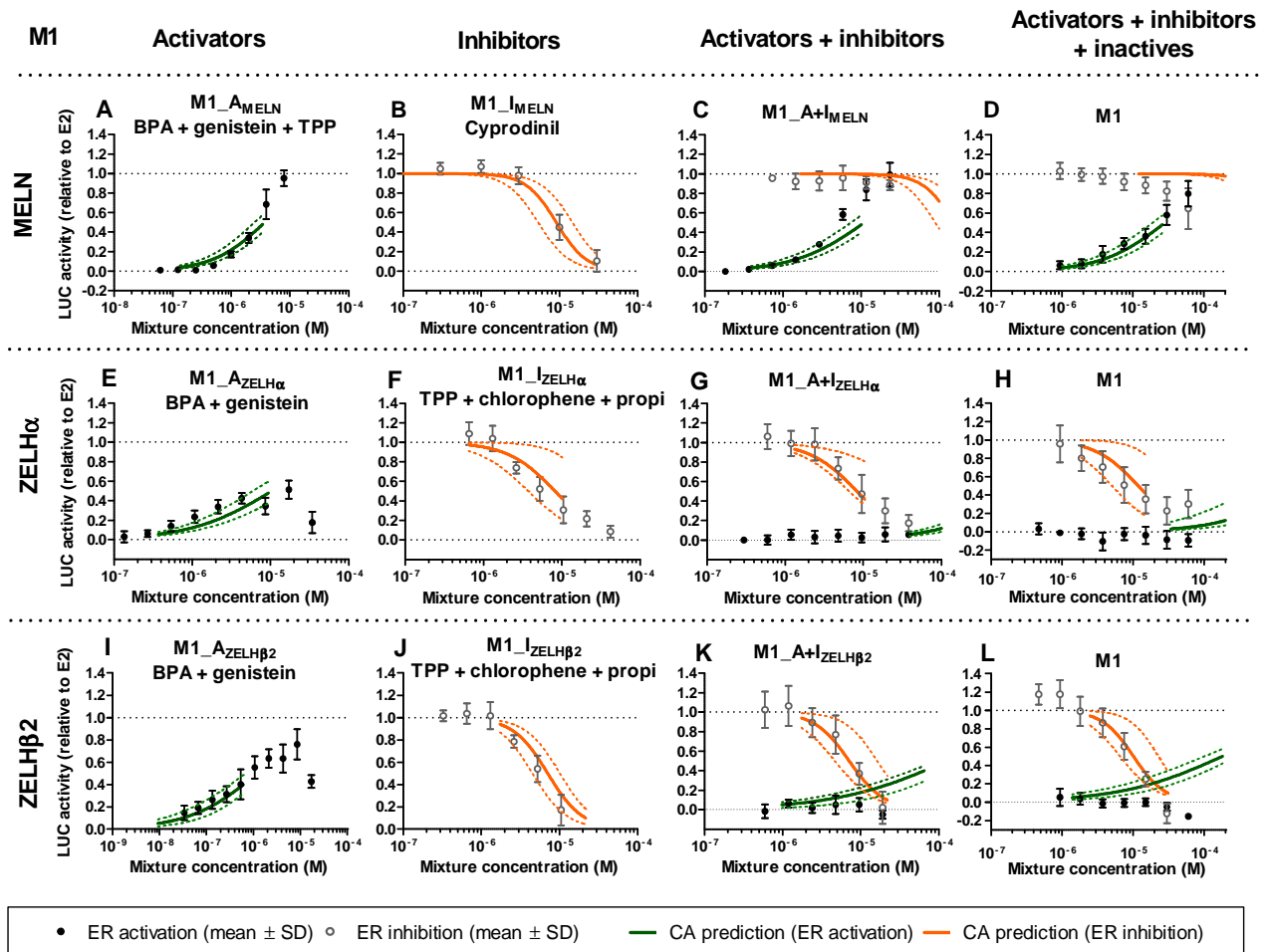
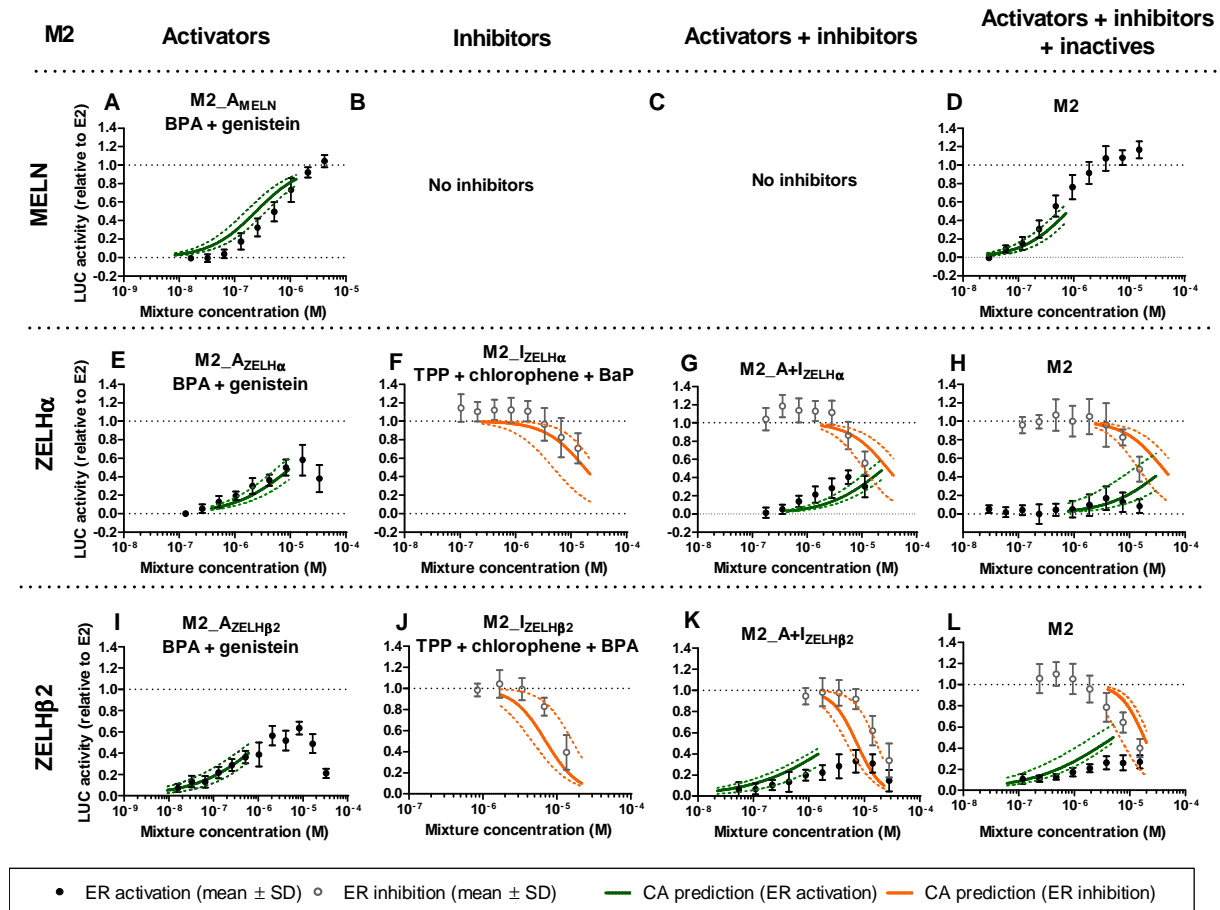


