Environmental Science & Technology

Article

Cite This: Environ. Sci. Technol. 2018, 52, 4367−4377

pubs.acs.org/est

High-Throughput Effect-Directed Analysis Using Downscaled in Vitro Reporter Gene Assays To Identify Endocrine Disruptors in Surface Water

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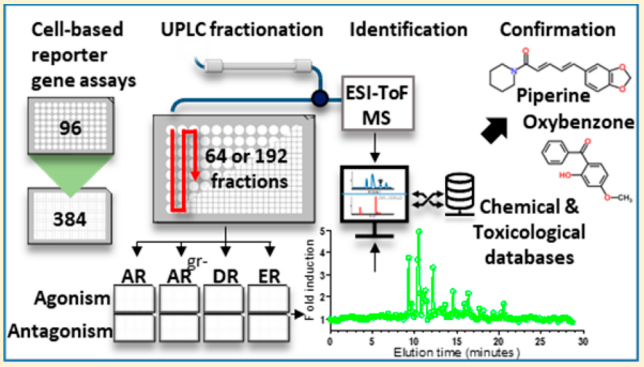
Supporting Information

ABSTRACT: Effect-directed analysis (EDA) is a commonly used approach for effect-based identification of endocrine disruptive chemicals in complex (environmental) mixtures. However, for routine toxicity assessment of, for example, water samples, current EDA approaches are considered time-consuming and laborious. We achieved faster EDA and identification by downscaling of sensitive cell-based hormone reporter gene assays and increasing fractionation resolution to allow testing of smaller fractions with reduced complexity. The high-resolution EDA approach is demonstrated by analysis of four environmental passive sampler extracts. Downscaling of the assays to a 384-well format allowed analysis of 64 fractions in triplicate (or 192 fractions without technical replicates) without affecting sensitivity compared to the standard 96-well format. Through a parallel exposure method, agonistic and antagonistic androgen and estrogen receptor activity could be measured in a single experiment following a single fractionation. From 16 selected candidate compounds, identified through nontargeted analysis, 13 could be confirmed chemically and 10 were found to be biologically active, of which the most potent nonsteroidal estrogens were identified as oxybenzone and piperine. The increased fractionation resolution and the higher throughput that downscaling provides allow for future application in routine high-resolution screening of large numbers of samples in order to accelerate identification of (emerging) endocrine disruptors.



INTRODUCTION

Endocrine disruption is an important end point in toxicological and environmental screening as well as the water quality control of the drinking water production process. Endocrine disruptive chemicals (EDCs) like estrogens[1] and androgens[2] have been detected in the aquatic environment as pollutants. A large portion of EDCs are emitted into the aquatic environment through urban (steroid hormones, flame retardants, and plasticizers) or industrial wastewater,[3] agricultural runoff,[4] or deposition after combustion (PAHs).[5] Subsequent exposure can lead to disrupted signaling of endogenous hormones. Further liver metabolism can enhance receptor binding potency of (inactive) pollutants and increase the risk of endocrine disruption.[6] While well-characterized potent EDCs are actively monitored, unknown compounds with endocrine disruptive potency, including their metabolites, transformation products, and degradation products, remain to be discovered. The ability to detect and identify relevant and yet unknown EDCs is essential to efforts aimed at reducing their presence in the aquatic environment and reducing human exposure.

Cell-based reporter gene assays have been used in effect-directed analysis (EDA) for the identification of (emerging) EDCs in environmental samples.[7−9] Via EDA, compounds not analyzed by routine (chemical) analysis are identified based on their biological activity in reporter bioassays. Activity measured by the reporter gene assays in particular fractions collected during chromatographic separation can be correlated to mass spectrometry data. A response in one or more fractions can direct efforts to identify the compound responsible for the observed activity to a limited number of corresponding masses on the mass chromatogram.

Reducing fraction complexity through high-resolution fractionation decreases the number of compounds and masses to be identified per fraction but increases the total number of fractions. Luciferase reporter gene cell lines, while showing high sensitive toward their respective (ant)agonists, are usually

Received: December 22, 2017
Revised: March 12, 2018
Accepted: March 16, 2018
Published: March 16, 2018

DOI: 10.1021/acs.est.7b06604
Environ. Sci. Technol. 2018, 52, 4367−4377

performed in a 96-well plate format, which limits the number of samples or fractions that can be analyzed simultaneously.

This study focused on improvement of the current EDA approach by increasing throughput and resolution to allow for faster identification as a step forward to a more routine application of EDA in future (surface) water quality assessments.

First, for high-resolution EDA of endocrine disruptive chemicals, an androgen receptor (AR) (AR-EcoScreen),[10] a recently developed AR-EcoScreen glucocorticoid receptor (GR) knockout mutant (AR-EcoScreen GR-KO),[11] aryl hydrocarbon receptor (AhR) receptor (DR-Luc),[12] and estrogen receptor (ER) (VM7Luc4E2, formerly known as BG1Luc4E2; termed ER-Luc in this work)[13] reporter gene assay were downscaled from a 96- to 384-well plate format. Throughput is further improved by introducing a method for parallel exposure of multiple end points with samples or fractions from a single source plate. In addition, a metabolic system was incorporated in the downscaled assays to allow for formation and detection of active metabolites from inactive or less active pollutants.

Second, downscaled assays, using the parallel exposure method, were applied in an EDA approach to analyze four passive sampler extracts which were separated by ultra-performance liquid chromatography (UPLC) and collected as either 64 or 192 fractions. The mass spectra (recorded in parallel) were analyzed at retention times that correlated with active fractions to select masses for identification. A qualitative nontargeted screening was performed, and selected candidates were confirmed chemically and biologically.

MATERIALS AND METHODS

Materials. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium with glutamax, phenol-free DMEM/F12 medium with L-glutamine, low-glucose phenol-free DMEM medium, and fetal bovine serum were obtained from Gibco (Eggenstein, Germany); penicillin/streptomycin, G418, hygromycin, zeocin, ATP, coenzyme A, formic acid, acetonitrile (HPLC grade), and methanol (Chromasolv) were obtained from Sigma (Zwijndrecht, The Netherlands); luciferin was obtained from Promega (Fitchburg, WI); DTT (dithiothreitol) was obtained from Duchefa (Haarlem, The Netherlands); and Aroclor 1254 induced rat liver S9 fraction was obtained from MP Biomedicals (Santa Ana, CA). Water was purified on a Milli-Q Reference A+ purification system (Millipore, Bedford, MA). Reference compounds used for validation of the downscaled test methods and candidate compounds for confirmation of hits were obtained from various suppliers (Table S1 in the Supporting Information) and were dissolved in DMSO (Acros, Geel, Belgium).

