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Identification of Unknown Antiandrogenic Compounds in Surface Waters by Effect-Directed Analysis (EDA) Using a Parallel Fractionation Approach

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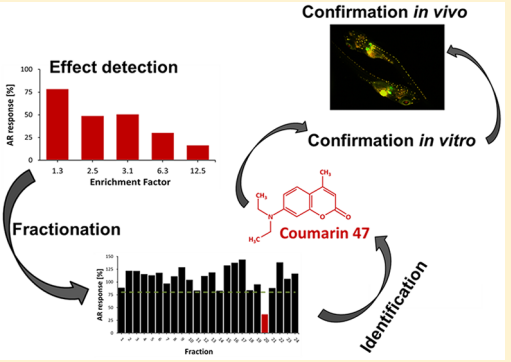
Supporting Information

ABSTRACT: Among all the nuclear-receptor mediated endocrine disruptive effects, antiandrogenicity is frequently observed in aquatic environments and may pose a risk to aquatic organisms. Linking these effects to responsible chemicals is challenging and a great share of antiandrogenic activity detected in the environment has not been explained yet. To identify drivers of this effect at a hot spot of antiandrogenicity in the German river Holtemme, we applied effect-directed analysis (EDA) including a parallel fractionation approach, a downscaled luciferase reporter gene cell-based anti-AR-CALUX assay and LC-HRMS/MS nontarget screening. We identified and confirmed the highly potent antiandrogen 4-methyl-7-diethylaminocoumarin (C47) and two derivatives in the active fractions. The relative potency of C47 to the reference compound flutamide was over 5.2, whereas the derivatives were less potent. C47 was detected at a concentration of 13.7 μg/L, equal to 71.4 μg flutamide equivalents per liter (FEq/L) in the nonconcentrated water extract that was posing an antiandrogenic activity equal to 45.5 (±13.7 SD) FEq/L. Thus, C47 was quantitatively confirmed as the major cause of the measured effect in vitro. Finally, the antiandrogenic activity of C47 and one derivate was confirmed in vivo in spiggin-gfp *Medaka*. An endocrine disrupting effect of C47 was observed already at the concentration equal to the concentration in the nonconcentrated water extract, underlining the high risk posed by this compound to the aquatic ecosystem. This is of some concern since C47 is used in a number of consumer products indicating environmental as well as human exposure.



1. INTRODUCTION

Aquatic organisms are exposed to numerous man-made androgens and antiandrogens.[1] Bioaccumulation leads to the presence of androgenic and antiandrogenic activity not only in water[2−4] and sediment[5−8] but also in aquatic biota[2,9−11] resulting in adverse effects on reproductive health. The first report on masculinization of mosquitofish caused by androgen-containing paper mill effluent dates back to 1980.[12] Moreover the observations of Mila et al.[13] indicate the impact of androgens on the immune system of fish. Other studies have demonstrated that feminization of nonmammalian vertebrate males was correlated to environmental concentrations of antiandrogens.[11,14] Laboratory studies have also confirmed that the skewed sex ratio toward females in fish can be induced by exposure to antiandrogens.[15,16] These facts raise concerns, since not only is the impact of this class of pollutants on wildlife well documented, but there is also evidence that wildlife might act as a sentinel for human health.[11]

Sources that have been demonstrated to release (anti)-androgens into freshwater systems are effluents from livestock feedlot,[3] pulp mills,[17,18] the leather industry[19] and wastewater treatment plants.[2,20−23] Identified androgens present in the aquatic environment include chlorinated pesticides such as dichlorodiphenyldichloroethylene (DDE) and lindane,[1] steroids such as androst-16-en-3-one or nandrolone[24] which are used as anabolic drugs to improve sport performance, and fungicides such as vinclozolin.[25] Antiandrogenic activity has been reported for example for the disinfectant chloroxylenol,[9] the fungicide dichlorophene,[9] and the insecticide isofenphos.[26] Bioassays frequently detect androgenic[7,27,28] and antiandrogenic[29−32] activity in environmental samples. However, in many cases, only a minor part of the effect can be explained by known androgen axis disruptors. Effect-directed analysis (EDA) is a powerful tool for identifying unknown toxicants in environmental samples exhibiting a specific mode of action.[33,34] It has been used successfully to identify estrogenic[35] and antiandrogenic[36] compounds. For instance, Weiss et al.[6] tentatively

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Table 1. Suppliers, Functionalization, and Dimension of Stationary Silica Phases and the Corresponding Guard Columns Applied in This Study

column name	supplier	functionalization	dimension	dimension precolumn
Nucleodur C18 Gravity	Macherey-Nagel	octadecyl	250 × 10.0 mm, 5 μm	10 × 10 mm, 5 μm
Hypersil Gold PFP	Thermo Fisher	pentafluorophenyl	250 × 10.0 mm, 5 μm	10 × 10 mm, 5 μm
Unison NH2	Imtakt	aminopropyl	150 × 10.0 mm, 3 μm	10 × 10 mm, 3 μm
Cosmosil PYE	Nacalai Tesque	pyrenyl ethyl	150 × 4.6 mm, 5 μm	20 × 4.6 mm, 5 μm

identified the antiandrogenic PAHs benzo[a]anthracene and fluoranthene in sediments, whereas Thomas et al.[36] identified petrogenic naphthenic acids in north sea offshore produced water discharges as antiandrogens. In EDA effect drivers are classically identified by effect testing, sequential fractionation and chemical analysis of active fractions. The application of EDA at hotspots of contamination identified by effect-based monitoring is a promising strategy for identifying environmentally relevant chemicals exhibiting a specific effect. Using this strategy, the River Holtemme (Saxony Anhalt, Germany) has been identified as a hotspot of antiandrogenic effects detectable in river water extracts with the anti-AR-CALUX assay (unpublished data).

