

Determination of Estrogen Activity in River Waters and Wastewater in Luxembourg by Chemical Analysis and the Yeast Estrogen Screen Assay

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Abstract

In many cases, only selected well-known target chemicals are analysed and used for a comparison with biological effects. Predicting the environmental impact of different chemical compounds does often fail. Effects are the result of mixture toxicity of single chemicals and their degraded products, which can have different biological potency and bioavailability. In vitro toxicity or mechanism-based assays are used as screening tools, prior to extended evaluation in animals or natural populations, or even prior to chemical monitoring. This study illustrates the use of the yeast bioassay to investigate the presence of compounds or chemicals with estrogenic activity in wastewater and surface water in Luxembourg and compares results with chemical measurements. Although the approach described in this paper has already been published in many case studies before, it confirms earlier findings and it delivers results for Luxembourg where similar analyses have not been documented so far. By comparison of the biological signal in the yeast assay, expressed as estrogen equivalents, with available results by liquid chromatography–mass spectrometry for steroid hormones we could only obtain a similar ranking for the majority of samples with low or high estrogenic activity. Measurements can therefore only be used as a screening tool for estrogenic activity. Seasonal changes as apparent for chemical results in surface waters were confirmed in the yeast assay. Dissolved estrone is diluted by higher discharge in the rivers during winter containing larger amounts of unpolluted soil water and groundwater runoff.

Keywords: endocrine disruptors, Luxembourg, steroid hormones, Yeast Estrogen Screen assay

1. Introduction

Since the last decade the growing availability of scientific data (Eertmans et al., 2003; Uzumcu et al., 2007; Meeker, 2010) on endocrine disrupting compounds (EDCs) has clearly demonstrated that their presence produces adverse effects in wildlife and humans, for example an influence on reproductive and sexual development and function. These anthropogenic or natural EDCs mimic the action of estrogens, androgens, and progestagens or of other hormones and can affect the endocrine system. Such effects depend on the level, the

duration and on the timing of exposure of the population. It was demonstrated that the maximum effect occurs in the period of “programming” the endocrine system, during the fetal development and childhood (The International Programme on Chemical Safety, 2002).

In order to assess environmental impact of chemicals distributed through industrial discharges, sewage water treatment plants or diffuse pollution extensive chemical monitoring is performed. These chemical monitoring programs are increasingly accompanied by a parallel monitoring of the ecological state of river systems (Vethaak et al., 2005; Jobling et al., 2005; Sanfilippo et al., 2010). However, analysis of selected chemicals does seldom allow to directly link the measured concentrations of chemicals to observed biological effects. In most cases, only a limited number of target chemicals are monitored. Moreover, prediction of toxicity and impact on populations do generally fail as effects are the result of “mixture” toxicity of single chemicals and their degraded products, which can have different biological potency (or activity) and bioavailability.

With respect to the evaluation of effects of chemicals, ecotoxicological tests with relevant organisms looking for acute or chronic effects can be used (Van den Belt et al., 2003). In addition to animal tests, as part of a tiered testing strategy (OECD 2003) *in vitro* tests that detect mechanism-based effects such as hormone receptor binding are used in first steps of hazard assessment. This approach of using mechanism-based *in vitro* tests for detection of estrogenic activity, genotoxicity and dioxin-like activity in complex environmental matrices, such as in surface waters, sediments or air samples has gained attention (Christiaens et al., 2005; Houtman et al., 2006; Cavanagh et al., 2009). Due to their ease of use, low cost and predictive value, *in vitro* assays are used as screening tools, prior to extended evaluation with *in vivo* assays (fish, invertebrates), or even prior to chemical monitoring and target analysis.

