EFFECT-BASED SCREENING OF CONTAMINANTS IN EFFLUENTS FROM WASTE WATER TREATMENT PLANTS IN THE DANUBE RIVER BASIN

Behnisch PA[1], Besselink H[1]; Alygizakis N[2]; Slobodnik J[2], Brouwer A[1, 3]

[1]BioDetection Systems BV (BDS), Science Park 406, 1098 XH Amsterdam, the Netherlands, peter.behnisch@bds.nl
[2]Environmental Institute, Okruzna 784/42, 97241 Kos, Slovak Republic
[3]Department of Ecological Sciences, VU University of Amsterdam, De Boelelaan 1089, 1081 HV, the Netherlands

Abstract
Proper water quality monitoring requires an array of sensitive and selective high-throughput monitoring tools to cover all relevant mode of actions (MoA) in a simple, time and cost-efficient way. Monitoring the quality of the aquatic environment by analysing a (limited) defined set of compounds is insufficient to effectively determine the biological effects of complex environmental mixtures. An alternative approach to enhance the effectiveness of environmental monitoring is the application of effect-based detection methods to characterize mixtures of endocrine disrupting compounds[1,2], PAH-like compounds[3], oxidative stressors[4] and/or POPs (e.g. dioxins/PCBs, pesticides, PBDEs)[5,6] in the environment. Here we report about testing of 12 waste water treatment plant (WWTP) effluents in nine European countries in the Danube River Basin (from Augsburg, Germany to Bucharest, Romania) using a panel of CALUX® bioassays for estrogen (by ERα CALUX®), anti-androgen (by AR CALUX®), labile AhR ligands (PAH CALUX®), oxidative stress (Nrf2 CALUX®), glucocorticoids by GR CALUX®), peroxisome proliferators (PPARɣ CALUX®) and pregnane x (PXR CALUX®) receptor activities. All WWTP effluent samples showed activities in the CALUX® panel. Effect-based trigger values (EBTs)[5-8] from the here used bioassays are derived and a response plan for operators of WWTPs is proposed.

Introduction
In the latest European watch list (EC/495/2015) several hormones are listed with in the low pg/l range. To allow for analysis at such low concentration levels, expensive and sophisticated chemical analysis methods are required according to the WFD guideline, whereas it has been documented several times that cost-efficient and easy-to-use bioassays can also reach such low detection limits[1-4]. Bioassays like CALUX® (ISO 19040-3; OECD TG455 approved) can quantify in estradiol equivalents (EEQs) such estrogenic hormone activities in water. In this study, the above described panel of CALUX® bioassays[1-6] was used to screen for endocrine disrupting chemicals as well as for oxidative stress and inducers of xenobiotic metabolism (Nrf2 and PXR CALUX®). WWTPs were selected in a way representing each country's predominant wastewater treatment technology including large plants in country capitals (e.g. Budapest, Ljubljana, Bucharest, Zagreb) as well as WWTPs in smaller cities (e.g. Žilina, Brno) and towns serving agricultural and industrialized regions (e.g. Krško, Amstetten). The same samples were subjected also to analysis by chemical analysis of more than 2,100 target organic substances and non-target screening. Basic physico-chemical parameters and information about the plants were provided by the WWTPs' operators. The information from wide-scope chemical screening is planned to be used for the follow up mass balance analysis, explaining the toxicity of individual bioassays by individual toxicants or their mixtures.

Materials and Methods
Water samples collection and extraction
Composite seven-day wastewater effluent samples were collected during dry weather and under normal operating conditions. Samples for analyses of organic substances remained in the freezer at -20°C in the WWTP and during transport. Samples were processed immediately after arrival to the laboratory. For application of bioassays, 500 ml water samples were extracted with solid phase extraction (SPE) using OASIS HLB SPE cartridges (500 mg, 6 cc, Waters 186000115) and then eluted with 10 ml of methanol followed by 10 ml of acetonitrile. Both fractions were

pooled and evaporated under a gentle stream of nitrogen. The final extracts were re-dissolved in 100 µl of DMSO after which serial dilution in DMSO were prepared.

