Chemosphere 201 (2018) 540–549

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Endocrine activity in an urban river system and the biodegradation of estrogen-like endocrine disrupting chemicals through a bio-analytical approach using DRE- and ERE-CALUX bioassays

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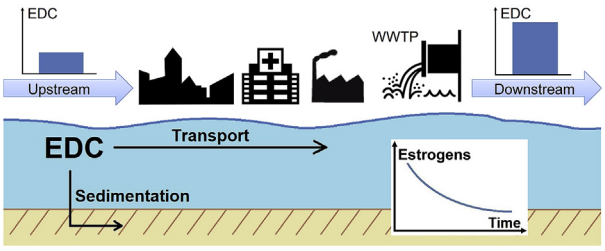
HIGHLIGHTS

- ERE-CALUX implementation on an urban river with domestic and industrial effluents.
- Sediment profiles differ significantly for dioxin- and estrogen-like compounds.
- Estrogenic compounds were found more abundantly in sediments.
- High correlations between SPM, POC, DOC and estrogenic EAS in water.
- Dose-response curves of EE2 change drastically during biodegradation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 December 2017
Received in revised form
12 February 2018
Accepted 3 March 2018
Available online 7 March 2018

Handling editor: Frederic Leusch

Keywords:
EDC
EAS
EE2
CALUX
Biodegradation
Water and sediment

ABSTRACT

The Zenne River, crossing the Brussels region (Belgium) is an extremely urbanized river impacted by both domestic and industrial effluents. The objective of this study was to monitor the occurrence and activity of Endocrine Active Substances (EAS) in river water and sediments in the framework of the Environmental Quality Standards Directive (2008/105/EC and 2013/39/EU). Activities were determined using Estrogen and Dioxin Responsive Elements (ERE and DRE) Chemical Activated Luciferase Gene Expression (CALUX) bioassays.

A potential contamination source of estrogen active compounds was identified in the river at an industrial area downstream from Brussels with a peak value of 938 pg E2 eq./L water (above the EQS of 0.4 ng/L) and 195 pg E2 eq./g sediment. Estrogens are more abundantly present in the sediments than in the dissolved phase. Principal Component Analysis (PCA) showed high correlations between Suspended Particulate Matter (SPM), Particulate (POC) and Dissolved Organic Carbon (DOC) and estrogenic EAS.

The dioxin fractions comply with previous data and all were above the United States Environmental Protection Agency (US EPA) low-level risk, with one (42 pg TCDD eq./g sediment) exceeding the high-level risk value for mammals.

The self-purifying ability of the Zenne River regarding estrogens was examined with an in vitro biodegradation experiment using the bacterial community naturally present in the river. Hill coefficient and EC_{50} values (Effective Concentration at 50%) revealed a process of biodegradation in particulate and

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https://doi.org/10.1016/j.chemosphere.2018.03.036

dissolved phase. The estrogenic activity was decreased by 80%, demonstrating the ability of self-purification of estrogenic compounds in the Zenne River.

1. Introduction

Awareness has been raised in the scientific community concerning substances called endocrine disrupting chemicals (EDCs) that interfere with hormone biosynthesis, metabolism, or action resulting in a deviation from normal homeostatic control or reproduction in human, wildlife and aquatic species (Diamanti-Kandarakis et al., 2009; Iwanowicz and Blazer, 2011; Mills and Chichester, 2005; Norris and Carr, 2006).

The group of molecules identified as estrogen-like EDCs is highly heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants and their by-products and contaminants (e.g. polychlorinated biphenyls, dioxins, furans), plastic/resin precursors (e.g. bisphenol A), plasticizers (phthalate esters and chloroparaffins), pesticides (e.g. methoxychlor, chlorpyrifos, dichlorodiphenyl-trichloroethane, vinclozolin …), pharmaceutical and personal care products (hormones, sterols, fragrances, disinfectants, antiseptics …), and flame retardants (e.g. the polybrominated biphenyls, polybrominated diphenyl ether and tetrabromobisphenol-A) (Birkett, 2002; Iwanowicz and Blazer, 2011; Norris and Carr, 2006; Roy et al., 2009). The peculiarity of EDCs is that these substances are extremely diverse and do not apparently share any structural similarity others than usually being of small molecular mass (<1000 Da), and often composed of fused aromatic rings that may contain halogen group substitutions by chlorine and bromine. This structural diversity suggests that EDCs can interact with a large number of nuclear receptors as analogues or antagonists (Diamanti-Kandarakis et al., 2009).

Water bodies currently contain thousands of chemicals about which there is little information regarding levels and occurrence since many EDCs are still unregulated and identification and characterization of those as EDCs is ongoing (Hering et al., 2010; Murray et al., 2010).

According to European Food Safety Authority (EFSA) and the World Health Organization (WHO), “Endocrine Active Substances (EAS) are chemicals that can interact or interfere with normal hormonal activity; when this leads to adverse effects they are called endocrine disruptors” (EFSA, 2013; WHO/UNEP, 2013). For samples measured with bioassays it is unclear which specific compounds are active and if they are currently known to be an endocrine disruptor. This is why they are referred to as EAS rather than EDC, expect for when the compounds measured are also identified and known as endocrine disruptors.

In the absence of regulation, EDCs and EAS are not routinely monitored, and they may or may not pose risks to organisms living in aquatic environments and to human health. For this reason, the Environmental Quality Standards Directive (2008/105/EC and 2013/39/EU) established a priority list with 33 new and 8 previously regulated chemical pollutants, some of which have shown to exhibit endocrine disrupting potential (octyl-, nonylphenol, DEHP, …). In addition, 15 compounds were also placed onto a watch list of estrogenic compounds, including 17α-ethinylestradiol (EE2) and 17 β-estradiol (E2) representing respectively the synthetic steroidal hormone from the contraceptive pills and the endogenous female sex hormone. The Annual Average Environmental Quality Standards (AA-EQS) proposed at European level for EE2 and E2 are 0.035 ng/L and 0.4 ng/L, respectively (European Commission, 2015). Dioxins and dioxin-like compounds were also listed as priority hazardous substances in this amendment (Commission of the European Communities, 2008; The European Parlament and the Council of the European Union, 2013).

