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M. Sonavane et al.

Effect-based analysis of WWTP impact on a river stream

**An Integrative Approach Combining Passive Sampling, Bioassays and Effect-Directed  
Analysis to Assess the Impact of Wastewater Effluent**

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**Abstract:** Wastewater treatment plant (WWTP) effluents are major sources of endocrine disrupting chemicals (EDCs) and of other chemicals of toxicological concern for the aquatic environment. In this study, we used an integrated strategy combining passive sampling (Chemcatcher®), developmental toxicity and mechanism-based *in vitro* and *in vivo* bioassays to monitor the impacts of a WWTP on a river. *In vitro* screening revealed the WWTP effluent as a source of estrogen (ER), glucocorticoid (GR), aryl hydrocarbon (AhR) receptor mediated activities, impacting the downstream river site where significant activities were also measured, albeit to a lesser extent than in the effluent. Effect-directed analysis (EDA) of the effluent successfully identified the presence of potent estrogens (estrone, 17 $\alpha$ -ethinylestradiol and 17 $\beta$ -estradiol) and glucocorticoids (clobetasol propionate and fluticasone propionate) as the major contributors to the observed *in vitro* activities, even though other unidentified active chemicals were likely present. The impact of the WWTP was also assessed using zebrafish embryo assays, highlighting its ability to induce estrogenic response through up-regulation of aromatase promoter-dependent reporter gene in the transgenic (cyp19a1b-GFP) zebrafish assay and to generate teratogenic effects at non-lethal concentrations in the zebrafish embryo toxicity test. This study argues for the use of such an integrated approach, combining passive sampling, bioassays and EDA, to comprehensively identify endocrine active compounds and associated hazards of WWTP effluents. This article is protected by copyright. All rights reserved

**Keywords:** WWTP effluent, Passive sampling, Endocrine disrupting chemicals (EDCs), Embryo toxicity, Effect-directed analysis (EDA), LC-MS/MS analysis

## INTRODUCTION

Wastewater treatment plant (WWTP) effluents are major sources of chemicals that contaminate surface waters and pose risks for exposed aquatic organisms. Among them, endocrine disrupting chemicals (EDCs) are of high (eco)toxicological concern due to their ability to interact with the functioning of hormonal systems and alter crucial physiological processes, such as development and reproduction in humans and wildlife (Hotchkiss et al. 2008). WWTP effluents are well identified sources of estrogenic EDCs in the aquatic environment (Desbrow et al. 1998; Aerni et al. 2004; Jarosova et al. 2014) and have been associated with the impairment of reproduction and sexual differentiation in wild fish (Jobling et al. 1998; Sanchez et al. 2011; Lange et al. 2012). In addition to estrogenic compounds, other endocrine activities such as androgenic, glucocorticoid or progestagenic activities have been reported in effluents (Chang et al. 2007; Van der Linden et al. 2008; Schriks et al. 2010; Creusot et al. 2014). However, the diversity of molecules with multiple endocrine activities, together with a lack of information on their effects on aquatic species, complicate the assessment of their environmental impact. There is therefore a need to identify and monitor different EDCs and their combined effects at WWTP effluent discharge sites for a better ecotoxicological assessment of water bodies.

Assessing water chemical contamination and associated hazards for aquatic systems is challenging because chemicals occur as complex mixtures and undergo biotic or abiotic transformations, therefore making prediction of their mixture effects difficult (Dévier et al. 2011). For a comprehensive assessment, integrated strategies should be developed, based on the combination of relevant sampling with chemical and biological analyses (Altenburger et al. 2015).

Proper sampling devices are necessary to gain a representative picture of water contaminants. Ideal sampling devices will be applicable for a wide range of polarities and



concentrations, since the characteristics of individual compounds can vary substantially (Vermeirssen et al. 2006). Grab or short-term composite samples provide single time points or an averaged short time period only, thereby potentially overlooking compounds with sporadic discharge patterns. Additionally, grab or composite samples often need to be enriched after sampling in order to ensure the detection of compounds present at low concentrations. Passive sampling has become a promising alternative for obtaining a time-integrated evaluation of the bioavailable contaminants in river waters and to realistically assess exposure of aquatic organisms (Vrana et al. 2005; Vermeirssen et al. 2005). Several studies have highlighted the usefulness of passive sampling for the biomonitoring of EDCs in surface waters using *in vitro* bioassays (Liscio et al. 2009, 2014; Tapie et al. 2011; Creusot et al. 2014).

Effect-based tools have been proposed in water quality assessment as they are considered integrative techniques for the detection of bioactive compounds (Eggen et al. 2004; Altenburger et al. 2015). A variety of *in vitro* receptor-based assays currently exists for the assessment of EDCs and many studies have reported their effectiveness for water quality assessment., e.g. for monitoring the endocrine disrupting potential of WWTP effluents (e.g. Schriks et al. 2010; Creusot et al. 2014; Macikova et al. 2014; Jia et al. 2016). Recently, mechanism-based *in vivo* assays have also been developed using transgenic fish embryos that allow sensitive detection of EDC within a living organism (Brion et al. 2012; Gorelick et al. 2014). Various studies have reported their relevance as bioanalytical tools to assess estrogenic activity of sediment and water samples (Fetter et al. 2014; Sonavane et al. 2016).