In a screening set up, *in vitro* test systems might give a first indication of the presence of different compounds in the environment and the biological activity of chemicals present in the mixture. This *in vitro* approach is available for detecting chemicals with (anti-)estrogen or (anti-)androgen like activity and has regularly been applied for environmental monitoring (Witters et al., 2001; Christiaens et al., 2005; Vethaak et al., 2006; Jobling et al., 2009). Comparison of transformed cell lines with reporter systems linked to human estrogen receptors (hERs), such as yeast cells or mammalian breast cancer cells has shown the robustness of the Yeast Estrogen Screen (YES) assay for complex environmental samples (Witters et al., 2001; Murk et al., 2002; Witters et al., 2003). However, hER-transformed assays based on human cells, e.g. MVLN, MELN or ER-Calux have shown more sensitivity than the yeast assay (Murk et al., 2002; Van Den Belt et al., 2004), which might be of interest to detect low estrogenic activity as expected by drinking water resources, rainwater, or groundwater.

This paper describes the analysis of three hormones (estrone, estradiol and ethinylestradiol) in wastewater treatment plant (WWTP) effluents and in river water sampled in Luxembourg throughout different seasons and analysed by LC/MS-MS and YES assay. LC/MS-MS quantified the amount of hormones compared to the YES assay that evaluates the estrogenic activity of the mixture. It is our goal to search for a potential seasonality of the estrogenic activities in local surface waters and to compare the data between the LC/MS-MS and YES assay to confirm the potential estrogenic activity in the sample extracts.

2. Materials and Methods

2.1 Sample Locations

The investigations are conducted in the Mess and the Pétrusse catchments in southern Luxembourg (Figure 1). The Mess basin has a total drainage area of 32.5 km² (measurement points 4 & 6). Land use in the basin consists of arable land (23 %) and grassland (58 %). Forest represents about 10 %. Urban areas amount to 6 % and the rail and road network is about 3 %. Surface runoff from different roads, untreated wastewaters from farms, and storm drainages of the combined sewer system influence river water quality. In the village of Reckange (outflow: measurement point 1) a mechanical-biological sewage water treatment plant is located, 3 500 inhabitants are connected with this purification plant. The villages are drained by combined sewage water systems including several storm-control reservoirs. Measurement point 6 is the outflow of the Mess basin at Pontpierre. In addition, samples are taken 50 meters downstream of point 6 (measurement point 4) because several illicit inflows were observed between the two locations. The river Pétrusse has a drainage area of 44 km². The covering by urbanized area amounts to 4.4 % road and rail network and 16.5 % urban and industrial areas. In the western part, a rural environment surrounds the stream, whereas in its lower part, the Pétrusse is actually a canalized watercourse, which flows through the intensively developed city of Luxembourg. Water quality is impacted by stormwater runoff because of harmful concentrations of substances from road surfaces and rooftops (Krein et al., 2007). Some major roadways of Luxembourg City drain into the watercourse leading to elevated contaminant load of heavy metals and organic pollutants. Measurement point 5 is the outflow of the Pétrusse catchment in the city of

Luxembourg. Two small sewage water treatment plants with less than 1 000 person equivalents are located in the Pétrusse catchment; only the sewage water treatment plant of Roedgen - consisting of a simple mechanical treatment (outflow: measurement point 2) - is monitored. Measurement point 3 is the effluent of the WWTP in Schiffingen, a modern plant with 90 000 person equivalents, not located in one of the catchments under investigation (Figure 1).

During dry weather, nineteen surface water samples have been taken by hand in one-liter glass bottles (brown colored) between September 2009 and November 2010. Furthermore, during September 2009 and November 2010, 36 samples were taken at the outflow of the local WWTPs of Reckange (1), Roedgen (2) and Schifflange (3). A selection of samples was used to conduct the yeast assays.