Scientific questions that arise about the impact of EDCs on ecosystems and on human health are justified given their diffuse and continuous release into the aquatic environment. Despite generally low concentrations (ng/L to μg/L), the continued concern about these EDCs argues that they have pseudo-persistent characteristics (Díaz-Cruz et al., 2009; Polar, 2007). The relationship between the dose of an EDC and its effect is usually not linear and while the general mechanism of endocrine disruption by some chemicals has been established, many aspects still remain to be elucidated. For example, a low chronic dose of a chemical can sometimes lead to a higher prevalence of endocrine or reproductive abnormalities than an acute high dose. This is also known as the “low dose effect” (De Coster and van Larebeke, 2012; Vandenberg et al., 2012) giving EDCs the potential to elicit negative effects on the endocrine systems of humans and wildlife at a low dose (Diamanti-Kandarakis et al., 2009; Gregoraszczuk et al., 2008).

In order to address these important environmental issues the Zenne River, which crosses the Brussels region (Belgium), was selected as the study area because this river is a good example of an extremely urbanized river impacted by both domestic and industrial effluents (Brion et al., 2015).

The first objective was to monitor the presence and activity of EAS in the Zenne River water and river sediments. Secondly, the potential self-purifying ability of the Zenne River relative to estrogenic EDCs was examined with an in vitro experiment to assess the biodegradation of the compounds responsible for the estrogenic activity in river water by the naturally occurring bacterial communities.

Although instrumental analysis can be used to identify and quantify known specific EDCs, hazard evaluation based on chemical monitoring is complicated. Because EDCs are structurally highly diverse compounds, mixture interactions have to be taken into account and the compounds responsible for estrogenic activity are still mainly unknown (Campbell et al., 2006). Dioxin Responsive Elements (DRE-) and Estrogen Responsive Elements (ERE-) CALUX assays (Chemical Activated Luciferase Gene Expression) were developed as mechanism-based, rapid and extremely sensitive in vitro reporter gene bioassays to assess dioxin-like and estrogenic activity (Kwon et al., 2012; Rogers and Denison, 2000; Sonneveld et al., 2005). These bioassays provide useful information about the total dioxin-like and estrogenic activity of complex mixtures of chemicals in environmental samples and are thus able to account for both unknown active compounds and mixture interactions in a sample.

In this study, we used the DRE and ERE CALUX bioassays and focused on two categories of endocrine active substances.

1. EAS interacting with the Aryl hydrocarbon Receptor (AhR) such as the halogenated aromatic hydrocarbons including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs).

2. EAS functioning as estrogens in organisms such as natural hormones, pharmaceutical estrogens, phytoestrogens, surfactants, as well as other industrial compounds like bisphenol A.

To date, there are no reports measuring estrogenic activity in the Zenne River using a CALUX technique. This study also investigated whether it was feasible to measure the activities of water and sediment samples in order to include these methodologies for routine monitoring in the future.

The main source of estrogen active compounds in the aquatic environment is the result of their use by patients and the treatment of domestic and hospital waste in wastewater treatment plants (Heberer, 2002; Miège et al., 2009; Polar, 2007). This estrogenic activity includes both natural and synthetic estrogens.

2. Materials and methods

2.1. Chemicals and standards

Dimethyl sulfoxide (DMSO) (Acros, minimum 99.9%) was purchased from Thermo Fisher Scientific and used to prepare stock and standard solutions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD 99% purity) as supplied by Campro Scientific, 17β-estradiol (E2, minimum 98%) and 17α-ethynylestradiol (EE2, minimum 98%), provided by Sigma-Aldrich (Germany). Toluene, n-hexane and acetone were purchased from Biosolve (Dioxin and PCB grade, The Netherlands) and ethyl acetate from Fluka (pestanal grade, Germany). Methanol was obtained from Sigma (purge and trap grade, Belgium). Powders were obtained from various suppliers; X-CARB™ was from XDS (Durham, USA), celite and silica powder were from Merck (Germany), Na_2SO_4 from Boom (Netherlands) and H_2SO_4 from JT Baker (trace metal analysis grade supplied by Achrom). All glassware was of borosilicate grade and baked at 450 °C in a muffle furnace for at least 4 h prior to use. HLB cartridges (6 cc, 200 mg, glass) were purchased from Waters and Glass fiber filters (47 mm, 0.7 μm) from Sartorius (Germany). All media and other cell equipments were supplied by Life Science Technology (United Kingdom): Dulbecco's Modification of Eagle's Medium (DMEM without phenol red), sodium pyruvate (100 mM, sterile-filtered), alpha-MEM, penicillin-streptomycin, FBS, FBS charcoal-stripped, PBS (Ambion), L-glutamine (200 mM), and trypsin with or without phenol red. Luciferin and lysis reagent were obtained from Promega (The Netherlands).

2.2. Sampling in the Zenne River crossing the Brussels region

The Zenne River basin covers an area of 1160 km^2 with important agricultural activity in the upstream part (51% of arable land and 18% of pasture land), minor presence of forests (10%), and extreme urbanization (19%) (Shrestha et al., 2013). The study area of the Zenne River, presented in Fig. 1, encompasses about 50 km between the upstream monitoring station Lembeek (reference station for measuring distances, designated as 0 km) and the confluence with the Dijle river. The Zuunbeek and the Woluwe River, are the two main tributaries of the Zenne River with confluence located at 19 km and 35 km from Lembeek, respectively. A navigation canal, parallel to the river, is used as a buffer in case of extreme flow events. The Zenne River enters the South of Brussels city at 20 km and leaves the North of the city at 34 km from Lembeek. A combined sewer system collects sewage and runoff waters from the Brussels' urban area into the river, doubling the discharge of the river from upstream to downstream. Currently, the water collected by the sewer system is treated by two Waste Water Treatment Plants (WWTPs) but before the year 2000 it was released in the river without any treatment. The Brussels South WWTP removes solids and organic matter through a secondary treatment technology and it has been active since 2000 for 360 000 Inhabitant Equivalents (IE). The Brussels North WWTP, active since 2007, uses a modern tertiary treatment removing solids, organic matter, nitrogen and phosphorus for 1.1 million IE (Brion et al., 2015).

Estrogenic activity was monitored in both the water and sediment samples while dioxin like compounds were only analyzed in sediments because of their known high hydrophobicity.

Samples were collected during the fall of 2012 at the following sites: 3 upstream from Brussels, 1 site inside Brussels (at 39 km from Lembeek) and 3 sites downstream from Brussels area. Sediments were collected on 5 of these sites (see Fig. 1) using a bottom sediment grab sampler. The samples were well mixed and aliquots were taken for physical, chemical and microbiological analyses.

