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

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HIGHLIGHTS

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

ABSTRACT

Most *in vitro* reporter gene assays used to assess estrogenic contamination are based on human estrogen receptor α (hERα) activation. However, fish bioassays can have distinct response to estrogenic chemicals and mixtures, questioning the relevance of human-based bioassays for assessing risk to this species. In this study, zebrafish liver cells stably expressing zebrafish ERβ2 (ZELHβ2) and human breast cancer cells expressing hERα (MELN) were used to quantify the estrogenic activity of 25 surface water samples of the Danube River, for which chemicals have been previously quantified. Most samples had a low estrogenic activity below 0.1 ng/L 17β-estradiol-equivalents that was more often detected by MELN cells, while ZELHβ2 response tend to be lower than predicted based on the chemicals identified. Nevertheless, both bioassays quantified well a higher estrogenic activity at two sites, which was confirmed *in vivo* using a transgenic zebrafish assay. The results are discussed considering the effect-based trigger values proposed for water quality monitoring.

1. INTRODUCTION

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

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

Most *in vitro* bioassays used in biomonitoring are mammalian or yeast-based system expressing the human ERα (hERα), which controls the expression of a reporter gene used to quantify the biological activity. Compared to the human genome that encodes only for two ER subtypes, hERα and hERβ, most teleost fish express at least 3 ER subtypes, ERα, ERβ1 and ERβ2 (Tohyama et al. 2016, Menuet et al. 2002) that can have distinct sensitivities to estrogenic chemicals compared to human isoforms (Cosnefroy et al. 2009, Matthews et al. 2000, Pinto et al. 2014). For instance, zebrafish zfERβ subtypes are one order on magnitude more sensitive to E2 than zfERα, while the reverse is observed for human ERs (Pinto et al. 2014). Among zfER subtypes, zfERβ2 is the most sensitive and responsive one to various estrogenic substances (Cosnefroy et al., 2012). In addition to structural differences at the receptor level, the response to estrogens is influenced by the cell context, such as the presence of transcriptional co-factors or metabolic pathways, which confer a tissue-specific response (Ohtake et al. 2003). In the aquatic environment, many studies reported that fish are among the most sensitive organisms to xenoestrogens (Tyler et al. 1998, Matthiessen et al 2018) and the question of the relevance of human-based bioassays to predict an effect in fish can thus be raised (Hotchkiss et al 2008).

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

quantitative differences between human and fish-based cell lines for surface water extracts, with some samples being selectively active in zebrafish liver cells expressing zfERβ2 (ZELHβ2 cells) and in an *in vivo* zebrafish assay but not in human MELN cells (Sonavane et al. 2016). Furthermore, ZELHβ2 and MELN cells have distinct sensitivity to anti-estrogenic chemicals, which can significantly influence the response to estrogenic chemicals in a cell-specific manner (Serra et al. 2019). These results suggest the presence of fish-specific factors influencing the *in vitro* estrogenic response to environmental extracts that warrants further research.

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

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

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

2.2. Study sites, sampling and extraction procedure

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

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

2.3. In vitro bioassays

The zebrafish *in vitro* assay derived from the zebrafish liver (ZFL) cell line. ZFL cells were stably transfected first by an ERE-driven luciferase gene, yielding the ZELH cell line, and then by zfERβ2 subtype yielding the ZELHβ2 cell lines (Cosnefroy et al. 2012). The establishment of this cell model and its response to different classes of well-known xenoestrogens have been previously described (Cosnefroy et al. 2012, Serra et al. 2019, Le Fol et al. 2017, Sonavane et al. 2016). In addition, we used the human-derived MELN cell line (Balaguer et al. 1999) kindly provided by Dr. Patrick Balaguer (INSERM Montpellier, France). The MELN cells are derived from the breast cancer MCF-7 cells that endogenously express the hERα, but no functional hERβ (P. Balaguer, *personal communication*). MCF-7 cells were stably transfected with an ERE-driven firefly luciferase reporter gene to yield MELN cells.