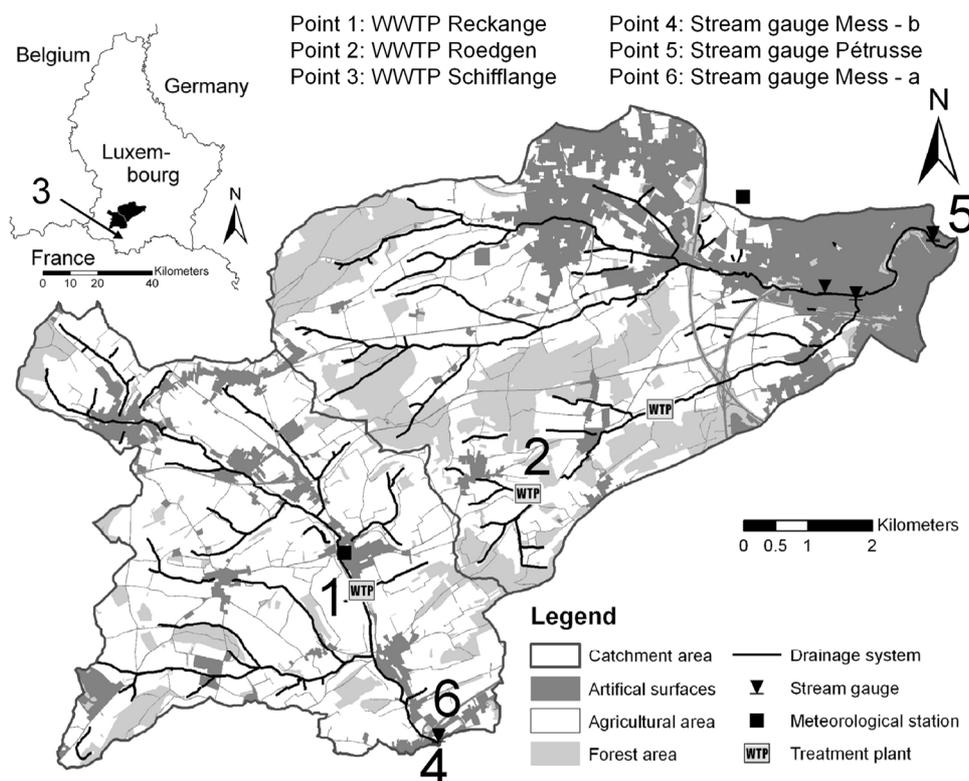


Figure 1. Localisation of sampling points at surface waters and at WWTP effluents; Measurement point 3 is the effluent of the sewage treatment plant in Schifflange not located in one of the catchments under investigation (please refer to small general map)

2.2 Chemicals

Analytical grade calibration standards for estrone (E1), estrone-d4 (E1-d4), β -estradiol (E2), 17- α -ethinylestradiol (EE2) were purchased (LGC Promochem, Molsheim, France), solvents and additives were purchased from Biosolve (Valkenswaard, The Netherlands), acetonitrile, methanol, ammonium acetate, formic acid, dimethyl sulfoxide from Sigma-Aldrich (Steinheim, Germany), Na₂-EDTA (ethylene diamine tetraacetic acid) from Biowhittaker (Maryland, USA) and sulfuric acid from Merck (Darmstadt, Germany). Ultra-pure water was prepared with a Milli-Q water purification system (Millipore, Bedford, USA).

2.3 Preparation of Samples and Extraction Method

Water samples were collected in 1-liter amber glass bottles and filtered through glass fiber filters (mesh size 3 μ m, Pall Corporation, Ann Arbor, USA) to eliminate the suspended matter and then filtered through cellulose acetate filters (mesh size 0.45 μ m, Sartorius, Göttingen, Germany). Afterwards, the one-liter filtrates were acidified (pH 4) with diluted sulfuric acid solution (25 %). Before storing the samples at 4°C until extraction, 3 ml of Na₂-EDTA 0.5 M were added. Estrogens were extracted by automated solid-phase extraction (SPE) using an Autotrace SPE workstation (Caliper, Teralfene, Belgium). Oasis[®] HLB (Waters, Milford, USA) was the sorbent used to concentrate all the analytes. 500 ml of surface water or 250 ml of wastewater samples were

loaded on 200 mg - 6 ml hydrophilic-lipophilic balanced SPE cartridges at a flow rate of 10 ml min⁻¹. Prior to that, the sorbents had been conditioned with 5 ml of methanol and 5 ml of water (pH 4). After loading, the cartridges were rinsed with 5 ml of a MeOH/Water mixture (5/95 v/v) and dried with a N₂ stream for 15 min. The selected compounds were eluted using methanol (2 x 5 ml). Extracts were concentrated to dryness with a stream of N₂ and redissolved in 1 ml of a water/methanol (80/20 v/v) mixture before LC-MS/MS analysis or substituted into 50 µl of 100 % DMSO for application in the YES assay.