In the present study, we aimed to implement an integrated strategy combining passive sampling and bioanalytical tools for characterizing the impact of a WWTP effluent discharge on the chemical quality and associated hazard in receiving streams. The studied site was located on the River Urtenen in Switzerland where the occurrence of EDCs and other contaminants using grab sampling has been reported (Tousova et al. 2017). This case-study

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site was selected as being representative of small streams impacted by WWTP effluents. The site has no wastewater discharge upstream of the WWTP, while total flow of the downstream consists of at least 20% of WWTP effluent in dry period. Passive sampling (Chemcatcher®) was performed upstream, downstream and at the effluent outlet. We first performed *in vitro* screening using reporter gene assays based on a suite of reporter gene assays including for steroid receptors and the AhR to establish the endocrine disrupting profile of the investigated WWTP. Then, the most active extracts (WWTP effluent) were fractionated in order to isolate the active chemicals and to identify them by liquid chromatography tandem mass spectrometry (LC-MS/MS). Finally, *in vivo* assays using the zebrafish embryo as a model were used to better predict the potential impact on aquatic species.

## MATERIALS AND METHODS

### *Chemicals and reagents*

All standard chemicals and reagents used in this study are listed in the Supplementary Information.

### *Study sites, sampling and extraction procedures*

The River Urtenen is a tributary of the River Emme in Switzerland, which eventually flows into the River Rhine. The WWTP Moossee-Urtenenbach discharges the treated wastewater of approximately 32,000 inhabitants into the River Urtenen. It is equipped with conventional activated sludge treatment, including anoxic and oxic zones, followed by a secondary clarifier (<http://www.ara-moossee.ch/>). Downstream of the point of discharge the river contains up to 40% treated wastewater. Three sites were sampled between September and October 2014, one located 200 m upstream of the WWTP, one 300 m downstream of the WWTP, and one directly at the outflow of the effluent pipe of the WWTP.

The occurrence of EDCs originating from the WWTP was investigated using Chemcatcher® SDB passive samplers. Complete procedures for conditioning, deployment,

and extraction of the SDB passive samplers have been previously described in Vermeirssen et al. (2013) and Moschet et al. (2015). Briefly, Empore™ SDB-RPS discs were conditioned on a rotary shaker, first in methanol for 30 min and then in nanopure water for 30 min. After conditioning, discs were transferred to stainless steel holders and stored in nanopure water until deployment. At each sampling location, ten SDB discs were placed perpendicularly to the flow direction for a period of 4 weeks. During collection, SDB discs were removed from the stainless steel holders, large particulate material was removed and discs were transferred to vials filled with 6 mL acetone. Each disc was extracted with 6 mL acetone followed by 6 mL methanol. The acetone fraction was dried to approximately 1 mL and then combined with the methanol fraction. The ten extracts per site were pooled and aliquoted for biological and chemical analyses. All aliquots were evaporated to dryness under nitrogen prior to transport. Experiments were carried out under strict protective laboratory conditions that minimize worker exposure to toxic solvents, such as methanol.

#### *In vitro assays for EDCs detection*

We used a panel of established *in vitro* reporter gene cell lines that allows the detection of estrogenic (MELN, Balaguer et al. (1999); ZELH-zfER $\alpha$  and ZELH-zfER $\beta$ 2, Cosnefroy et al. (2012)), androgenic (MDA-kb2, Wilson et al. (2002)), glucocorticoid (GR-CALUX®, Van der Linden et al. (2008)) and dioxin-like activity (PLHC-1, Louiz et al. (2008)). Conditions of routine culture and exposure of these cell lines have been described in detail previously (Cosnefroy et al. 2012; Creusot et al. 2014; Macikova et al. 2014). The cells were exposed in 96-well microplates to serial dilutions of sample extracts in DMSO, solvent controls (DMSO 0.5% v/v) and reference compounds (positive control). In each individual assay, three technical replicates per concentration were performed. All data was obtained in at least two independent experiments carried out on separate days. In the PLHC-1 assay, the EROD (7-ethoxyresorufin-O-deethylase) activity was used as a marker of AhR activation,



after cell exposure for 4 h (PAH-like activity) and 24 h (dioxin-like activity), as previously described (Louiz et al. 2008). For active samples in the MDA-kb2 assay, which expresses both AR and GR, cells were further co-exposed with sample extracts and RU846 (a GR antagonist) or flutamide (an AR antagonist) to inform which receptor pathway was involved in luciferase activation, *i.e.*, glucocorticoid and/or androgenic activity.

#### *Zebrafish embryo-based in vivo assays*

Zebrafish maintenance and breeding has been carried out as detailed previously (Brion et al. 2012). Fertilized eggs were selected at 0 dpf (days post-fertilization) and exposed to environmental extracts for up to 4 d. The assays based on zebrafish embryos used in this study are not subjected to animal experiment according to the European Directive 2010/63/EU and are to be considered as alternative methods for animal experiments.

The zebrafish embryo toxicity (FET) assay was performed according to OECD guideline 236 (OECD, 2013), using a wild-type zebrafish strain. In brief, freshly fertilized zebrafish embryos were exposed in 24-well plates, with one embryo per well in 1 mL of test media. In each plate, 20 embryos exposed to either solvent control (DMSO; 0.1% v/v), positive control (3,4-dichloroaniline (DCA); 4 mg/L), Chemcatcher® blank extract (0.1% v/v) or sample extract (serial dilutions), and 4 embryos were used as internal plate control. Exposures were performed from 0 dpf to 4 dpf in an incubator at 28°C with a light/dark cycle of 14 h/10 h, under semi-static conditions (50% renewal). Observations for lethal and sub-lethal effects were recorded every 24 h after the start of exposure, until the end of the test at 96 h. The observations used to determine lethality included coagulation of embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat (< 1 beat/ min). The different criteria used to determine sub-lethal effects are listed in **Table S2**; they include alteration of blood circulation and of heart rate, edema occurrence, morphological malformations, lack of hatching, as previously recommended (Frayssé et al. 2006).