The objective of this study was to unravel the causes of antiandrogenicity in the River Holtemme using an EDA approach, combining a new fractionation method using four columns with optimized orthogonal separation selectivity in parallel to separate mixtures of endocrine disruptors. Active fractions were identified with a downscaled luciferase reporter gene cell-based anti-AR-CALUX assay that has been recently developed.[37] For structure elucidation of nontarget peaks shared by the active fractions of the four orthogonal stationary phases, in-silico fragmentation prediction by Metfrag, pH-Dependent LC Retention and hydrogen−deuterium exchange (HDX)[38,39] were conducted. Finally the antiandrogenicity of the compounds identified in vitro was confirmed at a higher biological level[40] in vivo in the spiggin-gfp medaka model.[41]

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Information on all chemicals, reagents and solvents used is given in the Supporting Information (SI) Table S1.

2.2. Sampling, Extraction, and Fractionation of Surface Water. The water was sampled in the river Holtemme, 1.3 km downstream of the WWTP of Silstedt (60 000 person equivalents), Saxony-Anhalt, Germany. It was concentrated using a combination of four sorbents in a multilayer cartridge, eluted simultaneously from all four sorbents and fractionated by RP-HPLC in a parallel approach on four columns optimized for orthogonality (Table 1): octadecyl-, pentafluorophenyl-, aminopropyl, and pyrenyl ethyl-modified silica phase. One-minute fractions of the water extract were collected and combined according to peak clusters in the UV−vis chromatogram. For method blanks, LC-MS grade water was injected. More details on the extraction and fractionation procedure, as well as an overview on the gradients used for fractionation (Table S2) and collected fractions (Table S3) is given in SI Section S1.1.

2.3. Anti-AR-CALUX Assay. *Cell Culture and Assay Performance.* The human osteoblastic osteosarcoma U2OS cell line stably cotransfected with an expression construct for the human AR and a respective reporter construct was provided by BioDetection Systems BV (BDS, Amsterdam, The Netherlands) and cultured as previously described.[42,43]

Cells were exposed to a dilution series of the nonfractionated river water sample with a relative enrichment factor (REF) of 1.25−12.5 in 96-well plates in coexposure with a nonsaturating dihydrotestosterone (DHT) concentration equivalent to the EC50 in the agonistic assay (4.2×10^{-10} M) in a final volume of 200 μL exposure medium per well. Each plate contained a serial dilution of the reference antiandrogen flutamide ($10^{-9} - 10^{-5}$ M), solvent controls (1% DMSO) and DHT controls (4.2×10^{-10} M DHT). All conditions were performed in triplicate wells in each test. All exposure conditions and wells contained 1% DMSO. Exposure and measurement were performed according to regular protocols.[37] Tests to evaluate the water sample extract were repeated in three replicates. Nonspecific expression or inhibition of the luciferase gene, as well as luminescence of the candidate chemicals itself were excluded with the Cytotox CALUX assay.[44] For single and recombined fractions as well as method blanks, low-volume procedures for dosing and exposure were applied in dilution series to obtain a 75% reduction of the required sample volume compared to the regular protocol.[37]

Cell viability of at least 80% after exposure was verified by the MTT-assay[45] measuring the amount of formazan with a microplate spectrophotometer (Tecan Infinite M200, Tecan, Switzerland) at an absorbance wavelength of 492 nm. Details of the procedure are found in Di Paolo et al.[37,46]

Data Analysis. Results are presented as magnitude of AR response, obtained by normalizing the average of measured relative light units (RLU) from coexposed cells versus the average of RLU values of cells exposed in the DHT-control condition.[47] Sigmoidal dose−response fit of results was obtained by constraining top to 100 and bottom to zero using a two parameter logistic equation using GraphPad Prism version 7.01

Toxicity equivalent concentrations[48] were calculated to assess the biological recovery of activity after fractionation and for the evaluation of the portion of antiandrogenic effect caused by the identified compounds within the water extract. The chemically determined flutamide equivalent $FEq_{chem,i}$ (μg FEQ/L) of compound i was calculated from the simultaneously determined EC50 of flutamide ($EC50_{flutamide}$) and compound i ($EC50_i$) and the concentration c_i of compound i (eq 1).

The biologically determined flutamide equivalents of the sample FEq_{bio} was calculated from the simultaneously determined EC50 value of flutamide and the sample ($EC50_{sample}$) and the relative enrichment factor of the sample at its EC50 value ($REF_{sample\ EC50}$) (eq 2).

$$FEq_{chem,i} = EC50_{flutamide} \cdot \frac{c_i}{EC50_i} \quad (1)$$

$$FEq_{bio} = EC50_{flutamide} \cdot \frac{1}{REF_{sampleEC50}} \quad (2)$$

2.4. Structure Elucidation of the Antiandrogenic Compounds. Analysis was performed with an Ultimate 3000

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LC system connected to a Q-Exactive Plus HRMS instrument (all from Thermo). For separation, a Kinetex EVO C18 column (50 × 2.1 mm, 2.6 μm with precolumn 10 × 2.1 mm, Phenomenex) was used at 40 °C. Details of the gradient elution program and the eluents are given in SI Table S4. Full scan MS data was obtained by electrospray (ESI) and atmospheric pressure chemical ionization (APCI) in positive and negative mode (ESI+, ESI-, APCI+, and APCI-) in the mass range of m/z 100−1000 at a nominal resolving power of 140.000 (referenced to m/z 200).

