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► To cite this version:

Hélène Serra, François Brion, Clémence Chardon, Hélène Budzinski, Tobias Schulze, et al.. Estrogenic activity of surface waters using zebrafish- and human-based in vitro assays: The Danube as a case-study. *Environmental Toxicology and Pharmacology*, 2020, 78, pp.art. 103401. 10.1016/j.etap.2020.103401 . hal-03159755

HAL Id: hal-03159755

<https://hal.science/hal-03159755v1>

Submitted on 9 Aug 2021

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1 **Estrogenic activity of surface waters using zebrafish- and human-based *in vitro* assays:**
2 **the Danube as a case-study**

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13 **HIGHLIGHTS**

14

- 15 • *In vitro* evaluation of the estrogenicity of 25 Danube water samples
- 16 • Most samples have a low estrogenic activity
- 17 • Both human and zebrafish bioassays allow the identification of two hot spot sites
- 18 • Estrogenic effect is confirmed *in vivo* using the zebrafish EASZY assay
- 19 • Established effect-based trigger values for human bioassays allow prioritisation

20

21

22 **ABSTRACT**

23 Most *in vitro* reporter gene assays used to assess estrogenic contamination are based on
24 human estrogen receptor α (hER α) activation. However, fish bioassays can have distinct
25 response to estrogenic chemicals and mixtures, questioning the relevance of human-based
26 bioassays for assessing risk to this species. In this study, zebrafish liver cells stably expressing
27 zebrafish ER β 2 (ZELH β 2) and human breast cancer cells expressing hER α (MELN) were
28 used to quantify the estrogenic activity of 25 surface water samples of the Danube River, for
29 which chemicals have been previously quantified. Most samples had a low estrogenic activity
30 below 0.1 ng/L 17 β -estradiol-equivalents that was more often detected by MELN cells, while
31 ZELH β 2 response tend to be lower than predicted based on the chemicals identified.
32 Nevertheless, both bioassays quantified well a higher estrogenic activity at two sites, which
33 was confirmed *in vivo* using a transgenic zebrafish assay. The results are discussed
34 considering the effect-based trigger values proposed for water quality monitoring.

35

36 1. INTRODUCTION

37 The evidence that estrogenic chemicals occurring in the aquatic environment may adversely
38 affect the endocrine system of fish has led to a growing concern regarding their long-term
39 reproductive effects (Sumpter and Jobling 2013). In 2012, the European Commission
40 proposed to set environmental quality standards (EQS) for estradiol (E2), estrone (E1) and
41 ethynyl estradiol (EE2) at 0.4 ng/L, 3.6 ng/L and 0.035 ng/L, respectively (European
42 Commission 2015). However, most current analytical methods are not sensitive enough to
43 quantify E2 and EE2 at these very low concentrations, below the ng/L (Kase et al. 2018). For
44 instance, the results of the European Union (EU)-wide monitoring exercise for chemicals on
45 the Watch List showed that only half of EU member states achieved to implement a method
46 with a limit of quantification at the EQS level for EE2 (Loos et al. 2018). Consequently, the
47 lack of sufficiently sensitive and cost-effective analytical methods for monitoring estrogenic
48 chemicals considerably hinders their prioritization under the European Water Framework
49 Directive (WFD).

50 *In vitro* bioassays based on the activation of the estrogen receptor (ER) are sensitive effect-
51 based methods to assess estrogenic chemicals (Wernersson et al. 2015). Integrated with
52 analytical tools, they have been successfully applied to quantify the estrogenic activity of
53 surface and wastewater, and they showed to have a good sensitivity to detect E1, E2 or EE2 at
54 environmental levels, highlighting their potential to support chemical monitoring (Leusch et
55 al. 2010, Könemann et al. 2018, Kunz et al. 2015). Furthermore, they enable to detect active
56 chemicals in complex mixtures without *a priori* knowledge, as they take into account the
57 combined effects of both known and unknown substances targeting the ER, and, thus, they
58 provide a holistic evaluation of estrogenic mixtures (Altenburger et al. 2019).

59 Most *in vitro* bioassays used in biomonitoring are mammalian or yeast-based system
60 expressing the human ER α (hER α), which controls the expression of a reporter gene used to
61 quantify the biological activity. Compared to the human genome that encodes only for two ER
62 subtypes, hER α and hER β , most teleost fish express at least 3 ER subtypes, ER α , ER β 1 and
63 ER β 2 (Tohyama et al. 2016, Menuet et al. 2002) that can have distinct sensitivities to
64 estrogenic chemicals compared to human isoforms (Cosnefroy et al. 2009, Matthews et al.
65 2000, Pinto et al. 2014). For instance, zebrafish zfER β subtypes are one order on magnitude
66 more sensitive to E2 than zfER α , while the reverse is observed for human ERs (Pinto et al.
67 2014). Among zfER subtypes, zfER β 2 is the most sensitive and responsive one to various
68 estrogenic substances (Cosnefroy et al., 2012). In addition to structural differences at the
69 receptor level, the response to estrogens is influenced by the cell context, such as the presence
70 of transcriptional co-factors or metabolic pathways, which confer a tissue-specific response
71 (Ohtake et al. 2003). In the aquatic environment, many studies reported that fish are among
72 the most sensitive organisms to xenoestrogens (Tyler et al. 1998, Matthiessen et al 2018) and
73 the question of the relevance of human-based bioassays to predict an effect in fish can thus be
74 raised (Hotchkiss et al 2008).

75 Recent field studies showed that *in vitro* estradiol-equivalents (EEQs) measured in surface
76 and waste waters correlated to *in vivo* responses measured in fish models (Ihara et al. 2015,
77 Brion et al. 2019). Nevertheless, some studies suggested that a better correlation is found
78 with fish-based bioassays. Using wastewater samples, Ihara et al. (2015) showed that the
79 induction of vtg1/chgH in male medaka was better correlated with *in vitro* medaka ER α
80 activation than with human ER α . Interactions between chemicals leading to co-occurrence of
81 estrogenic and anti-estrogenic activities were suggested to drive the observed differences
82 (Ihara et al. 2014). In a previous field study, we also reported inter-specific qualitative and

83 quantitative differences between human and fish-based cell lines for surface water extracts,
84 with some samples being selectively active in zebrafish liver cells expressing zfER β 2
85 (ZELH β 2 cells) and in an *in vivo* zebrafish assay but not in human MELN cells (Sonavane et
86 al. 2016). Furthermore, ZELH β 2 and MELN cells have distinct sensitivity to anti-estrogenic
87 chemicals, which can significantly influence the response to estrogenic chemicals in a cell-
88 specific manner (Serra et al. 2019). These results suggest the presence of fish-specific factors
89 influencing the *in vitro* estrogenic response to environmental extracts that warrants further
90 research.