At the end of the FET assay, EROD activity was measured in exposed zebrafish to assess *in vivo* response to dioxin-like compounds. For this purpose, each individual living zebrafish larva was placed into a well of white 96-well microplates containing 200  $\mu$ L of water supplemented with 5  $\mu$ M 7-ethoxyresorufin (7-ERF). Kinetics of resorufin production was directly measured in living zebrafish for 20 h with a spectrofluorophotometer at 27°C (Saphire II, Tecan, Switzerland). EROD activity of a sample (fluorescent units per minute) was normalized to the EROD activity induced by the positive control (TCDD, 0.1 nM) included in the plate.

*In vivo* effect of organic extracts on ER-regulated brain aromatase was assessed by using the transgenic cyp19a1b-GFP zebrafish line (Brion et al. 2012, Sonavane et al. 2016). Briefly, each experimental group consisted of 20 embryos exposed for 96 h in 15 mL of acclimated water in glass crystallizing dishes, with daily renewal of 50% of spiked water and removal of any dead embryos. In each experimental series, positive control (E2) and solvent control (DMSO, 0.1% v/v) were included as separate experimental groups. After the end of the exposure period, live larvae were individually observed in dorsal view and the brain was photographed to measure GFP expression using a Zeiss AxioImager.Z1 fluorescence microscope equipped with an AxioCam Mrm camera (Zeiss, GmbH, Germany). Details of the parameters used and image analysis performed have been explained previously (Brion et al. 2012).

#### *Biological and chemical toxic equivalents calculation*

In all bioassays, the concentration unit of the sample extracts was expressed as relative enrichment factor (REF), which considers both the volume of water enriched by the passive sampler and sample processing and dilution in the bioassays. Dry extracts of SDB discs were reconstituted in DMSO and diluted in test media to reach final REFs from 0.1 to 100, *i.e.* the tested range included the original water concentration (REF = 1) and up to 100-times

concentrated levels (REF = 100). Water enrichment factor by passive sampling was estimated as a median sampling rate ( $R_s$ ) of 0.1 L/d, as proposed by Moschet et al. (2015) for semi-polar compounds such as estrogens and glucocorticoids. We used this sampling rate as a worst-case scenario; higher sampling rates can be expected since the SDB disc was deployed without a membrane, although for some compounds the integrative phase most probably was exceeded within the four-week deployment period.

*In vitro* and *in vivo* dose-response curves were modeled with the Hill equation model using the Excel Macro Regtox (freely available at <http://www.normalesup.org/~vindimian/>). This allows calculation of the 20% effect concentrations ( $EC_{20}$ ) of the tested samples by fixing the slope, the minimum and maximum effect values to that of the reference compound (e.g. E2 for MELN assay) (Villeneuve et al. 2002). Bioassay-derived toxic equivalents (Bio-TEQs) for hormone-like or dioxin-like activities were determined as the ratio of the  $EC_{20}$  of the reference chemical, expressed as g/L, to that of the environmental sample expressed as REF. Toxic equivalents derived from chemical analyses (Chem-TEQs) were defined as  $\text{Chem-TEQ} = \sum (C_i \times \text{REP}_i)$ , where, for a given compound  $i$ ,  $C$  is the measured concentration in a sample and REP is its effective potency relative to the reference compound in a given bioassay.

#### *Sample extract fractionation*

In order to isolate the compounds responsible for the observed estrogenic and glucocorticoid activities, the effluent extract was fractionated using high performance liquid chromatography (HPLC). This was achieved on a reverse phase C18 column (Poursuit C18, 5 $\mu$ m, 250 X 4.6 I.D, Varian, France) that has been previously calibrated for the isolation of chemicals covering a broad range of polarities (Creusot et al. 2013). Briefly, the run was performed at 25°C with a flow rate of 1 mL/min using a water/ACN gradient: 0-10 min (80/20), 10–60 min (80/20 to 55/45), 60–100 min (55/45 to 0/100), 100-120 min (0/100),

120.01-125 min (80/20). The collected fractions (40 fractions per run) were dried using an EZ-2 evaporator system and then redissolved in 200  $\mu$ l of ACN for storage until biological and chemical analyses.

#### *Targeted chemical analyses*

Targeted chemical analyses for estrogens and glucocorticoids were carried out for the active fractions using LC-MS/MS as described previously (Liscio et al. 2009; Ammann et al. 2014). The active fractions were received in aliquots of approximately 28  $\mu$ L ACN. Upon arrival, they were dried and reconstituted in 120  $\mu$ L with MeOH. From this, 30  $\mu$ L was used for the targeted estrogenic analysis and 30  $\mu$ L for the targeted glucocorticoid analysis. For the glucocorticoid analysis, aliquots were evaporated down to approximately 13  $\mu$ L under a gentle stream of nitrogen, and 27  $\mu$ L nanopure water (NP) was added to each aliquot. Then 10  $\mu$ L were injected from this mixture and analyzed by monitoring 18 GCs as described in Ammann et al. 2014.

Targeted chemical analysis for the following three estrogenic compounds was carried out on the fractionated aliquots of the effluent sample: E2, EE2 and E1. Internal standards (20 ng absolute) were added to each 30  $\mu$ L fraction, along with 125  $\mu$ L 25% ammonia. The fractions were dried, after which 25  $\mu$ L ethanol and 100  $\mu$ L NP was added, for a final volume of 125  $\mu$ L.