2.3. Biodegradation setup for estrogenic activity

Additional samples for biodegradation experiments were collected in the river downstream the Brussels North WWTP outlet during winter 2012, when the richness and evenness of the bacterial community composition were optimal and thus high metabolic potential was present (García-Armisen et al., 2014). To consume most of the biodegradable organic matter, naturally present in the samples, and increase the chance to see bacteria use EDCs as a carbon source, a pre-incubation step of 72 h in 5 L glass bottles was carried out. The samples were then spiked with 20 μg/L of 17α-ethinylestradiol (EE2) and incubated in the dark at 20 °C for one month. Regular sub-samples were taken at days 0, 2, 6, 9, 14, 22 and 29. Each sub-sample was analyzed for estrogenic activity in both dissolved and particulate phases. EE2 was used as a spike since this is one of the most abundant EDCs, recalcitrant to degradation in WWTP and compatible with the CALUX measurements.

2.4. Extraction and cleanup

Each water sample, stored in a glass pre-baked container at 4 C, was filtered using a glass fiber filter (47 mm diameter, 0.7 μm porosity). An aliquot of 50 mL of the filtrate was extracted with a Solid Phase Extraction (SPE) applying a HLB glass cartridge. This entailed a conditioning step with 3 mL of methyl-t-butyl ether (MTBE), followed by 3 mL methanol (MeOH) and 3 mL of ultra pure water. The sample was loaded onto the cartridge and washed with 3 mL 40% MeOH in ultra pure water, re-equilibrated with 3 mL of ultrapure water and washed again with 3 mL of 10% MeOH/2% NH_4OH in ultra pure water (PatriciaKearney and -G.E, 2007). The target compounds were eluted with 6 mL 10% MeOH/MTBE and redissolved in 5 mL hexane through a solvent exchange using purified air.

For non-spiked water samples, a 10 point sample dilution series was prepared starting with 2 mL of the hexane extract, representing 20 mL of filtered water, and using a dilution factor of two. Given the high concentration of EE2 in the spiked water samples the highest dilution point used only 0.125 mL of the hexane extract, equal to 1.25 mL of filtered spiked water. In every tube, 7 μL of DMSO was added and the hexane was evaporated using a vacuum centrifuge leaving only the DMSO containing the target compounds. The appropriate medium (693 μL) was added and the CALUX cells were exposed in triplicate to the extracts.

Sediments (10 g) and filtrate residues (or particulate phases) were extracted using an Accelerated Solvent Extractor (ASE), with the following conditions: 100 C; 1500 psi; 5 min static time; 2 cycles; 60% flush and a purge of 60 s with nitrogen gas. Hexane/acetone (1:1), (v/v) was used as extraction solvent. The solvent was changed to 10 mL of hexane for quantitative dilutions.

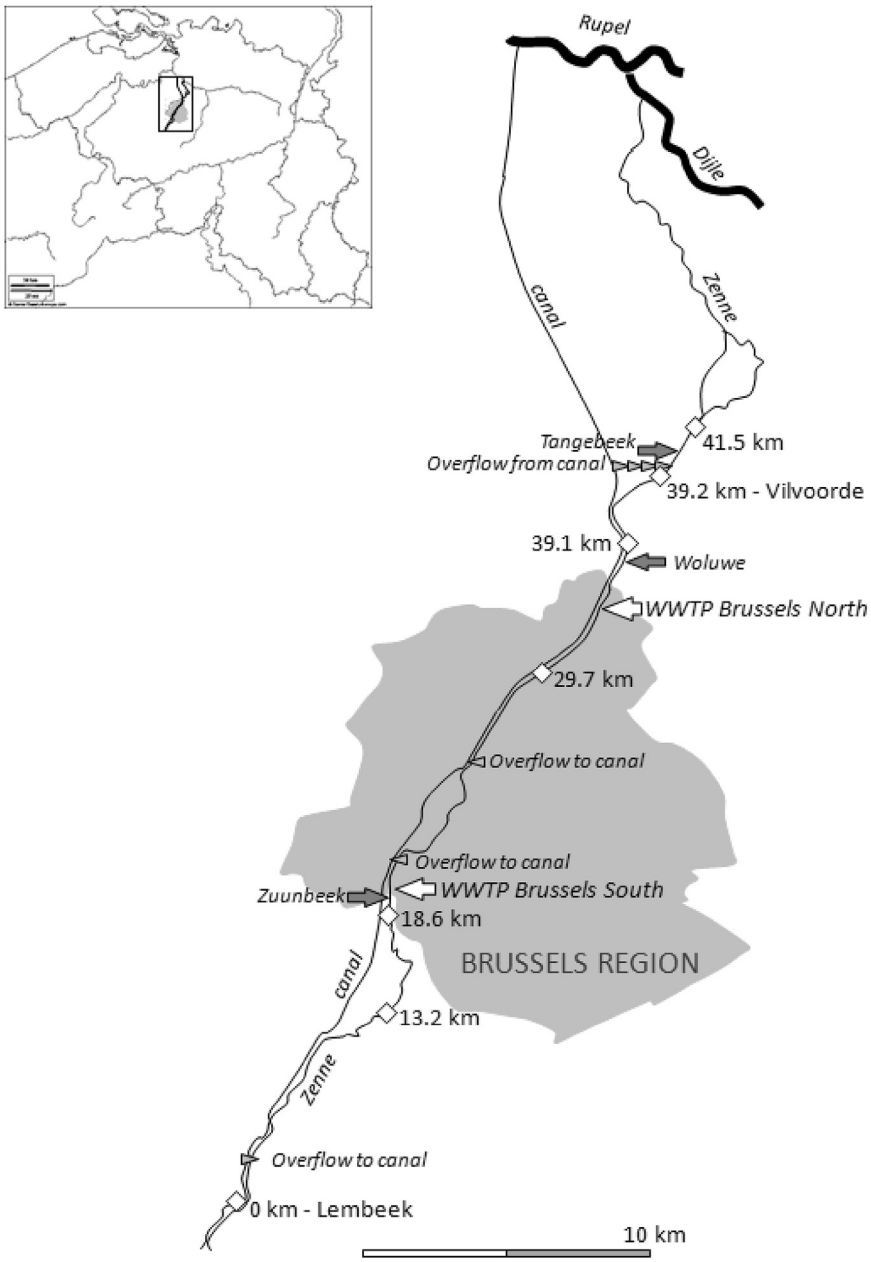

Fig. 1. The Zenne River in Belgium between Lembeek (reference station for measuring distances, designated as 0 km) and the confluence with the Dijle River with sampling stations indicated by ◇. The river crosses the Brussels city (in grey) between km 20 and km 34. In Brussels, the river has been covered for a stretch of 7 km since the nineteenth century for sanitation purposes (Brion et al., 2015).