Suspect screening for 302 known or suspected antiandrogens was carried out using Tracefinder Version 3.2 (Thermo). The suspect list is shown the accompanying Excel file, SI Table S7.

For nontarget screening, peak lists were obtained from the full scan chromatograms using the software MZmine 2.17.[49] Product ion spectra (MS/MS) were acquired with a data-dependent MS/MS acquisition with an inclusion list containing common masses of all active fractions from the preparative fractionation at a nominal resolving power of 35.000 using 50% or 55% normalized collision energy. Molecular formulas based on accurate mass and isotope patterns were assigned using XCalibur (Thermo). Experimental MS/MS spectra were compared against those predicted for candidate structures retrieved from ChemSpider (Royal Society of Chemistry) using the software MetFrag 2.2 (command line version 54).[50] Besides the match of predicted and experimental MS/MS spectra, hydrogen−deuterium exchange (HDX)[38] and pH-dependent LC retention time (RT) shift at pH 2.6, 6.4, and 10.0[38] were used for candidate selection. A literature survey for available data concerning antiandrogenic activity was carried out for candidates of potentially highest environmental relevance reflected by a high number of references in ChemSpider. For detailed information on the Settings of MzMine, HDX, and pH-Dependent LC Retention see SI Section S1.3.

2.5. Chemical and Effect Confirmation of Antiandrogens. The chemical identity of tentatively identified compounds was confirmed by comparison with RTs and MS/MS spectra of reference standards. To assess the contribution of the confirmed compounds to the observed antiandrogenicity, they were quantified and tested at the corresponding concentration level in the anti-AR-CALUX assay.

For internal matrix matched quantification in the water extract, an internal calibration standard containing verapamil-d6, atrazine-$^{13}C_3$ and bezafibrate-d4 was added to the water extract and the mixed analyte standards at concentration levels of 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 500 ng/mL. The concentrations of the internal calibration standards were 95 ng/mL. Peak integration, compound calibration and quantification were carried out in TraceFinder 3.2 (Thermo).

2.6. Rapid Androgen Disruption Adverse outcome Reporter (RADAR) assay. The RADAR assay was performed using a stable line of medaka eleuthero-embryos harboring the spiggin1-gfp transgene as previously described.[41] Briefly, day post hatch zero medaka fry were exposed to the test chemicals or control solutions in six-well plates. Five fry were exposed per well in four replicates for 96 h at 26 °C with a 14:10 light:dark cycle to stock solutions and controls in DMSO at a final concentration of 0.2% DMSO in all exposure solutions. All solutions were renewed every 24 h. For each experiment, fry were exposed to the test compounds or the solvent control in the presence or absence of 17α-methyltestosterone (17MT, 3 μg/L). Exposure in the presence of 17MT allowed inhibition of 17MT induced fluorescence to be quantified. The reference androgen receptor antagonist flutamide was also tested in the presence of 17MT for comparison.

Following 96 h of exposure fry were anaesthetized by immersion in a 200 mg/L solution of MS222 and positioned in order to reveal their dorsal surface. A Leica MZ10F fluorescent (Leica Microsystems) fitted with a TXD 14C camera (Baumer), ET-GFP long-pass filters (excitation 480/40, emission 510LP, Leica) and a 200 W Lumen fluorescence source (Prior Scientific) was used to capture a color image of the mesonephros of each fry. The GFP signal was quantified using ImageJ[51] as previously described.[41] Two independent replicate experiments were carried out for each set of conditions. In all cases the two replicate experiments gave similar results, therefore data from all groups were normalized to the mean of the solvent control group containing 17MT and pooled.

Data analysis and statistics were carried out in Prism version 5.04 (GraphPad Software). When the groups being compared followed a Gaussian distribution, a Students T-Test (pairwise comparison) or one-way ANOVA and Dunnett's post-test (comparing multiple groups) were carried out. When the data of one or more groups did not follow a Gaussian distribution, a Mann−Whitney test (pairwise comparison) or Kruskal−Wallis and Dunn's post-test (comparing multiple groups) were carried out. All unspiked groups were compared with the solvent control group, all spiked groups were compared to the solvent control containing 17MT.

3. RESULTS AND DISCUSSION

3.1. Identification of Antiandrogenic Active Fractions. The water extract reduced the magnitude of AR response of DHT to 79% ± 9% at REF 1.25 in the anti-AR-CALUX assay with a concentration-dependent decrease to 17% ± 2% at REF 12.5 (SI Figure S1). Extrapolation of the concentration−response relationship according to eq 2 indicates the presence of 46 ± 14 μg FEq/L antiandrogens in the nonconcentrated water extract.