RP-LC-MS/MS was carried out on an Agilent 1290 II HPLC system, coupled to an Agilent 6495 Triple Quad MS/MS in multiple reaction monitoring mode. LC separation was done on 50  $\mu$ L injected sample with a Waters XBridge BEH C18 column (2.5  $\mu$ m particle size, 2.1 x 75 mm), using a water/ACN gradient at room temperature: 0 min (90/10), 0.1 min (70/30), 0.1-2.1 min (50/50), 2.1-3.0 min (5/95). MS/MS spectra were acquired in negative electrospray ionization mode. Two transitions were monitored for each compound, with the



more intense fragment used for quantification. Quantification was done with internal standard calibration.

#### *Quality control*

Blanks were prepared at each step for quality control. Prior to sampling, a blank test was conducted with Chemcatcher® SDB discs and extracts analyzed with relevant bioassays. At the study sites, a field blank using the Chemcatcher® SDB samplers was produced. For biotesting, solvent blanks were prepared and run in parallel with samples. A fractionation blank was also prepared to identify any possible contamination during fractionation. All the tested blanks were found to be negative in the bioassays. Solvent blanks were also included in targeted chemical analyses to account for contamination or carry-over during measurement.

#### *Statistics*

Significant effect in GFP intensity in the *in vivo* cyp19a1b-GFP assay were determined by using the non-parametric Mann-Whitney test with  $\alpha$  set at 5%.

## **RESULTS AND DISCUSSION**

### *In vitro toxicological profiling: multiple endocrine activities are present*

*In vitro* screening of the Urtenen River sites revealed the WWTP effluent discharge site as a source of estrogenic, glucocorticoid and dioxin-like activities. These activities were the highest at the effluent discharge point and observed to a lesser extent at downstream and upstream sites (**Table 1, Figure S1**). Overall, *in vitro* bioactivity measured at the downstream site corresponded to 6% (MELN) and to 21% (GR-CALUX) of the concentration measured in the effluent, which fits well with the low dilution of the effluent in the stream (20-50% during the sampling period).

For *in vitro* estrogenic activity, E2-EQ values were measured in the hER $\alpha$  (human) and zfER $\beta$ 2 (zebrafish) assays at all three sites (Figures S1A-B). It is noteworthy that very similar E2-EQ values were obtained by the two bioassays, *i.e.* 0.1 and 0.2 ng E2-EQ/L for



upstream and downstream sites, respectively, and 0.5 ng E2-EQ/L for effluent site. The lack of species-specific estrogenic response suggests that both assays detected the same active compounds in the samples with similar sensitivity, as they do for steroidal estrogens (Cosnefroy et al. 2012; Pinto et al. 2014). Interestingly, no activity was detected at the three sampling points by the ZELH-zfER $\alpha$  assay, which is in line with the reported lower sensitivity of this assay to steroidal estrogens (Cosnefroy et al. 2012) and to environmental samples (Sonavane et al. 2016) as compared to MELN or ZELH-zfER $\beta$ 2 bioassays.

In regard to the MDA-kb2 assay, significant agonist response was detected both at the effluent and downstream sites, while no activity was detected upstream (**Figure S1C**). Co-exposure with RU486, a GR antagonist, almost completely blocked the sample-induced luciferase activity, showing the involvement of GR in the bioassay response. Conversely, co-exposure with flutamide (an AR antagonist) was ineffective at reversing the response induced by the samples. AR-active compounds were not detected by the bioassay, either because they were not present at bioactive concentrations in the sample and/or their activity on the AR was masked by high loads of glucocorticoids in the cells. Indeed, the presence of glucocorticoid activity was further confirmed using the GR-CALUX<sup>®</sup> assay, with higher levels of DEX-EQs (**Table 1**) being detected in the effluent extract (46 ng DEX-EQ/L). It is worth noting that the glucocorticoid activity quantified as DEX-EQs was 10-12 fold higher in the MDA-kb2 than the GR-CALUX<sup>®</sup> assay (Table 1). Such differences may be due to different sensitivities of the assays towards different GR ligands or, as mentioned above, to transcriptional interference between AR and GR signaling pathways in MDA-kb2 cells (Wilson et al. 2002). However, the measured DEX-EQ levels were in the same range as data reported in other field studies using GR-CALUX<sup>®</sup> (Macikova et al. 2014; Van der Linden et al. 2008) or MDA-kb2 cells (Creusot et al. 2014).

Finally, AhR-mediated activity was also detected at all three sites using the EROD assay in the PLHC-1 cell line, with higher activity observed in the effluent than at the other sites (**Table 1**). The higher EROD response after 4 h exposure than after 24 h suggests that the samples contained AhR ligands such as polycyclic aromatic hydrocarbons that, unlike halogenated aromatic hydrocarbons, are rapidly metabolized by the cells and induce transient EROD response (Louiz et al. 2008) (**Figures S1E and S1F**). Although the effluent was a source of BaP-EQs, the significant levels measured at the upstream site suggest different origins for these AhR active contaminants. The detection of low levels of mid- to non-polar dioxin-like compounds in the water-soluble phase using passive sampling, while surprising, confirms previous reports in other river studies (Tapie et al. 2011; Creusot et al. 2013b).

#### *Identification of endocrine-active compounds through EDA*

*In vitro* activity profiles in the effluent and to a lesser extent in the downstream site confirmed the effluent as a source of biologically active contaminants. To isolate the chemicals responsible for detected activities, the effluent extract was fractionated using RP-HPLC to yield 40 fractions of decreasing polarity that were tested *in vitro*. The resulting HPLC profiles showed well separated estrogenic and glucocorticoid active fractions (**Figure 1**).