Figure 3: Predicted and measured effects of multi-component mixtures based on M2 concentration ratios. Data represent the mean (+/- SD) of a minimum of 3 independent experiments done in triplicates and pooled together. The green line represents CA prediction for ER activation and the orange line ER inhibition, inhibition, and their respective dotted line represent the 95% CI belt. Cytotoxic concentrations (measured by MTT) were removed.



SUPPLEMENTARY INFORMATION

Combined effects of environmental xeno-estrogens within multi-component mixtures: comparison of *in vitro* human- and zebrafish-based estrogenicity bioassays

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Content:

Table SI 1: Composition of the 12 compound mixtures M1 and M2 and the highest substance concentration tested *in vitro*.

Table SI 2: Composition of mixtures of ER activator (M1_A), ER inhibitors (M1_I) or combined ER activators and inhibitors (M1_A+I) tested in MELN, ZELH α , ZELH β 2 and ZELH cells.

Table SI 3: Composition of mixtures of ER activator (M2_A), ER inhibitors (M2_I) or combined ER activators and inhibitors (M2_A+I) tested in MELN, ZELH α , ZELH β 2 and ZELH cells.

Table SI 4: Estrogenic activity of the M1 and M2 expressed in estradiol equivalent.

Figure SI 1: Response of the 12 chemicals on ER activation in MELN, ZELH α and ZELH β 2 cells.

Figure SI 2: Response of the 12 chemicals on E2-induced ER inhibition in MELN, ZELH α , ZELH β 2 and ZELH cells.

Figure SI 3: Cyprodinil response in MELN, ZELH α , ZELH β 2 and ZELH cells.

Figure SI 4: Predicted and observed effects of inhibiting chemicals on ZELH cells.

Table SI 1: Composition of the 12 compound mixtures M1 and M2 and the highest substance concentration tested *in vitro*.

	M1		M2	
	Concentration (M)	proportion ¹⁾	Concentration (M)	proportion ¹⁾
Benzo(a)pyrene	6E-08	0.05%	9.47E-09	0.06%
Benzo(b)fluorantene	1E-07	0.08%	9.51E-09	0.06%
Bisphenol A	7E-07	0.58%	4.17E-06	27.70%
Chlorophene	9E-06	7.50%	6.40E-06	42.51%
Cyprodinil	1E-06	0.83%	1.87E-07	1.24%
Diazinon	6E-09	0.00%	1.96E-08	0.13%
Diclofenac	3E-05	24.99%	2.90E-06	19.26%
Diuron	6E-07	0.50%	2.08E-07	1.38%
Genistein	1E-07	0.08%	4.47E-07	2.97%
Propiconazole	6E-05	49.97%	8.48E-08	0.56%
Triphenylphosphate	1.5E-05	12.49%	2.32E-07	1.54%
Triclosan	3.5E-06	2.92%	3.89E-07	2.58%
Mixture	1.2E-04	100%	1.51E-5	100%

¹⁾ mixture composition according to Altenburger et al., (2018)

Table SI 2: Composition of mixtures of ER activator (M1_A), ER inhibitors (M1_I) or combined ER activators and inhibitors (M1_A+I) tested in MELN, ZELH α , ZELH β 2 and ZELH cells. The mixture composition is based on their relative proportion in the 12-compound mixture M1 (Table SI 1).

Type of mixture	MELN			ZELH α / ZELH β 2			ZELH
	Activators	Inhibitors	Inhibitors + activators	Activators	Inhibitors	Inhibitors + activators	Inhibitors
Mixture name	M1_A _{MELN}	M1_I _{MELN}	M1_A+I _{MELN}	M1_A _{ZELHα} , M1_A _{ZELHβ2}	M1_I _{ZELHα} , M1_I _{ZELHβ2}	M1_I+A _{ZELHα} , M1_I+A _{ZELHβ2}	M1_I _{ZELH}
Genistein	1%	-	1%	13%	-	0.2%	-
Bisphenol A	4%	-	4%	87%	-	0.8%	-
Triphenylphosphate	95%	-	89%	-	18%	17.7%	20%
Cyprodinil	-	100%	6%	-	-	-	1%
Diclofenac	-	-	-	-	-	-	-
Chlorophene	-	-	-	-	11%	10.6%	-
Propiconazole	-	-	-	-	71%	70.8%	79%
Total	100%	100%	100%	100%	100%	100%	100%

