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ENDOCRINE ACTIVITY IN WASTE AND RIVER WATERS FROM THE BRUSSELS REGION, BELGIUM USING THE BG1LUC4E2 CALUX BIOASSAY.

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Introduction

Today, there is international concern regarding the effects of natural and synthetic chemicals on the health of humans and wildlife since these emerging pollutants are able to interfere and act upon the hormonal system. These so-called endocrine-disrupting chemicals (EDCs) or endocrine active chemicals (EACs) are of particular concern to aquatic ecosystems, because these compounds are present in almost all wastewater and treated wastewater effluents and in rivers receiving these effluents, ground water supplies, sea water, sediment and biota, and could be of major concern for urban river systems such as the Zenne River in Brussels, Belgium [1,2].

In this respect, the European Water Framework Directive (WFD) [3], whose main objective was to obtain a good ecological and chemical status for all European water bodies by 2015, established a priority list of 33 new and 8 previously regulated chemical pollutants presenting a significant risk to or via the aquatic environment. For the priority substances, environmental quality standards were set in 2008, and they have to be monitored by all EU-Member States [3]. Several of these substances are recognized endocrine disrupting chemicals such as the penta-bromodiphenylether (PBDE), octylphenol (OP), nonylphenol (NP) and the di(2-ethylhexyl)phthalate (DEHP), and are listed as hazardous priority substances [4]. In addition, several (natural) hormones have been included in the WFD Watch List [3], among them 17ß-estradiol (E2) and ethinylestradiol (EE2).

Rather than looking at individual concentrations of these compounds using hyphenated chromatography techniques coupled to mass detection, biologically relevant and integrative approaches exist to perform a first screening basis for endocrine activity [5,6]. For this reason, an in vitro trans-activational reporter gene assay, BG1Luc4E2 cell line [7,8], was employed to carry out endocrine activity determination in both Zenne River water, WWTP (waste water treatment plant) influents and effluents and also hospital effluent from the Brussels Region.

The Zenne River itself is a small-sized river that is the receiving environment of treated wastewater from 2 major WWTPs in the Brussels area, Brussels North & South station, which are treating wastewater of more than 1 million inhabitants. However, these WWTPs were not designed to deal with all the contaminating compounds, and are not able to protect the Zenne, its ecological status and its inhabitants against occasional contamination (such as point discharges, rain events…). This is especially true for the WWTP South, which does not have tertiary and/or advanced treatment to specifically reduce micropollutant load entering the Zenne ecosystem. The same applies for the WWTP North where tertiary treatment is available, but only for the removal of nitrogen and phosphorus as micronutrients.

Specifically with regards to EACs, large inflows are provided by hospitals which release vast quantities of wastewater containing EACs such as hormones, drugs and antibiotics and other pharmaceuticals and personal care products (PCPPs) [9] along with common municipal waste [10]. As a result, the Zenne River, downstream of Brussels, is composed by more than 50% of WWTP effluents, and since there is no active treatment for removal of these compounds from the Brussels WWTP South water, nor from the WWTP North, it is of crucial importance to have an insight about the occurrence and endocrine activity associated with these substances and following their path from hospital to Zenne River water.

Materials and methods
A 12 month sampling program (January 2015 to February 2016) was carried out at 4 locations in the Zenne River (Z3, Z5, Z9, Z11 according to literature [11]) taking 1L grab samples at each location. In addition, 24h composite samples were collected the day after at the WWTP North and South for in-/effluent as well as a grab sample of the raw hospital effluent (UZ Jette). The most upstream sampling site is Z3, followed by WWTP South, Z5, WWTP North, Z9, and Z11 as most downstream [11]. Hospital wastewater is diverted to WWTP North through the sewer system. In-situ analyses of pH, temp. (°C), O2 content (mg O2/L), and conductivity (µS/cm) were recorded and logged. Samples (150-800mL) were filtered within 24hrs using a 0.7µm glass fiber filter (GF/F Sartorius) and the filtrate was passed over an Oasis HLB (6cc 200mg glass) SPE cartridge to pre-concentrate estrogen target compounds according to vendor protocol [12]. Samples were solvent exchanged to n-hexane for storage. Filters were dried to perform suspended particulate matter analysis [11].