Similar estrogenic HPLC patterns were observed with the MELN (human) and ZELH-zfER $\beta$ 2 (zebrafish) *in vitro* assays (**Figure 1**), which corroborates the bioassay results of the raw extracts (**Table 1**). Two sets of active fractions were observed in both assays, with highest activities detected in fractions F14-F17 and F19-F21 (**Figure 1**), with marginal estrogenic activity in surrounding fractions, *i.e.*, F7 to F13 and F22. These two areas of high activity correspond to elution times of standard E2 (F16) and EE2 (F19) (Creusot et al. 2013a); the presence of steroidal estrogens was further investigated in the active fractions by LC-MS/MS. As seen in **Table 2**, EE2 and E1 were detected in the most active fraction F19.

Low levels of E1 were also present in F20, likely due to some carryover. E2 was also detected in multiple fractions, namely F16-F18, with the highest concentration in F16, which is in line with the standard calibration of the fractionation method (Creusot et al. 2013a). Comparison of chemical and biological data using mass-balance analysis showed that the biological activity in MELN cells was rather well explained by E2 in F16 and F17, and EE2 and E1 in F19, whereas E1 contributed only for a small part of the activity in F20 (Table 2). Overall, it cannot be excluded that other active chemicals such as bisphenol A and estrogenic alkylphenols co-eluted in these fractions which have been previously reported at this river site (Tousova et al. 2017). In ZELH-zfER $\beta$ 2 cells, as in MELN cells, steroidal estrogens also explained ER activity in F16, F17 and F19, while 90% of ER activity in F20 was likely due to other unknown compounds (Table 2).

For glucocorticoid activity, both MDA-kb2 and GR-CALUX<sup>®</sup> assays detected strong activity in F25, and two other less active fractions (F23 and F26) (**Figure 1**). These activities demonstrate good agreement between these two bioassays to detect GR-active compounds. Target chemical analysis of GR-active fractions revealed the presence of different compound classes, of which some contributed to biological activity (**Table 2**). In the most active fraction (F25), we successfully identified clobetasol propionate and fluticasone propionate accounting for up to 87 % of the total glucocorticoid activity in the GR-CALUX<sup>®</sup>. Some other chemicals were quantified in the fraction 26 but were inactive in the bioassay, *i.e.* pravastatine,  $\beta$ -naphthoflavone, and glycyrrhetic acid (**Table 2**). Other potent glucocorticoids, dexamethasone and prednisolone, which elute in fractions F10 and F13, respectively (Creusot et al. 2013), were not present at biologically active concentrations in these samples, although they have been reported previously in other Swiss rivers (Macikova et al. 2014).

Additionally, some weakly active fractions were found with the MDA-kb2 assay (*i.e.* F14, F18 and F19) which showed no activity in the GR-CALUX<sup>®</sup> assay. Since MDA-kb2

cells (but not GR-CALUX®) express both GR and AR, it is likely that the observed agonist activity in these fractions was due to the presence of androgenic compounds that were masked in the whole extract. However, the use of an AR specific bioassay would be needed to confirm this hypothesis. These results highlight the relevance of fractionation to reduce sample complexity and reveal active chemicals that are masked in the analysis of the whole mixture, a fact which has frequently been reported in EDA studies (Weiss et al. 2009; Creusot et al. 2014).

Overall, our findings identified target compounds as the main contributors to the ER and GR agonistic activity in the effluent extracts. These observations are in line with previous reports where the synthetic glucocorticoids clobetasol propionate and fluticasone propionate significantly contributed to the glucocorticoid activity in WWTP effluents (Macikova et al. 2014; Suzuki et al. 2015; Jia et al. 2016). Steroidal estrogens are well known major contributors of estrogenic activity in urban WWTP effluents (reviewed by Jarosova et al. 2014), as again confirmed at the studied site. Other active chemicals not quantified in the present study, *e.g.* bisphenol A or alkylphenols may also have contributed to a lower extent (Tousova et al. 2017).

#### *In vivo zebrafish embryo assays reveal developmental toxicity and endocrine activity*

To gain insight into the ecotoxicological hazard associated with the samples collected at the study site, the FET and cyp19a1b-GFP zebrafish assays were used to assess both general (lethal and sublethal) and mechanism-based (estrogenic and dioxin-like) toxicity at an early developmental stage. Overall, the effluent was identified as the most active site for all the investigated *in vivo* endpoints (**Table 3**), which confirmed the above described *in vitro* assay based results.

In the FET assay, acute toxicity was 7-fold higher at the WWTP effluent than the downstream site, *i.e.* LC<sub>50</sub> of 9 REF, while no toxicity was observed at the upstream site at



concentrations up to 100 REF (**Table 3, Table S1, Figure S2**). In parallel, sublethal and teratogenic effects were also monitored in living larvae and quantified at distinct stages (**Table S2**). The effluent sample showed a high percentage of concentration-dependent increases for phenotypic abnormalities at 48 hpf (*i.e.* decreased blood circulation, motility, heart rate and hatching) prior to the toxic effects observed after 72 and 96 hpf. For the downstream site, similar abnormalities were observed at 96 hpf. Knowing that this WWTP effluent discharge is from municipal sources (*i.e.*, households and industries), a diverse group of micropollutants is potentially responsible for such lethal and sublethal developmental effects. By using EDA based on *in vivo* zebrafish embryotoxicity assays, recent studies successfully identified teratogens in complex matrices (Legler et al. 2011; Bergmann et al. 2017). Such approaches, *i.e.* using *in vivo* teratogenic effect-directed analysis, could be used to attempt to identify the compounds responsible for the observed developmental toxicity effects in the present study. However, as the focus of our study was to identify EDCs in river water resulting from insufficient elimination by a WWTP, the observed developmental toxicity was not further investigated by EDA. Even so, our results indicate that compounds interfering with fish development are released from the WWTP.