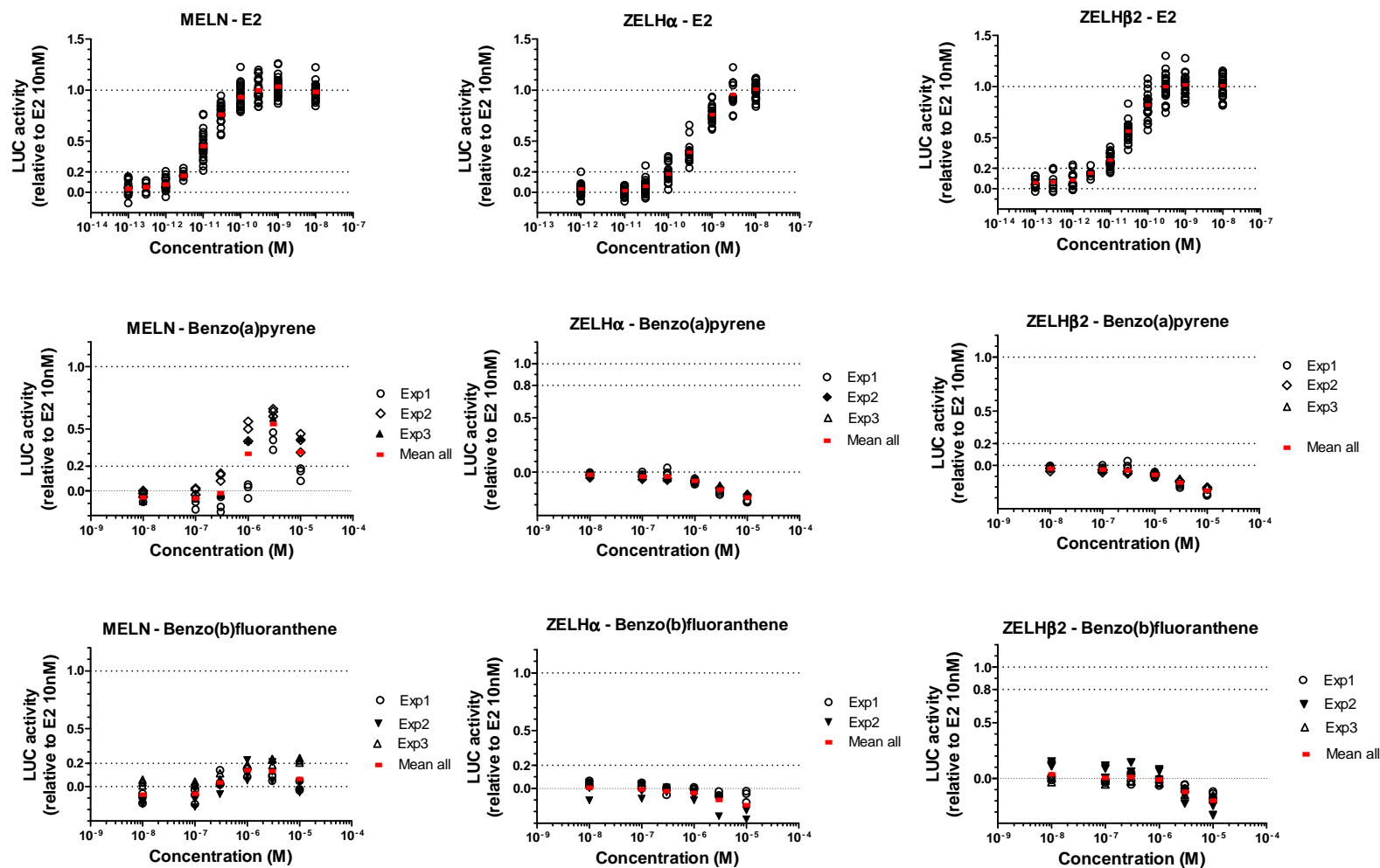
Table SI 3: Composition of mixtures of ER activator (M2_A), ER inhibitors (M2_I) or combined ER activators and inhibitors (M2_A+I) tested in MELN, ZELH α , ZELH β 2 and ZELH cells. The mixture composition is based on their relative proportion in the 12-compound mixture M2 (Table SI 1).

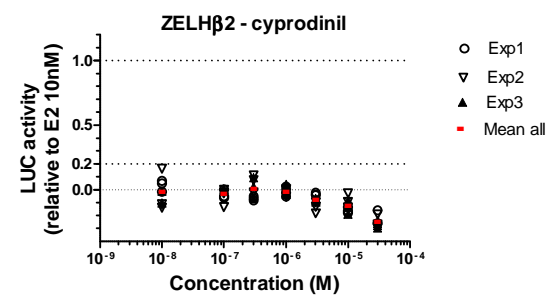
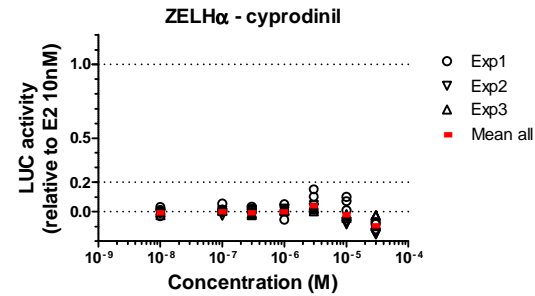
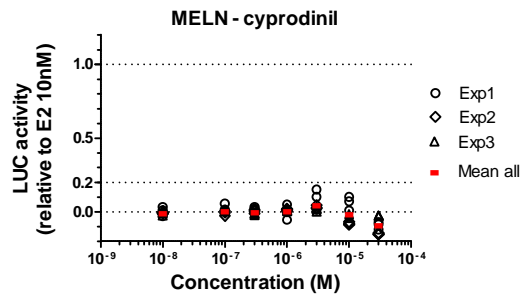
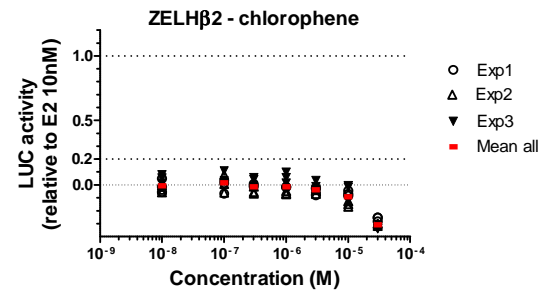
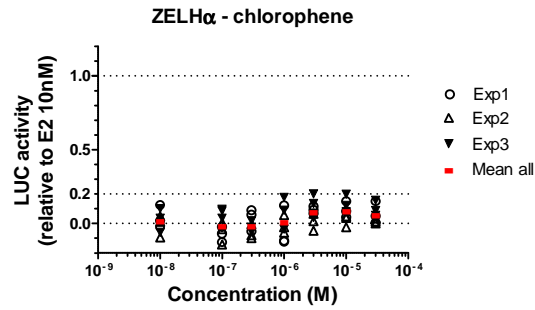
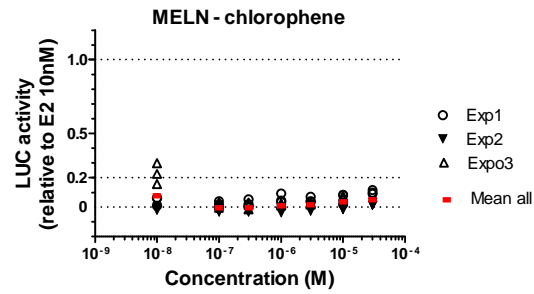
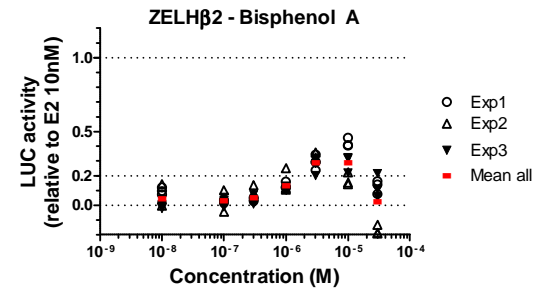
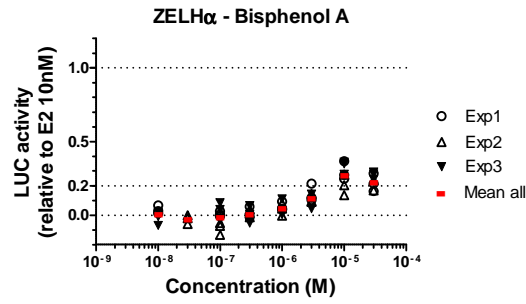
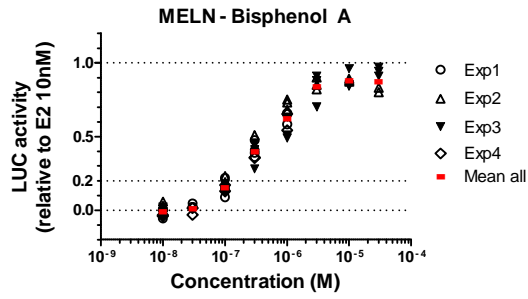
	MELN		ZELHα		ZELHβ2		ZELH	
Type	Activators	Activators	Inhibitors	Inhibitors + activators	Activators	Inhibitors	Inhibitors + activators	Inhibitors
Name	M2_A _{MELN}	M2_A _{ZELHα}	M2_I _{ZELHα}	M2_I+A _{ZELHα}	M2_A _{ZELHβ2}	M2_I _{ZELHβ2}	M2_I+A _{ZELHβ2}	M2_I _{ZELH}
Genistein	10%	10%		4.0%	10%		4.0%	
Bisphenol A	90%	90%		37.2%	90%	38.6%	37.2%	
Triphenylphosphate			3.5%	2.0%		2.1%	2.1%	77%
Chlorophene			96.4%	56.6%		59.3%	57.1%	
Propiconazole								23%
Benzo(a)pyrene			0.14%	0.08%				
Total	100%	100%	100%	100%	100%	100%	100%	100%