91 This study was carried out to gain further knowledge on possible differences between fish-
92 and human-based bioassays in the assessment of the estrogenic activity of river water sample
93 extracts, including the evaluation of the contribution of known estrogenic chemicals to the
94 measured activity. For that purpose, we used water extracts from the Danube River, which
95 have been previously characterized chemically and are representative of a large river with
96 high dilution capacity and highly diverse chemical contamination pattern (Liška et al. 2015).
97 The water was sampled using on-site large-volume solid phase extraction (LVSPE) during the
98 Join Danube Survey 3 (Neale et al. 2015) and in Novi Sad city (Serbia) that discharges
99 untreated wastewater directly into the Danube River (König et al. 2017). The biological
100 responses of both *in vitro* bioassays were compared with the estrogenic chemicals identified
101 by target chemical analyses. In addition, we assessed the anti-estrogenic activity of Novi Sad
102 samples and evaluated the *in vivo* estrogenic response in transgenic zebrafish embryos using
103 the EASZY assay (Brion et al. 2012). The response of the bioassays is discussed considering
104 the effect-based trigger values (EBT) proposed to implement the bioassays in a regulatory
105 context.

106

107 2. MATERIAL AND METHODS

108 2.1. Chemicals and reagents

109 17 β -estradiol (E2, CAS 50-28-2, purity of >98%), ethynyl estradiol (EE2, CAS n° 57-63-6,
110 purity of >98%) and hydroxy-tamoxifen (OH-TAM, CAS 68392-35-8, purity of >98%),
111 dimethylsulfoxide (DMSO), Leibovitz 15 culture medium (L-15), fetal calf serum (FCS), 4-
112 (2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), epidermal growth factor (EGF),
113 G418, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) and D-luciferin
114 were purchased from Sigma Aldrich (St-Quentin Fallavier, France). Dulbecco's Modified
115 Eagle Medium High Glucose (DMEM HG) powder, F-12 nutrient mixture (Ham's F12)
116 powder, penicillin and streptomycin were purchased from Gibco (France). Insulin,
117 hygromycin B and sodium bicarbonate were purchased from Dominique Dutscher (France).

118 2.2. Study sites, sampling and extraction procedure

119 Two sets of Danube River water samples were collected and prepared during the Joint
120 Danube Survey 3 (JDS, 22 samples) and Novi Sad (NS, 3 samples) campaigns as detailed
121 previously (Neale et al. 2015, König et al. 2017). In brief, sampling of the JDS was carried
122 out between August and September 2013 in Danube River and some tributaries, from Austria
123 to Romania (Liška et al. 2015). The city of Novi Sad city (300 000 inhabitants, Serbia),
124 located on the Danube River, was selected to investigate the impact of untreated municipal
125 effluent release on the Danube River (König et al. 2017) and to identify drivers of endocrine
126 disruption including estrogenicity and androgenicity (Hashmi et al. 2018) and progestogenic
127 and glucocorticoid receptor-mediated effects (Hashmi et al. 2020). The Novi Sad sites
128 investigated were selected as follows: NS1 site 2 km upstream the effluent release, NS2 site
129 200 m downstream the effluent release, and NS3 site 7 km downstream the effluent release. In

130 both case-studies, the samples were collected using an on-site solid phase extraction (SPE)
131 device designed to sample and extract large volume (LV) of water on site (LVSPE, Schulze et
132 al. 2017). About 500 L of water were extracted and concentrated at JDS sites, and 850 L to
133 1000 L at NS sites supporting a large set of investigations (Schulze et al. 2017) while for the
134 present study aliquots representing 6 to 12 L water equivalents were used. The organic
135 extracts were reduced to dryness before shipping and were then resuspended in DMSO and
136 stored at -20 °C before bioanalysis.

137 ***2.3. In vitro bioassays***

138 The zebrafish *in vitro* assay derived from the zebrafish liver (ZFL) cell line. ZFL cells were
139 stably transfected first by an ERE-driven luciferase gene, yielding the ZELH cell line, and
140 then by zfER β 2 subtype yielding the ZELH β 2 cell lines (Cosnefroy et al. 2012). The
141 establishment of this cell model and its response to different classes of well-known
142 xenoestrogens have been previously described (Cosnefroy et al. 2012, Serra et al. 2019, Le
143 Fol et al. 2017, Sonavane et al. 2016). In addition, we used the human-derived MELN cell
144 line (Balaguer et al. 1999) kindly provided by Dr. Patrick Balaguer (INSERM Montpellier,
145 France). The MELN cells are derived from the breast cancer MCF-7 cells that endogenously
146 express the hER α , but no functional hER β (P. Balaguer, *personal communication*). MCF-7
147 cells were stably transfected with an ERE-driven firefly luciferase reporter gene to yield
148 MELN cells.

149 Conditions for routine cell culture and exposure to chemicals have been detailed previously
150 (Sonavane et al. 2016). Briefly, ZELH β 2 cells were seeded in 96-well white opaque culture
151 plates (Greiner CellStar™, Dutscher, France) at 25,000 cells per well in phenol red free LDF-
152 DCC medium (containing L-15 50%, DMEM HG 35%, Ham's F12 15%, HEPES 15 mM,

153 0.15 g/L sodium bicarbonate, 0.01 mg/mL insulin, 50 ng/mL EGF, 50 U/mL penicillin and
154 streptomycin antibiotics, 5% v/v stripped FCS). MELN were seeded at 80,000 cells per well
155 in steroid- and phenol red-free DMEM medium. Cells were left to adhere for 24 h, and then
156 exposed for either 72 h at 28 °C for zebrafish cells or 16h at 37°C for MELN cells. Cells were
157 exposed in triplicates to serial dilutions of sample extracts to obtain a final DMSO
158 concentration in the well plate of 0.5% (v/v). To assess anti-estrogenic activity at selected
159 sites, ZELH β 2 and MELN cells were co-exposed to the sample in presence of 0.1 nM E2, i.e.
160 a concentration saturating 80% to 100% of ER. Each plate included both solvent (DMSO) and
161 positive (E2 for estrogenic activity, OH-TAM for anti-estrogenic activity) controls. After
162 exposure, culture medium was removed and replaced by 50 μ l per well of medium containing
163 0.3 mM D-luciferin. The luminescence signal was measured in living cells using a microtiter
164 plate luminometer (Synergy H4, BioTek). The effect of samples on cell viability was assessed
165 by using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) assay
166 (Mosmann 1983). After cell exposure, culture medium was removed and replaced by 100 μ L
167 of medium containing 0.5 mg/mL MTT. Cells were incubated for 1 h to 3 h. In metabolically
168 active cells, MTT is reduced onto a blue formazan precipitate, which is dissolved by adding
169 100 μ L of DMSO after removal of MTT containing medium. Plates were read at 570 nm
170 against a 640 nm reference wavelength on a microplate reader (KC-4, BioTek Instruments,
171 France) and results are expressed as absorbance relative to control cells.