Fractionation of the water extract on octadecyl- (C18), pentafluorophenyl- (PFP), aminopropyl- (NH2), and pyrenyl ethyl (PYE) silica and subsequent testing for antiandrogenic activity in a miniaturized anti-AR-CALUX assay[37] showed one active fraction with an AR response between 37 and 65% for each column (Figure 1). Only one additional fraction (F13 of the NH2 phase separation) showed a slight antiandrogenic response (75 ± 1.2%)(Figure 1c), but was not further considered due to its relatively low bioactivity.

At the same REF values, no cytotoxicity was observed in the MTT-assay in any fraction or the water extract. The fractionation method blanks did not show any antiandrogenic activity in the relevant concentration range between a REF of 1.25 and 12.5.

3.2. Suspect Screening for known Antiandrogens. Active fractions were screened with LC-HRMS for common antiandrogenic suspects addressing 302 of 385 known or suspected antiandrogens retrieved in the literature (see accompanying Excel file, SI Table S7) that are potentially ionizable in ESI or APCI.

Among others the river water extract was fractionated on a semipreparative octadecyl silica column. For chemical analysis an analytical scale octadecyl silica column was coupled online to a mass detector. A standard mixture containing 36 androgenic endocrine disrupting compounds (SI Table S5) was separated on both columns to derive a high correlation of retention times

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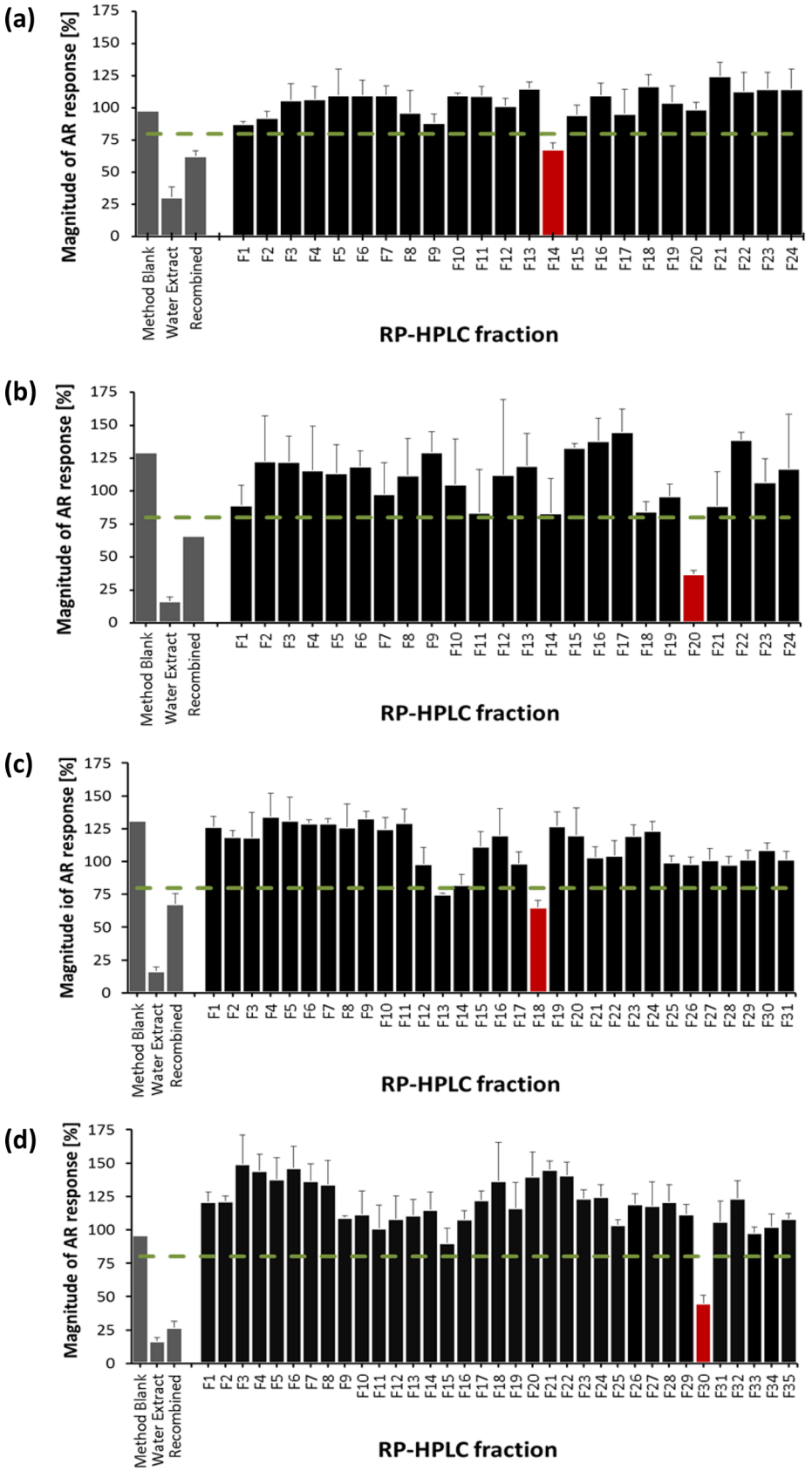

Figure 1. Magnitude of AR response in the downscaled anti-AR-CALUX assay of the water extract, recombined fractions and single fractions obtained with four different columns: (a) C18, (b) PFP (c) NH2 and (d) PYE. Red bars represent the identified active fractions. Fractions with values under the dashed line at 80% AR response are defined as antiandrogenic. REF was 6.25 for (a) and 12.5 for (b)−(d).