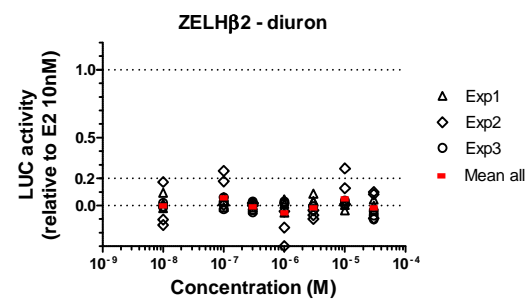
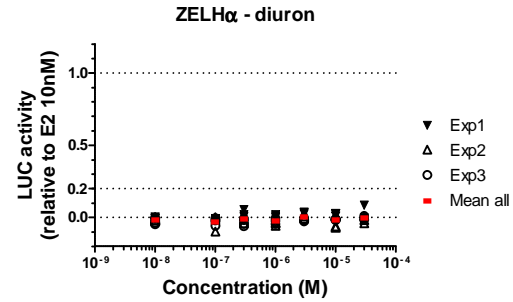
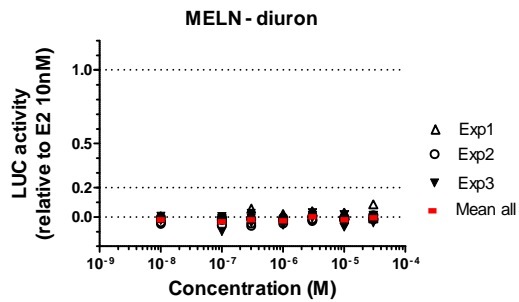
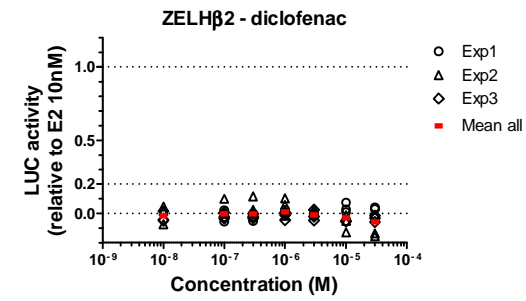
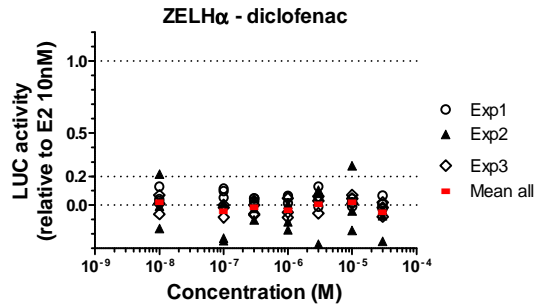
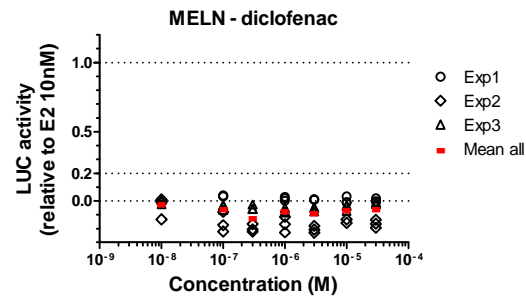
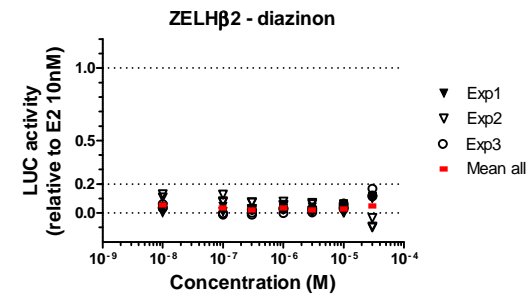
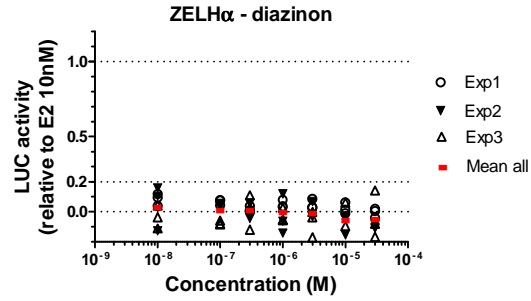
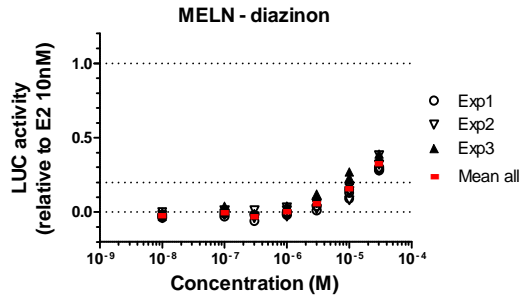
Table SI 4: Estrogenic activity of the M1 and M2 expressed in estradiol-equivalents (E2-Eq). E2-Eq (expressed in μM) were calculated for the 12-component mixtures on the basis of their predicted and observed EC20s (reported in Tables 3 and 4) in relation to the EC20 of E2 (in Table 2). The E2-Eq (observed) is the ratio between the EC20(E2) and the measured EC20(mixture), and E2-Eq(predicted) is the ratio between the EC20(E2) and the CA predicted EC20(mixture). n.a.: not applicable (no estrogenic activity measured).