Conditions for routine cell culture and exposure to chemicals have been detailed previously (Sonavane et al. 2016). Briefly, ZELHβ2 cells were seeded in 96-well white opaque culture plates (Greiner CellStar™, Dutscher, France) at 25,000 cells per well in phenol red free LDF-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, ZELHβ2 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 µl 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 µL
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 µL 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,

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

2.5. Data analysis

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

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

Concentration-response curves of bioassay data were fitted to all replicates pooled together with a minimum of two independent experiments (each with three technical replicates) using Hill equation in the RegTox 7.5 Microsoft Excel™ macro (freely available at 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 $$BioEEQ = \frac{EC20_{positive\ control}}{PC20_{sample}} \quad (2)$$

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 $$ChemEEQ = \sum_i^n REP_i \; x \; C_i \quad (3)$$

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

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

3. RESULTS

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

The results of the estrogenic activity of JDS and Novi Sad samples measured in MELN and ZELHβ2 bioassays are presented in Table 1, and the individual concentration-response curves are provided in supplementary information (figures SI 2 and SI 3 for MELN and ZELHβ2 cells, respectively).

Overall, the estrogenic activity measured in the JDS samples was relatively low, in both cell models. In MELN cells, 15 out of 22 JDS samples had an estrogenic activity above the LOQ (0.002 ng/L EEQ). The response measured was below 0.3 ng/L EEQ in all samples, except for JDS41 that had an estrogenic activity of 0.74 ng/L EEQ. A similar pattern was observed with the ZELHβ2 bioassay, with, however, only 8 samples being positively quantified. In all samples, the estrogenic activity measured in ZELHβ2 was below 0.08 ng/L EEQ, except for JDS41 that reached 2.3 ng/L EEQ. The comparison of both MELN and ZELHβ2 showed that 10 samples were equally detected in both cell lines (being either active or inactive), while 2

242 samples were selectively detected in ZELHβ2 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 ZELHβ2 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 ZELHβ2 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 ZELHβ2 cells was available
258 only for 36 substances, among which 7 were estrogenic on ZELHβ2 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 ZELHβ2
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 ZELHβ2 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 ZELHβ2 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 ZELHβ2 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 ZELHβ2 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).

309

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 ZELHβ2 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 ZELHβ2 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 ZELHβ2 cells. Indeed, the estrogenic activity predicted in ZELHβ2 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, ZELHβ2 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 ZELHβ2 cells. The lower response of
331 zebrafish cells might be explained by a higher sensitivity of ZELHβ2 cells over MELN cells

to non-ER chemicals present in the mixture and modulating the zfERβ2 response (Serra et al. 2019). In the highly diluted context of Danube, estrogenic compounds are at very low concentrations and in presence of a universe of other co-occurring compounds that are also caught and concentrated by LVSPE. Distinct sampling approaches and pollution patterns may account for the differences observed between the outcomes of both studies.

Despite different ability to pick up low estrogenicity levels, both bioassays quantified a higher estrogenic activity at JDS41 and at NS2. JDS41 site is located at the confluence of the Molika Morava (Serbia) and the Danube River. This site was identified as a highly contaminated site in the Danube survey (Liška et al. 2015), and had, indeed, the highest load in chemicals of all the JDS samples (Neale et al. 2015). The detected estrogenic chemicals together explained less than 30% of estrogenic activity at this site. This finding illustrates the complementarity of chemical and bioanalytical tools to assess environmental xenoestrogens, as the *in vitro* bioassays were able to detect chemicals not targeted by chemical analysis. In the Novi Sad case study, the detection of a higher estrogenic activity at NS2 site confirms that the wastewater release is a point source of environmental xenoestrogens (Könemann et al. 2018, Sonavane et al. 2018). For instance, the steroidal estrogens E1, E2 and E3 that drove the estrogenic activity of NS2 sample were well diluted few kilometres downstream of the release, as shown by both analytical and bioanalytical tools at NS3 site. Furthermore, our results are coherent with the estrogenic evaluation of the same Novi Sad samples performed using two hERα-based reporter gene bioassays (König et al. 2017). The estrogenic activity of NS2 of 1.52 ng/L EEQ measured in MELN cells was higher than the one reported in hERα-GenBLAzer (0.26 ng/L EEQ) and in BG1-Luc4E (0.67 ng/L EEQ) bioassays, although well explained by the chemicals quantified (mass balance of 115%). The tendency of MELN cells 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 ZELHβ2 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 zfERβ2
361 response to xenoestrogens. We previously showed that bisphenol A and genistein had additive
362 effects in both ZELHβ2 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 ZELHβ2 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 ERα bioassay was much more prevalent in
369 secondary wastewater effluents, while the primary wastewater effluent had much higher anti-
370 estrogenic activity on human ERα 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 ZELHβ2 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 ZELHβ2 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 zfERβ2, may have also contributed to lower the
384 estrogenic response of steroidal estrogens in ZELHβ2 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 at 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 ZELHβ2 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 ZELHβ2 cells, notably at trace levels, remains to be
434 addressed.