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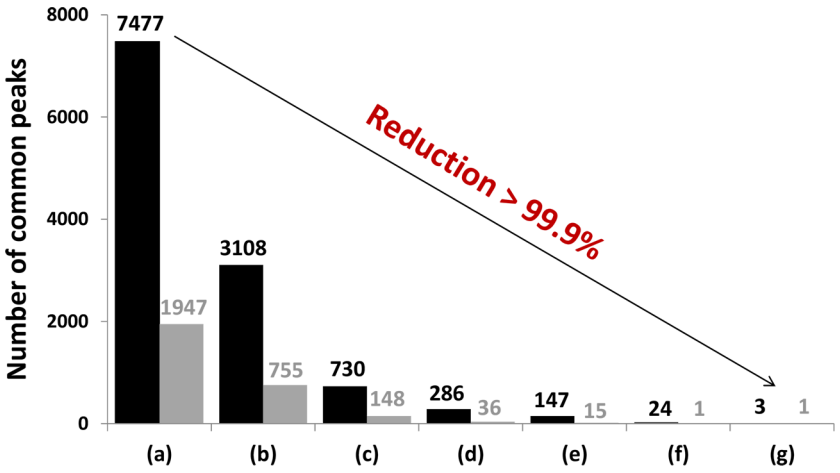

Figure 2. Overview of MS-data reduction by the parallel fractionation approach. Peaks were obtained by ESI (black bars) and APCI (gray bars) in positive mode. The average number of peaks in single active fractions (a) before and (b) after blank subtraction and the average number of peaks in common in any combination of (c) two, (d) three or (e) four active fractions is shown. Consideration of (f) the RT window (10 min ≤ RT ≤ 13 min) and (g) manual evaluation led to a further reduction of the number of common peaks.

Table 2. Overview on Five Non-Target Peaks Detected Commonly in All Active Fractions C18F14, PFPF20, PYEF30, and NH2F18[a]

#	m/z $[M + H]^+$	retention time	candidates in ChemSpider	compound name	molecular formula	CAS
1	232.1332	11.40 min	6931	4-methyl-7-diethylaminocoumarin (C47)	$C_{14}H_{18}NO_2$	91−44−1
2	233.1361	11.40 min		4-methyl-7-diethylaminocoumarin (C47)	$C_{13}{}^{13}C_1H_{18}NO_2$	91−44−1
3	208.1332	11.13 min	8569	Unknown	$C_{12}H_{18}O_2N$	
4	204.1019	9.75 min	3792	4-methyl-7-ethylaminocoumarin (C47T1)	$^{12}C_{12}H_{14}NO_2$	28821−18−3
5	176.0706	7.00 min	1190	4-methyl-7-aminocoumarin (C47T2)	$^{12}C_{10}H_{10}NO_2$	26093−31−2

[a]Three peaks were eluting within and two peaks outside of the retention time window between 10 and 13 min.

(R^2 = 0.94), as displayed in SI Figure S2. Peaks of the active fraction collected between 25 and 26 min were eluting between 11.37 and 11.54 min if separated on an analytical scale octadecyl silica column. Thus, the chemical analysis of the active fractions was constrained to peaks eluting between 10 and 13 min.

In total 34 masses assigned to 45 tentatively identified suspects were detected, as shown in SI Section S2.3. Ten masses were present within two and the remaining 24 masses solely within one active fraction. Thus, none of the suspects fulfilled the candidate selection criteria.

3.3. Non-Target Screening, Compound Identification and Confirmation. *3.3.1. Reduction of the Number of Peaks.* Nontarget screening (ESI± and APCI ±) identified common peaks with the same monoisotopic mass, retention time and isotope pattern within all active fractions eluting between 10 and 13 min from the analytical scale octadecyl silica column for structure elucidation. For ESI+, on average 7477 peaks were picked by the automated peak detection in MZmine in each active fraction before (Figure 2a) and 3108 after (Figure 2b) blank subtraction. Any combination of two and three active fractions shared on average 730 (Figure 2c) and 286 peaks (Figure 2d), respectively. All four active fraction had 147 peaks in common (Figure 2e) but solely 24 of them eluted within the RTs window between 10 and 13 min (Figure 2f). The manual evaluation of nontarget peaks in XCalibur led to an elimination of another 21 candidate peaks either due to similar or higher peak intensity in nonactive fractions and corresponding blanks despite the conducted blank removal or due to a false-positive automated peak picking by MZmine within in background noise. Thus, solely three peaks were present in all active fractions corresponding to a tremendous MS-data reduction of 99.96% underlining the power of the fractionation approach (Figure 2g).

The remaining three peaks (#1−3) are displayed in Table 2. Molecular formulas based on accurate mass and isotope patterns were assigned. With APCI+ only peak #1 was detected in all active fractions but with a lower intensity than in ESI+. In ESI/APCI- no common peaks were detected in all active fractions. Thus, the following nontarget analysis was conducted for the HRMS/MS data received by ESI+ for the active fraction collected from the fractionation on the C18 column.