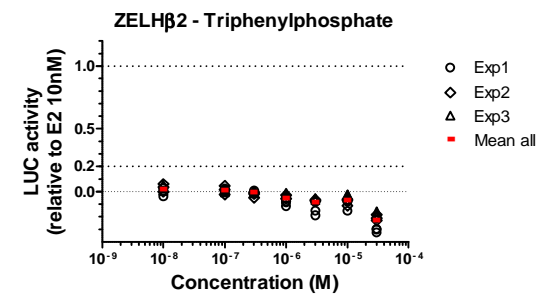
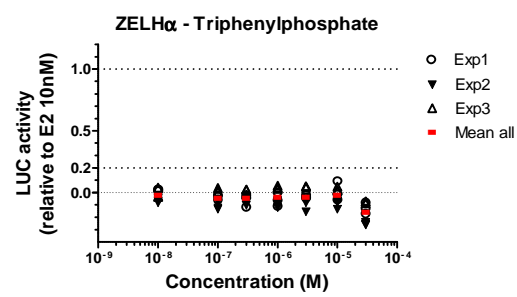
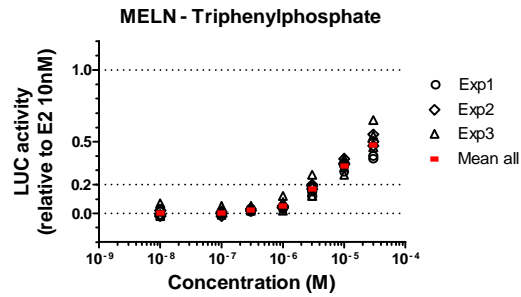
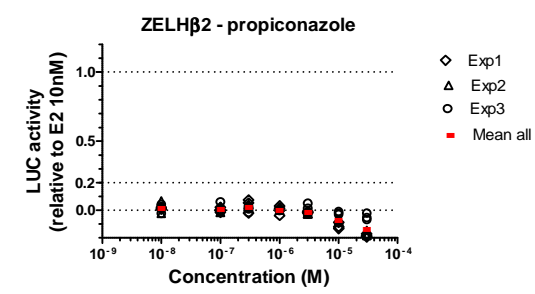
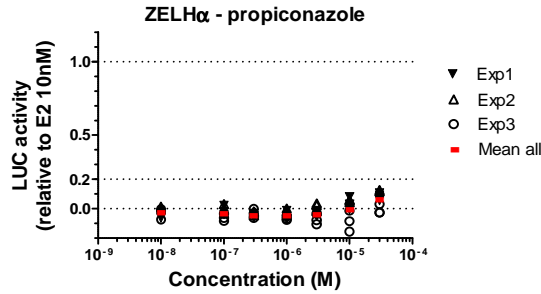
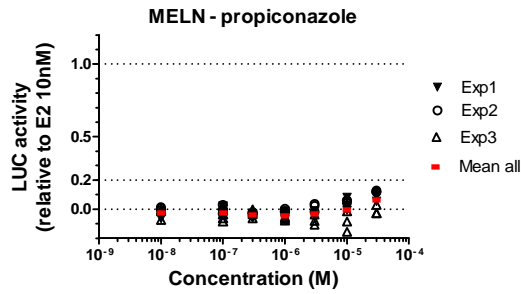
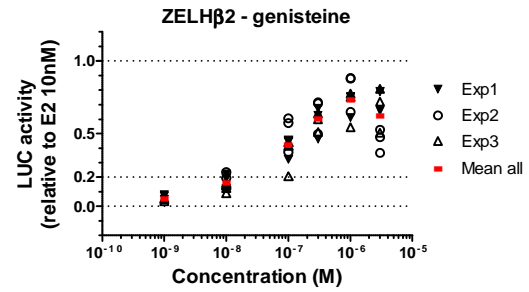
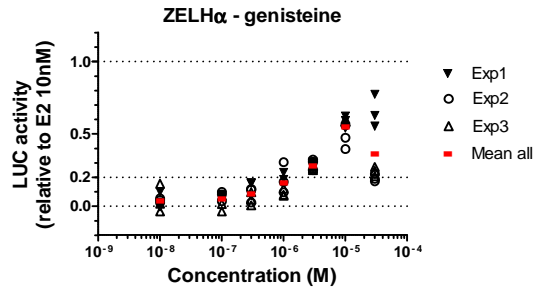
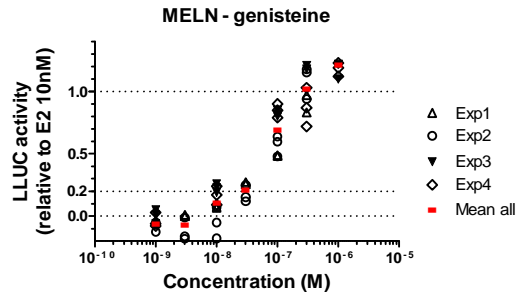
	M1 E2-Eq			M2 E2-Eq		
	Observed (μM)	Predicted (μM)	Observed/Predicted (%)	Observed (μM)	Predicted (μM)	Observed/Predicted (%)
MELN	0.56	0.51	110	22.7	16.3	139
ZELH α	n.a.	0.43	n.a.	n.a.	19.7	n.a.
ZELH β 2	n.a.	0.33	n.a.	3.33	12	28

Figure SI 1: Response of the 12 chemicals on ER activation in MELN, ZELH α and ZELH β 2 cells. Data represent each replicate and their mean (red dash) of at least 2 independent experiments done in triplicates. Chemicals were tested in the 10 nM - 30 μ M range, except for genistein (from 1 nM). 17 β -estradiol (E2) was used as positive control. The horizontal dotted line at 20% figures the threshold of effect.









