

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

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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

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

Most in vitro reporter gene assays used to assess estrogenic contamination are based on 23 24 human estrogen receptor  $\alpha$  (hER $\alpha$ ) activation. However, fish bioassays can have distinct response to estrogenic chemicals and mixtures, questioning the relevance of human-based 25 bioassays for assessing risk to this species. In this study, zebrafish liver cells stably expressing 26 zebrafish ER<sup>β</sup>2 (ZELH<sup>β</sup>2) and human breast cancer cells expressing hER<sup>α</sup> (MELN) were 27 used to quantify the estrogenic activity of 25 surface water samples of the Danube River, for 28 29 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 30 ZELH $\beta$ 2 response tend to be lower than predicted based on the chemicals identified. 31 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 considering the effect-based trigger values proposed for water quality monitoring. 34

# 36 1. INTRODUCTION

The evidence that estrogenic chemicals occurring in the aquatic environment may adversely 37 38 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 39 proposed to set environmental quality standards (EQS) for estradiol (E2), estrone (E1) and 40 ethynyl estradiol (EE2) at 0.4 ng/L, 3.6 ng/L and 0.035 ng/L, respectively (European 41 Commission 2015). However, most current analytical methods are not sensitive enough to 42 quantify E2 and EE2 at these very low concentrations, below the ng/L (Kase et al. 2018). For 43 instance, the results of the European Union (EU)-wide monitoring exercise for chemicals on 44 the Watch List showed that only half of EU member states achieved to implement a method 45 46 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 47 chemicals considerably hinders their prioritization under the European Water Framework 48 Directive (WFD). 49

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 analytical tools, they have been successfully applied to quantify the estrogenic activity of 52 surface and wastewater, and they showed to have a good sensitivity to detect E1, E2 or EE2 at 53 environmental levels, highlighting their potential to support chemical monitoring (Leusch et 54 al. 2010, Könemann et al. 2018, Kunz et al. 2015). Furthermore, they enable to detect active 55 56 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 57 provide a holistic evaluation of estrogenic mixtures (Altenburger et al. 2019). 58

Most in vitro bioassays used in biomonitoring are mammalian or yeast-based system 59 60 expressing the human ER $\alpha$  (hER $\alpha$ ), 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 61 62 subtypes, hER $\alpha$  and hER $\beta$ , most teleost fish express at least 3 ER subtypes, ER $\alpha$ , ER $\beta$ 1 and  $ER\beta 2$  (Tohyama et al. 2016, Menuet et al. 2002) that can have distinct sensitivities to 63 estrogenic chemicals compared to human isoforms (Cosnefroy et al. 2009, Matthews et al. 64 2000, Pinto et al. 2014). For instance, zebrafish zfERβ subtypes are one order on magnitude 65 more sensitive to E2 than zfERa, while the reverse is observed for human ERs (Pinto et al. 66 2014). Among zfER subtypes, zfER $\beta$ 2 is the most sensitive and responsive one to various 67 estrogenic substances (Cosnefroy et al., 2012). In addition to structural differences at the 68 receptor level, the response to estrogens is influenced by the cell context, such as the presence 69 of transcriptional co-factors or metabolic pathways, which confer a tissue-specific response 70 (Ohtake et al. 2003). In the aquatic environment, many studies reported that fish are among 71 the most sensitive organisms to xenoestrogens (Tyler et al. 1998, Matthiessen et al 2018) and 72 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 with fish-based bioassays. Using wastewater samples, Ihara et al. (2015) showed that the 78 79 induction of vtg1/chgH in male medaka was better correlated with in vitro medaka ERa activation than with human ERa. Interactions between chemicals leading to co-occurrence of 80 81 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 82