2.4 Chemical Analysis by LC/MS-MS

Chromatography has been conducted with an Ultimate 3000 Intelligent LC (Dionex, Sunnyvale, USA) with a high-pressure gradient pump HPG-3200, automatic injector WPS-3000, and a column oven TCC-3100. The chromatographic column consisted of a ZORBAX Eclipse Plus C-18, 150 x 2.1 mm internal diameter, 3.5 µm particle size with a guard column of 12.5 x 2.1 internal diameter, 5 µm particle size (Agilent, Germany). Estrogens (estrone, β-estradiol and 17-α-ethinylestradiol) were analysed in negative electrospray ionization mode (-ESI). For chromatography, solvent X and solvent Y consisted of water containing 10 mM ammonium acetate and acetonitrile, respectively. The gradient started with 20 % of Y during the first minute (80% X), increased to 70 % at 15 minutes, to 95 % at 16 minutes, returned to its initial composition within 1 minute and equilibrated during 3 minutes for a total run time of 20 minutes. The mobile phase flow rate was 0.25 ml min⁻¹ and the column was kept at 40°C. The injection volume was 25 µl and all molecules were eluted within 13 minutes. The MS-MS analyzer consisted of a triple quadrupole mass spectrometer API 3200 (Applied Biosystem/MDS Sciex, Rotterdam, The Netherlands) equipped with an ESI interface using the Analyst 1.4.1 software (Applied Biosystems). The capillary voltage was set at -4500 V. N₂ was used as nebulizer gas (GS1 = 55 psi and GS2 = 40 psi), curtain gas at 10 psi, collision gas at 5 psi, and the desolvation temperature was fixed at 350°C. The mass spectrometer was running in Selected Reaction Monitoring mode (SRM) for an increased sensitivity, with two SRM transitions for each molecule to improve the selectivity (except for the internal standard where a single transition is sufficient). Optimal compound-dependant parameters were chosen for the detection. Table 1 summarizes, for each analyte, the precursor ion used, the quantifier and the qualifier product ions, the corresponding collision energy in V (between brackets) and the retention time (RT). The performance of the SPE-LC/MS-MS method, investigated in our previous work (Pailler et al., 2009) is improved by the addition of a deuterated internal standard E1-d4 and briefly summarized as follows: for qualitative purposes, the combination of the retention time and the selection of two specific fragments resulted in successful determination of each hormone, the blank samples did not react positively. Regarding quantification, calibration curves were linear in the range 1-100 ng ml⁻¹ with r² > 0.998 and recoveries for hormones were better than 80 % over a 5 months period. The method shows a limit of detection (LOD) of 0.5, 1 and 2 ng l⁻¹ and a limit of quantification (LOQ) of 1, 3 and 6 ng l⁻¹ for E1, E2 and EE2, respectively (Meyer et al., 2011).

Table 1. Selected Reaction Monitoring transitions of target compounds: precursor, quantifier product, qualifier product, collision energy in V (between brackets), and retention time (RT)

Analyte	m/z Precursor	m/z Quantifier	m/z Qualifier	RT (min)
E1	269.1	145.1 (-50)	143.1 (-74)	12.03
E2	271.1	145.2 (-52)	143.1 (-70)	10.72
EE2	295.1	145.0 (-56)	143.1 (-74)	11.70
E1-d4	273.1	147.2 (-50)	145.0 (-74)	12.00

2.5 The Yeast Estrogen Screen (YES assay)

A screening assay consisting of an estrogen-inducible expression system in yeast has been used. The Genetics department at Glaxo (U.K.) developed the *Saccharomyces cerevisiae* yeast species. The screen has been validated and shows high specificity for estrogen-like compounds. Androgens, progesterone, and corticosteroids are completely inactive, unless high concentrations are present which show weak activity (Routledge and Sumpter 1996; Harris et al., 1997). The DNA sequence of the human estrogen receptor (hER) is integrated into the yeast genome, also containing expression plasmids carrying estrogen-responsive sequences (ERE) controlling the expression of the reporter gene lac-Z. Upon binding an active ligand, the estrogen-occupied receptor modulates gene transcription, the reporter gene lac-Z is expressed producing the enzyme β-galactosidase, secreting into the medium. It then metabolises the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), from normally yellow into a red product, which can be measured by

absorbance. Brunel University, laboratory of J. Sumpter, kindly provided yeast cells. Routledge and Sumpter (1996) give more details on maintenance of the yeast strain, preparation of medium components and on the general test protocol. The entire medium components were purchased from Sigma Aldrich.