To determine the dioxin (PCDD/Fs) and dioxin-like PCB potency of the sediment samples the ASE extracts were cleaned up on an acid silica column (33%, w/w) to remove labile interfering compounds. PCDD/Fs and dl-PCBs were differentially eluted and separated on an X-CARB column (1 cc of X-CARB) (Mihale et al., 2013). For measurement of estrogenic potency of sediments, a dilution range of the ASE extract was made starting with 0.5 mL of the hexane extract, corresponding to 0.5 g of sediment, and dilutions by a factor two for a 10 point dilution curve.

A solvent exchange takes place prior to dosing by adding 7 μL DMSO to each tube, evaporating hexane and adding the appropriate medium (693 μL). After this, the CALUX cells were exposed in triplicate.

2.5. CALUX measurements

For the semi-quantitative screening of estrogenic potencies the ERE-CALUX (Estrogen Responsive Elements Chemically Activated LUciferase gene eXpression) bioassay is used. This bioassay uses a human breast carcinoma cell line, VM7Luc4E2, stably transfected with an ER responsive luciferase reporter gene (NIEHS and (The National Institute of Environmental Health), 2016; Rogers and Denison, 2000). Cell treatments and measurements were based on the XDS LUMI-CELL® agonist protocol from 2009 (Xenobiotic Detection Systems Inc, 2009) and previously described in detail by Vandermarken et al. (2016).

Briefly, cells were maintained in alpha Minimum Essential Medium Eagle (αMEM), supplemented with 8% FBS and 0.9% Pen-

Strep, in an incubator at 37 C and 5% CO_2. The cells were transferred into an estrogen-free medium (DMEM supplemented with 4.5% charcoal stripped FBS, 1.9% L-glutamine and 0.9% Pen-Strep) 48 h before seeding in 96-well plates. Upon reaching ~80% confluence, phenol red free trypsin was used to harvest the cells and the DMEM cell suspension was diluted to 200 000 cells/mL to attain a final amount of 40 000 cells/well when seeding 200 μL in every well. After 24 h of incubation, cells attained confluence and dosed in triplicate with 190 μL DMEM containing 1% of DMSO with standard solutions of 17β-estradiol (E2) or, in the case of biodegradation, standard solutions of 17α-ethynylestradiol (EE2) and dilutions of the water or sediment extracts.

DRE-CALUX (Dioxin Responsive Elements Chemically Activated LUciferase gene eXpression) analysis of PCBs and dioxin-like compounds in the sediments (Denison et al., 2004) was carried out using a highly responsive and sensitive recombinant mouse hepatoma cell line H1L7.5c1. These cells are essentially identical to the previously described AhR-responsive CALUX cell line H1L7.5c3 (He et al., 2011), in that they are simply another clonal cell line that was isolated concurrently with the H1L7.5c3 cells from the same hepa1c1c7 cell transfection experiment with the plasmid pGudLuc7.5. Thus, both the H1L7.5c1 and H1L7.5c3 cell lines contain the exact same vector in the same mouse hepatoma cell line and we have also previously reported that H1L7.5c1 cells respond similarly to AhR agonists in a time-, concentration- and chemical-specific manner (Van Langenhove et al., 2011; Vandermarken et al., 2018). The previously described method for analysis using these cells (Mihale et al., 2013), is quite similar to the ERE-CALUX except that there is no need to eliminate phenol red from medium or trypsin.

After dosing with standards and sample dilutions, the 96-well plate was placed in the incubator for 19–24 h before further handling. After removing the medium, the wells were rinsed with 75 μL PBS buffer and the cells in each well were visually inspected under the microscope to evaluate possible toxicity of the extract. Stressed cells round up and detach from the 96-well plate causing holes in the confluent layer. Wells showing this effect are excluded from the analysis. Afterwards, 50 μL of Promega cell lysis reagent was added to each well and the plate was shaken for 5 min, placed in the luminometer and incubated for 10 min before analysis. Luciferin reagent (50 μL) was automatically injected into each well and light output was measured, in Relative Light Units (RLUs), after a lag time of 6 s and an integration time of 5 s for the ERE-CALUX and 6 s and 3 s respectively for the DRE-CALUX.

A four parameter non-linear equation was fitted to the data points (RLUs of the standard solutions or dilutions of the samples as a function of the concentrations, in amount per well) using a weighted least squares regression (Elskens et al., 2011; Gottschalk and Dunn, 2005). When plotted on a log scale, the non-linear curves follow S-shaped functions when the concentration range is wide enough. To characterize these curves, 3 geometric descriptors were used: (i) the lower and upper asymptotes (amplitude of the fold induction or efficacy), (ii) the abscissa of the mid-height point (Effective Concentration at 50% of the maximum or EC_{50}), and (iii) the slope parameter (steepness). Each of these properties is mathematically formulated in Equation (1). From the software application, explained in statistical data treatment, the background value "a" is obtained with a Standard Deviation (SD). The Limit of Detection (LOD) is defined by adding three times the SD to the background value as described previously by Elskens et al., 2011) (Elskens et al., 2011).

$$y = a+(d-a)/(1+(x/c)^b) \quad (1)$$

Equation (1): Formula of Gottschalk and Dunn, where a and d represent the lower and upper asymptotes, c is the Effective Concentration or EC_{50}, and b is the slope parameter, sometimes referred as the Hill coefficient.

The estrogenic or dioxin-like activity of each sample was expressed as a Bio-analytical EQuivalent concentration (BEQ) and calculated by dividing the Effective Concentration (EC) at 50% of the full dose-response curve's maximum (parameter k or also EC_{50}) of the standard curve by the EC_{50} of the sample curve, as indicated in Equation (2).

$$BEQ = EC_{50}(standard)/EC_{50}(sample) \quad (2)$$

Equation (2): Bio-analytical EQuivalent concentration (BEQ) expressed in pg standard (E2, EE2 or PCDD) per g sediment or per L water.

For reasons of clarity the estrogenic BEQ results were expressed in pg E2 eq./L for water samples and pg E2 eq./g for sediment samples assessed with the VM7Luc4E2 cells (ERE-CALUX), while results using the AhR-responsive H1L7.5c1 cell line (DRE-CALUX) were expressed in pg TCDD eq./g. A higher BEQ value means a higher potency of the sample and thus a higher estrogenic or dioxin-like activity.