435

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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)** ZELHβ2 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)** ZELHβ2 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.

Table 1: Estrogenic activity of the Joint Danube Survey (JDS) and Novi Sad (NS) samples on MELN and ZELHβ2 cells. The Chem-EEQ for MELN cell line originate from Neale *et al.* (2017). The Bio-EEQ and Chem-EEQ are expressed in ng/L EEQ. The % effect indicates the contribution of identified chemicals (Chem-EEQ) to the biological response (Bio-EEQ). CI95: 95% confidence intervals, n.a.: not active, LOQ: limit of quantification.

		MELN			**ZELHβ2**		
		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%

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**	**ZELHβ2**	EASZY Assay
Estrone	0.110 [a]	0.136 [a]	0.78 [e]
Estriol	0.077[a]	0.039 [a]	0.06 [d]
Bisphenol F	1.43×10-5 [b]	5.14×10^{-6} [c]	4.00×10^{-4} [c]
Bisphenol S	1.18×10^{-6} [a]	6.69×10^{-6} [c]	7.80×10^{-6} [c]
Bisphenol A	3.50×10^{-5} [a]	3.57×10^{-6} [c]	1.44×10^{-4} [c]
Daidzein	6.50×10^{-5} [a]	9.22×10^{-6} [a]	Inactive [a]
Diazinon	2.80×10^{-7} [a]	Inactive [a]	Inactive [a]
Genistein	2.00×10^{-4} [a]	3.47×10^{-4} [a]	1.91×10^{-4} [a]
Terbutylazine	1.60×10^{-7} [a]	Inactive [a]	Inactive [a]