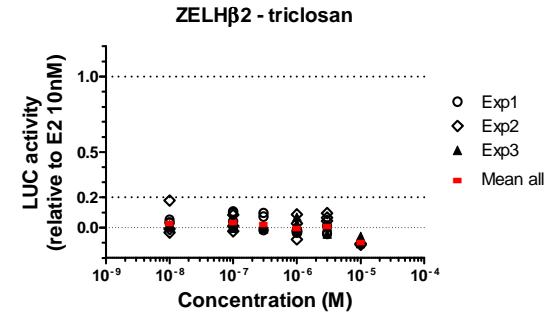
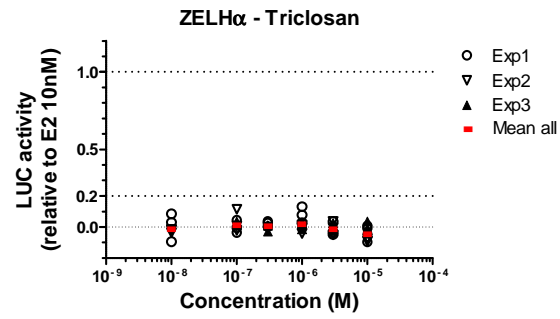
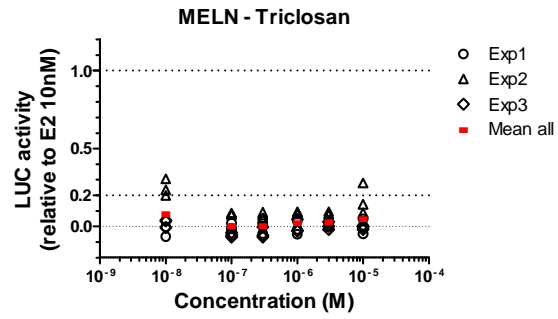
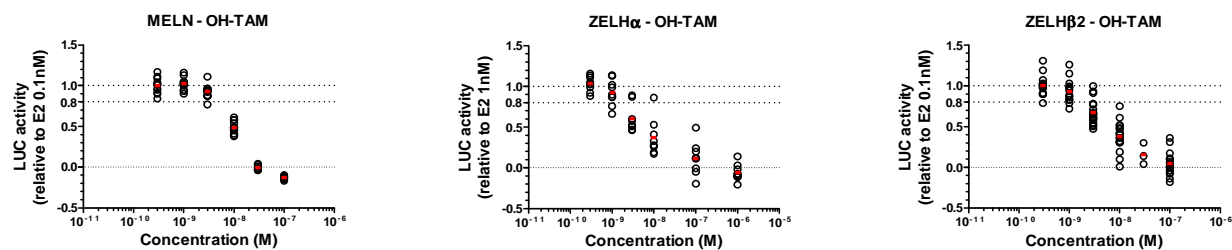
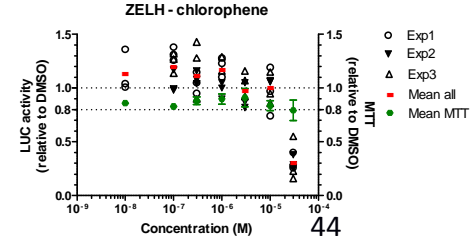
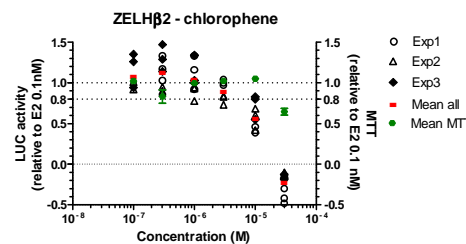
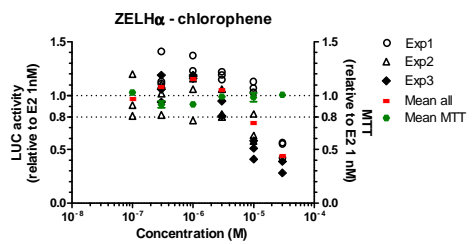
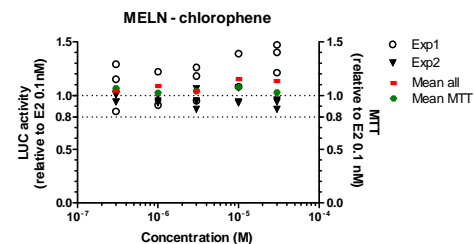
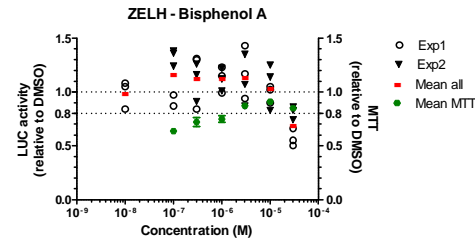
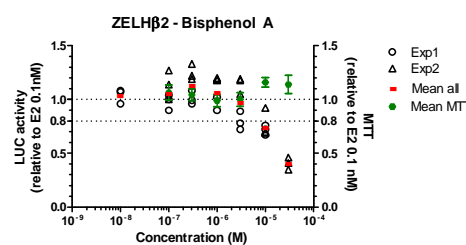
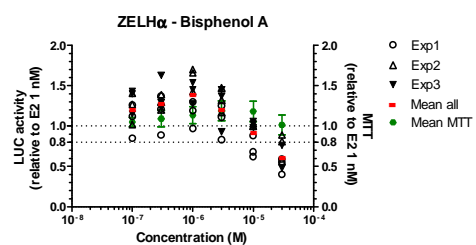
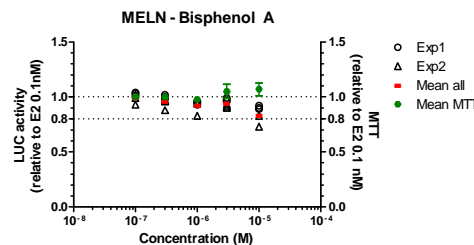
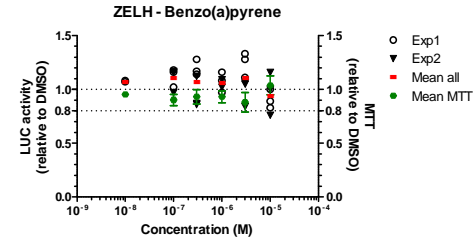
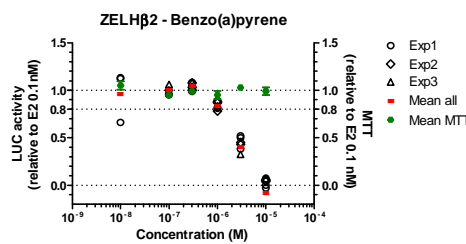
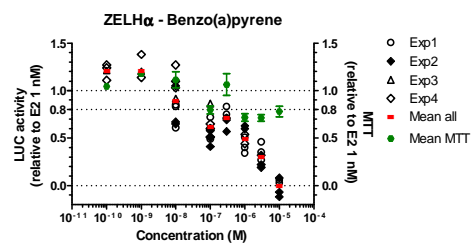
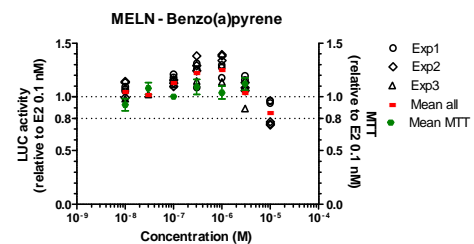
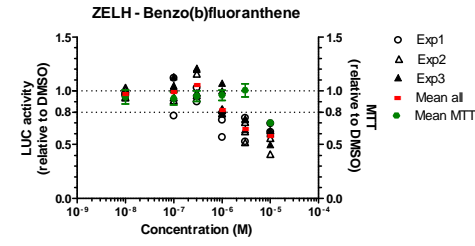
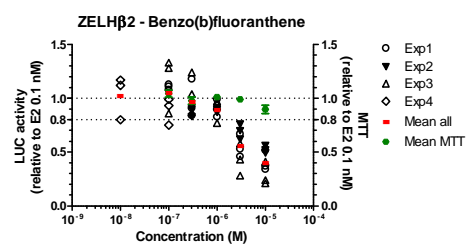
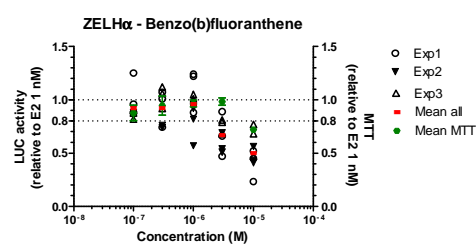
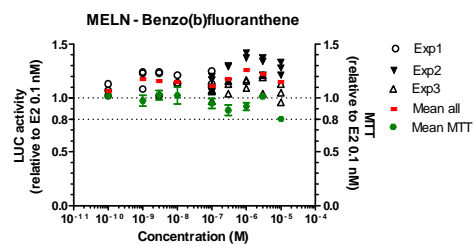