At non-toxic concentrations, *in vivo* up-regulation of GFP in transgenic cyp19a1b-GFP embryos was detected at all three sites. Significant and concentration-dependent induction of GFP expression was observed by the effluent and downstream site samples with a weak effect by the upstream site sample (**Figure 2**). As expected from the *in vitro* data, the order of estrogenic activity based on *in vivo* E2-EQ values (**Table 2**) demonstrated similar estrogenic profile, *i.e.*, effluent > downstream > upstream. However, there was a more pronounced difference between upstream and downstream sites in the *in vivo* assay. Such a difference may be explained by the occurrence of EE2 in the effluent, and likely at the downstream site (Table 2), which is about 90 times more active than the natural estrogens E1



and E2 in the cyp19a1b-GFP *in vivo* assay (Brion et al. 2012), while they are almost equipotent in MELN and ZELH- zER $\beta$ 2 cells (Cosnefroy et al. 2012, Sonavane et al. 2016). It is also likely that other sex steroids (Brion et al. 2012) or synthetic hormones, such as progestins (Cano-Nicolau et al. 2016) discharged from the WWTP may have contributed to higher *in vivo* estrogenic activity at the downstream site compared to the upstream site. However, further chemical analyses would be required to confirm this hypothesis.

A similar *in vitro* and *in vivo* strategy was used for AhR ligands. Measurement of EROD activity on living zebrafish larvae revealed the presence of AhR ligand activity at all three sites (**Table 3, Figure S3**). This can be explained by the fact that in early-life stages, zebrafish embryos can metabolize and respond to exposure to typical AhR ligands such as PAHs (Billard et al. 2006, Otte et al. 2010, Creusot et al 2015). Quantification of TCDD-EQ values confirmed similar AhR activity profiles as depicted by the PLHC-1 cells, with the highest levels measured in the effluent.

Overall, the mechanism-based *in vivo* assays confirmed the ER and AhR activities detected *in vitro* while adding further toxicological information at the whole organism level. For instance, the cyp19a1b-GFP assay provides information on the capacity of xenoestrogens to target radial glial cells and to induce brain aromatase, which are key cellular and molecular actors in neurodevelopmental processes (Diotel et al. 2011). In this fish model, the high estradiol equivalent levels measured at both effluent and downstream sites raised the question of longer term effects in exposed fish, such as male feminization (Purdom et al. 1994), abnormal reproduction (Brion *et al.*, 2004), or altered secondary sexual characteristics (Lange et al. 2012).

In the present study, such an *in vitro* / *in vivo* comparison could not be performed with regard to glucocorticoids. In the future this missing link could be addressed by the use of the recently developed GRIZLY assay, based on transgenic GRE-luciferase zebrafish embryos

(Weger et al. 2012), although its application for environmental monitoring still needs to be demonstrated. However, to our knowledge, there is a lack of appropriate bioanalytical *in vivo* tools to assess endogenous GR-mediated responses. Furthermore, unlike estrogens, there is very limited information on the effects of glucocorticoids on freshwater fish, even though higher quantities of these compounds may reach the aquatic environment compared to the often investigated (xeno)estrogens (Creusot et al. 2014; Runnalls et al. 2010). In our study, the total glucocorticoid concentration was in the ng/L range in rivers, which can potentially alter reproductive or developmental functions in fish (Leatherland et al. 2010), or cause immunosuppression (Margiotta-Casaluci et al. 2016; Hidasi et al. 2017). Finally, physiological effects of glucocorticoids on aquatic organisms can also be investigated by identifying potential glucocorticoid-sensitive protein biomarkers in fish using targeted proteomics (Hidasi 2016).

## CONCLUSIONS

This study successfully demonstrated the suitability of an integrated approach combining Chemcatcher® SDB discs, bioassays and EDA to characterize chemical contamination and hazards associated with a WWTP discharge. At the studied site, WWTP discharge was shown to be a source of multiple endocrine disrupting activities. The steroidal estrogens E1, E2 and EE2, and the pharmaceutical glucocorticoids clobetasol propionate and fluticasone propionate were identified as major causative agents for *in vitro* activities, stressing the need to increase our understanding of their effects on aquatic organisms. *In vivo* fish embryo assays confirmed the presence of compounds interacting with ER and AhR signaling pathways and demonstrated that water soluble chemical contaminants occurring at the downstream river site could affect embryonic development. This study supports the improvement of bioassay-based strategies by combining *in vitro* and *in vivo* assessment for a comprehensive monitoring and evaluation of the impact of effluents. Furthermore, it

highlights the need to better characterize and identify compounds in complex mixtures that interfere with crucial signaling pathways and developmental processes in order to predict and limit their impacts on fish health. Coupling EDA with *in vivo* bioassays is certainly a major improvement to provide in the future.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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*Data Availability*— All data are provided in the manuscript and in Supplementary info files. Any further request on raw data not explicitly presented in the paper can be requested to the senior authors who directed the study, namely Juliane Hollender (eawag) or Selim Aït-Aïssa (INERIS).

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Figure 1. RP-HPLC profiles of Urtenen effluent extract showing estrogenic (MELN and ZELH- $\beta$ 2 assays) and glucocorticoid (MDA-kb2 and GR-CALUX® assays) active fractions. DMSO: solvent control; PC: positive control.