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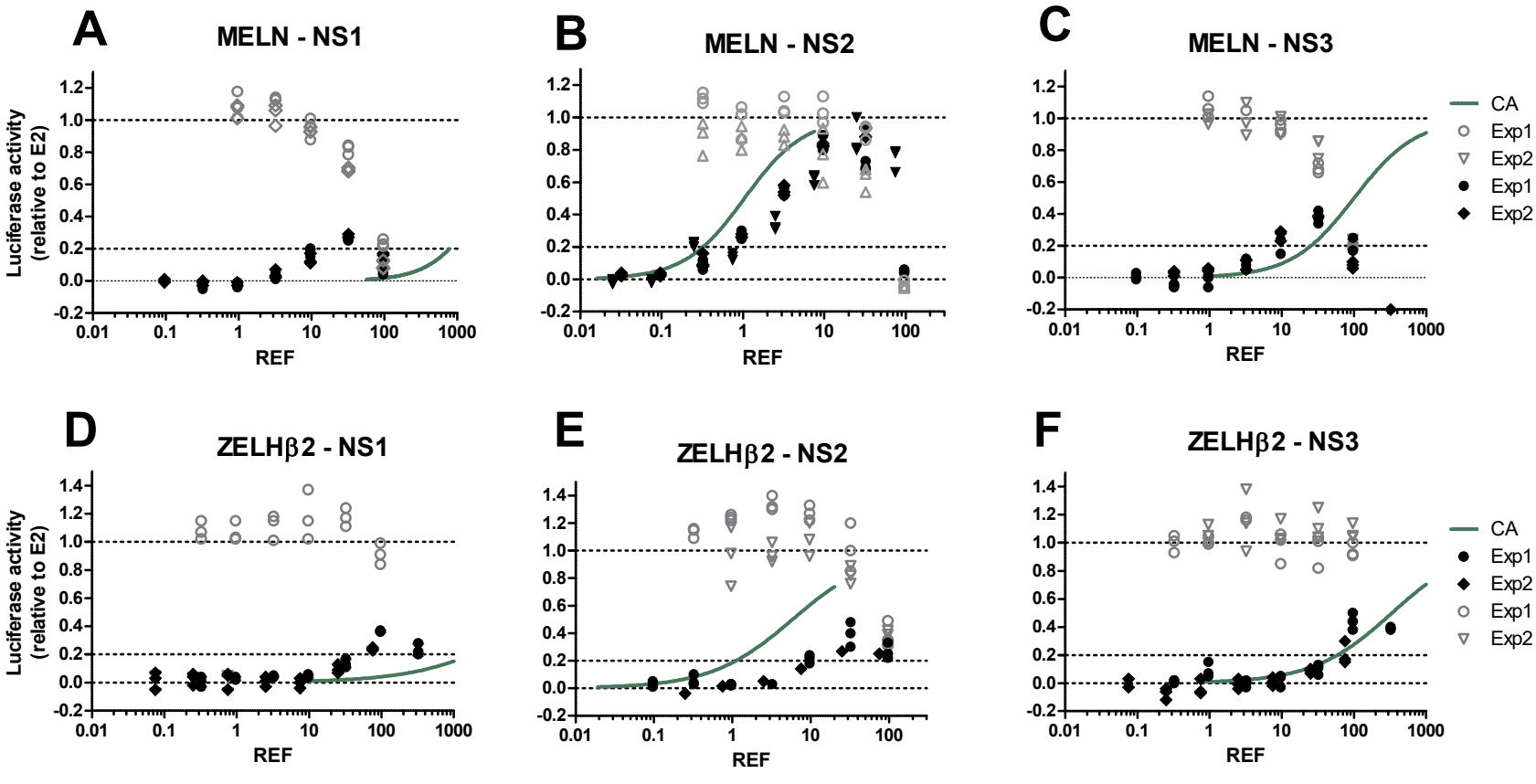
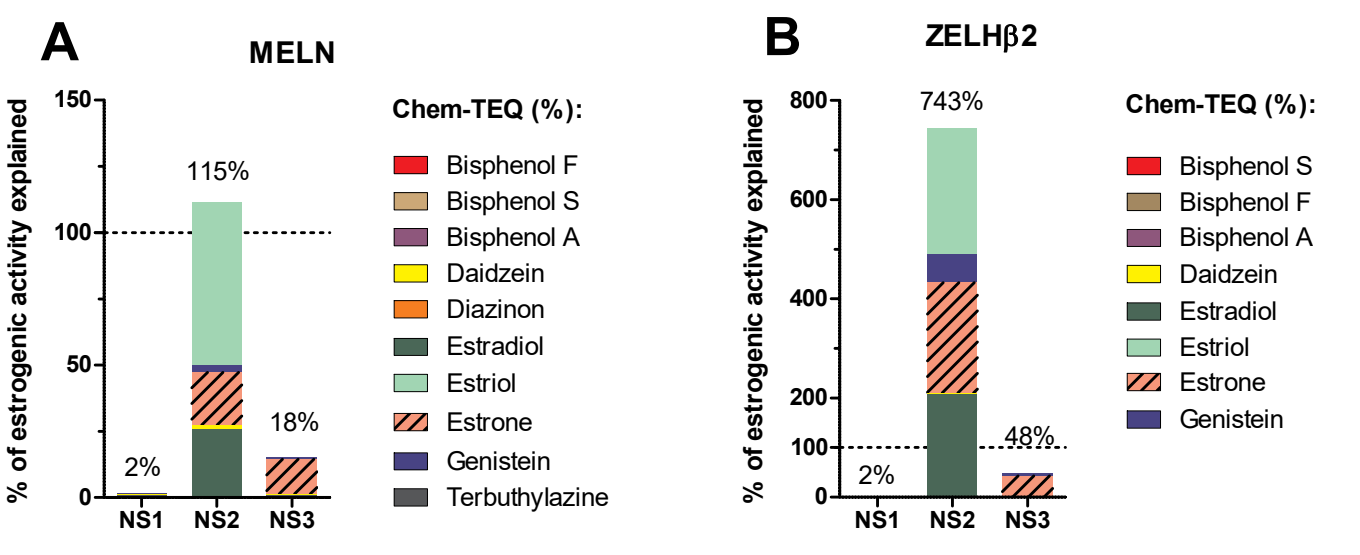
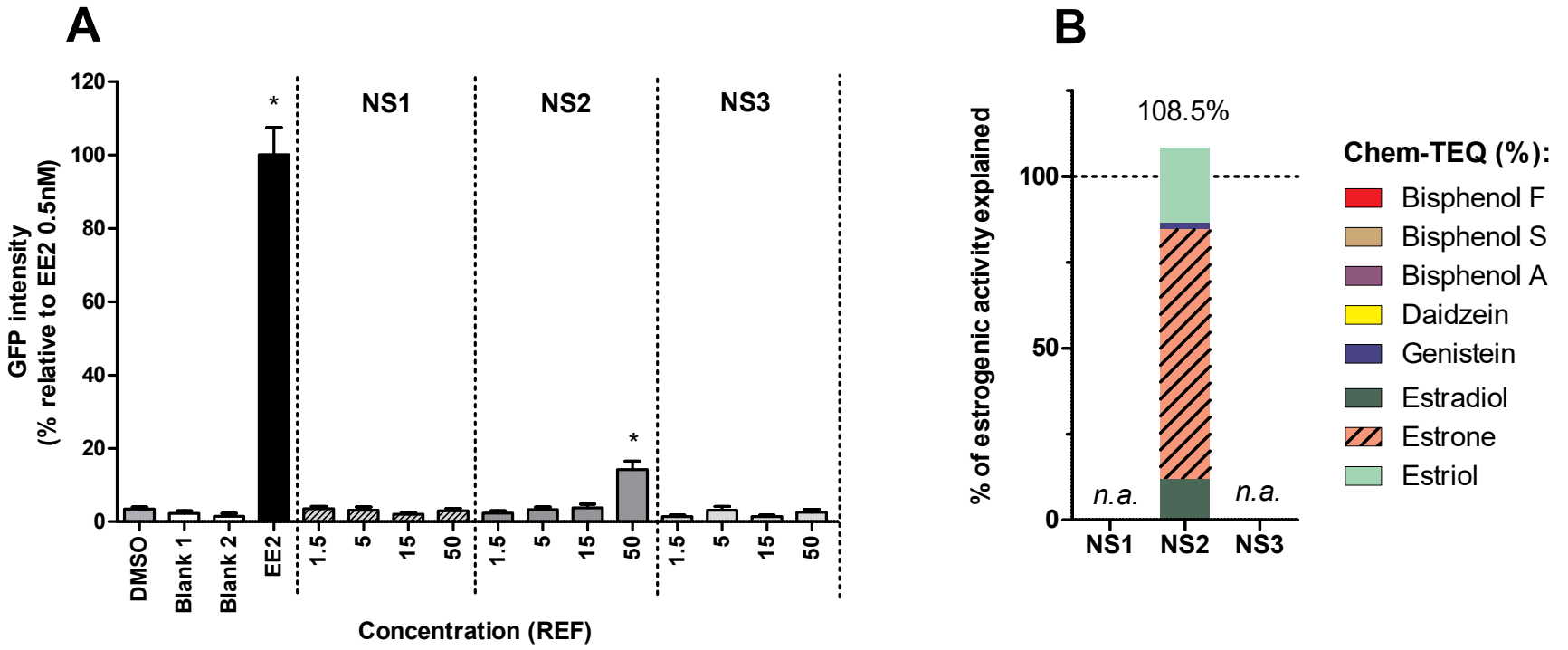
Table 3: Comparison of effect-based trigger values proposed for estrogenic activity. The characteristics of each EBT are presented with a focus on the point of departure, inclusion of bioassay-specific and mixture considerations. n.a.: not applicable, n.i.: not included, REP: relative potency, CI: confidence 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 - NS1
B
MELN - NS2
C
MELN - NS3
D
ZELHβ2 - NS1
E
ZELHβ2 - NS2
F
ZELHβ2 - NS3
Luciferase activity (relative to E2)
REF
CA
Exp1
Exp2
Exp1
Exp2
CA
Exp1
Exp2
Exp1
Exp2


A
MELN
% of estrogenic activity explained
150
100
50
0
115%
18%
2%
NS1
NS2
NS3
Chem-TEQ (%):
Bisphenol F
Bisphenol S
Bisphenol A
Daidzein
Diazinon
Estradiol
Estriol
Estrone
Genistein
Terbuthylazine
B
ZELHβ2
% of estrogenic activity explained
800
600
400
200
100
0
743%
48%
2%
NS1
NS2
NS3
Chem-TEQ (%):
Bisphenol S
Bisphenol F
Bisphenol A
Daidzein
Estradiol
Estriol
Estrone
Genistein


A
GFP intensity
(% relative to EE2 0.5nM)
120
100
80
60
40
20
0
*
*
NS1
NS2
NS3
DMSO
Blank 1
Blank 2
EE2
1.5
5
15
50
1.5
5
15
50
1.5
5
15
50
Concentration (REF)
B
% of estrogenic activity explained
108.5%
100
50
0
n.a.
n.a.
NS1
NS2
NS3
Chem-TEQ (%):
Bisphenol F
Bisphenol S
Bisphenol A
Daidzein
Genistein
Estradiol
Estrone
Estriol