Cell Culture Conditions. AR-EcoScreen (CHO-K1), exhibiting residual GR sensitivity, and AR-EcoScreen GR-KO (CHO-K1) cells, with exclusive AR sensitivity, were maintained as described by Satoh et al.[10] ER-Luc (MCF7 human breast carcinoma) cells were maintained as described by Rogers and Denison.[13] Briefly, cells were cultured at 37 °C with 5% CO_2 in DMEM/F12 medium (with 10% fetal bovine serum and 1% penicillin/streptomycin), termed culture medium in the following, and subcultured twice weekly. DR-Luc cells were maintained and exposed as described in section S1.1 of the Supporting Information.

Reporter Gene Assay Protocols. Reporter gene assays were performed in transparent polystyrene CellStar 96-well plates (655160) (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) (as the standard format to compare against the downscaled format) or in white μclear polystyrene CellStar 384-well plates (781098) (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) (downscaled format) using the same cell lines. To maintain the cell-to-surface-area ratio during seeding and compensate for smaller well size, reaction volumes used in the downscaled format were approximately 3-fold lower compared to the standard format (Table S2). Potential effects of medium evaporation on the measurement were reduced by filling the outer ring with 100 μL (96-well plates) or two outer rings with 34 μL (384-well plates) of sterile Milli-Q water which resulted in 60 or 240 wells available for measurements, respectively. Prior to exposure, trypsinated cells were resuspended in phenol-free DMEM/F12 L-glutamine (AR-EcoScreen and AR-EcoScreen GR-KO), low-glucose phenol-free DMEM (ER-Luc) medium (with 5% charcoal stripped fetal bovine serum), termed AR-EcoScreen or ER-Luc assay medium, respectively, and seeded at 200.000 cells/mL in 100 μL (96-well plates) or 34 μL aliquots (384-well plates). Plates were incubated for 24 h at 37 °C and 5% CO_2. In each experiment (n = 1), seeded cells were exposed at t = 0 in triplicate to compounds dissolved in 100 μL (96-well plates) or 34 μL of assay medium (384-well plates), at a final DMSO concentration of 1 or 5 μL/mL for the AR-EcoScreen (and GR-KO) or ER-Luc cells, respectively, using a single (96-well plates) or 8-channel (384-well plates) manual pipet. Reference compound dilution series were prepared in DMSO and, prior to exposure, diluted to exposure concentrations (Table S1) in the respective assay medium for each cell type at a final DMSO concentration of 1 or 5 μL/mL for the AR-EcoScreen (and GR-KO) or ER-Luc cells, respectively. In antagonism experiments, cells were additionally exposed to compounds or fractions in the presence of 200 pM DHT in AR-EcoScreen (and GR-KO) or 4 pM 17β-estradiol (E2) in ER-Luc by spiking assay medium with 5 μM DHT and 0.1 μM E2 in DMSO, respectively. Cytotoxicity was measured in the exposed cells by adding resazurin dissolved in PBS at t = 22 h, leading to a final concentration of 21 nM and measuring fluorescence (λ_{ex} = 530 nm; λ_{em} = 590 nm) immediately after addition and at t = 24 h. Conversion rate of resazurin into resorufin in cells exposed to test substances was compared to the conversion rate in cells exposed to vehicle (DMSO) only. At t = 24 h, the medium was aspirated and cells were lysed for 10 min on an orbital shaker at 700 rpm with 50 μL (96-well plates) or 17 μL of lysis mix [25 mM TRIS (pH 7.8), 2 mM DDT, 2 mM 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA), 10% glycerol, and 1% Triton-X100] (384-well plates). Luminescence was measured on a Varioskan luminometer (Thermo Fisher Scientific, Waltham, MA) for one second after injection of 100 or 34 μL of glow-mix (2 mM Trycin, 1.07 mM $C_4H_2Mg_5O_{14}$, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, 270 mM Coenzyme A, 470 mM Luciferin, and 530 mM ATP) followed by quenching of the reaction with 100 μL (96-well plates) or 34 μL of 0.1 M NaOH (384-well plates).

Biotransformation. Prior to exposure of cells seeded on a 384-well plate in the downscaled format, compounds or fractions were incubated for 90 min at 37 °C in 50 μL of DMEM phenol-free low-glucose medium (with a final volume of 3.6% DMSO (v:v) to ensure solubility of the concentrated compounds during metabolism) in 96-well plates with or, as a

DOI: 10.1021/acs.est.7b06604
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control, without addition of 1.7 μL of S9-mix (300 μL rat liver S9 fraction per mL, 33 mM KCl, 8 mM $MgCl_2 \cdot 6H_2O$, 6.5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate buffer pH 7.4), to the 50 μL reaction volume at a final concentration of 33 μL S9-mix per mL reaction volume (0.2 mg protein per mL reaction mixture), for generation of metabolites. Incubations were performed on single compounds at concentrations from 1.81 to 109 μM (BPA), 0.181 to 181 μM (flutamide), or 1.81 to 181 μM (tamoxifen). After incubation, 216 μL of DMEM phenol-free low-glucose medium was added to the reactions, reducing DMSO concentrations to 0.7% and S9-mix concentrations to 6.4 μL/mL. Cells seeded on 384-well plates were prepared for exposure by adding 24 μL of their respective assay medium to the aspirated cells. In each experiment (n = 1), 10 μL of diluted biotransformation reaction mixtures were added to the cells in triplicate to reach the final volume of 34 μL with a maximum DMSO concentration of 0.2% and an S9-mix concentration of 1.9 μL per mL exposure medium.

Sample Preparation. Adsorption-based Speedisk (SD) and partitioning-based silicone rubber (SR) passive samplers were deployed for a period of 6 weeks between August and October 2014 in the river Meuse at Eijsden and in the effluent stream of an ultralow loaded activated sludge municipal wastewater treatment plant (WWTP) with 25% industrial contribution serving a population equivalent of approximately 300 000 in The Netherlands. Briefly, sample preparation consisted of dichloromethane extraction of SD and hexane extraction of SR samplers. SD and SR extracts were solvent-exchanged from dichloromethane and hexane, respectively, by evaporation under a flow of nitrogen at room temperature and redissolving the samples in 1:2:3 (v:v:v) MeOH:ACN:H_2O to 8.4 SD/mL and 60 g SR/mL, respectively.