172 ***2.4. Zebrafish embryo-based bioassay (EASZY assay)***

173 The estrogenic activity of Novi Sad samples was assessed *in vivo* using the EASZY assay
174 based on transgenic *cyp19a1b*-GFP zebrafish line (Brion et al. 2012). The assay procedure for
175 sample testing has been described in Sonavane et al. (2016) and Brion et al. (2019). In brief,

176 15 newly fertilized transgenic eggs (< 4h post fertilization) were selected for each
177 experimental group and exposed for 96 h in 15 mL of acclimated water in glass crystallizers.
178 Serial dilutions were tested with a final volume of solvent (DMSO) of 0.1% v/v, a
179 concentration without effects on embryo development or GFP expression. In each
180 experimental series, positive (EE2 0.05 nM) and DMSO controls were included as separate
181 experimental groups. Exposed embryos were incubated at 28 °C, under semi-static conditions
182 with daily complete renewal of medium. At the end of the exposure period, each zebrafish
183 embryo was photographed using a Zeiss AxioImager.Z1 microscope equipped with an
184 AxioCam Mrm camera (Zeiss GmbH, Gottingen, Germany) to measure GFP expression in the
185 brain. Image analysis was performed using the ImageJ software, and fluorescence data was
186 treated exactly as previously described (Brion et al. 2012).

187 **2.5. Data analysis**

188 Sample concentration data are expressed in relative enrichment factor (REF) that considers
189 both sample enrichment in LVSPE and further dilution in the test system. For all *in vitro* data,
190 the luciferase activity was normalized between 0 and 1, as described in equation (1), with
191 $signal_{sample}$ the signal of the tested sample, $signal_{control}$ the signal of the solvent control and
192 $signal_{positive}$ the signal of the positive control (E2):

$$193 \quad Response = \frac{signal_{sample} - signal_{control}}{signal_{positive} - signal_{control}} \quad (1)$$

194 Concentration-response curves of bioassay data were fitted to all replicates pooled together
195 with a minimum of two independent experiments (each with three technical replicates) using
196 Hill equation in the RegTox 7.5 Microsoft Excel™ macro (freely available at
197 http://www.normalesup.org/~vindimian/fr_download.html). Positive control 20 (PC20, a

198 concentration corresponding to 20% of the response of the positive control) were estimated
199 for samples reaching at least 20% effect by fixing the minimum and the maximum of the
200 sample response to 0 and 1, respectively, and fixing the slope to the one of E2. The biological
201 estradiol-equivalent (Bio-EEQ) was then calculated as the ratio of EC20 of positive control to
202 the PC20 of the sample, expressed in ng/L EEQ, as presented in equation (2):

$$203 \quad BioEEQ = \frac{EC20_{positive\ control}}{PC20_{sample}} \quad (2)$$

204 The MELN raw data have been previously analyzed using PC10 value and linear regression
205 model (Neale et al. 2015). The data were re-evaluated in the present study using PC20 and
206 Hill model and a very good agreement between both Bio-EEQ calculation methods was
207 observed (Figure SI 1).

208 To assess the contribution of detected chemicals to the observed biological response, chemical
209 equivalents (Chem-EEQ) were calculated for the estrogenic chemicals as the sum of the
210 concentration of each active chemical weighted by their relative estrogenic potency (REP),
211 based on the equation (3):

$$212 \quad ChemEEQ = \sum_i^n REP_i \times C_i \quad (3)$$

213 The REP is defined as the ratio of PC20 of E2 to the PC20 of the chemical, or as the ratio of
214 EC50s, when no PC20 is available. The contribution of the quantified chemicals to the
215 biological response was assessed by dividing the Chem-EEQ by the Bio-EEQ, expressed in
216 %.

217 In addition, the concentration addition (CA) model was used to predict the additive effects of
218 identified estrogenic chemicals along the entire dose-response curve, to facilitate graphical

219 comparison of observed and predicted estrogenic activity. For that purpose, single chemical
220 data were fitted with logit non-linear regression model in GraphPad Prism v.5 and the logit
221 parameters derived were entered into an Excel template to predict the additive response using
222 the concentrations and mixture ratios of the active chemicals identified in each sample (Serra
223 et al. 2019, Altenburger et al. 2018). For simplification purpose, only the active chemicals
224 contributing to more than 5% of the predicted response at REF of 1 were included in CA
225 prediction. Logit equation and the Excel template were kindly provided by Martin Scholze
226 (Brunel University, UK).

227

228 **3. RESULTS**

229 *3.1. The estrogenic activity of Danube water extracts is overall low*

230 The results of the estrogenic activity of JDS and Novi Sad samples measured in MELN and
231 ZELH β 2 bioassays are presented in Table 1, and the individual concentration-response curves
232 are provided in supplementary information (figures SI 2 and SI 3 for MELN and ZELH β 2
233 cells, respectively).

234 Overall, the estrogenic activity measured in the JDS samples was relatively low, in both cell
235 models. In MELN cells, 15 out of 22 JDS samples had an estrogenic activity above the LOQ
236 (0.002 ng/L EEQ). The response measured was below 0.3 ng/L EEQ in all samples, except for
237 JDS41 that had an estrogenic activity of 0.74 ng/L EEQ. A similar pattern was observed with
238 the ZELH β 2 bioassay, with, however, only 8 samples being positively quantified. In all
239 samples, the estrogenic activity measured in ZELH β 2 was below 0.08 ng/L EEQ, except for
240 JDS41 that reached 2.3 ng/L EEQ. The comparison of both MELN and ZELH β 2 showed that
241 10 samples were equally detected in both cell lines (being either active or inactive), while 2

242 samples were selectively detected in ZELH β 2 cells (JDS63, JDS67) and 10 samples only in
243 MELN cells (e.g. JDS22, JDS27, JDS30).