The 50- μ l sample extracts in DMSO prepared from surface waters (500 ml) or WWTP effluents (250 ml) were further diluted in DMSO (Labscan) with a maximum concentration in the yeast assay medium of 0.5 %. Taking into account the concentration factor of the water sample by SPE (10 000 x for surface waters, 5 000 x for WWTP effluents) and a 1:200 dilution of the extract in medium, the highest test concentration was 10 ml equivalent/well. The YES-assay has been performed as described by Witters et al. (2001). In each series of experiments one plate with a serial dilution (1:2) of 17 β -estradiol in DMSO (0.5 %) in a concentration range of $2 * 10^{-9}$ M (108.96 pg/well) to $1.56 * 10^{-1}$ M (0.87 pg/well) was included to get a standard curve. On this same plate, one row of 6 wells was provided for the procedural control or extraction blank applied at 10 ml equivalent/well. DMSO blanks of the CRP-Gabriel Lippmann and VITO have been tested on the same plate, as well as the procedural extraction blank, in comparison with the solvent control.

The standard curve for estradiol was fitted (sigmoidal function) using Graphpad Prism (version 5, 2007), which allowed calculating EC50 values and 95 % confidence limits. The detection limit of the yeast assay for the E2-standard was calculated as absorbance elicited by the solvent control plus three times the standard deviation, and ranged between 2.58 and 3.12 ng l⁻¹ E2. In order to determine estradiol equivalents (E2-equivalents) as a measure of total estrogenic activity in water samples, the absorbance of the sample extracts (calculated as percent of solvent control) was interpolated in the linear range of the corresponding estradiol standard curve. This allowed converting the percent induction for sample extracts into a concentration of estrogenic activity, which is similar to the observed signal for E2 at the standard curve. The sample concentration factor and dilution in well plates were taken into account and the result was then expressed as an amount of estrogenic activity of ng l⁻¹ E2 equivalents. Considering the concentration factor and dilution of sample extracts in the test medium, the limit of detection (LOD) in extracts was 0.06 ng l⁻¹ E2 equivalents for surface water and 0.12 ng l⁻¹ E2 equivalents for WWTP effluents, while the lowest limit of quantification (LLOQ) was 0.34 ng l⁻¹ E2 equivalents for surface water and 0.68 ng l⁻¹ E2 equivalents for WWTP effluents.

3. Results and Discussion

3.1 Results from YES Assay and Chemical Analysis

In 6 of 66 samples, estrogenic activity was below the limit of detection (LOD; 3 samples in sampling campaign December 1st 2009 and 3 samples in sampling campaign December 8th 2009). For all other sample extracts, estrogenic activity was present above detection limit. For another 6 sample extracts a low signal was seen, which could not be quantified (< LLOQ). For most of the samples, clear concentration response curves were obtained in the YES assay, which did allow calculating the estrogenic activity in the samples (Figure 2). Only for one sample (June 29th 2010 – Roedgen WWTP), slight toxicity to the yeast culture was observed. As this sample is the effluent of a WWTP where only mechanical treatment of the influent was performed, the presence of toxic compounds is not unexpected.