2.6. Statistical data treatment

All statistical analyses were performed using the XLSTAT software, Version 2017.6 from Addinsoft. Principal component analysis (PCA) were used to assess the spatial distribution of estrogenic and dioxin-like compounds in surface water and sediments. The extraction procedure was based on a normalized PCA using the correlation matrix with Varimax rotation and a Kaiser normalization. The model parameters (parameter value and standard error) in the dose-response curves were estimated using a four parameter logistic model as described by Equation (1). All the statistical tests were performed at a significance level of 5% ($\alpha = 0.05$).

3. Results and discussion

3.1. Water and sediment samples

The estrogenic activity or the Bio-analytical EQuivalent concentration (BEQ) of all water samples, expressed as pg E2

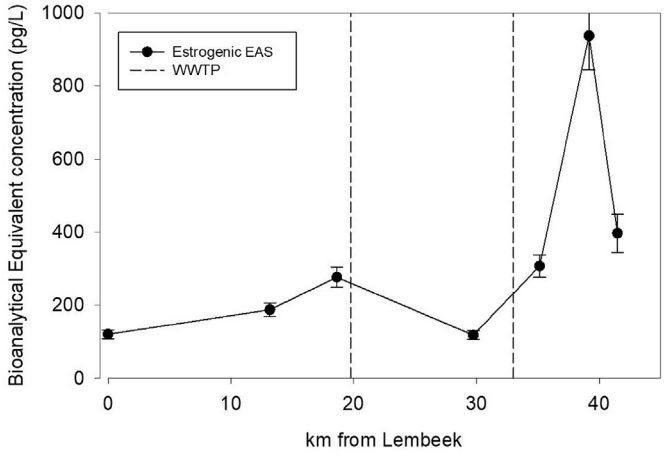

Fig. 2. Bio-analytical Equivalent (BEQ) concentration for estrogenic Endocrine Active Substances (EAS) in water of the Zenne River (Belgium) in October 2012 in pg E2 equivalents per L river water. The values represent the mean ± standard error of triplicate determinations and the locations of the Waste Water Treatment Plants (WWTPs) are presented by a dashed line.

equivalents per L, are shown in Fig. 2. The values oscillated around 200 pg E2 eq./L until km29 (inside Brussels) to increase sharply up to 938 pg E2 eq./L at km39 (Vilvoorde, an industrial area). The activity then decreased to 397 pg E2 eq./L at km41 (downstream Brussels).

These values are somewhat lower than the Flemish (Belgium) river values previously reported using an in vitro recombinant assay with yeast cells (Witters et al., 2001), although some water samples were below the detection limit of 2.75 ng E2 equivalents/L for the yeast assay. Other studies in the Netherlands (applying the in vitro reporter assay ER-CALUX based on a human T47D breast cancer cell line) and in the Swiss midland rivers (using a recombinant yeast bioassay) indicated estrogenic activity of similar magnitude (Vermeirssen et al., 2005; Vethaak et al., 2005).

When comparing these data with the proposed Environmental Quality Standard (EQS) for E2 of 0.4 ng/L, it can be noted that most samples are compliant. However, the industrial area of Vilvoorde (km39) exceeds this limit with a value of more than the double EQS value and the station more downstream Brussels is very close to the EQS level. Besides the many factories at the industrial area of Vilvoorde, a hospital also releases effluents into the Zenne River on this site.

While most data in literature deals with the occurrence of estrogenic chemicals in water, estrogenic activity was also detected in sewage sludge and sediments (Díaz-Cruz et al., 2009; Nieto et al., 2010; Thomaids et al., 2012). In particular, although steroids are relatively lipophilic, these molecules can be adsorbed on suspended solids and sediments where they can contaminate benthic organisms (Lai et al., 2000).

The measured estrogenic activity in sediment samples of the Zenne River are presented in Fig. 3, where a peak in activity (195 pg E2 eq./g sediment) was found in the industrial area of Vilvoorde (km39) corresponding to the peak in estrogenic activity in the tested water samples. When comparing the BEQ data for the dissolved phase and for the sediment phase on a weight-weight ratio, it appears that most estrogen active compounds reside in the sediment phase, rather than in the dissolved phase. BEQ ratios indicated that 200- to 1300-times more pg E2 equivalents were present per kilogram in the sediment than in the dissolved phase.

In contrast, a totally different pattern was observed for the dioxin-like compounds. The Beersel station located at km13 showed a markedly higher PCDD/F activity (42 pg TCDD eq./g sediment) compared to the other sampling stations which range from 5 to 16 pg TCDD eq./g sediment. As can be seen in Fig. 3, the potency of PCBs tended to decrease from upstream (1.0 pg TCDD eq./g sediment) to downstream of Brussels (0.6 pg TCDD eq./g sediment). A previous study states that the input of dioxin-like compounds in soil and sediments is highly related to the atmospheric dioxin and PCB emissions (Elskens et al., 2013).

Data on dioxin and especially estrogenic activities in river sediment are very scarce. Most of the available BEQ data originate from sewage sludge analysis and have been determined using the DRE-CALUX. A study on PCDD/Fs and dl-PCBs in sediments from the Scheldt mouth yielded results comparable to what was found in our study (Sanctorum et al., 2007). In another study on sewage sludge in Belgium, PCDD/F values ranged from 10 to 132 pg BEQ/g with a median of 30 pg/g (compared to15 pg TCDD eq./g in this study). Dl-PCB levels were much lower and ranged from 0.8 to 4 pg BEQ/g with a median of 1.8 pg BEQ/g (compared to 0.8 pg TCDD eq./g in this study) (Van Langenhove et al., 2012).

Two benchmarks concentrations for TCDD in sediments are used by the Environmental Protection Agency of the United States (USEPA): a low risk value of 2.5 pg/g sediment and a high risk value of 25 pg/g sediment for mammals (USEPA, 1993). All samples tested here exceeded the low risk value for PCDD/Fs and 1 sample (km 13) exceeded the high risk value with 42 pg TCDD eq./g sediment.