244 The concentration-response curves of Novi Sad samples are presented in Figure 1, and the
245 Bio-EEQs are given in Table 1. The estrogenic activity pattern of Novi Sad samples was
246 similar between MELN and ZELH β 2 bioassays, with a peak of activity detected just after the
247 effluent release at NS2 site. However, the NS2 sample had a higher estrogenic activity in
248 MELN cells (1.52 ng/L EEQ) compared to the one measured in ZELH β 2 cells (0.19 ng/L
249 EEQ).

250 When quantified by the two assays, Bio-EEQs provided by the two models were overall
251 correlated (Figure SI 4) although MELN assay tended to quantify higher levels (Table 1).

252 *3.2. The identified estrogenic chemicals only partially explained the fish-specific* 253 *response*

254 We further assessed the contributions of estrogenic chemicals identified in the samples to the
255 activity measured in fish and human-based bioassays. More than 100 chemicals were
256 quantified in JDS and Novi Sad samples (König et al. 2017, Neale et al. 2015), and
257 information about the activity of the chemicals on MELN and ZELH β 2 cells was available
258 only for 36 substances, among which 7 were estrogenic on ZELH β 2 cells and 9 on MELN
259 cells. The Relative Potency (REP) of these xenoestrogens for each cell line are indicated in
260 Table 2, and the concentrations of the active chemicals across all sampling sites are presented
261 in Table SI 1. These chemicals include natural steroid estrogens, bisphenols, phytoestrogens
262 and pesticides.

263 The Chem-EEQs of the 25 samples and their contribution to the biological response (Bio-
264 EEQs) are presented in Table 1. Overall, the same main contributors to the estrogenic activity
265 were detected for both cell lines: estrone and genistein, confirming our previous report (Neale
266 et al. 2015). It is worth to note that EE2 was included in the analyses but was never detected
267 (LOQ 0.4 ng/L). Regarding JDS samples, estrone and genistein together explained 19% to
268 124% of the biological response observed in MELN cells for most samples, except at JDS27
269 and JDS60 (<1%, no estrone detected), and at JDS64 (710%, very weak estrogenic response
270 measured). Furthermore, 5 samples were expected to have an estrogenic activity ranging from
271 0.18 to 0.58 ng/L EEQ in MELN cells based on the chemical quantified, but were not detected
272 by the bioassay (JDS35, JDS57, JDS59, JDS63 and JDS65), as confirmed by comparing CA
273 prediction curve with the observed response (Figure SI 2). Conversely, JDS27 and JDS60
274 were active in MELN cells albeit no significant contributors could be identified. In ZELH β 2
275 cells, estrone and genistein explained 11% to 136% of the observed biological response of
276 JDS samples, except at 3 sites for which the biological activity was lower than expected based
277 on the estrogenic chemicals quantified (JDS29: 503%, JDS39: 306% and JDS67: 214%). In
278 contrast, 11 samples were predicted to have an estrogenic activity in ZELH β 2 cells, ranging
279 from 0.022 to 0.065 ng/L EEQ, as predicted by CA model, but were not detected by the
280 bioassay (Table 1 and Figure SI 3).

281 Because estrone is a major driver of estrogenicity in JDS samples, it may thus contribute to
282 estrogenic activity of the extracts even if present at concentration below or at its LOQ (i.e. 0.1
283 ng/L). Thus, for those few sites with very low contribution of Chem-EEQ in MELN cells,
284 namely JDS 27 and JDS60, including estrone LOQ in the mass balance calculation increases
285 the contribution of Chem-EEQs up to 13% and 16%, respectively. In all cases, maximized

286 Chem-EEQs remain below Bio-EEQ and suggest that other non-detected active compounds
287 are present in the samples at these sites.

288 As for most JDS samples, genistein was detected in all Novi Sad samples but explained only a
289 small fraction (less than 10%) of the observed response, in both MELN and ZELH β 2 cells
290 (Figure 2). In NS2 sample, the steroidal estrogens (E1, E2, E3) were the main drivers
291 identified. They were adequately detected in MELN cells (contribution of 88% to the
292 biological response); however, the activity of NS2 measured in ZELH β 2 cells was about 7
293 times lower than predicted based on Chem-EEQ (Table 1, Figure 1). To investigate possible
294 negative interactions on the estrogenic response, we assessed the anti-estrogenic activity of
295 Novi Sad samples by co-exposing the cells to the samples with E2 at a concentration inducing
296 80% of E2 maximal response. As presented in Figure 1, a decrease in E2-induced luciferase
297 activity was noted but only at the highest non-cytotoxic concentration tested. Thus, no
298 significant anti-estrogenic activity could be evidenced, especially regarding NS2 sample
299 extract in ZELH β 2 cells.

300 ***3.3. In vivo estrogenic activity of Novi Sad samples***

301 In order to further investigate the toxicological relevance of detected estrogenic activities at
302 Novi Sad sites, we assessed *in vivo* effect of the samples in zebrafish embryos that express
303 GFP under control of the ER-regulated *cyp19a1b* promoter, applying the EASZY assay
304 (Brion et al., 2012, Brion et al., 2019). As presented in Figure 3A, a significant response was
305 observed at NS2 site, while no effect was detected at the upstream and downstream sites. The
306 NS2 sample had an estrogenic activity of 3.3 ng/L EEQ in transgenic zebrafish embryos, in
307 very good agreement with the chemicals identified, E1, E2 and E3 being the main
308 contributors *in vivo* (Figure 3B).

310 4. DISCUSSION

311 4.1. Comparative evaluation of estrogenic activity in fish and human-based bioassays

312 Using an integrated analytical-bioanalytical method, we assessed the estrogenic activity of the
313 Danube River combining zebrafish ZELH β 2 and human MELN cells. Our results show that,
314 with the exception of two sites, the estrogenic activity of the Danube River was overall low.
315 The majority of samples had a Bio-EEQ below 0.1 ng/L EEQ both in MELN cells (18/25
316 samples) and in ZELH β 2 cells (23/25 samples). The low estrogenicity measured is consistent
317 with the high dilution capacity of the large Danube River, resulting in trace levels of
318 contaminants. Estrone was the main estrogenic chemical detected in the Danube samples, in
319 line with previous studies evaluating surface water estrogenicity in other contexts (Conley et
320 al. 2017, Alvarez et al. 2013). Overall, we show that, although both cell lines are sensitive,
321 levels of estrogenic activity below 0.1 ng/L EEQ were more often detected by MELN cells
322 than ZELH β 2 cells. Indeed, the estrogenic activity predicted in ZELH β 2 cells tend to be
323 higher than the biological response observed, whenever the sample was detected.