LC-MS Analysis and Fractionation. Fractionation of SD and SR sample extracts was performed on a Kinetex C18 (100 × 2.1 mm, 1.7 μm particle size) column using an Agilent Infinity 1290 UPLC pump and autosampler. Extracts were injected (10 μL) at a flow rate of 250 μL/min in 80% mobile phase A (100% H_2O + 0.1% formic acid) and 20% mobile phase B (100% ACN + 0.1% formic acid). The solvent gradient increased to 80% mobile phase B over 15 min and was subsequently kept as such for 13 min. Postcolumn, the flow was split in a 9:1 ratio on a Quicksplit adjustable flow splitter (ASI, Richmond, CA) with 9 parts being diverted to a nanofraction collector and 1 part to a microTOF II time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA). The MS was equipped with an electrospray ionization (ESI) source set to positive mode and scanned masses from 50 m/z to 3000 m/z at 10 Hz. Corona and capillary voltages were set to 500 and 4500 V, respectively. Nebulizer pressure was kept at 2 bar, and nitrogen drying gas flow was kept at 6 L/min.

Exposure of Fractionated Extracts. In each experiment (n = 1), sample fractions were collected in either 96 (64 fractions collected at 26 s intervals) or 384-well plates (192 fractions collected at 9 s intervals) filled with 10 or 4 μL of 10% DMSO in Milli-Q water, respectively, as keeper to increase recoveries.[14] Collected fractions were dried in a Centrivap concentrator (Labconco Corp., Kansas City, MO) for 5 h at 25 °C and redissolved in 50 or 12 μL of ER-Luc assay medium supplemented with 3.6% DMSO added to improve solubility of compounds, in 96- or 384-well plates, respectively, for 10 min at 700 rpm. Redissolved fractions were diluted with 216 or 50 μL of ER-Luc assay medium, lowering the DMSO concentration to 0.7% (considering potential residual DMSO used as keeper negligible), in 96- or 384-well plates, respectively. Cells seeded on 384-well plates in the downscaled format were prepared for exposure to fractions by aspirating the medium and adding 24 μL of AR-EcoScreen assay medium to AR-EcoScreen (and GR-KO) cells or 24 μL of ER-Luc assay medium with 0.43% DMSO to ER-Luc cells. Cells were exposed to a single concentration by adding 10 μL of the redissolved fraction to prepared cells, in triplicate for measuring 64 fractions or in a single well for measuring 192 fractions, using a digital stepper or manual 8-channel pipet. This resulted in a final DMSO concentration of 0.2% (2 μL/mL) in AR-EcoScreen (and GR-KO) and 0.5% (5 μL/mL) in ER-Luc cells during exposure compared to 0.1% and 0.5% DMSO during exposure, respectively, described in the reporter gene assay protocol. As a result from the addition of 10 μL of dissolved fraction in ER-Luc assay medium to 24 μL of AR-EcoScreen assay medium, AR-EcoScreen (and GR-KO) cells were exposed in 29.4% ER-Luc and 70.6% AR-EcoScreen assay medium. From each fraction plate, multiple seeded 384-well plates were exposed to measure different end points in parallel. Reference compound dilution series prepared in DMSO were diluted in the same assay medium composition and at the same DMSO concentration at which cells were exposed to fractions. Cells were exposed, in triplicate, to reference compounds by adding 34 μL of the diluted compounds to aspirated cells.

Data Analysis. Bioassay results were analyzed in Prism 5.04 (Graphpad Software Inc., San Diego, CA). For each serial dilution data set, a D'Agostino−Pearson test was used to test data normality, and Levene's test was used to test homogeneity of variance (significance $P < 0.05$). Dose response curves of reference compounds and candidate compounds were fitted with a four parametric logistic function $[Y = A + (B - A)/(1 + (x/C)^D)]$, where A and B denote minimal and maximal response respectively; C is the EC50 or IC50; D is the hillslope, and x represents the tested concentration. Significant differences ($P < 0.05$) between responses in assays in the 96-well plate format and responses in the 384-well plate format were determined by performing an F-test on fitted curves based on shared EC50/IC50 and hillslope parameters. Responses in fractions were calculated as the induction factor (average fold induction) relative to the response in the first fraction. Responses to compounds were reported as EC50/IC50 concentrations or as PC50 concentrations, at which luciferase induction corresponds to 50% of the maximum response (EC50/IC50) by the reference agonist or antagonist measured in the corresponding assay.

Identification and Confirmation. Nontarget analysis was performed on masses correlating with active fractions (Figure 1) measured in AR-EcoScreen (and GR-KO), DR-Luc, and ER-Luc. Total ion chromatograms were calibrated using internal calibration (high-precision calibration method) using sodium formate clusters. Molecular formulas were determined by the maximum scoring, based on the lowest combined exact mass difference (<0.002 mDa) and isotopic distribution difference (mSigma <20), calculated with the SmartFormula module in the Bruker DataAnalysis software (Bruker Daltonics, Billerica, MA). The elements C, H, N, O, P, S, F, Cl, and Br were selected as allowed elements in determining the molecular formula.[15] Compound IDs (CIDs) matching molecular formulas (of accurate masses within a 0.002 mDa range) were retrieved from Chemspider (https://www.chemspider.com/) or Pubchem (https://pubchem.ncbi.nlm.nih.gov/) data-

DOI: 10.1021/acs.est.7b06604
Environ. Sci. Technol. 2018, 52, 4367−4377

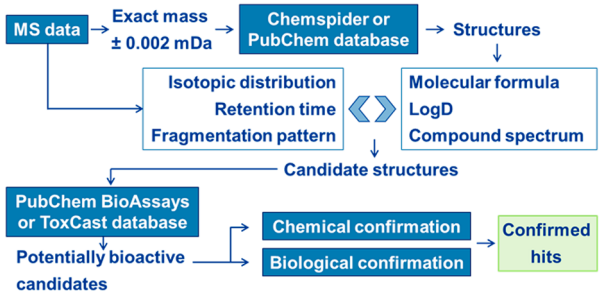


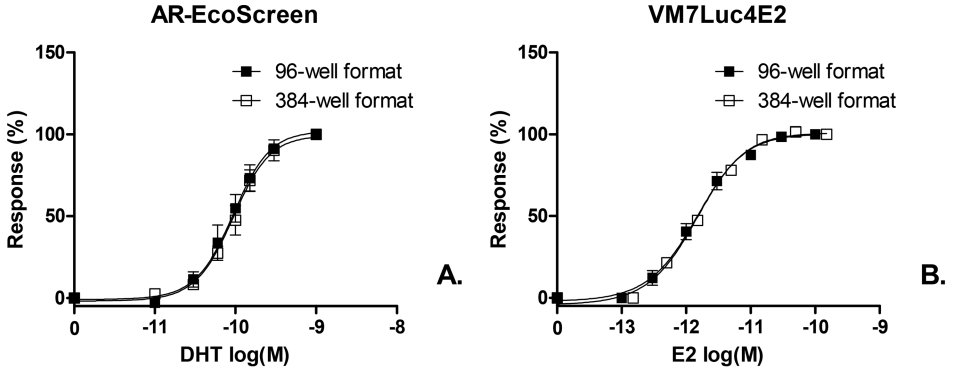
Figure 1. Schematic representation of the identification strategy. Structures matching with an exact mass are retrieved from online databases, and known or calculated properties of that structure are compared with features observed in the MS data to select candidate structures. Candidates are confirmed chemically and biologically, leading to confirmed hits.