3.3.2. Structure Elucidation of Three Common Peaks within the Active Fractions. Peaks #1 and #2 are the monoisotopic and the $^{13}C_1$ isotopologue peak of the same compound. For peak #1 6931 candidates were found in total in the Chemspider database with 195 structures exhibiting both a Metfrag score of at least 0.7 and a minimum of 10 reference compounds. Within this group 18 compounds displayed a score of higher than 0.9. By far the highest number of references (155) and a high score of 0.94 were assigned to the candidate 4-methyl-7-diethylaminocoumarin (C47). The in silico fragmentation prediction by Metfrag explained 17 of the obtained 19 most abundant fragment peaks. Moreover, the predicted logK_{OW} value of 3.22 fitted to the observed RT, as shown in SI Table S5. In addition, peak #1 was detected in positive but not in negative ionization mode. This is plausible since C47 can only be ionized by protonation.

Two nontarget peaks (#4 and #5), exhibiting a mass difference to peak #1 corresponding to one, respectively two ethyl groups, were considered since they were not only detected at 9.75 (peak #4) and 7.00 min (peak #5) respectively

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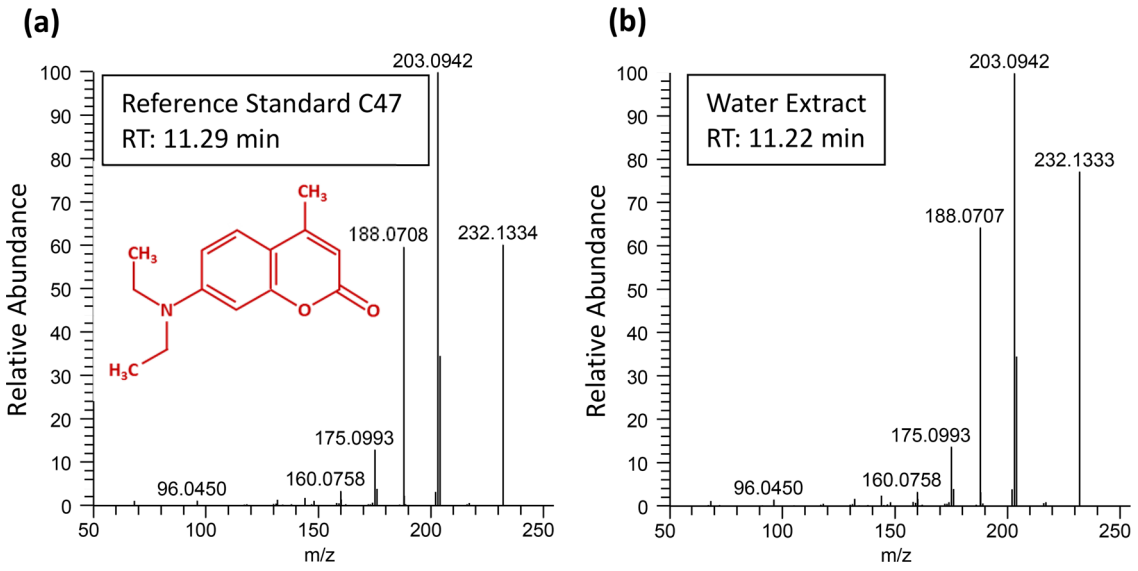

Figure 3. Fragment ion spectra (HCD 50) of (a) C47 and (b) peak #1 with m/z 232.1332 at RT 11.22 min from the water extract (REF 76). The mass spectrum is available in MassBank (https://massbank.eu/MassBank) with accession UA006401 (splash10−0gz0000000-db9037e4c-ce830af7e43).

(Table 2), but also with the same RT as peak #1 due to in-source fragmentation of C47. This is shown in the extracted ion chromatogram of the active fraction (SI Figure S4). The fractionated sample was collected in the effluent of a WWTP. N-Deethylation is a well-known transformation reaction of micropollutants in activated sludge[52] supporting the evidence for C47 as a candidate for peak #1 which contains two ethyl groups at the terminal aryl core. The chemical identity of the nontarget peak #1 and its dealkylated transformation products 4-methyl-7-ethylaminocoumarin (C47T1) and 4-methyl-7-aminocoumarin (C47T2) was confirmed using authentic reference standards, as shown in Figure 3 for C47 and for its derivatives in SI Section S2.4. The presence of peak #4 and #5 in the active fractions despite their lower hydrophobicity compared to C47 suggests some transformation during sample processing after fractionation. However, by far the highest signal intensity within the fractions of the octadecyl silica phase was observed in fraction C18F6 for peak #4 and in fraction C18F1 for peak #5, as shown in SI Figure S8.

Among the 8569 candidates for peak #3 (Table 2) a total number of 338 exhibit a Metfrag score higher than 0.7 and at least 10 references in ChemSpider which are believed to reflect reasonable cutoff values.[38] Ciclopirox, the candidate having the most references in ChemSpider (277) was the only candidate with a suggested environmental relevance and literature indications for antiandrogenic potency.[53] Moreover its predicted logK_{OW} value of 2.73 is in the same range of other antiandrogens eluting in a RT window between 10 and 13 min, as shown in SI Table S5. However, the identity of the nontarget peak #3 could not be confirmed using the authentic reference standard of ciclopirox due to different fragment ion patterns of peak #3 and ciclopirox in LC-HRMS/MS, even if identical RTs were observed (SI Figure S7). A possible coelution of ciclopirox with an isobaric compound was disproved by different common fragment intensities derived from the standard and the environmental sample.