CALUX® reporter gene assays
CALUX® reporter gene assays were performed as described elsewhere[1-4]. In short, stably transfected human U2OS-cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supplemented with 7.5 % fetal calf serum (FCS) and 200 µg/ml G418. The stable transfected H4IIE-cell line was cultured in α-MEM medium supplemented with 10% FCS. Cells were seeded into 96-well plates in medium supplemented with hormone-stripped serum. The next day, the medium was replaced with medium containing the water extracts (0.1 % DMSO). After 24 hours exposure, the medium was removed and the cells were lysed. The substrate luciferin was added to the wells to quantify the amount of luciferase produced by the cells by measuring the amount of light using a luminometer. For equivalents calculation, a dose-response curve of the reference compound was included in the analysis. The reported reference compounds equivalents used for the different bioassays were: 17ß-estradiol, flutamide, rosiglitazone, B[a]P, dichlorvos and DEHP for the ERα, anti-AR, PPARγ, PAH, Nrf2 and PXR CALUX, respectively. To test for possible cytotoxic effects of the samples analyzed, the cytotox CALUX® activity was also determined. Cytotox CALUX cells constitutively express luciferase. In case cytotox CALUX® cells are exposed to sample extracts causing cytotoxicity, a decrease in luminescence is observed. A reduction of 20% in luminescence is considered as a cytotoxic response.

Results and Discussion
To facilitate water quality assessment, effect-based trigger values (EBTs) are being established above which bioassay responses of water extract indicate a potential risk for aquatic fauna and flora or adverse health effects[5-7]. In Table 1, EBTs for various CALUX bioassays are given based on recommendations for estrogens (EAWAG, CH)[7] and for the other endpoints based on available literature studies[5, 6, 8]. Based on these EBTs, a typical response plan of actions for WWTP operators could be as following:
The sample location and frequency (proposed once in six months) for these bioassays should be linked to specified monitoring requirements in the WWTPs. An exceedance of the above proposed trigger values should initiate the following actions:

- If the measured value/EBT < 1: no further action required.
- If 1 < measured value/EBT < 3: quality check data, continue to monitor every three months, until 1 year and until the EBT < 1.
- If 3 < measured value/EBT < 10: data check, immediate re-sampling and quantify specific target compounds which are known to cause the effects observed in the respective bioassay (toxicity drivers). Continue to monitor every three months, until 1 year and the EBT < 1.
- If 10 < measured value/EBT < 100: all of the above plus enhance source identification program. Also monitoring of influent waste water to confirm the magnitude of assumed safety factors associated with removal efficiency by the available WWT technology and dilution in the receiving water body.
- If measured value/EBT > 100: all of the above plus immediately confer with the local environmental authority to determine the required response action. Confirm WWTP corrective actions through additional monitoring that indicates the measured value/EBT ratio is below at least 100.

This would mean for the here applied bioassays, listed Mode of Actions and proposed EBTs:

Table 1: List of bioassays, with their mode of action, reference compounds, their proposed effect-based trigger values and a potential response plan (labelled with different font styles as described above) for operators of these 12 WWTPs in 9 EU countries in the Danube river basin.

Mode of Action	Reference compound	Cell TA assay e.g.	Below EBT (standard)	1 to 3-times EBT level *(italic)*	3- to 10-times EBT level (underlinded)	10- to 100-times EBT level **(bold)**	Above 100-times EBT level ***(italic bold underlined)***
Estrogenicity (ER)[a]	ng eq E2/l	Human or Yeast	0.4	*0.4-1.2*	1.2-4.0	**4.0-40**	***>40***
Inhibition Androgenicity (anti-AR)[b]	µg eq Flutamide/l	Human or Yeast	3.3	*3.3-9.9*	9.9-33	**33-330**	***>330***
Glucocorticoid receptor activation (GR)	ng eq Dexamethasone/l	Human	100	*100-300*	300-1000	**1000-10000**	***<10000***
Activation of peroxisome proliferator-activated receptor (PPARɣ)	ng eq Roziglitazone/l	Human	36	*36-108*	108-360	**360-3600**	***>3600***
AhR receptor activation (PAH)[c]	ng eq B(a)P/l	Rat	6.2	*6.2-18.6*	18.6-62	**62-620**	***>620***
Adaptive Stress (Nrf2)	µg eq dichlorvos/l	Human	26	*26-78*	78-260	**260-2600**	***>2600***
Activation pregnane x receptor (PXR)	µg eq DEHP/l	Human	272	*272-816*	816-2720	**2720-27200**	***>27200***

[a]: Estrogenicity testing according to ISO19040 and OECD TG455; [b]: Anti-androgenicity testing according to OECD TG458; [c]: AhR receptor activation testing[6, 8]

