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

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- 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

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ABSTRACT

Most *in vitro* reporter gene assays used to assess estrogenic contamination are based on human estrogen receptor α (hER α) 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 ER β 2 (ZELH β 2) and human breast cancer cells expressing hER α (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 17 β -estradiol-equivalents that was more often detected by MELN cells, while ZELH β 2 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, Könnemann 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 ER α (hER α), 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, hER α and hER β , most teleost fish express at least 3 ER subtypes, ER α , ER β 1 and ER β 2 (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 zfER β subtypes are one order on magnitude more sensitive to E2 than zfER α , while the reverse is observed for human ERs (Pinto et al. 2014). Among zfER subtypes, zfER β 2 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 ER α activation than with human ER α . 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 zfER β 2 (ZELH β 2 cells) and in an *in vivo* zebrafish assay but not in human MELN cells (Sonavane et al. 2016). Furthermore, ZELH β 2 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

17 β -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 zfER β 2 subtype yielding the ZELH β 2 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 hER α , but no functional hER β (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, ZELH β 2 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,

0.15 g/L sodium bicarbonate, 0.01 mg/mL insulin, 50 ng/mL EGF, 50 U/mL penicillin and streptomycin antibiotics, 5% v/v stripped FCS). MELN were seeded at 80,000 cells per well in steroid- and phenol red-free DMEM medium. Cells were left to adhere for 24 h, and then exposed for either 72 h at 28 °C for zebrafish cells or 16h at 37°C for MELN cells. Cells were 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 sites, ZELH β 2 and MELN cells were co-exposed to the sample in presence of 0.1 nM E2, i.e. 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 exposure, culture medium was removed and replaced by 50 μ L per well of medium containing 0.3 mM D-luciferin. The luminescence signal was measured in living cells using a microtiter plate luminometer (Synergy H4, BioTek). The effect of samples on cell viability was assessed 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 μ L 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 100 μ L of DMSO after removal of MTT containing medium. Plates were read at 570 nm against a 640 nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France) and results are expressed as absorbance relative to control cells.

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 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 $signal_{positive}$ 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

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

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

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):

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

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

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 ZELH β 2 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 ZELH β 2 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 ZELH β 2 bioassay, with, however, only 8 samples being positively quantified. In all samples, the estrogenic activity measured in ZELH β 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 β 2 showed that 10 samples were equally detected in both cell lines (being either active or inactive), while 2

samples were selectively detected in ZELH β 2 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 β 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 β 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 activity measured in fish and human-based bioassays. More than 100 chemicals were quantified in JDS and Novi Sad samples (König et al. 2017, Neale et al. 2015), and information about the activity of the chemicals on MELN and ZELH β 2 cells was available only for 36 substances, among which 7 were estrogenic on ZELH β 2 cells and 9 on MELN cells. The Relative Potency (REP) of these xenoestrogens for each cell line are indicated in 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 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

Chem-EEQs remain below Bio-EEQ and suggest that other non-detected active compounds 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 small fraction (less than 10%) of the observed response, in both MELN and ZELH β 2 cells (Figure 2). In NS2 sample, the steroidal estrogens (E1, E2, E3) were the main drivers identified. They were adequately detected in MELN cells (contribution of 88% to the biological response); however, the activity of NS2 measured in ZELH β 2 cells was about 7 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 Novi Sad samples by co-exposing the cells to the samples with E2 at a concentration inducing 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 significant anti-estrogenic activity could be evidenced, especially regarding NS2 sample extract in ZELH β 2 cells.

3.3. In vivo estrogenic activity of Novi Sad samples

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

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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

to non-ER chemicals present in the mixture and modulating the zfER β 2 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 hER α -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 hER α -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

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

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

Finally, the ZELH β 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 β 2, may have also contributed to lower the estrogenic response of steroidal estrogens in ZELH β 2 cells.

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 environmental samples (Könemann et al. 2018). The implementation of bioassays in a regulatory context, such as the WFD, relies upon the derivation of a biological threshold of 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 for *in vitro* estrogenicity bioassays, with different protection goals and construction methods, as presented in Table 3. Most of the EBT are constructed using primarily fish toxicity data. 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 published long-term PNEC for E1, E2, E3 and EE2 in fish. Brion et al. (2019) compared 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 ER-CALUX as part of SIMONI strategy is based on all acute and chronic data on fish, 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 effects and average environmental occurrence ratio (Escher et al. 2018) or assuming all

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 to 0.56 ng/L EEQ (Table 3). In the current study, only JDS41 (0.74 ng/L EEQ) and NS2 (1.52 ng/L EEQ) samples exceeded the EBT, including the highest one of 0.56 ng/L EEQ. Thus, the application of these thresholds for MELN cells would have enabled to identify JDS41 and NS2 as polluted sites. The risk quotient, defined as the sum of ratios of measured concentrations of E1, E2 and EE2 to their respective EQS, reaches 0.95 for NS2 sample, as 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 0.196 ng/L, below the EQS level proposed of 3.6 ng/L (risk quotient of 0.05). Thus, JDS41 site would have not been prioritised based only on the steroidal estrogens identified, as proposed in the WFD.

It is noticeable that *in vivo* estrogenic activity at the three NS sites was correctly predicted by the *in vitro* bioassays in the present study. Since the EBT value of 0.56 ng/L was built based on a comparison of MELN and EASZY data (Brion et al. 2019), the present dataset confirms 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 sample at the organism level. It showed that the estrogenic pollutants could target the ER and induce the expression of the brain aromatase in the developing zebrafish, hence adding further toxicological relevance to the environmental diagnosis.

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

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Figure captions

Figure 1: Estrogenic and anti-estrogenic activities of Novi Sad samples on (A) MELN and (B) ZELH β 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).

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

Figure 3: In vivo estrogenic activity of Novi Sad sites in transgenic zebrafish embryos using the EASZY assay. (A) *In vivo* induction of GFP in 4-days old zebrafish embryos (results of one experiment with n=15 embryos per condition, representative of two independent 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 statistically significant induction in GFP intensity compared with the DMSO control group (Mann-Whitney test, α : 5%). (B) Contribution of identified xeno-estrogens to *in vivo* estrogenic activity of Novi Sad samples. 100% corresponds to Bio-EEQ measured experimentally. n.a.: no activity.

Table 1: Estrogenic activity of the Joint Danube Survey (JDS) and Novi Sad (NS) samples on MELN and ZELH β 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				ZELH β 2		
		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	ZELH β 2	EASZY Assay
Estrone	0.110 ^a	0.136 ^a	0.78 ^c
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 (%):

B

ZELHβ2

Chem-TEQ (%):