bases. Resulting CIDs were converted to structures (SMILES) and predicted log *P* or log *D* (5.5) values were retrieved for each structure using the ALOGPS 2.1 software (http://www.vcclab.org/web/alogps/) or from the ChemSpider database manually, respectively. Exclusively structures with log *P* or log *D* values that corresponded with the retention time of the mass peak within 2 times the log *P* standard deviation (SD = 4.0) or 3 times the log *D* standard deviation (SD = 0.41), based on the log *P* or log *D*/retention time correlation of known compounds tested on the LC gradient, were further analyzed. From the remaining structures, candidate structures with a specific compound name and/or that were described in the literature were manually selected. Fragmentation patterns associated with the exact masses were manually matched with fragmentation patterns of the suspected structures retrieved from the mzCloud database (https://www.mzcloud.org/) or analyzed with MetFrag.[16] Toxicological data of matching structures was retrieved from the Pubchem bioassay database (https://pubchem.ncbi.nlm.nih.gov/) or ToxCast database (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data; INVITRODB V2 SUMMARY database; gain-loss model data; AR_LUC_MDAKB2 and ERa_LUC_BG1 assay data) and compound structures with confirmed (ant)agonistic activity or missing activity data were ordered for confirmation. Candidates were chemically confirmed by LC/MS and biologically confirmed at concentrations from 0.1 to 100 μM in the downscaled bioassays.

RESULTS AND DISCUSSION

Downscaling Reporter Gene Assays. Downscaling of the AR and ER reporter assays to a 384-well plate format increased the number of wells available for measurements on a single well plate to 240 wells compared to 60 wells on a 96-well plate. This allowed for measurement, in triplicate, of a standard dilution curve consisting of 10 concentrations with either 8 sample dilution curves consisting of 8 sample concentrations or 64 fractions (compared to a standard curve and a single sample curve or 10 fractions in triplicate on a 96-well plate). Responses in the downscaled AR and ER reporter assays to their respective agonists compared to responses in the 96-well plate format did not differ significantly (F-test), and curves of both formats could be fit with the same EC50 and hillslope parameters (Figure 2). Additionally, the dioxin and dioxin-like compound responsive DR-Luc was downscaled (section S1.1 of the Supporting Information). Similarly, responses in the downscaled DR-Luc reporter assay did not differ significantly from responses in the 96-well plate format (Figure S1). The downscaled assay formats have fewer cells per well and as such produce a lower light intensity during measuring of luciferase activity. However, this is compensated for by the use of the white opaque 384-well plates which, compared to the transparent 96-well plates used in the original assay protocols, reflect more light toward the detector. By using low-volume reactions, the downscaled 384-well plate format, compared to the standard 96-well plate format, benefits from reduced reagent consumption and an increased number of samples that can be measured on a single plate reducing assay costs per test. These properties allow for the downscaled reporter assays to be used for screening large numbers of samples or fractions in high-resolution EDA.

Bioactivation of Compounds by Rat Liver S9 Fraction. Metabolic activation of compounds prior to exposure of AR



	96-well format		384-well format		F-test
	EC50 (pM)	Slope	EC50 (pM)	Slope	P-value
AR-EcoScreen	150 ± 23	-45.3	170 ± 23	-46.18	0.7717
VM7Luc4E2	4.7 ± 1.0	-27.51	3.5 ± 0.2	-34.43	0.0746

Figure 2. Dose−response curves of androgen (panel A) and estrogen (panel B) responsive cell lines exposed to their respective agonists in 96- (filled squares) and 384-well plate format (empty squares) with errors bars representing the SD (n = 3). No significant differences could be detected by F-test (P = >0.005) based on the EC50 and hillslope parameters. EC50 values are expressed as the averaged EC50 value ± standard deviation.

DOI: 10.1021/acs.est.7b06604
Environ. Sci. Technol. 2018, 52, 4367−4377

and ER reporter gene assays was investigated using rat liver S9 fraction. Responses of AR-EcoScreen and ER-Luc cells to DHT and E2, respectively, in the presence of 1.9 μL preincubated (in the absence of DHT or E2) S9-mix per mL exposure volume, did not significantly differ from exposures in the absence of S9-mix (data not shown), suggesting that the concentration of S9-mix, after incubation and dilution, does not interfere with the reporter gene assay read-out.

Exposure of AR-EcoScreen cells (in the presence of 200 pM DHT) to antiandrogen flutamide preincubated in the presence of S9-mix increased its potency and lowered IC50 values approximately 30-fold from 582 nM to 20 nM compared to flutamide incubated in the absence of S9 (Figure 3A). CHO-K1

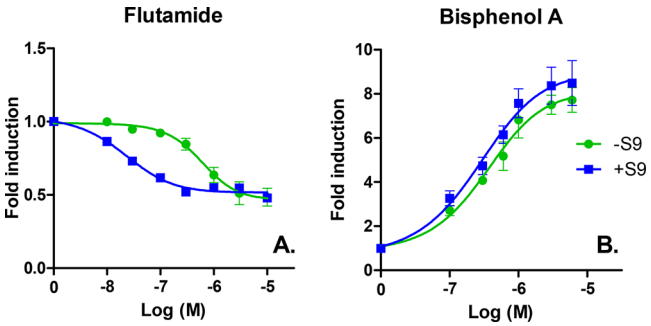


Figure 3. Response of AR-EcoScreen cells, in the presence of 200 pM DHT, to flutamide treated in the absence (green circles) or presence (blue squares) of S9 metabolic enzymes (panel A) and the response of ER-Luc cells to BPA incubated in the absence (green circles) or presence (blue squares) of S9 (panel B). The flutamide EC50 shifted from 5.8×10^{-7} to 2.0×10^{-8} M, respectively, indicating significant activation of flutamide (F-test, p = <0.0001). Error bars indicate standard deviation between averaged response of three experiments (n = 3). The BPA EC50 shifted from 4.0×10^{-7} to 3.2×10^{-7} M, respectively, indicating the activation of BPA (F-test, p = 0.0066). Error bars indicate the standard deviation between three replicates within an experiment (n = 1).

