Supplementary Materials

Dynamics of estrogenic activity in an urban river receiving wastewater effluents: effect-based measurements with CALUX.

Marc Elskens*, Kersten Van Langenhove, Vincent Carbonnel, Natacha Brion, Steven J. Eisenreich

***Correspondence to:** Prof. Marc Elskens, Analytical, Environmental and Geochemistry Group, Chemistry Department, Vrije Universiteit Brussel (VUB), Pleinlaan 2, Brussels 1050, Belgium. E-mail: marc.elskens@vub.be

ORCHID: https://orcid.org/0000-0002-8862-9422

Assessment of estrogenic activity using ERα-CALUX

After solid-phase extraction on Hydrophilic-Lipophilic-Balanced (HLB) Oasis glass cartridge (Waters), the solvent was vacuum-evaporated, and the samples re-suspended in 5mL of n-hexane (Dioxins-grade from Biosolve, Netherlands) and stored in the dark at 4 °C until analysis. The ERα-CALUX (Estrogen Responsive Elements Chemically Activated LUciferase gene eXpression) was used to assess the estrogenic activity of extracts of wastewaters (WWTP influents and effluents and Hospital effluents) and river water samples. The ERα-CALUX bioassay uses a recombinant human breast cancer (variant MCF7) cell line (VM7luc4E2), which contains a stably transfected estrogen-responsive luciferase reporter gene, and these cells respond to estrogenic chemicals with the induction of luciferase gene expression. This activation leads to the production of luciferase, with the amount of induced luciferase being directly proportional to the potency of the inducing chemical. Since VM7Luc4E2 cells do not contain any functional ERβ, the estrogenic activity measured with these cells directly results from ERα activation[1,2]. Cell culture and luciferase measurements were performed as previously described using the natural female sex hormone 17β-Estradiol (E2) as a reference standard. Briefly, VM7Luc4E2 cells were grown and maintained in α-MEM (alpha Minimum Essential Medium), supplemented with 1% pen-strep and 10% FBS (Fetal Bovine Serum). Cells were incubated at 37 °C in an atmosphere of 5% CO2 and 80% humidity. At least 48 h before dosing, the cells were transferred into DMEM (Dulbecco's Modification of Eagle's Medium), supplemented with 4.5% charcoal-stripped FBS, 2% L-

glutamine, 1% pen-strep, and 1% sodium pyruvate. The cells were collected using phenol-red free trypsin and seeded into 96-ell plates (40.000 cells/well). After incubation for 24 h, 200 µL of media containing the desired concentration of standard or water sample extract was added to wells in triplicate (final concentration 1% (v/v) DMSO in medium). After 19-22 h of incubation, the medium was removed, the wells were rinsed with phosphate-buffered saline, and the cells were visually examined by microscopy for signs of changes in morphology (indicative of toxicity) or cell death. Afterward, 50 µL of lysis reagent was added to each well, the plate was shaken for 15 min and placed in the luminometer (Tristar2S Multimode Reader, Berthold Technologies), and light output (relative light units [RLUS]) was measured after the automatic injection of 50 µL of luciferin.

Hydrometric data

Continuous hydrometric measurements (water height, velocity, and flow) were provided by Flowbru (http://www.flowbru.be/fr, July 2017) for Zenne-Paepsem [inside the city; Figure 1], Zenne-Buda (leaving the city), Woluwe-outlet region, Aa Zenne-Canal overflow, and combined sewer overflow (CSO)-Lion. Continuous hydrometric measurements (water height, velocity, and flow) were provided by Waterinfo (https://www.waterinfo.be/, 15/07/2017) at Zenne-Lot (upstream station), Zenne-Eppegem (downstream station), and at the outlet of Zuunbeek and Tangebeek, respectively, [Figure 1] to the Zenne River. Daily water volumes at the outlet of the WWTPS were provided by Aquafin (pers. Comm.) for Beersel and Grimbergen and by SBGE (Société Bruxelloise de Gestion des Eaux (pers. comm.) for Brussels-South and North. Daily CSO water volumes were provided by Aquafin (pers. Comm.) for CSO-SPLeeuw, and CSO-Grimbergen 1, 2, and 3 [Figure 1].