CALUX (Chemically Activated Luciferase gene eXpression) analyses were carried out using the BG1 ERE TA cell assay (BG1Luc4E2 cell line) as described elsewhere [6–8]. Briefly, cells were maintained in alpha minimal essential medium (α-MEM) supplemented with 10% (v/v) foetal bovine serum (FBS). Media was exchanged to DMEM supplemented with L-Glutamine, Sodium Pyruvate, Penicillin-Streptomycin and charcoal stripped FBS (i.e. estrogen free media) 48 hours prior to seeding in 96-well plates (200µL, density of 40,000 cells/well). Incubator settings were set at 37°C, 85% relative humidity and 5% CO2. After an incubation time of 24hrs and cells reaching a monolayer, sample extract dilutions and E2 treatment solutions (both 1% DMSO as final concentration) were dosed in triplicate (188µL). Cells were again incubated over a 24-hour period after which lysis and measurement were performed using Luciferase assay substrate and a Glomax 96-well plate reader (both from Promega, The Netherlands). Data analysis was performed in Excel® where statistical analysis and BEQ50/EC50 quantification [13] involved fitting the 4-parameter Hill equation. BEQ (as BEQ50) values are expressed in ng EEQ (Estrogen Equivalence)/L or otherwise as ng E2-eq./L (whereby E2 is the reference ligand for this specific assay).

Results and discussion
Initial experiments conducted were aimed at optimizing and validating the method for estrogen analysis using the in vitro reporter gene assay. Solvent blanks, matrix blanks (water), and recovery tests are represented in figure 1. These tests demonstrated that selecting appropriate solvent lots and water blanks may be crucial in order to attain low background levels void of endocrine agonists. From figure 1 (left graph) it became apparent that the methanol contributed up to 100fg E2-eq./mL used. For comparison reasons, the EC50 of E2 is approx. 526fg E2/well (n:180), indicating the need for a different solvent lot that would meet the criterion of low agonist activity. A similar case is seen with blank water where some commercially available water displays high agonist activity (figure 1, left graph), even for MilliQ treated water. However, carefully designing experiments (total volume of solvent or water) during blank and recovery tests with the lowest contributing solvents allows their combined agonist activity to be $<LOD_{DMSO}$ of CALUX (approx. 64fg E2-eq./well (n:180)).

Recovery testing of E2 (figure 1 on the right) dissolved in water and extracted using Oasis HLB SPE cartridges shows excellent results (individual recoveries ranging from 86-109%) and this over an environmentally relevant level (2-16ng E2/L).

After method optimization, a 12 month sampling program was performed with 9 samples analyzed every month (4 Zenne River samples, 2 WWTP influent and effluent samples (4 in total), and 1 hospital grab sample). During a first series of tests, SPM results were analyzed (figure 2). As expected, Zenne River values show the lowest amounts of suspended particulate matter (median of 21.4mg/L, average of 34.6mg/L, and a Min-Max ranging from 2.31 to 314mg/L), followed by WWTP effluent (median of 9.93mg/L, average of 36.46 mg/L, and ranging from 3.44 to 574mg/L). Both hospital and WWTP influent yield high SPM content (respectively 252 and 329mg/L on average). The average reduction of SPM content amounts to 93% in WWTP South and up to 96% in the North plant. However, an interesting phenomenon is encountered during the 4th sampling campaign (figure 2), whereby extremely high levels of SPM were measured in the WWTP influent and effluent and location Z5 (downstream of the WWTP South). The former results are indicative of a combined sewer overflow (CSO) event [11] whereby the sewer discharge exceeds the treatment capacity of the WWTP South and untreated wastewater is diverted into the Zenne River.

Such a CSO event is also noticeable when comparing BEQ values for endocrine activity among the different sites (spatial) and sampling dates (temporal). Applying the optimized extraction protocols on the various water samples results in graph 3.

In figure 3 we can clearly observe a corresponding high BEQ of endocrine activity (~17ng E2-eq./L) coinciding with the CSO event in June 2015 at location Z9. Interestingly enough, the highest SPM content at Z5 does not yield a high BEQ suggesting that most endocrine agonists enter the Zenne river after Z5 and before Z9. Within the various Zenne locations there is also a clear trend of increasing BEQ level when moving from the upstream part (Z3) to the most downstream sampling point (Z11), see also figure 3. BEQ levels are highest in the hospital raw waste (average 137ng E2-eq./L), followed by the wastewater received by the treatment plants South (av. 88ng E2-eq./L influent and 1.5ng E2-eq./L as effluent) and North (averaging, respectively, 40 and 2.5ng E2-eq./L for in- and effluent). The latter show, respectively, removal capacities of approx. 97.5% and 93.6% resulting in a disposal of treated water that is similar in BEQ level as Zenne River water from upstream locations (Z3).