A Principal Component Analysis (PCA) was applied as an explanatory tool to highlight possible relations between quality parameters and endocrine activities. The PCA loading plot for the water samples (Fig. 4) displays the relationships between 10 variables, visualised in a two dimensional plane defined by the first two principal components. The first component axis explains 68% of the variation and the second component axis 21%. The coordinates of the original variables in the plane express their correlations with the new principal components. Variables carrying similar information or varying in a comparable way are grouped together, i.e., they are correlated. When negatively correlated, they are positioned on opposite sides of the plot origin, in diagonally opposed quadrants. The further away a variable lies from the origin, the stronger the influence of that variable has on the PCA model. For example, temperature (T°C), conductivity (COND), Dissolved Organic Carbon (DOC), Particulate Organic Carbon (POC) and to a lesser extent Suspended Particulate Matter (SPM) and estrogenic EAS are strongly correlated with Principal Component 1 (PC1),

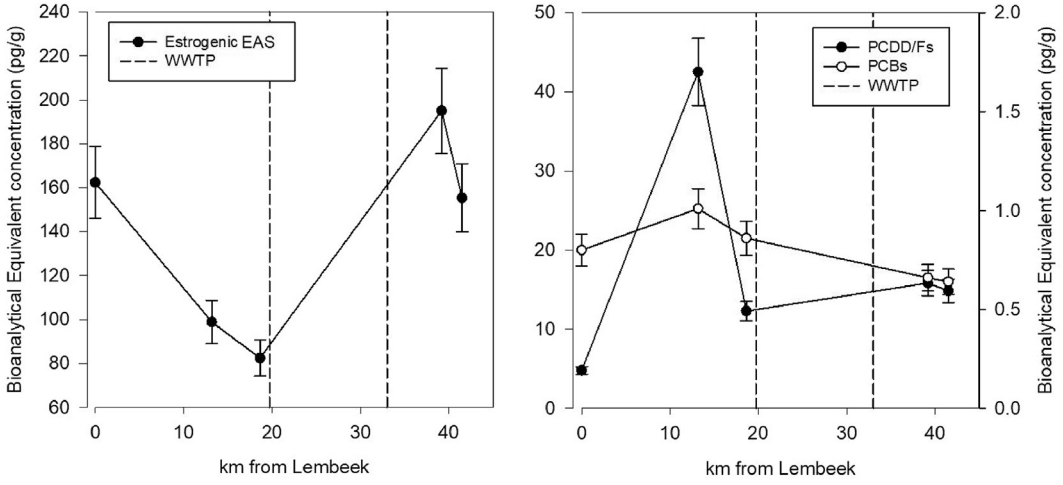

Fig. 3. Bio-analytical Equivalent (BEQ) concentration for estrogenic Endocrine Active Substances (EAS) in sediment of the Zenne River (Belgium) in October 2012 (left) and dioxin-like compounds (right). Values represent the mean ± standard error of triplicate determinations and the locations of the Waste Water Treatment Plants (WWTPs) are presented by a dashed line.

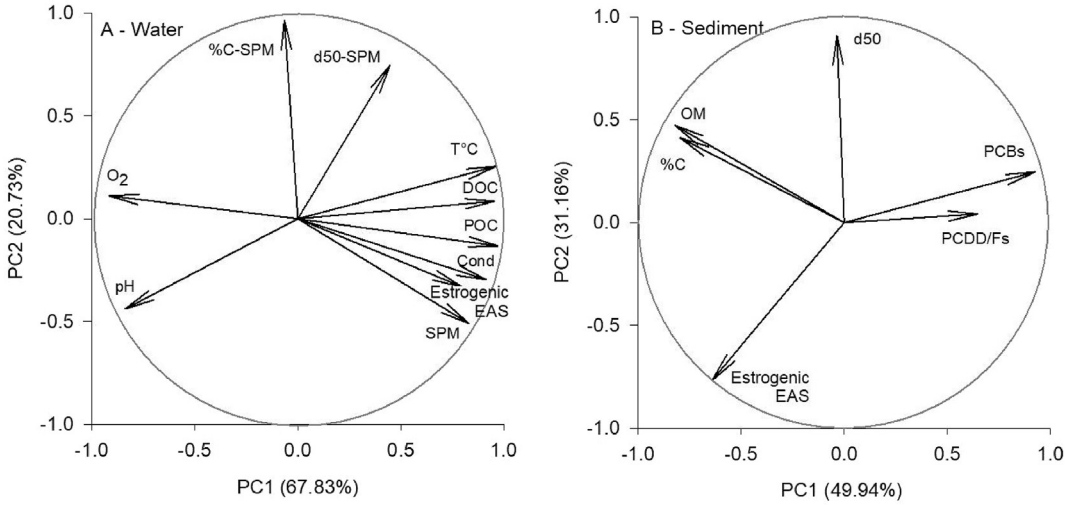

Fig. 4. PCA loading plots obtained after Varimax rotation for the whole dataset. The factor loadings represent the correlations between the original variables and the principal components. On the left the water samples (A) and on the right the sediment samples (B).

while O_2 and pH are anti-correlated. In contrast, the percentage of carbon in the SPM (%C-SPM) is mainly correlated with Principal Component 2 (PC2) and the median diameter of the particles in the SPM (d50-SPM) is in an intermediate situation. Orthogonal vectors in the loading plot indicate low correlations between variables.

For instance, it is observed that d50-SPM is not related to estrogenic EAS in water samples; neither is %C-SPM. On the other hand, COND, SPM, POC and DOC are water quality parameters of potential interest for estrogenic EAS with Pearson's correlation coefficients ranging from 0.80 to 0.87, $p < 0.05$. It is not surprising that POC and DOC show covariance with estrogenic EAS given the lipophilic nature of many of these compounds.

For sediment, the PCA loading plot (Fig. 4) indicates that dioxins like compounds (PCDD/Fs and dl-PCBs) and estrogenic EAS behave differently, since these vectors are located in opposite quadrants. PCA does not explain why they behave differently; it just explains that a different correlation is seen between both. Estrogens are mostly rather small molecules coming from urban, agricultural or domestic discharges (Diamanti-Kandarakis et al., 2009; Murray et al., 2010). Dioxin-like compounds are much larger components and originate mainly from atmospheric depositions through incineration (Elskens et al., 2013). Furthermore, none of these compounds exhibits significant correlation with the other environmental variables measured in this study, such as Organic Matter (OM), %C or the mean diameter of particles (d50).

3.2. Biodegradation by natural bacterial communities

The estrogenic activity of each subsample taken at days 0, 2, 6, 9, 22 and 29 are shown in Fig. 5, with the dissolved and particulate phase results indicated separately.

To characterize these non-linear curves, 3 geometric descriptors were used as mathematically formulated in Equation (1).

A paired *t*-test indicates that there is no significant difference in the efficacy (d - a) between the standard (EE2) and sample curves (dissolved phase $p = 0.157$; particulate phase $p = 0.098$). At most it can be noted that the background (lower asymptote) is slightly higher for the samples curves. Fig. 6 illustrates the outcome for the two other descriptors. These results clearly show that the slope parameter (b) tends to increase during the incubation and that the sample curves move to the right (i.e. the value of the EC_{50} rises significantly) leading to a lower BEQ. These observations are applicable to both the dissolved and particulate phases.