The consistency of water discharge data was checked for the Zenne River between Z3 and Z5, Z5 and Z9, and Z9 and Z11 by constructing hydrological budgets. For each of the three subsections below, the outlet flow (Q) was compared with the sum of all incoming contributors (rivers, WWTP, CSO, and canal diversions).

i. For Z3-Z5: QZ5 is compared to QZ3 + QZuunbeek + QCSO-SPLeeuw + QWWTP-South
ii. For Z5-Z9: QZ9 is compared to QZ5 + QAA-in - QAA-out + QCSO-Lion + QWWTP-North
iii. For Z9-Z11: QZ11 is compared to QZ9 + QWoluwe + QCSO-Grimbergen1 + QWWTP-Grimbergen + QTangebeek + QCSO-Grimbergen2 + QSiphons-Vilvoorde + QCSO-Grimbergen3.

This comparison was performed for each sampling day and all days of 2015. Differences between outflow and the sum of inflows are presented in Table SI-1. On a yearly scale, the water budget can be considered closed for each of the three river segments and thus on the entire studied river stretch (between Z3 and Z9). On a shorter timescale (daily), the variability is higher. However, an important imbalance of over 30% of the missing or excessive flow is only observed for a few flow periods: July, September, and October 2015 for the upstream stretch; May, June, and July 2015 for the central stretch; and May, June, and August 2015 for the downstream stretch.

Estrogenic activity in the surface water of the Zenne: Principal component analysis

A multivariate analysis was carried out, including the water quality parameters (pH, conductivity, dissolved oxygen, temperature, and concentration of suspended matter) and the BEQ E2 concentrations. Figure S1-1 shows the factorial planes corresponding to the first two axes of a Principal Component Analysis (PCA) bi-plot. These components account for most of the system variance, with PC1 and PC2 axes explaining 47% and 25% of the variations, respectively. The loadings of variables (vectors) in the plane express their correlations with the new principal components. Variables carrying similar information or varying in a comparable way are grouped together (such as BEQ E2 concentration and conductivity). There is a segregation of variables along the PC axes with BEQ E2 concentration, conductivity, and temperature mainly associated with PC1 and pH and SPM with PC2. The positive correlation between BEQ E2 concentration and conductivity (Pearson correlation = 0.76, $p < 0.01$) suggests that ES behave conservatively, at least in the studied river stretch, in agreement with [3]. The score plot (dots) indicates four sample clusters based on their similarity:

iv. Right upper quadrant: hospital data with SPM, pH, conductivity, and BEQ values above average;
v. Right lower quadrant: essentially downstream Z9 and Z11 stations during summer and early autumn with temperatures above average and low dissolved oxygen content;
vi. Left lower quadrant: essentially upstream Z3 and Z5 stations with BEQ, conductivity, and pH values below average;
vii. Left upper quadrant: a mix of stations in winter conditions, low temperature, and dissolved oxygen concentration above average.

Table 1. The Zenne River water budget between Z3 and Z5, Z5 and Z9, and Z9 and Z11 on the sampling days and for the entire year 2015 as computed by the difference between the output flow and the sum of all input flows, relative to the output flow. An imbalance of more than 30% is presented in bold.

date	% of QZ5 imbalance Z3-Z5	% of QZ9 imbalance Z5-Z9	% of QZ11 imbalance Z9-Z11
20-01-15	10	-8	-1
13-04-15	9	-23	28
19-05-15	15	**-37**	**32**
22-06-15	7	**35**	**-38**
27-07-15	**-55**	**37**	-18
24-08-15	-17	24	**-32**
28-09-15	**-60**	22	-18
20-10-15	**-104**	22	14
23-11-15	-2	12	13
14-12-15	10	10	2
25-01-16	14	7	-9
29-02-16	16	5	-8
2015	-2	6	0