cells do not metabolize steroid hormones,[10] and no expression of CYP P450 enzymes was detected in CHO cells;[17] this indicates that metabolites were exclusively formed by enzymes provided by the S9-mix. While not further tested, the most likely metabolite is 2-hydroxyflutamide (OH-flutamide) which is the major bioactive metabolite of flutamide, used as a therapeutic in prostate cancer therapy, in humans.[18] A similar 12−13 fold increase in potency of 2-hydroxyflutamide compared to flutamide was reported by Ma et al.[19] in the androgen responsive MDA-kb2 cell line.

The presence of S9 increased the potency of BPA on ER-Luc cells approximately 1.25 fold from 397 nM to 319 nM (Figure 3B). A similar, S9 enzyme-dependent, 2- to 5-fold increase in potency was observed in an alternative MCF-7 cell-based ERE-luciferase reporter assay following metabolization of BPA with S9 enzymes[20] and involved the formation of the BPA metabolite 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP), which was 200-fold more potent than BPA.[21] While MCF-7 cells used to develop the ER-Luc cells express metabolic enzymes from the cytochrome P450 superfamily (and thus possess the ability for endogenous biotransformation of chemicals), formation of MBP was dependent on the presence of S9 enzymes.[22,23] However, incubation of the antiestrogenic precursor drug tamoxifen did not lead to an increased antiestrogenic response (data not shown) despite the presence of CYP2D6 in S9-mix which increases the potency of tamoxifen 30−100 fold through formation of the active metabolite 4-hydroxytamoxifen.[24,25] Therefore, formation of 4-hydroxytamoxifen by endogenously expressed CYP2D6 in MCF-7 cells[22,23] could explain the lack of increased potency following treatment with S9-mix.

The combined application of downscaled AR-EcoScreen and ER-Luc reporter gene assays with an S9-mix-based metabolic system provided a quick test of compounds and may prove a useful tool for high-throughput screening of compound libraries. Testing of complex (environmental) samples, however, needs to be further investigated.

Application of Downscaled EDA to Passive Sampler Extracts. SR and SD passive sampler extracts from the river Meuse at Eijsden and WWTP effluent in The Netherlands were fractionated using UPLC, and the collected fractions were tested in the AR and ER reporter gene assays. Additionally, fractions were tested on the downscaled DR-Luc assay (Figure S2) and AR-EcoScreen GR-KO[11] (Figure S3). Fractions from chromatographically separated samples could be analyzed in parallel on eight different end points consisting of four different reporter gene assays in agonistic (Figures 4, 5, S2, and S3) and in antagonistic mode (Figures S3 and S4). In all four assays, agonistic activities that were previously detected in all unfractionated extracts (Hamers et al., in prep) were also found in the fractionated samples (Figures 4, 5, S2, and 3). Antagonistic activity on the AR receptor, previously detected in the unfractionated extract from SR deployed in the river Meuse at Eijsden (Hamers et al., in preparation), could not be observed in collected fractions (Figure S4). Compounds captured by SD tended to elute at an earlier retention time compared to compounds from silicone rubber corresponding with the affinity of the passive sampler material for more polar or nonpolar compounds, respectively. Metabolic activation, while successful with exposure of single compounds, did not result in an observable increase but instead a decrease in agonist response during preliminary experiments on fractions and was not further attempted (n = 1) (data not shown). The lack of an increase of agonistic response after bioactivation in fractions may be explained by (1) the co-occurrence of metabolic inactivation of (steroid hormone) agonists present in the same fraction, (2) an insufficient increase in activity to exceed the limit of detection, and (3) too low concentrations of compounds that may be bioactivated. Co-elution with agonistic steroid hormones into the same fraction(s) can occur as compounds that undergo bioactivation have to share some structural similarity to steroid hormones, required to bind to hormone receptors.[26] Therefore, the described method for metabolic activation may work best in EDA studies on samples that are not expected to contain steroid hormones (e.g., industrial effluents and agricultural runoff from crops). The current method is compatible with HT-EDA; however, further investigation will be required to determine optimal sample type and compound concentrations for the detection of agonistic and antagonistic metabolites.

Exposure of fractionated extracts was performed in either three technical replicates (64 fractions) or single (192 fractions) wells per experiment. Replicates were incorporated in the original assays to improve accuracy during quantification. For a qualitative EDA approach, however, single exposures were considered sufficient and allowed for more fractions to be collected and analyzed. The pipetting of cells and fractions onto 384-well plates was performed manually. Implementation of