Interestingly, the Hill coefficient (b) increases from ~1 to values > 3–4. This slope parameter may provide information on the number of interacting sites at the receptor level. The theory of cooperative binding indicates that when $b \approx 1$, the affinity of the receptor for a ligand molecule is independent of whether or not other ligand molecules are present or bound (non-cooperative binding). This situation is encountered at time 0 for the sample curves and always noticed for the standard EE2 curves. When $b > 1$, the theory states that once one ligand molecule is bound to the receptor, its affinity for other ligand molecules increases (positively cooperative binding) (Weiss, 1997). These results suggested that ligand molecules other than EE2 are generated in the incubation, resulting in an apparent cooperative binding. The concomitant increase of the EC_{50} values may indicate that the newly generated compounds have a lower affinity for the receptor than EE2 or there has been a process of degradation with these two possibilities being not mutually exclusive.

The consequence is a decrease of the estrogenic activity over time of about 80% when compared to the starting value for the BEQ. This trend is very similar in the dissolved and particulate phases (Fig. 7).

Various studies have examined the role of bacterial degradation of estrogenic steroids in environments under aerobic and anaerobic conditions (Polar, 2007; Snyder et al., 2003). Some of these compounds are easily removed and degraded during sewage treatment, mainly by adsorption and biodegradation, with the chemical structure (polar vs. non-polar compounds, acidic or basic functional groups) playing a leading role in determining the efficiency of removal. Overall sludge activated treatments apparently reduce significantly the influent concentrations of hormones, but conjugation reactions, oxidation of metabolites and regeneration of the parent compounds in the effluent are still possible (Miège et al., 2009). These statements are fully consistent with the results obtained in our study.

Furthermore, a mean dissolved EE2 concentration of 4 ng/L in the influent of the WWTP and a mean dissolved concentration of 0.8 ng/L in the effluent was observed, reflecting a reduction of 80% in the EE2 concentration (Miège et al., 2009). Miège and coworkers also reported that triclosan, norfloxacin, 17β-estradiol and estriol

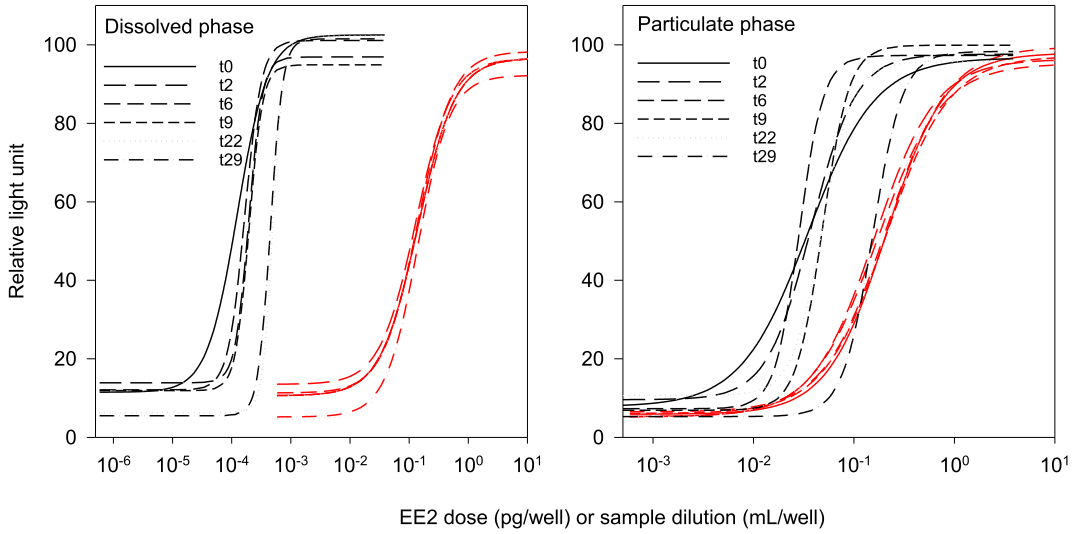

Fig. 5. Full dose-response curve of subsamples taken at days 0, 2, 6, 9, 22 and 29 of the incubation. The concentration of EE2 (red) or the concentration of the subsample (black) is plotted on a log scale against the light response in relative light unit, where the highest output is placed at 100%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