324 The latter outcome contrasts with the results of a previous study that compared the estrogenic
325 activity of POCIS extracts deployed on 20 French river sites (Sonavane et al. 2016). In the
326 latter, ZELH β 2 cells revealed an estrogenic activity at 8 sites that was not detected by MELN
327 cells. The authors hypothesized the presence of fish-selective ER active chemicals, although
328 the samples were not characterized chemically. In the current study, we cannot argue in
329 favour of bioassay-specific estrogenic substances, as genistein and estrone, identified as main
330 estrogenic drivers, are active in both MELN and ZELH β 2 cells. The lower response of
331 zebrafish cells might be explained by a higher sensitivity of ZELH β 2 cells over MELN cells

332 to non-ER chemicals present in the mixture and modulating the zER β 2 response (Serra et al.
333 2019). In the highly diluted context of Danube, estrogenic compounds are at very low
334 concentrations and in presence of a universe of other co-occurring compounds that are also
335 caught and concentrated by LVSPE. Distinct sampling approaches and pollution patterns may
336 account for the differences observed between the outcomes of both studies.

337 Despite different ability to pick up low estrogenicity levels, both bioassays quantified a higher
338 estrogenic activity at JDS41 and at NS2. JDS41 site is located at the confluence of the Molika
339 Morava (Serbia) and the Danube River. This site was identified as a highly contaminated site
340 in the Danube survey (Liška et al. 2015), and had, indeed, the highest load in chemicals of all
341 the JDS samples (Neale et al. 2015). The detected estrogenic chemicals together explained
342 less than 30% of estrogenic activity at this site. This finding illustrates the complementarity of
343 chemical and bioanalytical tools to assess environmental xenoestrogens, as the *in vitro*
344 bioassays were able to detect chemicals not targeted by chemical analysis. In the Novi Sad
345 case study, the detection of a higher estrogenic activity at NS2 site confirms that the
346 wastewater release is a point source of environmental xenoestrogens (Könemann et al. 2018,
347 Sonavane et al. 2018). For instance, the steroidal estrogens E1, E2 and E3 that drove the
348 estrogenic activity of NS2 sample were well diluted few kilometres downstream of the
349 release, as shown by both analytical and bioanalytical tools at NS3 site. Furthermore, our
350 results are coherent with the estrogenic evaluation of the same Novi Sad samples performed
351 using two hER α -based reporter gene bioassays (König et al. 2017). The estrogenic activity of
352 NS2 of 1.52 ng/L EEQ measured in MELN cells was higher than the one reported in hER α -
353 GenBLAzer (0.26 ng/L EEQ) and in BG1-Luc4E (0.67 ng/L EEQ) bioassays, although well
354 explained by the chemicals quantified (mass balance of 115%). The tendency of MELN cells
355 to provide higher Bio-EEQ than other reporter gene assays has been evidenced in previous

356 studies, and is believed to account for a higher sensitivity of MELN cells to estrone
357 (Könemann et al. 2018).

358 Compared to MELN cells, the Bio-EEQ of NS2 sample in ZELH β 2 cells was about 7 times
359 lower than the Chem-EEQ. The lower biological activity in zebrafish cells suggests the
360 occurrence of negative interactions within the environmental mixture modifying the zfER β 2
361 response to xenoestrogens. We previously showed that bisphenol A and genistein had additive
362 effects in both ZELH β 2 and in MELN cells, however certain environmental contaminants,
363 such as propiconazole and triphenyl phosphate, were able to selectively decrease the
364 estrogenic response of ZELH β 2 cells but not MELN cells, resulting in deviation from
365 expected additive response (Serra et al. 2019). These results demonstrated a different
366 sensitivity to anti-estrogenic chemicals between zebrafish and human ER cell bioassays. Ihara
367 et al. (2014) documented a similar outcome using wastewater effluents, by showing that the
368 anti-estrogenic activity detected by medaka ER α bioassay was much more prevalent in
369 secondary wastewater effluents, while the primary wastewater effluent had much higher anti-
370 estrogenic activity on human ER α activation. In this study, we did not evidence a strong anti-
371 estrogenic activity of NS2 extract, and the anti-estrogenic chemicals identified in Serra et al.
372 (2019) on ZELH β 2 cells were not occurring at concentrations expected to have an effect in
373 the sample, whenever detected. However, the co-occurrence of both ER agonists and
374 antagonists within the same sample could impede a clear assessment of anti-estrogenic
375 compounds that may be masked by ER active compounds.

376 Further experiments using sample fractionation and biotesting of the fractions, for instance,
377 would be warranted to unravel the mixture effect and to evidence possible masking effects, as
378 previously demonstrated for (anti)androgens in sediments (Weiss et al. 2009),

379 (anti)mineralocorticoids in surface water (Creusot et al. 2014) and progestogen and
380 glucocorticoid-receptor mediated effects in the same samples (Hashmi et al. 2020).

381 Finally, the ZELH β 2 cells originate from zebrafish liver cells that remain metabolically active
382 (Creusot et al. 2015, Le Fol et al. 2015). Thus, bioassay-specific factors, such as distinct
383 metabolic capacity and sensitivity of zfER β 2, may have also contributed to lower the
384 estrogenic response of steroidal estrogens in ZELH β 2 cells.