Figure 2. *In vivo* estrogenicity of the Urtenen River sites on zebrafish larvae using transgenic cyp19a1b-GFP line (n=20 larvae per condition). *X*-axis: concentrations are expressed as enrichment factor (REF). Only the concentrations at which no toxicity (i.e. lethality) was observed are given. Exposure was performed for 96 h with semi-static renewal (50% sample renewal every 24 hours). \*: statistically significant effect in GFP intensity as compared to DMSO control (Mann-Whitney test,  $p < 0.05$ ).

**Table 1.** *In vitro* toxicological profiles of the Urtenen River sites<sup>a</sup>.

Activity (unit)	Receptor (cell line)	Urtenen sites		
		Upstream	Effluent	Downstream
Estrogenic (ng E2-EQ/L)	Human ER $\alpha$	0.13	0.48	0.16
	(MELN)	(0.10-0.15)	(0.38-0.62)	(0.13-0.18)
	Zebrafish ER $\alpha$	<0.3	<0.3	<0.3
	(ZELH- $\alpha$ )			
	Zebrafish	0.10	0.54	0.19
	ER $\beta$ 2 (ZELH- $\beta$ 2)	(0.08- 0.12)	(0.41-0.81)	(0.15-0.23)
Androgenic (ng DHT-EQ/L)	Human AR (MDA-kb2)	<0.05	<0.05	<0.05
Glucocorticoid (ng DEX-EQ/L)	Human GR (MDA-kb2)	<12.5	534.1 (515.3-592.7)	85.8 (78.2-88.0)
	Human GR (GR-CALUX)	<LOQ	46.3 (32.5-50.5)	9.5 (8.8-9.7)
Dioxin-like (ng TCDD-EQ/L)	Fish AhR (PLHC-1)	0.40 (0.36-0.45)	0.67 (0.59-0.78)	0.52 (0.45-0.59)
PAH-like (ng BaP-EQ/L)	Fish AhR (PLHC-1)	83.4 (67.2-110.0)	261.0 (200.1-373.1)	105.0 (82.1-165.0)

<sup>a</sup>Results are expressed as estradiol- (E2-EQ), dihydrotestosterone- (DHT-EQ), dexamethasone- (DEX-EQ), 2,3,7,8-tetrachloro-dibenzodioxin- (TCDD-EQ), benzo[a]pyrene- (BaP-EQ) equivalents in ng/L in the stream. The 95% confidence intervals (in brackets) are derived from the dose response curves presented in Figure S2.

**Table 2.** Steroidal estrogens and glucocorticoid compounds identified using targeted analysis (LC-MS/MS) and their contribution to biological activities of the ER-active (MELN and ZELH-beta2 assays) and GR-active (GR-CALUX®) fractions<sup>a</sup>.

F#	Chemical name	REP	Concentration <sup>b</sup> (ng/mL) <sup>b</sup>	Chem-TEQ (ng/mL)	Bio-TEQ <sup>b</sup> (ng/mL)	Chem-TEQ/ Bio-TEQ ratio (%)
F13	17 $\beta$ -estradiol (E2)	1	< LOQ	-	ND	-
F16	E2	1	1.33 <sup>c</sup>	1.33	2.0 [1.1-3.1]	67% [43%-131%]
F17	E2	1	0.80 $\pm$ 0.04	0.80	1.3 [1.0-1.7]	61% [49%-84%]
F18	E2	1	0.70 $\pm$ 0.04	0.70	ND	-
F19	17 $\alpha$ -ethinylestradiol (EE2)	0.93	0.51 $\pm$ 0.01	0.5	6.5 [4.5-8.8]	41% [30%-59%]
	estrone (E1)	0.28	7.86 $\pm$ 0.24	2.2		
F20	E2	1	< LOQ	-	2.5 [1.9-3.3]	6% [5%-8%]
	E1	0.28	0.58 $\pm$ 0.11	0.2		
Human ER fractions (MELN)						
F13	E2	1	< LOQ	-	ND	-
F16	E2	1	1.33 <sup>c</sup>	1.33	1.1 [ 0.9-3.0]	119% [45%-151%]
F17	E2	1	0.80 $\pm$ 0.04	0.80	1.0 [0.6-2.1]	77% [38%-123%]
F18	E2	1	0.70 $\pm$ 0.04	0.70	ND	-
F19	EE2	1.53	0.51 $\pm$ 0.01	0.78	1.4	114%
Zebrafish ER fractions (ZELH-ER $\beta$ 2)						

	E1	0.11	$7.86 \pm 0.24$	0.86	[1.3-1.6]	[101%-131%]
	E2	1	<LOQ	-	ND	-
F20	E1	0.11	$0.58 \pm 0.11$	0.06	0.6 [0.57-0.73]	10% [9%-11%]

(Table 2 to continued)



Table 2 (end)

F#	Chemical name	REP	Concentration (ng/mL)	Chem-TEQ (ng/mL)	Bio-TEQ (ng/mL)	Chem-TEQ/Bio-TEQ ratio (%)
F23	fluorometholone	0.98	<LOQ	-	16.2 [11.5-20.4]	0%
	clobetasol propionate	38	5.4 <sup>c</sup>	205.8		
	fluticasone propionate	57	1.3 <sup>c</sup>	74.2	321.8 [208.4-352.4]	87% [78-132%]
	mifepristone	GR antagonist	<LOQ	-		
F26					14.9 [10.6-22.6]	0%

Human GR fractions (GR-CALUX®)

<sup>a</sup> REP: relative potency; ND: not detected, LOQ: limit of quantitation. LOQ values of the presented compounds in ng/mL: E2: 0.04, E1: 0.015, EE2: 0.017, fluorometholone: 1.9, clobetasol propionate: 0.1, fluticasone propionate: 0.7, mifepristone: 1.1;

<sup>b</sup> no replicate measurement available;

<sup>c</sup> For Bio-TEQ values, 95% confidence intervals are provided [in bracket] and were derived from the dose response curves performed for each active fraction. For chemical concentration, values are reported as the mean and standard deviation of replicate measurements (n=2).