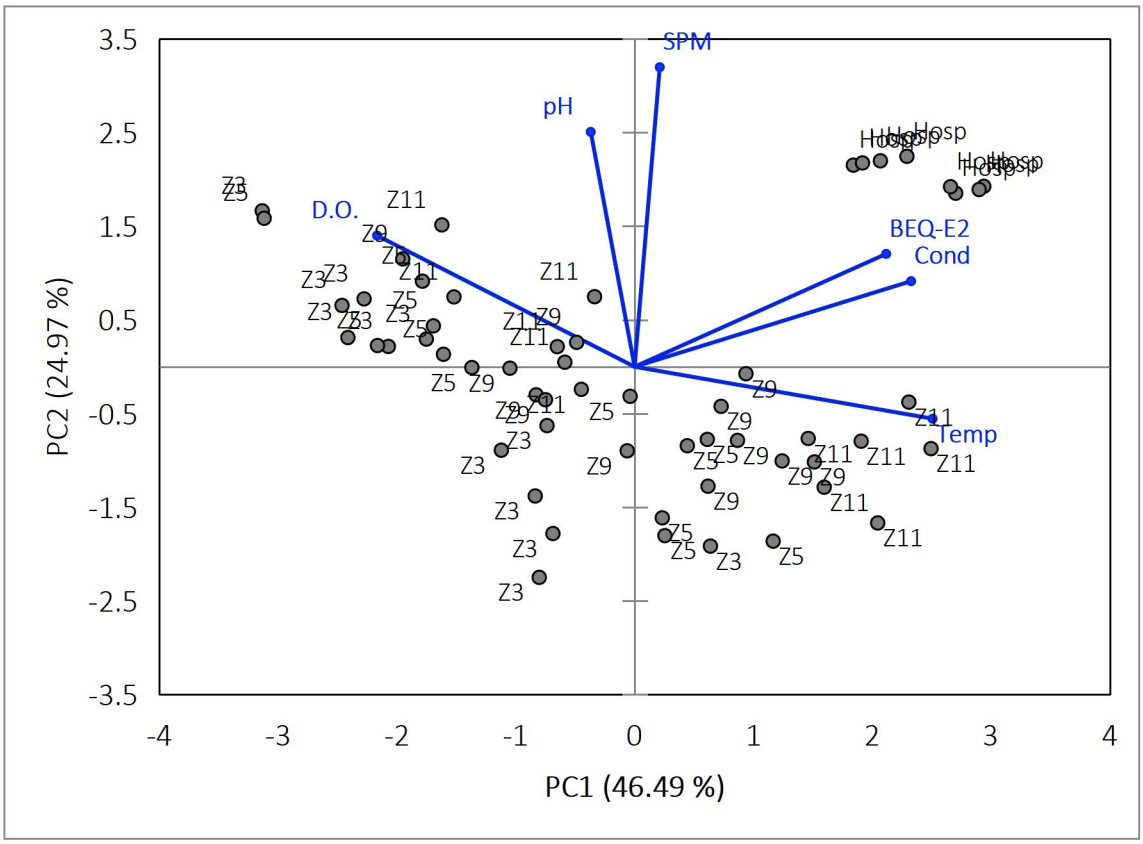

Figure 1. PCA bi-plot obtained after Varimax rotation. The factor loadings represent the correlations between the original variables and the principal component. The dots represent the sampling stations.

REFERENCES

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[2] Brennan J.C, Bassal, A., He, G., Denison, M.S. 2016. Development of a recombinant human ovarian (bg1) cell line containing estrogen receptor α and β for improved detection of estrogenic/antiestrogenic chemicals. *Environ Toxicol Chem* 35(1):91–100. DOI: 10.1002/etc.3146

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