quantitative differences between human and fish-based cell lines for surface water extracts, 83 with some samples being selectively active in zebrafish liver cells expressing zfER<sup>β</sup>2 84 (ZELH<sub>β2</sub> cells) and in an *in vivo* zebrafish assay but not in human MELN cells (Sonavane et 85 86 al. 2016). Furthermore, ZELHB2 and MELN cells have distinct sensitivity to anti-estrogenic chemicals, which can significantly influence the response to estrogenic chemicals in a cell-87 specific manner (Serra et al. 2019). These results suggest the presence of fish-specific factors 88 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 extracts, including the evaluation of the contribution of known estrogenic chemicals to the 93 measured activity. For that purpose, we used water extracts from the Danube River, which 94 have been previously characterized chemically and are representative of a large river with 95 high dilution capacity and highly diverse chemical contamination pattern (Liška et al. 2015). 96 97 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 98 untreated wastewater directly into the Danube River (König et al. 2017). The biological 99 100 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 samples and evaluated the *in vivo* estrogenic response in transgenic zebrafish embryos using 102 103 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 104 105 context.

#### 107 2. MATERIAL AND METHODS

# 108 2.1. Chemicals and reagents

109 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%), 110 111 dimethylsulfoxide (DMSO), Leibovitz 15 culture medium (L-15), fetal calf serum (FCS), 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), epidermal growth factor (EGF), 112 G418, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) and D-luciferin 113 were purchased from Sigma Aldrich (St-Quentin Fallavier, France). Dulbecco's Modified 114 115 Eagle Medium High Glucose (DMEM HG) powder, F-12 nutrient mixture (Ham's F12) powder, penicillin and streptomycin were purchased from Gibco (France). Insulin, 116 117 hygromycin B and sodium bicarbonate were purchased from Dominique Dutscher (France).

#### 118

# 2.2. Study sites, sampling and extraction procedure

Two sets of Danube River water samples were collected and prepared during the Joint 119 Danube Survey 3 (JDS, 22 samples) and Novi Sad (NS, 3 samples) campaigns as detailed 120 121 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 122 123 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 effluent release on the Danube River (König et al. 2017) and to identify drivers of endocrine 125 disruption including estrogenicity and androgenicity (Hashmi et al. 2018) and progestogenic 126 127 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 128 200 m downstream the effluent release, and NS3 site 7 km downstream the effluent release. In 129

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.

# 137 *2.3. In vitro bioassays*

The zebrafish in vitro assay derived from the zebrafish liver (ZFL) cell line. ZFL cells were 138 stably transfected first by an ERE-driven luciferase gene, yielding the ZELH cell line, and 139 then by  $zfER\beta2$  subtype yielding the ZELH $\beta2$  cell lines (Cosnefroy et al. 2012). The 140 establishment of this cell model and its response to different classes of well-known 141 xenoestrogens have been previously described (Cosnefroy et al. 2012, Serra et al. 2019, Le 142 143 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, 144 France). The MELN cells are derived from the breast cancer MCF-7 cells that endogenously 145 express the hER $\alpha$ , but no functional hER $\beta$  (P. Balaguer, *personal communication*). MCF-7 146 cells were stably transfected with an ERE-driven firefly luciferase reporter gene to yield 147 MELN cells. 148

149 Conditions for routine cell culture and exposure to chemicals have been detailed previously 150 (Sonavane et al. 2016). Briefly, ZELH $\beta$ 2 cells were seeded in 96-well white opaque culture 151 plates (Greiner CellStar<sup>TM</sup>, 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,

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

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# 2.4. Zebrafish embryo-based bioassay (EASZY assay)

The estrogenic activity of Novi Sad samples was assessed *in vivo* using the EASZY assay based on transgenic *cyp19a1b*-GFP zebrafish line (Brion et al. 2012). The assay procedure for 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 176 experimental group and exposed for 96 h in 15 mL of acclimated water in glass crystallizers. 177 Serial dilutions were tested with a final volume of solvent (DMSO) of 0.1% v/v, a 178 concentration without effects on embryo development or GFP expression. In each 179 180 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 181 with daily complete renewal of medium. At the end of the exposure period, each zebrafish 182 183 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 184 brain. Image analysis was performed using the ImageJ software, and fluorescence data was 185 treated exactly as previously described (Brion et al. 2012). 186