For estrogenic activity in the surface water, the overall view showed lower concentrations in winter and increasing estrogenic activity in spring and summer. This observation is an indication for seasonal influences on estrogenic activity, which is in line with season-dependent changes of E1 concentrations shown in Figure 3. During the two sampling campaigns in December 2009 and during one sampling campaign in January 2010, the measured values for estrogenic activity were below LOD or below LLOQ, while levels quantified in surface waters sampled 5 January 2010 were all low (< 1 ng l⁻¹ E2 eq.). Subsequent sampling of surface waters during springtime (March and April, 2010) showed moderate levels of estrogenic activity (0.63 - 2.93 ng l⁻¹ E2 equivalents). Higher values of estrogenic activity up to 20.77 ng l⁻¹ E2 equivalents were present in surface water samples taken during 4 campaigns in the months May and June 2010. It was observed that the estrogenic activity of the surface water samples taken at point 4 and 6 (Mess at Pontpierre) were rather comparable and higher than the surface water samples taken at point 5 (Pétrusse). The comparable values for points 4 and 6 can be explained due to the fact that the points are located on the same river. The distance between both points is only 50 meters. Between points 6 and 4 several illicit inflows are known, but since the estrogenic activity at both sampling points is rather comparable, these inflows appear to have no additional contribution to the measured estrogenic activity downstream the river (point 4). Both sampling points 6 and 4 do receive the effluent of the WWTP of Reckange (point 1), which could explain the slightly higher values measured for sampling points 4 and 6, compared to the other surface water samples at point 5, a distinct location in another catchment.

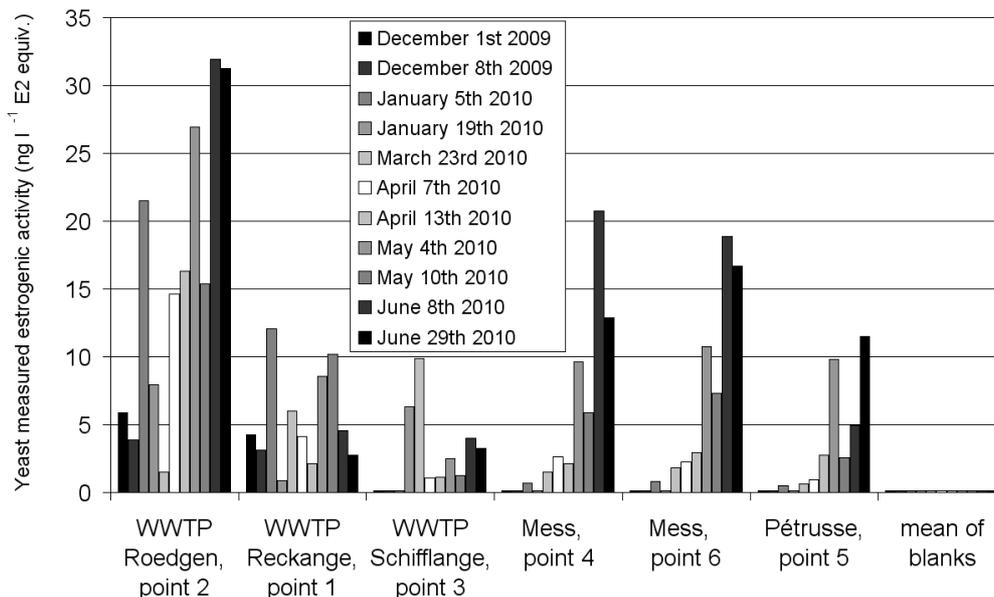


Figure 2. Results of yeast analysis (mean values of 3 to 6 replicates). Extraction blanks gave a signal lower than the limit of detection (LOD) or lower than the lower limit of quantification (LLOQ). For surface water samples and for one of the WWTP effluents, signals were sometimes lower than LOD or LLOQ