385 **4.2. Identification of hot spots and comparison with effect-based trigger values**

386 *In vitro* bioassays are sensitive and specific tools to assess the estrogenic activity of
387 environmental samples (Könemann et al. 2018). The implementation of bioassays in a
388 regulatory context, such as the WFD, relies upon the derivation of a biological threshold of
389 response to enable water managers to discriminate between water bodies that are at low or
390 high risk (Escher et al. 2018). Several effect-based trigger values (EBT) have been proposed
391 for *in vitro* estrogenicity bioassays, with different protection goals and construction methods,
392 as presented in Table 3. Most of the EBT are constructed using primarily fish toxicity data.
393 For instance, Kase et al. (2018) and Escher et al. (2018) read across from the proposed EQS
394 derived from chronic toxicity of E1, E2 or EE2 on fish, and Jarošová et al. (2014) used
395 published long-term PNEC for E1, E2, E3 and EE2 in fish. Brion et al. (2019) compared
396 empirically the *in vitro* activity of environmental samples with the *in vivo* ER-regulated
397 *cyp19a1b* expression in transgenic zebrafish embryos. Alternatively, the proposed EBT for
398 ER-CALUX as part of SIMONI strategy is based on all acute and chronic data on fish,
399 insects, algae and crustaceans (van der Oost et al. 2017). It is worth noticing that only two
400 approaches actually take into account xenoestrogen mixture effects, either assuming additive
401 effects and average environmental occurrence ratio (Escher et al. 2018) or assuming all

402 possible mixture effects based on empirical response of fish embryos to environmental
403 samples (Brion et al. 2019). With the exception of the EBT proposed by Kunz et al. (2017)
404 which is a direct translation of the EQS of E2 into EEQ (0.4 ng/L EEQ), all other approaches
405 consider the bioassay-specific sensitivity (REP) to estrogenic chemicals in deriving the EBT.

406 The EBTs proposed for MELN cells are very consistent one to another, ranging from 0.3 ng/L
407 to 0.56 ng/L EEQ (Table 3). In the current study, only JDS41 (0.74 ng/L EEQ) and NS2 (1.52
408 ng/L EEQ) samples exceeded the EBT, including the highest one of 0.56 ng/L EEQ. Thus, the
409 application of these thresholds for MELN cells would have enabled to identify JDS41 and
410 NS2 as polluted sites. The risk quotient, defined as the sum of ratios of measured
411 concentrations of E1, E2 and EE2 to their respective EQS, reaches 0.95 for NS2 sample, as
412 both estrone and estradiol were at concentrations very close to their EQS. In comparison,
413 estrone was the only steroidal estrogen quantified in JDS41 sample and had a concentration of
414 0.196 ng/L, below the EQS level proposed of 3.6 ng/L (risk quotient of 0.05). Thus, JDS41
415 site would have not been prioritised based only on the steroidal estrogens identified, as
416 proposed in the WFD.

417 It is noticeable that *in vivo* estrogenic activity at the three NS sites was correctly predicted by
418 the *in vitro* bioassays in the present study. Since the EBT value of 0.56 ng/L was built based
419 on a comparison of MELN and EASZY data (Brion et al. 2019), the present dataset confirms
420 the suitability of this EBT value in another environmental context. Furthermore, the use of the
421 *in vivo* EASZY assay enabled us to confirm the estrogenic activity observed *in vitro* for NS2
422 sample at the organism level. It showed that the estrogenic pollutants could target the ER and
423 induce the expression of the brain aromatase in the developing zebrafish, hence adding further
424 toxicological relevance to the environmental diagnosis.

425 The prioritization of steroidal estrogens under the WFD relies upon the development of
426 enough sensitive and cost-effective monitoring methods. In the current study, we show that
427 both the MELN and ZELH β 2 bioassays performed well in detecting a higher estrogenic
428 activity at two hot spot sites. Furthermore, this study showed that MELN cells detected both
429 sites exceeding the proposed EBT, allowing a correct prioritisation of sites with a risk. In
430 contrast, JDS41 site would not have been prioritized based only on the steroidal estrogens
431 detected, as estrone was quantified at a level 20-time lower than the proposed EQS. Although
432 both human and fish-based bioassays identified well the two hot spot sites, the relevance of
433 the deviation from additivity observed in ZELH β 2 cells, notably at trace levels, remains to be
434 addressed.

435

436 **ACKNOWLEDGMENTS**

437 This study was supported by the EU Seventh Framework Programme as a part of
438 SOLUTIONS project (FP7-ENV-2013-two-stage) under grant agreement number 603437, and
439 by the French Ministry of Ecology (P190-Ecotoxicologie, P181-DRC60). We wish to thank
440 Emmanuelle Maillot-Maréchal for her excellent technical support with the cell lines, Martin
441 Scholze for providing tools and methods to predict additive effects of xenoestrogens, Margit
442 Petre, Melis Muz and Riccardo Massei for sample preparation and Peter Tarabek and Jörg
443 Ahlheim for technical help with sample collection. We are grateful to Jaroslav Slobodnik and
444 his team at Environmental Institute who organised all logistics with the JDS3 sampling.

445

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639

640 **Figure captions**

641

642 **Figure 1:** Estrogenic and anti-estrogenic activities of Novi Sad samples on (A) MELN and
643 (B) ZELH β 2 cells. The filled symbols represent the estrogenic activity, and the empty
644 symbols represent the anti-estrogenic activity. The green line represents concentration
645 addition (CA) prediction based on the main active xenoestrogens identified in the sample
646 (contribution >5% of the response at REF=1).

647

648 **Figure 2:** Contribution of identified xeno-estrogens to the estrogenic activity measured in
649 Novi Sad samples for (A) MELN and (B) ZELH β 2 cells. 100% corresponds to Bio-EEQ
650 measured experimentally.

651

652 **Figure 3:** In vivo estrogenic activity of Novi Sad sites in transgenic zebrafish embryos using
653 the EASZY assay. (A) *In vivo* induction of GFP in 4-days old zebrafish embryos (results of
654 one experiment with n=15 embryos per condition, representative of two independent
655 experiments). The concentrations are expressed in Relative Enrichment Factor (REF). The
656 positive control is 0.05 nM EE2. The mortality was below 20% in all groups. (*) denotes a
657 statistically significant induction in GFP intensity compared with the DMSO control group
658 (Mann-Whitney test, α : 5%). (B) Contribution of identified xeno-estrogens to *in vivo*
659 estrogenic activity of Novi Sad samples. 100% corresponds to Bio-EEQ measured
660 experimentally. n.a.: no activity.