# 187 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<sub>sample</sub> the signal of the tested sample, signal<sub>control</sub> the signal of the solvent control and signal<sub>positve</sub> the signal of the positive control (E2):

193 
$$Response = \frac{signal_{sample} - signal_{control}}{signal_{positive} - signal_{control}}$$
(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<sup>TM</sup> 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 
$$Bio EEQ = \frac{EC20_{positive\ control}}{PC20_{sample}}$$
(2)

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

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

212 
$$ChemEEQ = \sum_{i}^{n} REP_i \ x \ C_i$$
(3)

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

In addition, the concentration addition (CA) model was used to predict the additive effects of
 identified estrogenic chemicals along the entire dose-response curve, to facilitate graphical
 11

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

227

#### 228 **3. RESULTS**

# 229 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 234 models. In MELN cells, 15 out of 22 JDS samples had an estrogenic activity above the LOQ 235 236 (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 237 the ZELH<sup>β</sup>2 bioassay, with, however, only 8 samples being positively quantified. In all 238 239 samples, the estrogenic activity measured in ZELH<sup>β</sup>2 was below 0.08 ng/L EEQ, except for JDS41 that reached 2.3 ng/L EEQ. The comparison of both MELN and ZELH<sup>β</sup>2 showed that 240 241 10 samples were equally detected in both cell lines (being either active or inactive), while 2 12

samples were selectively detected in ZELHβ2 cells (JDS63, JDS67) and 10 samples only in
MELN cells (e.g. JDS22, JDS27, JDS30).

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

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

# 3.2. The identified estrogenic chemicals only partially explained the fish-specific response

We further assessed the contributions of estrogenic chemicals identified in the samples to the 254 activity measured in fish and human-based bioassays. More than 100 chemicals were 255 256 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<sup>β</sup>2 cells was available 258 only for 36 substances, among which 7 were estrogenic on ZELH $\beta$ 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 in Table SI 1. These chemicals include natural steroid estrogens, bisphenols, phytoestrogens 261 262 and pesticides.

The Chem-EEQs of the 25 samples and their contribution to the biological response (Bio-263 EEQs) are presented in Table 1. Overall, the same main contributors to the estrogenic activity 264 were detected for both cell lines: estrone and genistein, confirming our previous report (Neale 265 266 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 124% of the biological response observed in MELN cells for most samples, except at JDS27 268 and JDS60 (<1%, no estrone detected), and at JDS64 (710%, very weak estrogenic response 269 270 measured). Furthermore, 5 samples were expected to have an estrogenic activity ranging from 0.18 to 0.58 ng/L EEQ in MELN cells based on the chemical quantified, but were not detected 271 by the bioassay (JDS35, JDS57, JDS59, JDS63 and JDS65), as confirmed by comparing CA 272 273 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<sup>β</sup>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<sup>β</sup>2 cells, ranging 279 from 0.022 to 0.065 ng/L EEQ, as predicted by CA model, but were not detected by the bioassay (Table 1 and Figure SI 3). 280

Because estrone is a major driver of estrogenicity in JDS samples, it may thus contribute to estrogenic activity of the extracts even if present at concentration below or at its LOQ (i.e. 0.1 ng/L). Thus, for those few sites with very low contribution of Chem-EEQ in MELN cells, namely JDS 27 and JDS60, including estrone LOQ in the mass balance calculation increases 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 compounds287 are present in the samples at these sites.