A combination of HDX and pH-dependent retention, which was successfully applied for the identification of unknown mutagens in surface waters,[38,39] was utilized for a further reduction of the candidate list. Out of the 338 candidates, a total number of 252 structures were selected after HDX. The consideration of pH-dependent retention led to a final list of 126 compounds without any outstanding candidate. Thus, the compound related to peak #3 remained unidentified.

3.4. Contribution of Identified Compounds to the Samples Antiandrogenicity. The assumed antiandrogenic activity of C47 was supported by the literature. Several derivatives, all joining a coumarin scaffold, have been already reported as antiandrogens[54−56] with up to 50 fold higher antiandrogenic potency than bicalutamide which is a major antiandrogen in clinical use worldwide.[56] Additionally C47 was already assessed as antiandrogenic in the Tox21 AR-LUC MDAkb2 antagonist assay using a human breast cell line.[53]

The antiandrogenic activity of the identified compounds was verified in the anti-AR-CALUX assay. The corresponding concentration−response curves are shown in SI Figure S9. The contribution of the compounds to the antiandrogenic activity of the water extract was estimated from their concentrations determined by internal matrix-matched quantification by LC-HRMS and their relative potency values to flutamide in the anti-AR-CALUX assay. All values are displayed in SI Table S6.

C47 was detected at a concentration of 13.7 μg/L, equal to 71.4 μg FEq/L, in the water extract (REF = 1). The corresponding concentration−response curves of the water extract and C47 are well in agreement (Figure 4). At the EC50 the compound was calculated to explain 157% of the water extracts total antiandrogenic activity, which is within the uncertainty of the method. The bioassay method uncertainty is estimated to be within 1 order of magnitude, in accordance with the variation expected for the main acceptance criteria of the bioassay, that is, the flutamide IC50 (1.1−10.7 × 10−7 M).[43]

This is the first study reporting the high antiandrogenic activity of this compound with an EC50 of 23.2 μg/L and thus 5.2-fold higher potency than the reference compound flutamide.

For the transformation product C47T1 an EC50 of 30.8 μg/L displayed a slightly lower antiandrogenic potency than its

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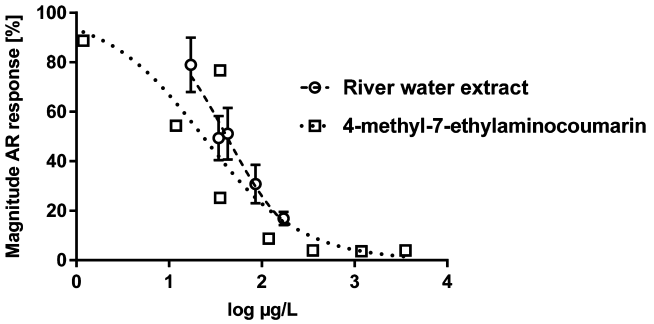

Figure 4. Magnitudes of AR response (%) versus concentrations of C47 in the antagonistic AR-CALUX assay. The C47 reference standard was tested in one and the water extract in three tests (error bars indicate the 95% confidence interval).

parent compound C47 but still exhibited a 3.7-fold higher potency than flutamide. The observed concentration in the water extract (REF = 1) was 3.9 μg/L, equal to 14.5 μg FEq/L. It contributed to 31.8% of the extract total antiandrogenic activity. The potency of the transformation product C47T2 (EC50 371.1 μg/L) is 5.8-fold lower than the one of flutamide. It contributed only to 0.4% of the extract total antiandrogenic activity due to its comparatively low concentration of 1.1 μg/L, equal to 0.2 μg FEq/L, in the nonconcentrated water extract.

3.5. spiggin-gfp medaka (RADAR Assay). The antiandrogenic activity of C47, C47T1 and C47T2 was confirmed at the organism level using the spiggin-gfp medaka model.[41] An initial range-finder experiment indicated that C47 at 10 mg/L was lethal to 90% of the tested medaka fry after 24 h. C47T1 (10 mg/L) was lethal to 10% of fry after 96 h and 10% of fry were immobile following 96 h exposure to C47T2 at 10 mg/L. No toxicity was observable after 96 h exposure to any of the three molecules at 1 mg/L or 0.1 mg/L.

With the observed toxicity in mind, spiggin-gfp medaka fry were exposed to C47, C47T1 or C47T2 at 1 mg/L alone or at a range of concentrations from 1 mg/L to 0.1 mg/L in the presence of 17MT (Figure 5a). The two transformation products, but not C47, showed very weak pro-androgenic activity at 1 mg/L in the absence of 17MT. In the presence of 17MT, the transformation product C47T2 showed no antiandrogenic activity at any of the concentrations tested. However, both C47 and C47T1 demonstrated almost total inhibition of 17MT induced androgen axis activity at 0.1 mg/L and 0.5 mg/L respectively.

In light of the potent antiandrogenicity observed for C47 and C47T1, full concentration response curves were performed for these two molecules in addition to the reference androgen receptor antagonist flutamide (Figure 5b). The concentrations tested covered the full effect range from inactivity to complete inhibition of the 17MT induced fluorescence. Using the modeled curves, EC50 values were determined as 62 μg/L, 32 μg/L, and 69 μg/L for flutamide, C47 and C47T1 respectively, indicating that C47 is a more powerful inhibitor of androgen axis activity than flutamide. These results confirm the evaluation via the antiAR-CALUX assay. C47T1 showed a concentration-dependent antiandrogenic effect and a weaker antiandrogenic activity than C47. C47 displays an antiandrogenic effect already at concentrations equal to the concentration in the water extract at REF = 1, thus suggesting an adverse impact of this compound on the aquatic ecosystem.