In Table 2, CALUX® bioanalysis results and a proposal for typical response plan of actions for operators of the WWTPs (presented by different font style coding) are presented: The results show that the CALUX® panel can successfully be applied to complex mixtures of pollutants in typical WWTP effluents. Almost all types of hormone receptor agonistic activity could be detected in the effluents. Highest ER CALUX® activities were detected in the WWTP effluents from Ljubljana, Bucharest and Varazdin (between 5 to 7,4 ng EEQ/l). Highest PAH CALUX® activities were detected in WWTPs effluents from Vipap, Amstetten and Brno (122 to 240 ng BaP-EQ/l). PPARɣ receptor agonist's activities were only found in Varazdin. The levels of glucocorticoids (GR CALUX®) and oxidative stressors (Nrf2 CALUX®) were in the range from below 20 (LOQ) to 120 ng DEX-EQ/l and below 20 (LOQ) to 200 µg eq dichlorvos/l. Highest results in PXR CALUX® were found in Vipap, but all other effluents

ranged between 100 to 420 µg eq DEHP/l. Presence of one type of bioassay/MoA activity does not seem to predict the presence of activity for the other types.

Table 2: Bioanalysis results from the panel of CALUX® bioassays and a proposal for response plan of actions for operators of WWTPs.

	ERα CALUX	**anti-AR CALUX**	**GR CALUX**	**PPARγ CALUX**	**PAH CALUX**	**Nrf2 CALUX**	**PXR CALUX**
1 – Varazdin (HR)	**5**	*5,7*	<19	**640**	**72**	*64*	210
2 – Amstetten (AT)	*1,1*	22	<20	<520	**122**	*71*	*320*
3 – Cluj (RO)	<0,06	31	34	<420	52	<79	*210*
4 – Augsburg (DE)	*1*	10	72	<410	**72**	*71*	*330*
5 – Vipap (SI)	*0,65*	32	<25	<460	**242**	120	1200
6 – Budapest (HU)	*0,56*	11	<23	<430	**62**	*73*	*360*
7 – Ljubljana (SI)	**6,6**	*8,4*	*120*	<350	**62**	*78*	*240*
8 – Bucharest (RO)	**7,4**	*5,7*	38	<340	**82**	200	*250*
9 – Zilina (SK)	2,2	*8,9*	78	<480	**72**	94	130
10 – Sabac (RS)	*1,1*	14	<41	<490	**72**	*71*	100
11 – Brno (CZ)	*0,54*	13	47	<1100	**122**	130	*430*
12 – Zagreb (HR)	*0,8*	*6*	<42	<1100	52	<26	*420*

Note: The different font style-codings are described in the text above; Results are expressed as ng- (ERα: in 17ß-estradiol, PAH: in B[a]P; GR: in Dexamethasone; PPARɣ: in Roziglitazone) or µg- (anti-AR: in Flutamide, Nrf2: in Dichlorvos, PXR: in DEHP) of these reference compounds in toxic equivalents per litre of water.

In conclusion, the here used panel of CALUX® bioassays adds information about the presence of currently unknown biologically active compounds in the aquatic environment. Compounds are present that have measurable estrogen, anti-androgen, PXR, PAH, Nrf2, PPARɣ and glucocorticoid-like activities. More information is needed about the levels, identity and removal processes of these nuclear hormone receptor mimics, to assess the possible risk that they might pose to the environment and to human health.

References

1. Murk AJ, Legler J, Brouwer A. et al. (1996); *Toxicol. Sci.* 33, 149–160.
2. Van der Linden SC, Heringa M, Man HY. et al. (2008); *Environ. Sci. Technol.* 42, 5814–5820.
3. Pieterse P, Felzel E, Winter R. et al. (2013); Environ. Sci. Technol. 47, 11651–11659.
4. Van der Linden SC, von Bergh A, Van Vugt-Lussenburg B. et al. (2014); *Mutat Res Genet Toxicol Environ Mutagen.* 760:23-32.
5. Van der Oost R, Sileno G, Suárez-Muñoz M. et al. (2017); *Environ. Toxicol. Chem* 36, 2385-2399.
6. Escher BI, Aït-Aïssa S, Behnisch PA. et al. (2018); *Sci. Tot. Environm.* 628-629, 748-765.
7. Kase R, Javurkova B, Simon E. et al. (2018); *TrAC Trends in Analytical Chemistry*; 102, 343-358.
8. Drewes JE, Anderson P, Denslow N. et al (2018). SCCWRP Technical Report 1032.