DOI: 10.1021/acs.est.7b06604
Environ. Sci. Technol. 2018, 52, 4367−4377

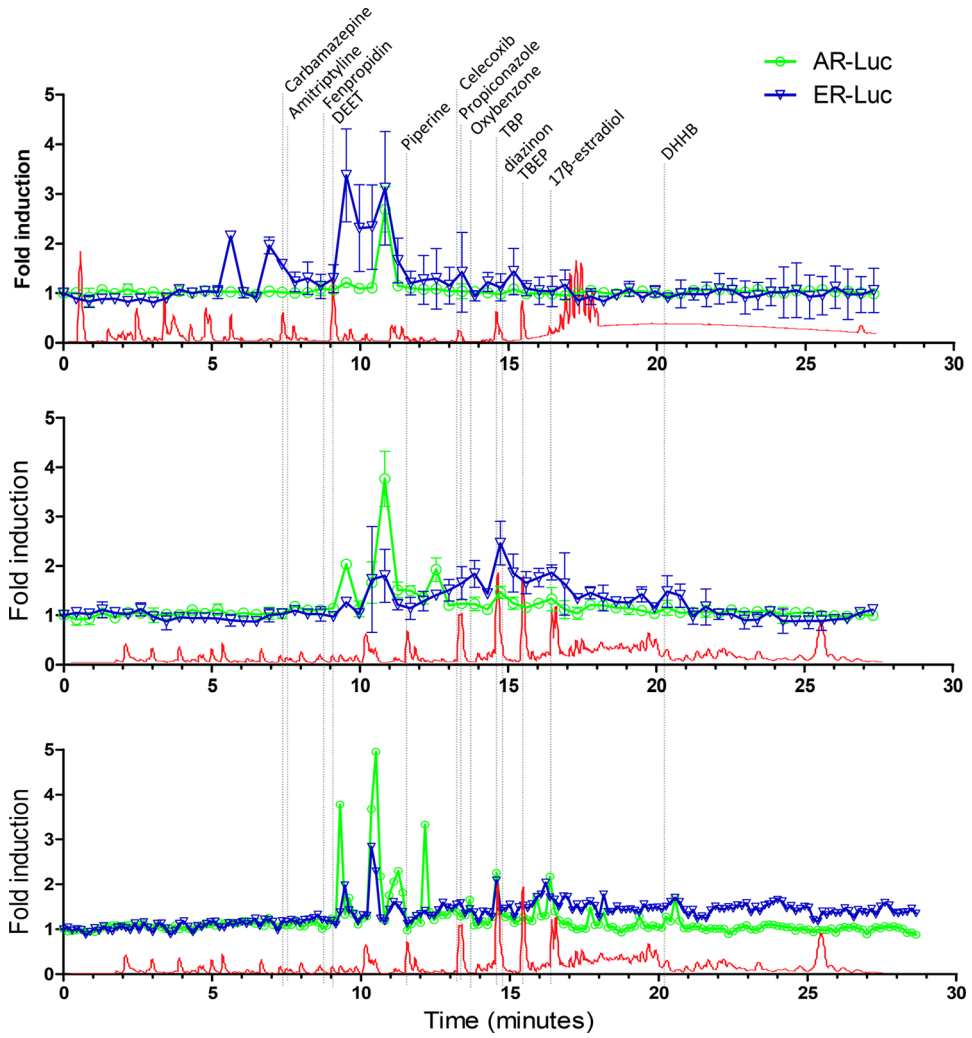


Figure 4. Responses of AR-EcoScreen (green circles) and ER-Luc cells (blue inverted triangles) exposed in parallel to 64 fractions (measured in triplicate in each experiment) from SD (panel A) and SR (panel B) (n = 2) or 192 fractions (measured once in each experiment) from SR (panel C) (n = 1) passive sampler extracts collected at the River Meuse expressed as the average fold induction ± standard deviation between experiments. The MS base peak chromatogram recorded in parallel is shown below the bioassay response to the respective samples (red). The retention times of compounds chemically confirmed are marked with a dotted vertical line.

automated pipetting during further HT-EDA development will increase the number of samples that can be processed daily.

A limited number of agonists have been identified for the AR with the majority of ligands being antagonists.[27] The majority of the agonists consist of endogenous hormones and synthetic derivatives used as therapeutics, which was mirrored by the limited number of high-intensity AR-agonistic responses in collected fractions (Figures 4 and 5). Steroid hormones share similar polarity and subsequently retention time. Therefore, clusters of intense AR-agonistic peaks suggest potential steroid hormone activity. Further investigation of the androgen responsiveness in fractions using the glucocorticoid (GC) insensitive AR-EcoScreen GR-KO mutant indicated the presence of GCs in fractions from WWTP effluent SD extract at 8.3 and 13.5 min and from WWTP SR extract at 12.5−14 min as a higher response was observed in the original, GC sensitive, AR-EcoScreen compared to the GR-KO mutant (Figure S3). While no masses corresponding to GCs commonly detected in the aquatic environment[28] could be observed in the mass spectrum, the retention times correlated with predicted log D (pH 5.5) values (Figure S5) at 8.3 min (1.87) and at 12.5−14 min (3.32−3.63), corresponding with more polar GCs like cortisol (1.66) and dexamethasone (1.92) or less polar GCs like budesonide (3.02) and beclomethasone-17-mono-propionate (3.46).[29]

The estrogen receptor, like the AR, is targeted by endogenous hormones or synthetic derivatives used as therapeutic compounds. However, many environmental pollutants, like BPA, have been reported to have agonistic potency as well.[21] Compared to the AR, fewer receptor antagonists have been reported for the ER. Ethynyl estradiol (EE2) is a well-known agonistic estrogenic pollutant of surface water but could not be detected based on the MS data. Like many other estrogenic steroid hormones, the application of ESI in the negative mode is expected to facilitate detection.[30,31]

Identification and Confirmation of Active Compounds. Masses were linked to active fractions and subjected to identification (Table S3). The use of high-resolution fractionation in 64−192 fractions allowed for faster and more focused identification by reducing the average number of peaks per fraction 4- to 38-fold compared to earlier studies in which 5−18 fractions were collected.[8,32,33] Further increase in the

DOI: 10.1021/acs.est.7b06604
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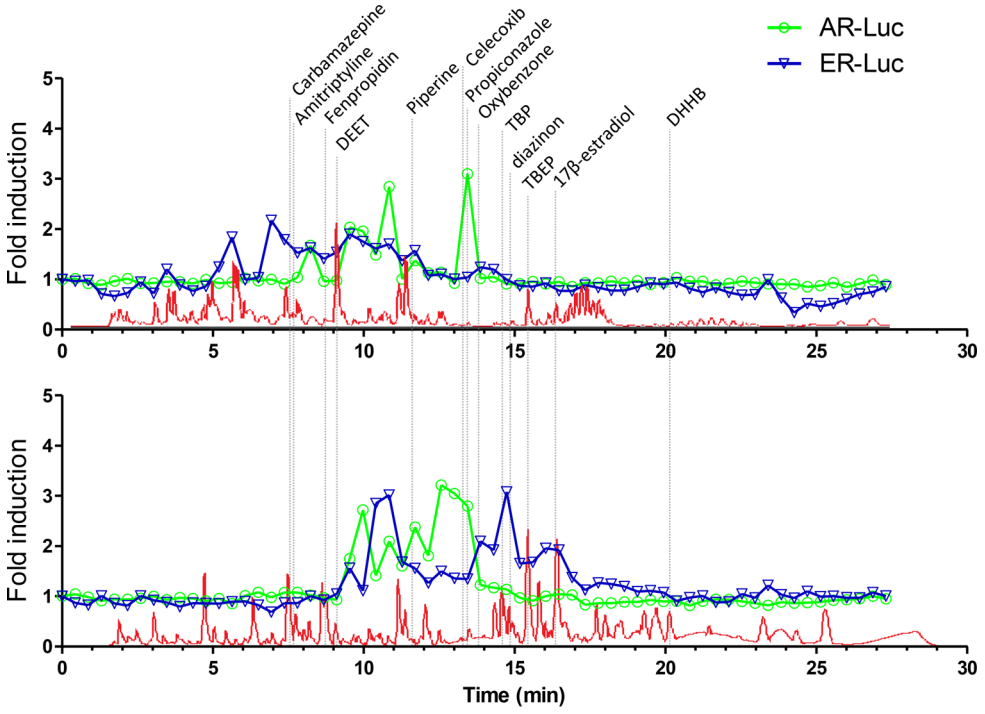

Figure 5. Responses of the AR-EcoScreen (green circles) and ER-Luc cells (blue inverted triangles) exposed in parallel to 64 fractions from SD (panel A) and SR (panel B) (n = 2) passive sampler extracts collected from WWTP effluent expressed as the average fold induction ± standard deviation between experiments. The MS base peak chromatogram recorded in parallel is shown below the bioassay response to the respective samples (red). The retention times of compounds chemically confirmed are marked with a dotted vertical line.