661 **Table 1: Estrogenic activity of the Joint Danube Survey (JDS) and Novi Sad (NS)**
662 **samples on MELN and ZELH β 2 cells.** The Chem-EEQ for MELN cell line originate from
663 Neale *et al.* (2017). The Bio-EEQ and Chem-EEQ are expressed in ng/L EEQ. The % effect
664 indicates the contribution of identified chemicals (Chem-EEQ) to the biological response
665 (Bio-EEQ). CI95: 95% confidence intervals, n.a.: not active, LOQ: limit of quantification.

| | MELN | | | ZELH β 2 | | | |
|----------|------------|-----------------------|----------|----------------|-----------------------|----------|----------|
| | LOQ | Bio-EEQ [CI95] | Chem-EEQ | % effect | Bio-EEQ [CI95] | Chem-EEQ | % effect |
| JDS | LOQ | 0.002 | - | - | 0.002 | - | - |
| | 8 | 0.015 [0.015 ; 0.020] | 0.019 | 124% | 0.031 [0.031 ; 0.031] | 0.024 | 77% |
| | 22 | 0.048 [0.047 ; 0.061] | 0.021 | 43% | n.a. | 0.026 | - |
| | 27 | 0.10 [0.096 ; 0.13] | <LOQ | 1% | n.a. | < LOQ | - |
| | 29 | 0.11 [0.11 ; 0.17] | 0.050 | 44% | 0.012 [0.010 ; 0.014] | 0.062 | 503% |
| | 30 | 0.22 [0.19 ; 0.32] | 0.040 | 19% | n.a. | 0.051 | - |
| | 32 | 0.064 [0.061 ; 0.086] | 0.040 | 62% | n.a. | 0.054 | - |
| | 33 | 0.033 [0.032 ; 0.042] | 0.040 | 122% | n.a. | 0.051 | - |
| | 35 | n.a. | 0.034 | n.a. | n.a. | 0.042 | - |
| | 36 | 0.054 [0.052 ; 0.066] | 0.021 | 39% | n.a. | 0.026 | - |
| | 37 | n.a. | 0.024 | n.a. | n.a. | 0.030 | - |
| | 39 | 0.15 [0.14 ; 0.21] | 0.051 | 34% | 0.021 [0.017 ; 0.026] | 0.063 | 306% |
| | 41 | 0.74 [0.65 ; 0.84] | 0.20 | 27% | 2.3 [2.05 ; 2.9] | 0.244 | 11% |
| | 44 | 0.070 [0.069 ; 0.089] | 0.026 | 37% | 0.039 [0.039 ; 0.063] | 0.032 | 82% |
| | 53 | n.a. | 0.053 | n.a. | n.a. | 0.065 | - |
| | 55 | n.a. | <LOQ | n.a. | n.a. | <LOQ | - |
| | 57 | n.a. | 0.018 | n.a. | n.a. | 0.022 | - |
| | 59 | 0.053 | 0.050 | 94% | n.a. | 0.062 | - |
| | 60 | 0.078 [0.066 ; 0.092] | <LOQ | 0% | n.a. | <LOQ | - |
| | 63 | n.a. | 0.058 | n.a. | 0.053 [0.039 ; 0.064] | 0.072 | 136% |
| | 64 | 0.0026 [0.26 ; 0.33] | 0.019 | 710% | n.a. | 0.023 | - |
| | 65 | 0.12 [0.11 ; 0.13] | 0.066 | 55% | 0.071 [0.062 ; 0.082] | 0.082 | 116% |
| | 67 | n.a. | 0.045 | n.a. | 0.026 [0.023 ; 0.034] | 0.056 | 214% |
| Novi Sad | LOQ | 0.005 | - | - | 0.008 | - | - |
| | NS1 | 0.079 [0.073 ; 0.092] | <LOQ | 2% | 0.050 [0.044 ; 0.053] | <LOQ | 2% |
| | NS2 | 1.52 [1.44 ; 1.83] | 1.76 | 115% | 0.19 [0.17 ; 0.22] | 1.39 | 743% |
| | NS3 | 0.12 [0.11 ; 0.15] | 0.021 | 18% | 0.051 [0.039 ; 0.060] | 0.025 | 48% |

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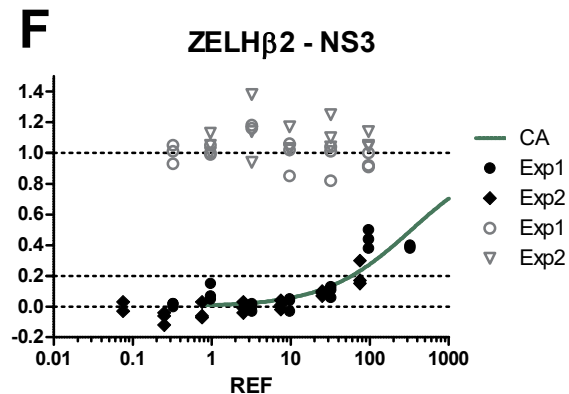
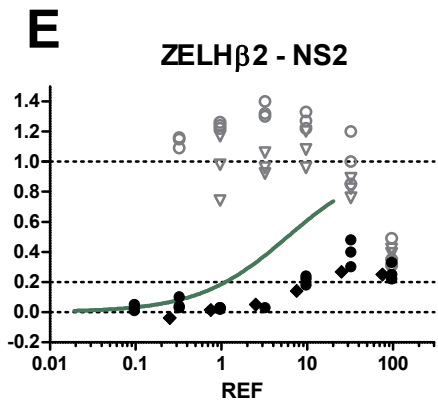
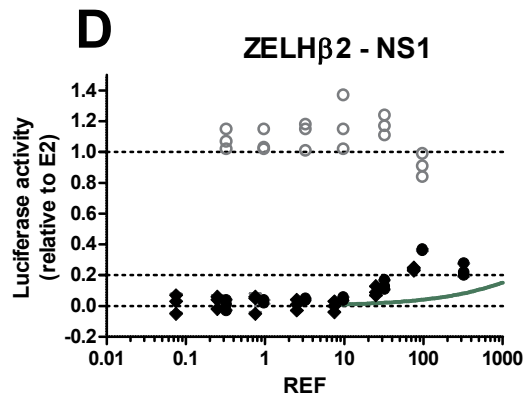
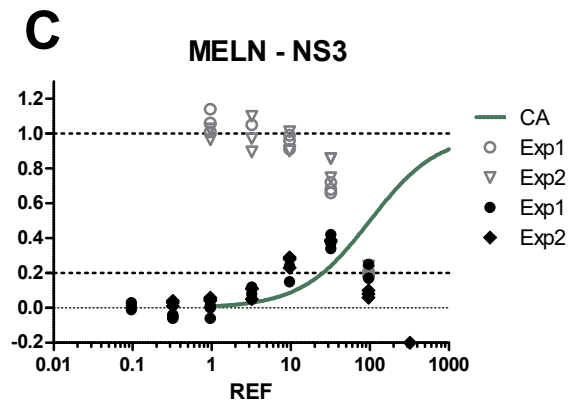
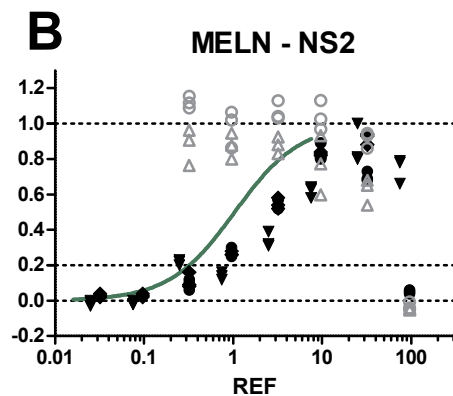
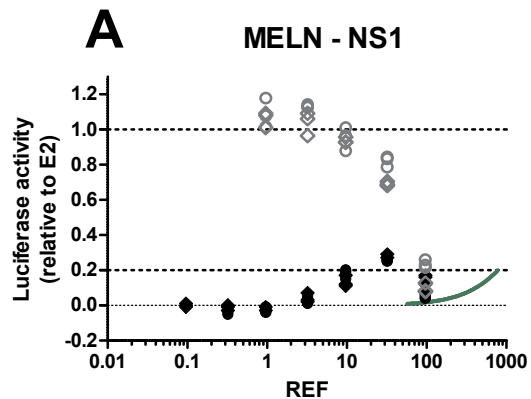
668 **Table 2: Relative potency (REP) of the chemicals quantified for each bioassay.** The REP
 669 values were calculated as the ratio of EC20 of E2 to that of test compound. They were derived
 670 from initial data published by ^(a)Neale et al. (2017), ^(b)Molina-Molina et al. (2008) and
 671 Molina-Molina et al. (2013), ^(c)LeFol et al. (2017), ^(d)Brion et al. (2012), ^(e)Brion et al. (2019).