Comparison of the values observed during this study revealed that levels in the river waters were high compared to other literature where values ranged from 0.2-0.5ng E2-eq/L for surface waters and 0.4-1.0ng E2-eq./L for municipal effluents [5]. However, ranges have also been reported from 0.53 to 17.9ng EEQ/L [14] similar to our findings, with 2/3 of the samples remaining below 0.5ng E2-eq./L indicating the higher and constant load present in the Zenne River water samples during this study (all samples ≥0.7ng E2-eq./L). Under the WFD the Environmental Quality standards have been proposed at 0.4ng/L for E2 and 0.035ng/L for EE2 following chemical specific analysis [3]. Although BEQs are presented here, which take into account all endocrine active agonists along with their possible mixture effects, their levels can be considered a concern [15]. For instance, PNECs have been established at levels as low as 1ng E2/L (molecule specific) [15]. Given that our results are in vitro and endpoint-specific, with no clear-cut extrapolation to in vivo effects (pharmacokinetics, in vivo metabolism, …) and keeping in mind that BEQ values are presented here as E2 equivalents (without knowing the exact contribution of pure E2 to the BEQ result, if any), future work will thus focus on carrying out compound specific analyses to determine concentrations of E2 and EE2 and identify as well as characterize other estrogenic chemicals.

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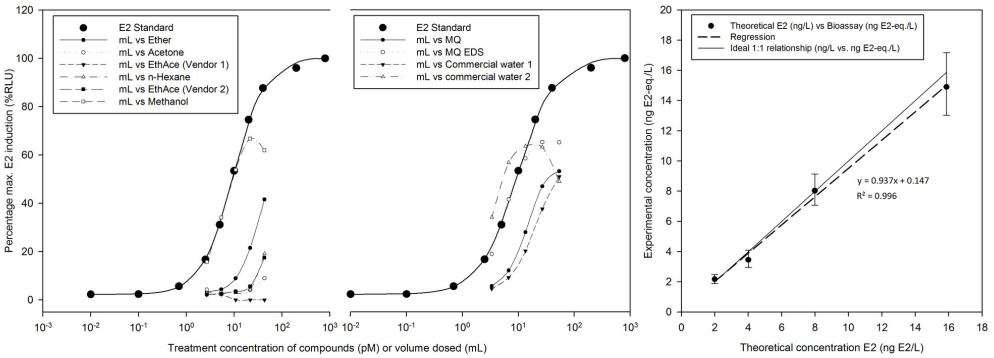

Figure 1: Agonist activity of various solvent and water blank samples (left) and recovery testing of E2 with Oasis HLB cartridges over a range of concentrations (right). Abbreviations of EthAce represent Ethyl Acetate, MQ for MilliQ water, and EDS for Endocrine Disrupters (-free) MQ water.

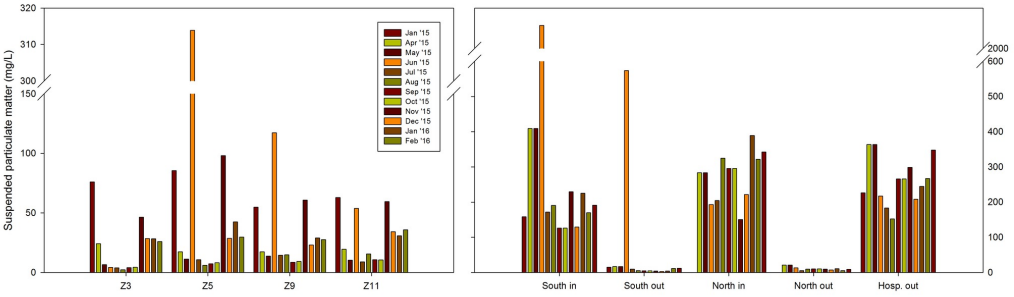

Figure 2: Suspended particulate matter (mg/L) for Zenne River grab samples (left) and WWTP and hospital samples (right).

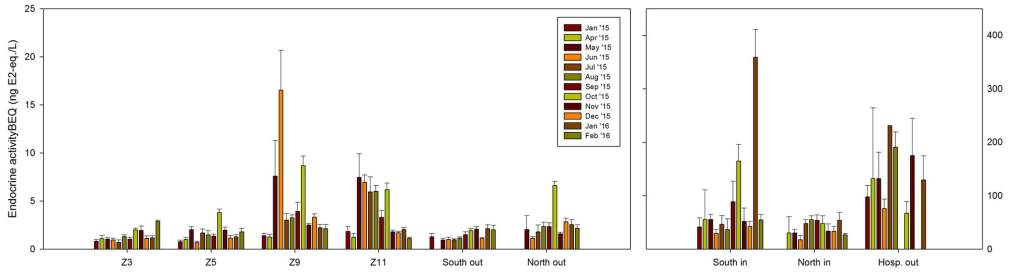

Figure 3: BEQ values (ng E2-eq./L) for Zenne river and treated effluent waters (left) and untreated wastewaters (right) accompanied with error bars (+/- 1SD).