Table 1. EC50 and PC50 Values of Reference Estrogen E2 and ER-Agonist Candidates Oxybenzone and Piperine Confirmed in the ER-Luc Assay and IC50 and PC50 Values of Reference Anti-androgen Flutamide and Six Candidate Compounds Measured in the AR-EcoScreen Assay[a]

	AR-agonism		AR-antagonism		ER-agonism		ER-antagonism	
compound	EC50 (M)	PC50 (M)	IC50 (M)	PC50 (M)	EC50 (M)	PC50 (M)	IC50 (M)	PC50 (M)
E2	ND	ND	ND	ND	2.72×10^{-12}	n/a	ND	ND
oxybenzone	1.36×10^{-5}	–	–	–	2.52×10^{-6}	1.79×10^{-6}	–	–
piperine	3.33×10^{-7}	–	–	–	4.50×10^{-7}	1.88×10^{-6}	–	–
fenpropidin	–	–	–	–	6.70×10^{-7}	–	–	–
4-dimethylamino-benzophenone[b]	6.50 X10^{-7}	–	–	–	8.77×10^{-7}	1.24×10^{-6}	–	–
miconazole[b]	–	–	1.22×10^{-6}	7.91×10^{-7}	–	–	–	–
amitriptyline	–	–	1.99×10^{-5}	1.38×10^{-5}	–	–	2.54×10^{-5}	2.28×10^{-5}
celecoxib	–	–	8.20×10^{-6}	5.49×10^{-6}	1.08×10^{-6}	–	–	–
DHHB	–	–	2.36×10^{-6}	2.40×10^{-6}	–	–	4.76×10^{-6}	5.01×10^{-6}
propiconazole	–	–	8.22×10^{-6}	3.40×10^{-6}	–	–	–	–
TBP	–	–	2.51×10^{-5}	2.47×10^{-5}	–	–	–	–
TBEP	–	–	2.59×10^{-5}	3.09×10^{-5}	–	–	–	–
flutamide	ND	ND	0.50×10^{-6}	n/a	ND	ND	ND	ND
fulvestrant	ND	ND	ND	ND	ND	ND	1.00×10^{-11}	n/a

[a]ND, not determined; –, no response; n/a not applicable. [b]Not chemically confirmed.

number of fractions, however, can lead to loss of sensitivity as eluting compounds become divided over an increasing number of fractions resulting in concentrations below the detection limit of the bioassay. Therefore, high-resolution fractionation is limited to highly sensitive bioassays like reporter gene assays used in the current study. The elution of compounds over multiple wells, however, can aid identification of biologically active compounds by matching dose response relations observed for the bioassay response peak and MS ion peak over multiple fractions at varying eluent concentrations.[34] Alternatively, the extract concentration can be increased to negate the dilution effect at the expense of sample material. When high concentrations overload the LC column, multiple fractionations can be performed on the same plate or a single fractionation plate can be used to expose fewer assays in parallel at the expense of throughput.

Molecular formulas were determined for 56 masses, observed in fractions that produced an agonistic response in AR-EcoScreen (and GR-KO), DR-, and/or ER-Luc cells, in four samples. Structures have been (tentatively) identified for 46

DOI: 10.1021/acs.est.7b06604
Environ. Sci. Technol. 2018, 52, 4367−4377

masses in active fractions of which 16 were selected as candidates for confirmation (Table S3). The candidates consisted of predominantly known (characterized) compounds for which fragmentation data was available. Development of an automated method which predicts ion source-specific fragments and compares this to observed fragment patterns will allow identification of unknown compounds and realize faster identification. Furthermore, retention time predictions based on log *D* (pH 5.5) (SD = 0.41), when compared to log *P* values (SD = 4.0), were more accurate (data not shown). Calculating log *D* values for the specific buffer pH used during analysis can further increase the accuracy of retention time prediction, narrow the number of candidates to analyze, and result in shorter data analysis time. Current candidates have in part been selected based on toxicological data from the PubChem BioAssays and ToxCast databases. While not commonly used in EDA,[35] more effective application of available activity data can further reduce identification time by eliminating inactive candidates during the selection process. Combined with lists of inactive (common) masses observed during routine EDA, mass libraries can be developed that can be used for an automated prescreen of MS data.

From candidates selected manually, the presence of 13 compounds could be chemically confirmed based on their retention times, isotopic pattern, and fragmentation pattern (Figures 4 and 5). Agonist responses were detected for 5 of the 13 compounds in AR-EcoScreen (and GR-KO) and/or ER-Luc (but not DR-Luc) reporter assays including the steroid hormone 17ß-estradiol (E2) (Table 1). However, no agonist response was observed in AR-EcoScreen or ER-Luc cells when exposed to carbamazepine, diazinon, DEET, diethylamino hydroxybenzoyl hexyl benzoate (DHHB), propiconazole, TBP (tributyl phosphate), and TBEP (Tris(2-butoxyethyl)-phosphate), which confirms earlier observations in the reporter gene assays.[36−38] A weak partial agonistic response was observed with celecoxib and fenpropidin, and an antagonistic response was observed for amitriptyline and DHHB in the ER-Luc (Table 1 and Figure 6). The most potent nonsteroidal compounds, piperine and oxybenzone, acted as partial and full ER-agonists, respectively, with a relative potency approximately million-fold lower than that of E2 (Table 1 and Figure 6).

Piperine (log Kow 2.66) is an alkaloid found in black and long pepper (*Piper nigrum* and *Piper longum*) detected in SR (Eijsden and WWTP effluent) at high intensity and SD (Eijsden) at low intensity, which was earlier detected in communal wastewater.[39] The presence in wastewater might be explained by its presence in consumer products including food, supplements, and care products as well as its use as a pesticide.