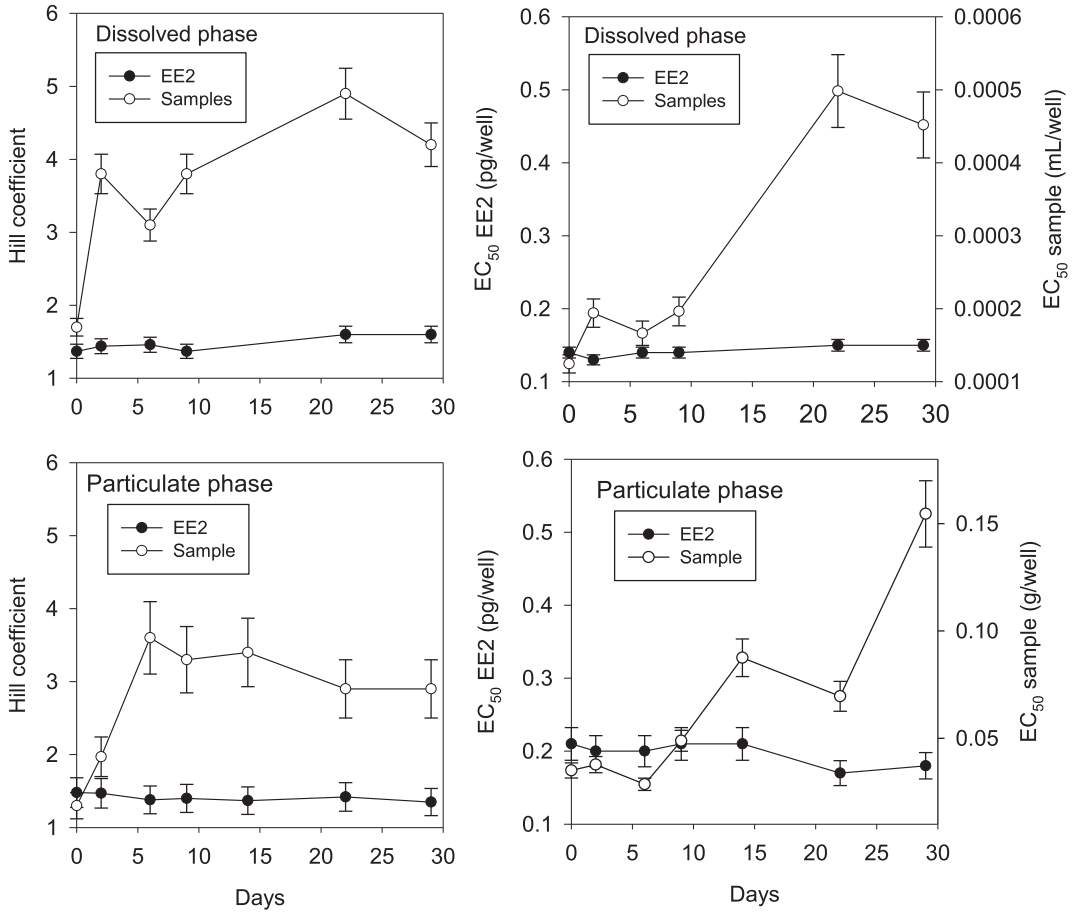

Fig. 6. Time evolution of slope parameter (Hill Coefficient) and effective concentration EC_{50} for samples and EE2 curves in the dissolved and particulate phases. Values are expressed as the mean ± standard error of triplicate determinations.

were denoted as highly removed contaminants when Pharmaceuticals and Personal Care Products (PPCPs) in the dissolved phase were reduced by more than 80% (Miège et al., 2009).

Our in vitro results show a similar reduction in estrogenic activity in both the dissolved and particulate phase demonstrating the self-purifying ability of the Zenne River, relative to estrogenic EDCs, by the naturally present bacterial communities.

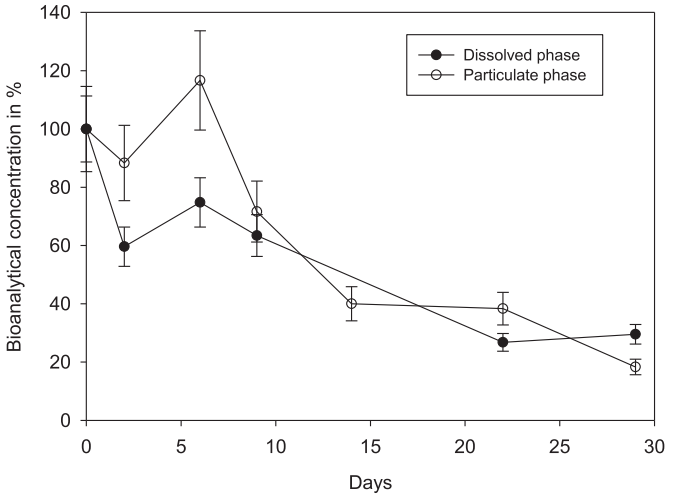

Fig. 7. Time evolution during the biodegradation experiment of the Bio-analytical Equivalent (BEQ) concentration where the concentration at day 0 is put at 100% and values are expressed as the mean ± standard error of triplicate determinations.

4. Conclusions

In this study, the implementation of the ERE-CALUX (Estrogen Responsive Elements Chemically Activated LUciferase gene eXpression) bioassay for the analysis of water and sediment was successful when used on samples from an urban river. Our results showed that it could be a useful tool to include in routine monitoring in the future.

Most water samples (values oscillating around 0.2 ng E2 eq./L) were compliant to the proposed Environmental Quality Standard (EQS) of 0.4 ng/L, but one sample exceeded this value with 0.938 ng E2 eq./L near the industrial area of Vilvoorde (km39). A corresponding peak in estrogenic activity was found in the sediments of the same location with a value of 0.195 ng E2 eq./g sediment. Further investigation is necessary to see if there is an active source of estrogenic EAS just upstream this site.

In the studied urban river, estrogenic EAS were found more abundantly in the sediments than in the dissolved phase when looking at the weight ratios of the measured activities. The principal component analysis showed that water quality parameters such as Suspended Particulate Matter (SPM), Particulate Organic Carbon (POC) and Dissolved Organic Carbon (DOC) were highly correlated with estrogenic EAS activities with Pearson's correlation coefficients ranging from 0.80 to 0.87 ($p < 0.05$). It will be further investigated if these water quality parameters, due to the lipophilic nature of the investigated compounds, can be used as predictor variables or proxies for estrogenic activities in river water.

A totally different pattern was observed for the dioxin-like compounds. For both the PCDD/Fs and the dl-PCBs fractions, the median results comply with what has been previously reported in the Scheldt mouth and sewage sludge data (Sanctorum et al., 2007; Van Langenhove et al., 2012). All samples were above the low-level risk value for TCDD in sediments set by the Environmental Protection Agency of the United States (USEPA). One sample (km 13) even exceeded the high-level risk value for mammals of 25 pg/g sediment with a PCDD/F potency of 42 pg TCDD eq./g sediment.

PCA is applied as an explanatory tool to see possible correlations between quality parameters and endocrine activities. Through the loading plot it became clear that, for sediments, dioxin-like compounds and estrogenic EAS behave differently. This is not surprising since they both originate from different sources and their molecule size is quite different. No other measured environmental factors exhibited a significant correlation with the dioxin- or estrogen-like compounds. Although interpretations have to be handled with care, due to a limited data set, our results show that combining an ERE and DRE-CALUX with a principal component analysis can be a powerful future investigation strategy.

From the in vitro biodegradation experiment with the natural bacterial communities of the Zenne River, we noticed some remarkable changes. First, results suggested that ligand molecules other than EE2 were produced during the incubation, leading to an apparent cooperative binding. Secondly, the EC_{50} values increase, possibly indicating that new generated compounds have a lower affinity for the receptor than EE2 and/or that there has been a process of degradation.

These results are fully consistent with those that have been previously reported in literature concerning the role of bacterial communities in the degradation of estrogenic steroids in the environment (Polar, 2007; Snyder et al., 2003). The decrease of estrogenic activity by 80% in the dissolved and particulate phase is comparable to the decrease in EE2 concentration found by a molecule specific study (Miège et al., 2009). This demonstrates the possibility of self-purification of estrogenic EDCs by the Zenne River and the power of using bioassay analysis to evaluate this phenomenon.

Acknowledgements

This work was realized in the framework of the research project “GESZ - Towards the Good Ecological Status of river Zenne” with the financial support of INNOVIRIS (Brussels) through the IMPULSE-Environment 2009 and 2012 initiatives. We thank Professor M. Verbanck and Professor M. Chen for providing part of the physicochemical dataset.

The VM7Luc4E2 and H1L7.5 cell lines were developed by the University of California-Davis (USA) with funding from a Superfund Research Program grant (ES04699) from the National Institute of Environmental Health Sciences.

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