As for most JDS samples, genistein was detected in all Novi Sad samples but explained only a 288 small fraction (less than 10%) of the observed response, in both MELN and ZELHB2 cells 289 (Figure 2). In NS2 sample, the steroidal estrogens (E1, E2, E3) were the main drivers 290 291 identified. They were adequately detected in MELN cells (contribution of 88% to the biological response); however, the activity of NS2 measured in ZELH<sup>β</sup>2 cells was about 7 292 293 times lower than predicted based on Chem-EEQ (Table 1, Figure 1). To investigate possible negative interactions on the estrogenic response, we assessed the anti-estrogenic activity of 294 Novi Sad samples by co-exposing the cells to the samples with E2 at a concentration inducing 295 296 80% of E2 maximal response. As presented in Figure 1, a decrease in E2-induced luciferase activity was noted but only at the highest non-cytotoxic concentration tested. Thus, no 297 298 significant anti-estrogenic activity could be evidenced, especially regarding NS2 sample 299 extract in ZELH $\beta$ 2 cells.

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

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

Using an integrated analytical-bioanalytical method, we assessed the estrogenic activity of the 312 Danube River combining zebrafish ZELH<sup>β</sup>2 and human MELN cells. Our results show that, 313 314 with the exception of two sites, the estrogenic activity of the Danube River was overall low. The majority of samples had a Bio-EEQ below 0.1 ng/L EEQ both in MELN cells (18/25 315 316 samples) and in ZELH $\beta$ 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, levels of estrogenic activity below 0.1 ng/L EEQ were more often detected by MELN cells 321 322 than ZELH<sub>β2</sub> cells. Indeed, the estrogenic activity predicted in ZELH<sub>β2</sub> 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 activity of POCIS extracts deployed on 20 French river sites (Sonavane et al. 2016). In the 325 326 latter, ZELH<sup>β</sup>2 cells revealed an estrogenic activity at 8 sites that was not detected by MELN cells. The authors hypothesized the presence of fish-selective ER active chemicals, although 327 328 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 estrogenic drivers, are active in both MELN and ZELHB2 cells. The lower response of 330 zebrafish cells might be explained by a higher sensitivity of ZELHβ2 cells over MELN cells 331

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 337 estrogenic activity at JDS41 and at NS2. JDS41 site is located at the confluence of the Molika 338 339 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 the JDS samples (Neale et al. 2015). The detected estrogenic chemicals together explained 341 342 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 343 bioassays were able to detect chemicals not targeted by chemical analysis. In the Novi Sad 344 345 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, Sonavane et al. 2018). For instance, the steroidal estrogens E1, E2 and E3 that drove the 347 estrogenic activity of NS2 sample were well diluted few kilometres downstream of the 348 349 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 350 351 using two hERa-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 hERa-353 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 354 355 to provide higher Bio-EEQ than other reporter gene assays has been evidenced in previous 17

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

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

Further experiments using sample fractionation and biotesting of the fractions, for instance,
would be warranted to unravel the mixture effect and to evidence possible masking effects, as
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).

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

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

In vitro bioassays are sensitive and specific tools to assess the estrogenic activity of 386 environmental samples (Könemann et al. 2018). The implementation of bioassays in a 387 388 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 high risk (Escher et al. 2018). Several effect-based trigger values (EBT) have been proposed 390 for *in vitro* estrogenicity bioassays, with different protection goals and construction methods, 391 as presented in Table 3. Most of the EBT are constructed using primarily fish toxicity data. 392 393 For instance, Kase et al. (2018) and Escher et al. (2018) read across from the proposed EQS derived from chronic toxicity of E1, E2 or EE2 on fish, and Jarošová et al. (2014) used 394 published long-term PNEC for E1, E2, E3 and EE2 in fish. Brion et al. (2019) compared 395 396 empirically the in vitro activity of environmental samples with the in vivo ER-regulated cyp19a1b expression in transgenic zebrafish embryos. Alternatively, the proposed EBT for 397 ER-CALUX as part of SIMONI strategy is based on all acute and chronic data on fish, 398 399 insects, algae and crustaceans (van der Oost et al. 2017). It is worth noticing that only two approaches actually take into account xenoestrogen mixture effects, either assuming additive 400 effects and average environmental occurrence ratio (Escher et al. 2018) or assuming all 401 19

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

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

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

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

435

# 436 ACKNOWLEGMENTS

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

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640 Figure captions

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

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Figure 2: Contribution of identified xeno-estrogens to the estrogenic activity measured in
Novi Sad samples for (A) MELN and (B) ZELHβ2 cells. 100% corresponds to Bio-EEQ
measured experimentally.