**Table 3.** Summary of *in vivo* effects in zebrafish embryo assays exposed to Urtenen River extracts<sup>a</sup>.

Toxicity endpoint	<i>In vivo</i> assay	Urtenen sites		
		Upstream	Effluent	Downstream
Lethality LC50 (in REF)	FET	>100	9	63
Sublethal effect <sup>b</sup>	FET	0	++	+
Estrogenicity EC20 (in REF) ng E2-EQ/L	tg( <i>cyp19a1b</i> -GFP)	218 0.7 [0.5-0.9]	6 19.6 [17.2-23.2]	13 8.5 [7.4-9.6]
Dioxin-like EC20 (in REF) ng TCDD-EQ/L	EROD activity in living zebrafish embryos	20 0.10 [0.08-0.11]	3 0.68 [0.55-0.70]	16 0.13 [0.10-0.15]

<sup>a</sup> LC50 (lethal sample concentration affecting 50% of exposed embryos) and EC20 (sample concentration inducing 20% effect in GFP and EROD assays) concentrations were expressed as enrichment factor (REF). E2-EQ and TCDD-EQ refer to the concentrations in the stream. The 95% confidence intervals are provided [in bracket]. The data were derived from the dose-response curves presented in Figures 1 (*cyp19a1b*-GFP), S2 (FET) and S3 (EROD).

<sup>b</sup> Sublethal effect classification: 0: no detectable effect after 96 h exposure; +: sublethal effect detected after 4 d exposure, ++: sublethal effect detected as from 2 d of exposure. Detailed observations after 2 and 4 d and for the different sample concentrations are provided in Table S2.

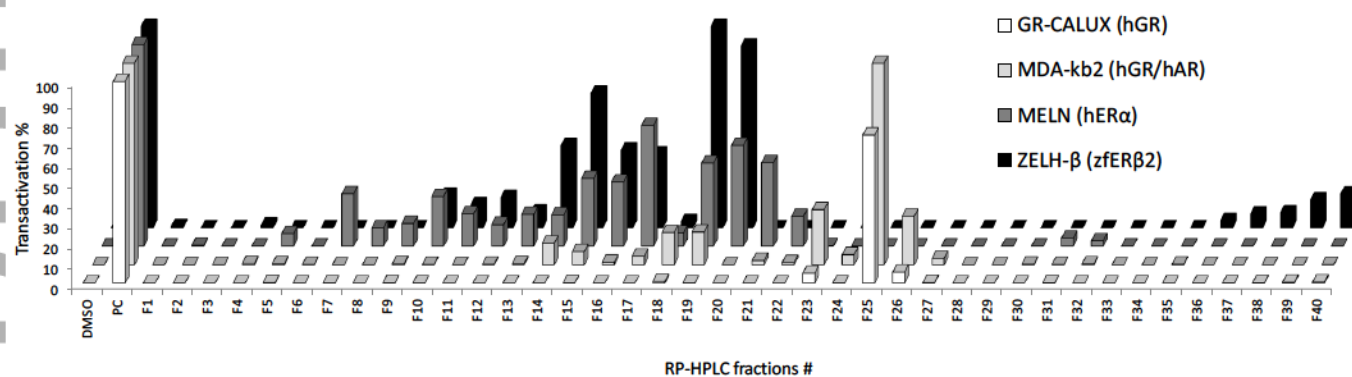


Figure 1.

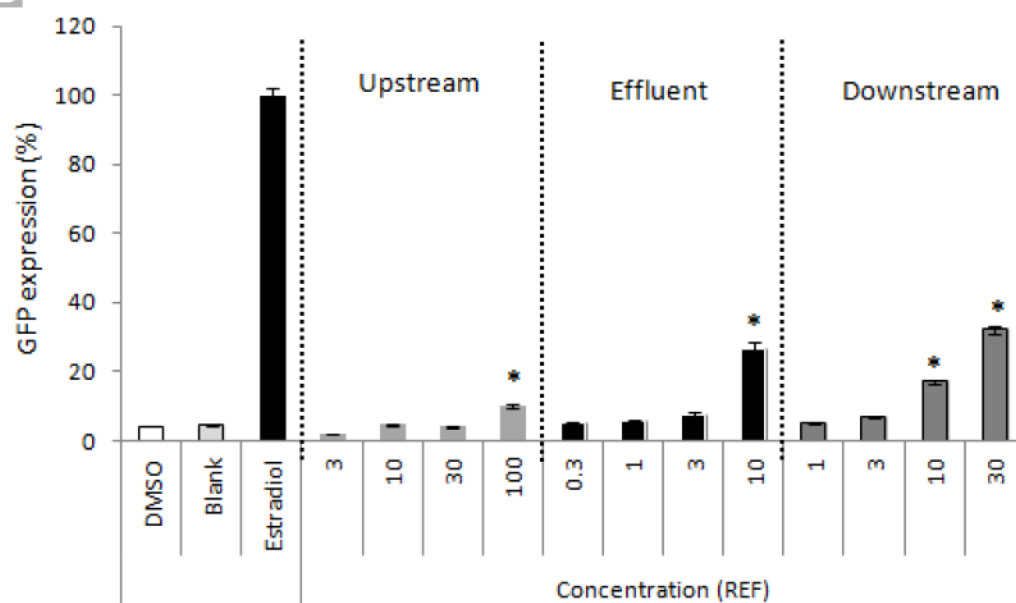


Figure 2.