| Chemical | MELN | ZELH β 2 | EASZY Assay |
|---------------|------------------------------------|------------------------------------|------------------------------------|
| Estrone | 0.110 ^a | 0.136 ^a | 0.78 ^e |
| Estriol | 0.077 ^a | 0.039 ^a | 0.06 ^d |
| Bisphenol F | 1.43 $\times 10^{-5}$ ^b | 5.14 $\times 10^{-6}$ ^c | 4.00 $\times 10^{-4}$ ^c |
| Bisphenol S | 1.18 $\times 10^{-6}$ ^a | 6.69 $\times 10^{-6}$ ^c | 7.80 $\times 10^{-6}$ ^c |
| Bisphenol A | 3.50 $\times 10^{-5}$ ^a | 3.57 $\times 10^{-6}$ ^c | 1.44 $\times 10^{-4}$ ^c |
| Daidzein | 6.50 $\times 10^{-5}$ ^a | 9.22 $\times 10^{-6}$ ^a | Inactive ^a |
| Diazinon | 2.80 $\times 10^{-7}$ ^a | Inactive ^a | Inactive ^a |
| Genistein | 2.00 $\times 10^{-4}$ ^a | 3.47 $\times 10^{-4}$ ^a | 1.91 $\times 10^{-4}$ ^a |
| Terbutylazine | 1.60 $\times 10^{-7}$ ^a | Inactive ^a | Inactive ^a |

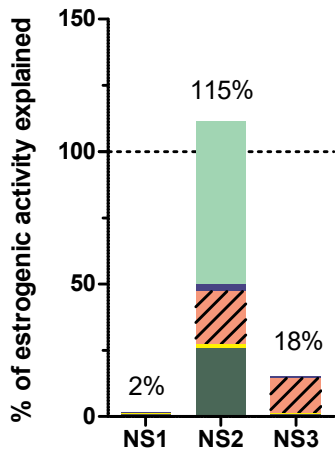
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Table 3: Comparison of effect-based trigger values proposed for estrogenic activity. The characteristics of each EBT are presented with a focus on the point of departure, inclusion of bioassay-specific and mixture considerations. n.a.: not applicable, n.i.: not included, REP: relative potency, CI: confidence interval, EQS: environmental quality standard. ^(a): according to option G in Escher et al. (2018).

| Point of Departure | | Special considerations | | | EB |
|---|--|---|------------------------------------|---|------------|
| Hazard | Chemicals | Occurrence | REP | Mixture effects | All |
| Chronic toxicity data on fish and amphibian (based on EQS) | E2 | n.i. | n.i. | n.i. | 0.4 |
| Acute and chronic toxicity data on fish, crustacean, algae, insects | 7 chemicals with REP > 0.001 in ER-CALUX | n.i. | Yes, only ER-CALUX | n.i. | 0.5 (95) |
| Chronic toxicity data on fish (PNEC) | E1, E2, E3, EE2 | Proportion in waste water and surface water | Yes, 8 in vitro bioassays compared | n.i. | 0.1 (me) |
| Chronic toxicity data on fish and amphibian (based on EQS) | E1, E2, EE2 | Proportion in waste water and surface water | Yes, 9 in vitro bioassays compared | Yes (additive effects) | 0.1 (me) |
| in vitro-in vivo comparison of 33 water samples using cyp19a1b-GFP transgenic embryos | All active chemicals in the samples | Based on empirical data | Yes, 5 in vitro bioassays compared | Yes (additive and non-additive effects) | 0.1 (me) |



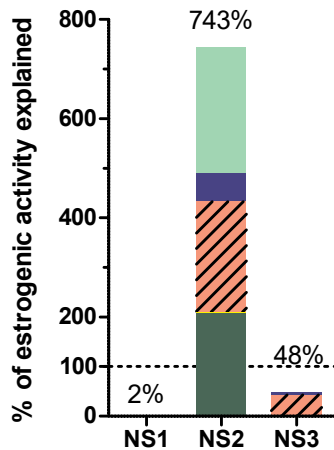
A MELN



Chem-TEQ (%):

- Bisphenol F
- Bisphenol S
- Bisphenol A
- Daidzein
- Diazinon
- Estradiol
- Estriol
- Estrone
- Genistein
- Terbutylazine

B ZELH β 2



Chem-TEQ (%):

- Bisphenol S
- Bisphenol F
- Bisphenol A
- Daidzein
- Estradiol
- Estriol
- Estrone
- Genistein