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Figure 3: In vivo estrogenic activity of Novi Sad sites in transgenic zebrafish embryos using 652 653 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 positive control is 0.05 nM EE2. The mortality was below 20% in all groups. (\*) denotes a 656 657 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 ZELH $\beta$ 2 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< td=""><td>1%</td><td>n.a.</td><td>&lt; LOQ</td><td>-</td></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< td=""><td>n.a.</td><td>n.a.</td><td><loq< td=""><td>-</td></loq<></td></loq<>	n.a.	n.a.	<loq< td=""><td>-</td></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< td=""><td>0%</td><td>n.a.</td><td><loq< td=""><td>-</td></loq<></td></loq<>	0%	n.a.	<loq< td=""><td>-</td></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	LOQ	0.005	-	-	0.008	-	-
Sad	NS1	0.079 [0.073 ; 0.092]	<loq< td=""><td>2%</td><td>0.050 [0.044 ; 0.053]</td><td><loq< td=""><td>2%</td></loq<></td></loq<>	2%	0.050 [0.044 ; 0.053]	<loq< td=""><td>2%</td></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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Table 2: Relative potency (REP) of the chemicals quantified for each bioassay. The REP
values were calculated as the ratio of EC20 of E2 to that of test compound. They were derived
from initial data published by <sup>(a)</sup>Neale et al. (2017), <sup>(b)</sup>Molina-Molina et al. (2008) and
Molina-Molina et al. (2013), <sup>(c)</sup>LeFol et al. (2017), <sup>(d)</sup>Brion et al. (2012), <sup>(e)</sup>Brion et al. (2019).

Chemical	MELN	ΖΕĹΗβ2	EASZY Assay
Estrone	0.110 <sup>a</sup>	0.136 <sup>a</sup>	0.78 <sup>e</sup>
Estriol	0.077 <sup>a</sup>	0.039 <sup>a</sup>	0.06 <sup>d</sup>
Bisphenol F	1.43×10-5 <sup>b</sup>	5.14×10 <sup>-6 c</sup>	4.00×10 <sup>-4 c</sup>
Bisphenol S	1.18×10 <sup>-6 a</sup>	6.69×10 <sup>-6 c</sup>	7.80×10 <sup>-6 c</sup>
Bisphenol A	3.50×10 <sup>-5 a</sup>	3.57×10 <sup>-6 c</sup>	1.44×10 <sup>-4 c</sup>
Daidzein	6.50×10 <sup>-5 a</sup>	9.22×10 <sup>-6 a</sup>	Inactive <sup>a</sup>
Diazinon	2.80×10 <sup>-7 a</sup>	Inactive <sup>a</sup>	Inactive <sup>a</sup>
Genistein	2.00×10 <sup>-4 a</sup>	3.47×10 <sup>-4 a</sup>	1.91×10 <sup>-4 a</sup>
Terbutylazine	1.60×10 <sup>-7 a</sup>	Inactive <sup>a</sup>	Inactive <sup>a</sup>

**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. (<sup>a</sup>): 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 (m
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 (m
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 (m



![](_page_32_Figure_0.jpeg)

Che	m-TEQ (%):
	Bisphenol F
	Bisphenol S
	Bisphenol A
	Daidzein
	Diazinon
	Estradiol
	Estriol
	Estrone
	Genistein
	Terbuthylazine

![](_page_32_Figure_2.jpeg)

Chem-TEQ (%): **Bisphenol S Bisphenol F Bisphenol A** Daidzein Estradiol Estriol Estrone Genistein

![](_page_33_Figure_0.jpeg)