Figure SI 2: Response of the 12 chemicals on E2-induced ER inhibition in MELN, ZELH α , ZELH β 2 and ZELH cells. Data represent each replicate and the mean (red dash) of at least 2 independent experiments done in triplicates. Chemicals were tested in the 10 nM - 30 μ M range. MELN and ZELH β 2 cells were co-exposed with 0.1 nM E2, and ZELH α and ZELH cells with 1 nM E2. Cell viability (MTT) was measured for at least one experiment and is represented in green full circles (mean \pm SD) on the right Y axis. The horizontal dotted line at 80% figures the threshold of effect (IC20). Hydroxy-tamoxifen (OH-TAM) was used as positive control.





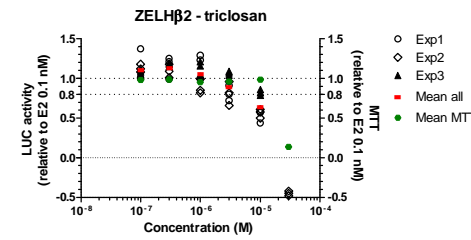
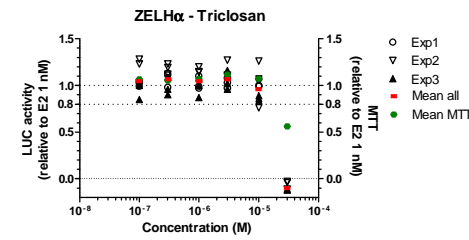
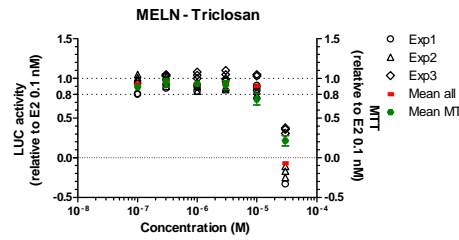
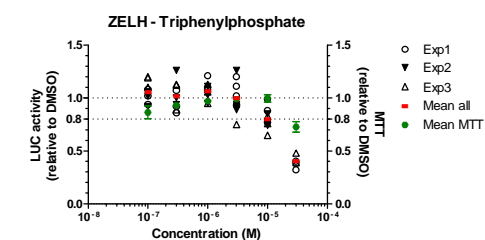
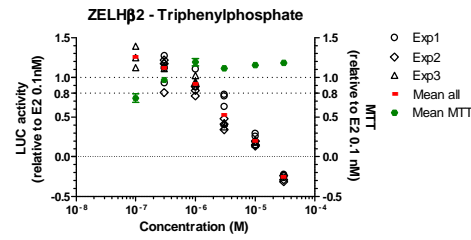
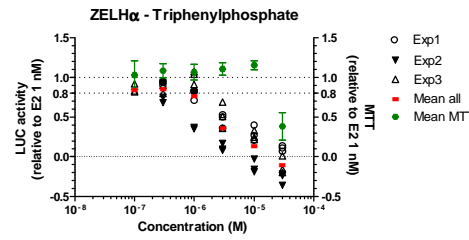
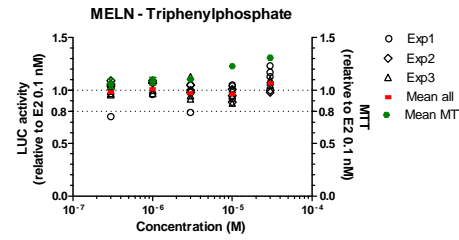
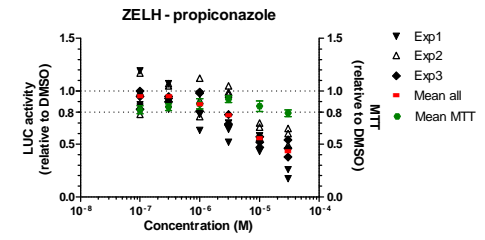
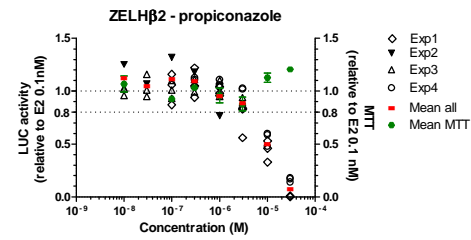
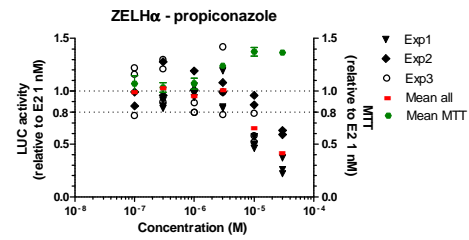
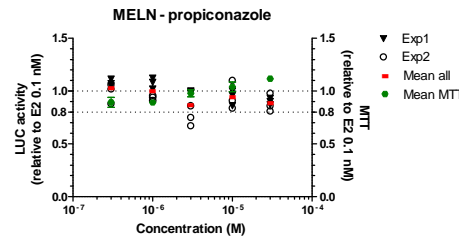
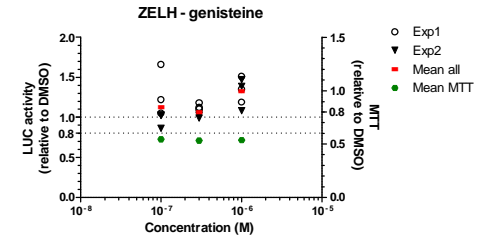
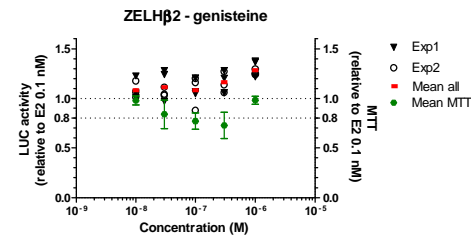
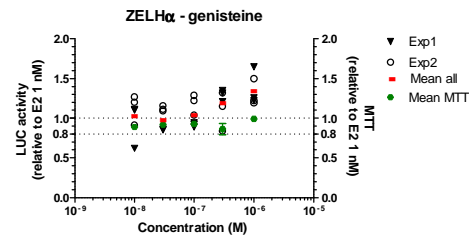
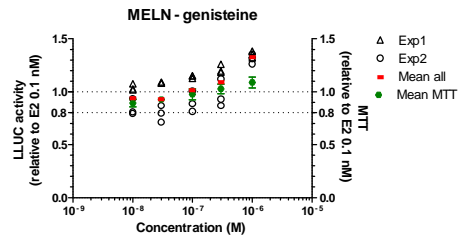


Figure SI 3: Cyprodinil response in MELN, ZELH α , ZELH β 2 and ZELH cells. The response was measured with cyprodinil alone (ER, luciferase induction relative to DMSO control) or in presence of E2 (antiER, luciferase induction relative to E2 positive control). Data represent the mean (+/- SD) of a minimum of 2 independent experiments done in triplicates and pooled together.