Oxybenzone (log Kow 3.64) is an ultraviolt filter used in sun lotion and plastics[40] and was detected in SD (WWTP effluent) extract and at 5-fold higher intensity in WWTP SR extract. It has earlier been detected in sediment.[41] Estrogenic activity of oxybenzone was observed in zebrafish (*Danio rerio*)[41,42] and was predicted by quantitative structure−activity relationship analysis. Confirmation of estrogenicity in ER-Luc cells revealed full agonism on the ER (Figure 6).

Compounds identified as ER-agonists were present in active fractions in the ER response for both SD and SR samples from Eijsden (piperine 11.6−11.7 min) and WWTP effluent (oxybenzone, 10.2−10.6 min) (Figures 4 and 5) but could not explain the total observed activity. This can be due to the presence of more potent or more abundant compounds in the same fractions. Masses were recorded in positive ESI mode because many pharmaceuticals, pesticides, and additives can be protonated at low pH and subsequently be detected. Therefore, positive ESI mode increases the chance of detecting bioactive pollutants.[43−45] However, typical steroid hormone ligands of the AR and ER ionize better at high pH in negative ESI[46] and were not detected in the current study. Likewise, typical nonpolar ligands of the AhR used in the DR-Luc reporter assay often contain no ionizable groups and remain undetected by "soft" ionization methods like ESI. Alternatively, GC/MS was successfully applied in EDA aimed at detection and identification of AhR ligands.[47] Therefore, further development of a complete identification strategy requires investigation of different ionization methods and implementation of a combination of chemical analysis techniques to detect the wide variety of compounds present in a sample. However, the presence of steroid hormones could be estimated by comparing calculated retention times to observed retention times of compounds measured on the used UPLC conditions (Figure S5). Calculated retention times of common steroid hormones corresponded with the highest bioassay activity around 13 min (Figures 4 and 5). While changes in chromatographic conditions may further separate compounds' peaks, overlap of unknown bioactive compounds, with masking by potent steroid hormones, and the poor detectability of steroid hormones using ESI-MS remain a technical limitation. By focusing EDA on suspected sources of nonsteroidal EDCs, such as industrial effluent[27] or plastic leachate,[48] before they reach (urban) waste or surface water, where steroid hormones are present, could greatly improve chances to identify emerging EDCs using the sensitive reporter gene assays.

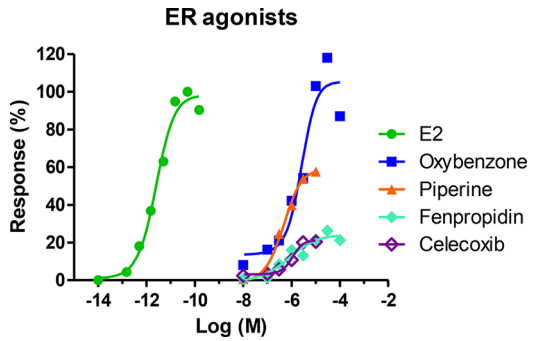

Figure 6. Response of ER-Luc cells to reference compound 17*β*-estradiol (E2) (green circles), two candidate agonists piperine (orange triangles) and oxybenzone (blue squares) (n = 1), and weak estrogens fenpropidin (cyan diamonds) and celecoxib (purple empty diamonds). The maximum response (100%) corresponds to maximum induction by E2.

Antiandrogenic Activity of Confirmed Compounds. The 13 chemically confirmed compounds were tested with the AR-EcoScreen (and GR-KO) and ER-Luc for antagonistic potency regardless of bioassay activity in collected fractions, and six compounds (amitriptyline, celecoxib, DHHB, propiconazole, TBP, and TBEP) acted as AR-antagonists. Full antagonism was observed for all compounds with relative potencies of 5- to 50-fold lower than that of reference antiandrogen flutamide (Tables 1 and S4).

While AR-antagonism was previously described for celecoxib, propiconazole, and TBP,[36,49,50] at the time of writing it had not been reported for amitriptyline, DHHB, and TBEP. AR-antagonism was observed in the unfractionated SR [15.5 and

DOI: 10.1021/acs.est.7b06604
Environ. Sci. Technol. 2018, 52, 4367−4377

1.40 μg flutamide equivalent per gram SR at Eijsden and in WWTP effluent, respectively, (Hamers et al., in prep)] but not in SD extracts. However, AR-antagonism was not observed in the collected fractions. This might be explained by (1) low concentrations of antagonists present in individual fractions and (2) a lower potency of antagonists compared to agonists present in the same fraction. AR-antagonists are abundant in the environment, consisting of pesticides,[36] brominated flame retardants,[51] and pharmaceuticals,[52] but have a relatively low (micromolar range) potency. Because of the large variety of compound classes, AR-antagonists have different retention times and may elute over many different fractions. While the total concentration of antiandrogens present in unfractionated samples may be sufficient to induce an antagonistic response, the concentrations of a limited number of AR-antagonistic compounds in separate fractions can be insufficient to induce an antagonistic response. Furthermore, potent agonists, like steroid hormones, that are present in the same fraction as antagonists may cause masking of potent antagonist responses of individual compounds, further reducing the chance of detecting antagonism in fractions. This was true for all identified AR-antagonists, which eluted among a strong agonistic response (±13 min), except amitriptyline and DHHB which eluted at 7.6 and 20.2 min, respectively, but were unable to induce a response at concentrations present in the wells.

Implementation of the HT-EDA approach described in the current study can be realized at any laboratory equipped with cell-culturing facilities, a LC-ToF MS setup, and a well-plate compatible fraction collector. However, further development is required to incorporate automated pipetting and additional ionization modes and automate the identification process. Further optimization of exposure methods, implementation of increasingly sensitive reporter gene assays, and use of various mass spectrometry techniques with high-resolution fractionation will allow us to detect antagonism, reveal a wider range of compounds, and ultimately make EDA available for the routine identification of bioactive compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b06604.

Additional AR-EcoScreen GR knockout mutant, DR-Luc, and antagonistic response data (PDF)

Tables of used compounds and of identified masses and structures (XLSX)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The described research was funded by the Dutch Technology Foundation (STW), Project Number 12396. The authors acknowledge Dr. Michael Denison for providing the VM7Luc4E2 and H4IIE DR-Luc cell lines, Dr. Iida Mitsuru and Dr. Hiroyuki Kojima for providing the AR-EcoScreen, and the TIPTOP project funded by CEFIC-LRI (Eco023) for providing passive sampler extracts.

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