SI Figure S10 shows that C47 also exhibits a weak estrogenic activity in medaka, confirming results previously obtained in vitro with the Tox21 Era-BLA antagonist assay.[53] This indicates that in addition to the androgen receptor antagonism observed in vitro with the antiAR-CALUX assay, a second mechanism of antiandrogenicity may be present in vivo. Estrogens have previously been shown to inhibit androgen axis activity in vivo,[41,57,58] presumably through their well-documented ability

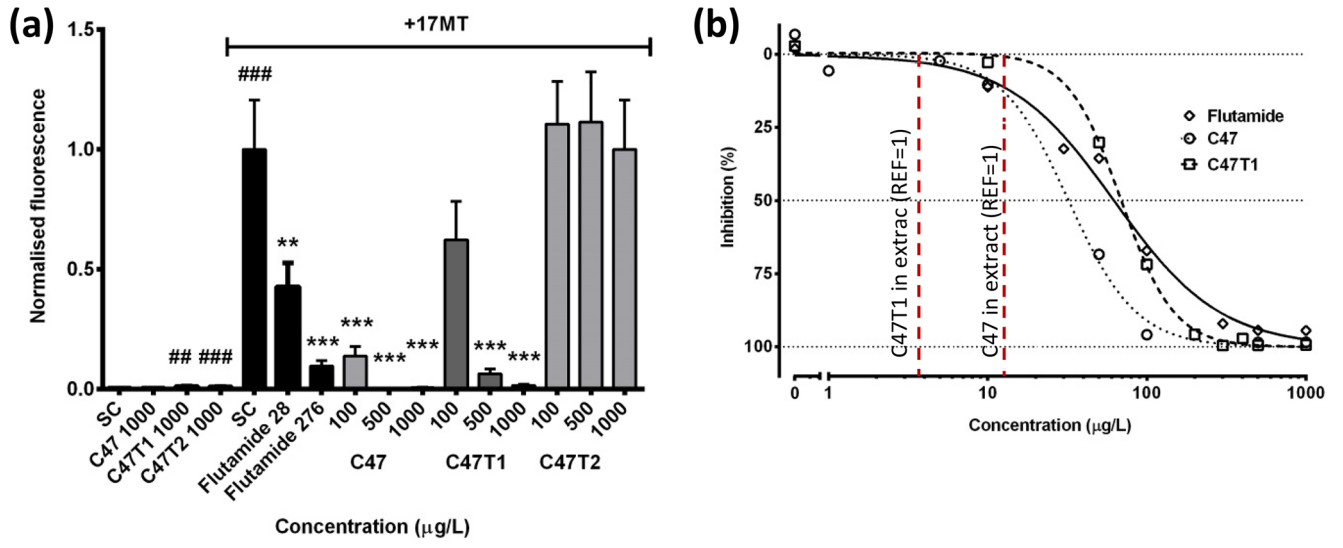

Figure 5. Fluorescent detection of antiandrogenic activity with the RADAR assay. A: Confirmation of the in vivo antiandrogenic activity of C47 in spiggin-gfp medaka by inhibition of fluorescence production. Statistical significance in the absence of cotreatment, compared to the solvent control group (SC), is indicated by ## ($P < 0.01$) and ### ($P < 0.001$). Statistical significance in the presence of 17-methyltestosterone (17MT), compared to the 17MT alone group (SC+17MT), is indicated by ** ($P < 0.01$) and *** ($P < 0.001$). Mean and standard error of the mean are shown. B: Full concentration−response curves for the two antiandrogenic contaminants (C47 and C47T1) compared to the reference antiandrogenic pharmaceutical flutamide. The graph indicates similar EC50s for flutamide and C47T1, but a lower EC50 for C47. All three molecules achieved complete inhibition of androgen dependent fluorescence production. The concentration of the two compounds in the nonconcentrated water extract is marked by vertical lines.

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to upregulate aromatase expression[59] resulting in increased conversion of androgens to estrogens and reducing the concentration of circulating androgens. Further studies will help to shed light on the relative importance of the two mechanisms of antiandrogenicity.

The present study demonstrated the power of the novel EDA approach using parallel orthogonal fractionation procedures, miniaturized in vitro tests, state-of-the-art LC-HRMS/MS nontarget techniques and organism level confirmation procedures to identify and confirm environmentally relevant endocrine disruptors in complexly contaminated water samples. This study is clearly underlining that not only effect-based monitoring of pro- and antiandrogenic activity should be integrated into the water framework directive but also EDA as an important tool to identify endocrine disruptors in pollutes sites.[60] The identified compound C47 was demonstrated to be a highly potent environmental contaminant that acts as an androgen receptor antagonist. The antiandrogenic potency of C47 in vivo was demonstrated to be higher than that of the reference antiandrogenic pharmaceutical flutamide. This is of some concern since C47 is used in a number of consumer products, including bathroom air fresheners at concentrations up to 1%. Screening of other molecules belonging to the coumarin class for endocrine activity is therefore recommended.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04994.

Detailed information on sample extraction, fractionation, LC-HRMS analysis, biotest results, spectra of C47T1, C47T2 and the unidentified nontarget peak #3 (PDF)

A table containing 385 known or suspected antiandrogens (XLSX)

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Notes

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