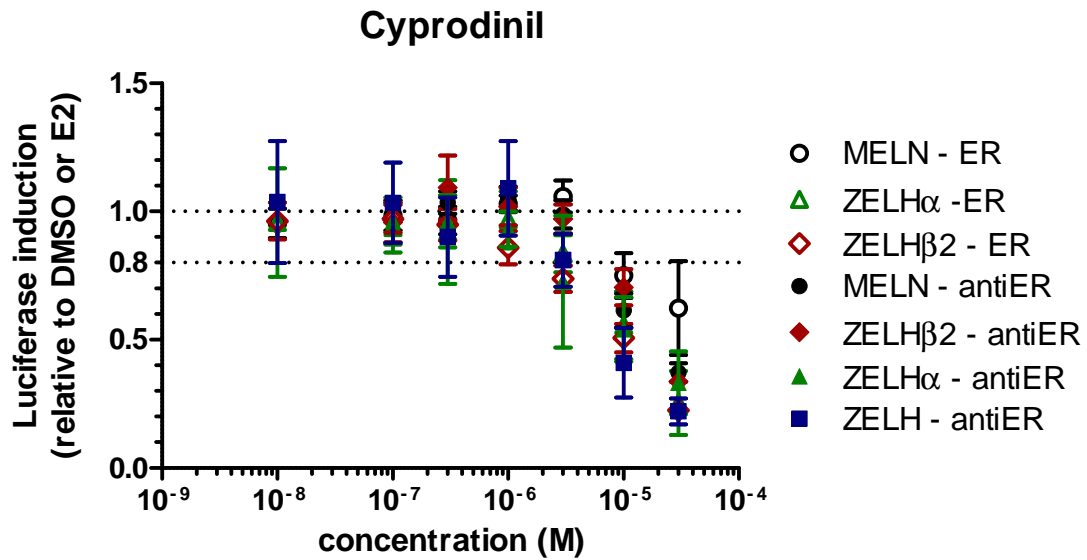
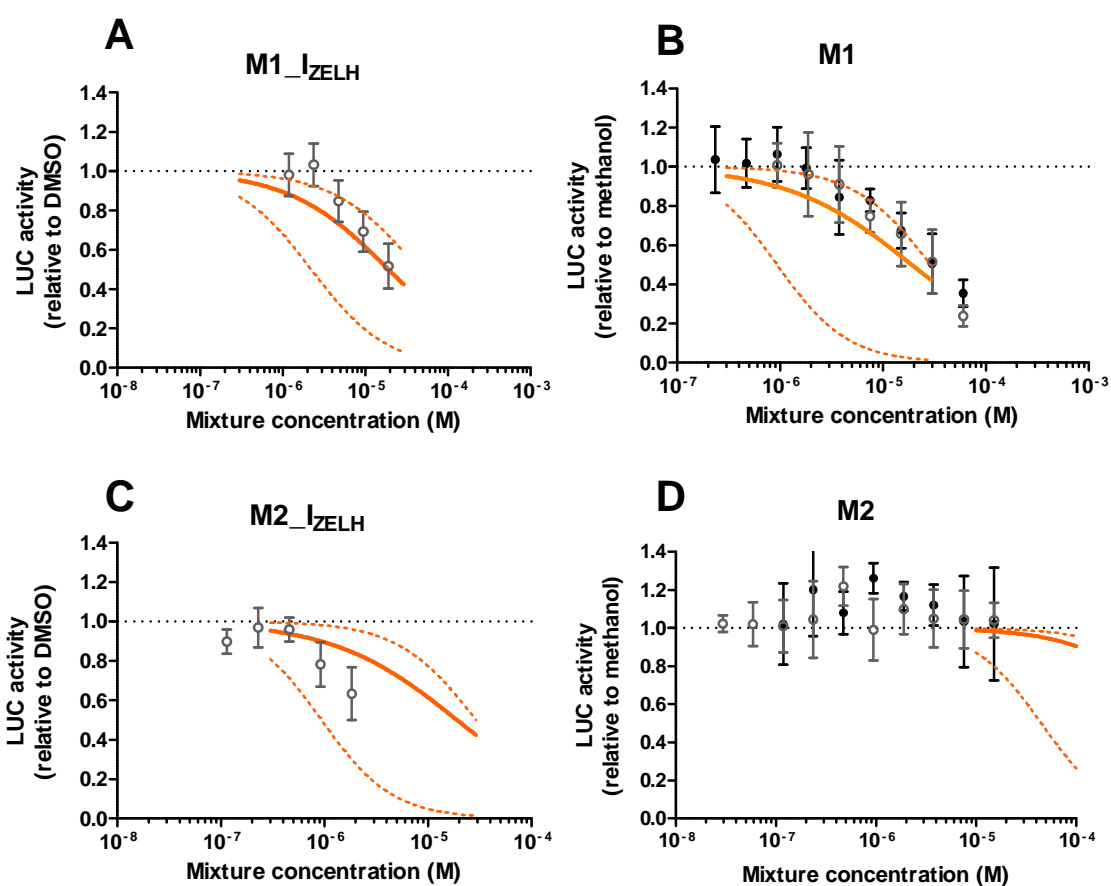


Figure SI 4: Predicted and observed effects of inhibiting chemicals on ZELH cells.

Results of subgroup mixtures M1_I_{ZELH} (A), M2_I_{ZELH} (B), and 12-component mixtures M1 (B) and M2 (D). Mixture effects were predicted according to CA model (orange line, 95% CI belt). Luciferase (LUC) activity was measured in absence (black circles) or in presence of E2 (co-exposure with E2 at 1 nM, grey open circles). The data (mean +/- SD) originate from at least 2 independent experiments done in triplicates and pooled together. Cytotoxic concentrations (measured by MTT) were removed.



Highlights:

- 12-chemical mixtures including xenoestrogens were tested in ER-reporter gene assays
- Human and zebrafish cells had distinct estrogenic response to the mixtures
- Several ER inhibitors were identified but in zebrafish cells only
- Inhibitors decreased the ER response in zebrafish cells compared with expected CA
- Non-estrogenic chemicals influenced ER mixture response in a cell-specific manner