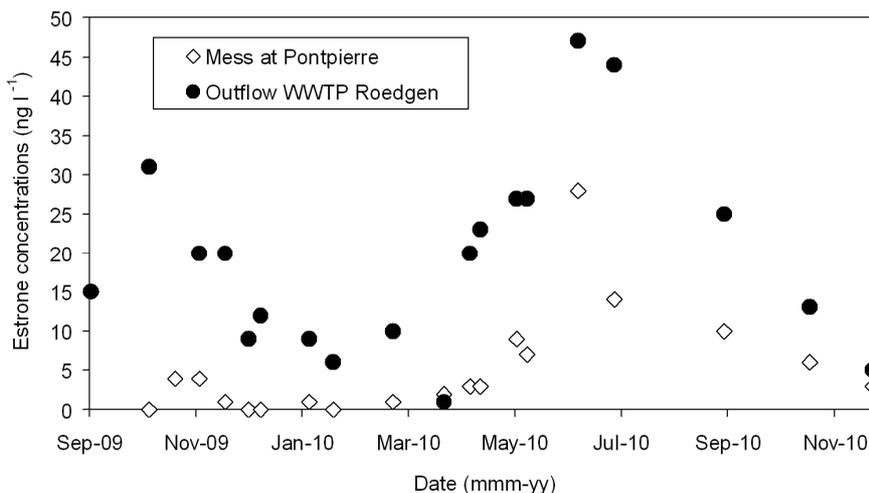


Figure 3. Concentrations of dissolved estrone measured in the Mess catchment at Pontpierre (point 6) and at the outflow of the WWTP of Roedgen (point 2)

These levels of estrogenic activity in surface waters in Luxembourg are somewhat higher than those obtained by Vethaak et al. (2005) in the Netherlands ($LOD = 0.61 \text{ pmol EEQ l}^{-1}$) or Vermeirssen et al. (2005) in Swiss midland rivers ($0.3\text{-}7 \text{ ng l}^{-1} \text{ E2 equiv.}$). Studies in Belgium (Witters et al., 2001; 2003) and in UK (Jobling et al., 2009) indicate estrogenic activity in surface waters, which is of similar magnitude as presented in this study. The measured estrogenic activity could contribute to reproductive disturbances of natural populations of fish as suggested by Vermeirssen et al. (2005) who demonstrated increased vitellogenin levels in feral male brown trout. Jobling et al. (2005) pointed to medium to high risk for occurrence of intersex in wild roach when estrogenic activity was $1\text{-}10$ or $> 10 \text{ ng l}^{-1} \text{ E2 equivalents}$, respectively.

The estrogenic activity measured in the WWTP effluent of Roedgen showed highest activities in this monitoring campaign and ranged between 1 and $32 \text{ ng l}^{-1} \text{ E2 equivalents}$. This observation is in line with chemical measurements of E1 (Figure 3). Comparison between WWTPs for each of the sampling dates demonstrated that measurements with YES assay were always higher than the values measured for the WWTP effluents of Reckange and Schifflange, except in one case for the sampling campaign March 23rd 2010. The high values of

the effluent of the WWTP of Roedgen, despite low person equivalents (1 000), can probably be explained by the fact that this is a WWTP with only a mechanical treatment, which likely does remove or reduce estrogenic activity less efficiently than the other WWTPs. The estrogenic activity measured in the WWTP effluent of Schiffflange (90 000 person equivalents, modern plant) ranged between LOD and 6 ng l⁻¹ E2 equivalents, with only one higher value (9.89 ng l⁻¹ E2 eq.) measured during the sampling campaign of March 23rd 2010. The estrogenic activity measured in the WWTP effluent of Schiffflange was in most campaigns lower than the values measured for the WWTP effluents of Roedgen and Reckange, both with lower person equivalents. These measurements indicate that the WWTP of Schiffflange, which likely has a high estrogenic activity at incoming water due to high person equivalents, successfully reduces the estrogenic activity compared to the other WWTPs thanks to the application of modern (mechanical and biological) treatment. The estrogenic activity measured in the WWTP effluent of Reckange ranged between 0.9 and 12 ng l⁻¹ E2 equivalents.

The results of the chemical analysis have shown the ubiquity of E1 present in 80 % of the samples. The highest activities were obtained for the outflow of the Roedgen WWTP (measurement point 2), a small mechanical wastewater treatment plant. For E2 and EE2 few samples with levels above LOD were found (presence in 7 % and 3 % of the total measurements, respectively). Figure 3 highlights the concentrations and the seasonality of dissolved estrone for the Mess surface water (measurement point 6) and the cleaned sewage water in the outflow of the Roedgen WWTP (point 2). The highest concentrations are measured during summer. Vonbank et al. (Seine, France, 2004), Laganà et al. (Tiber, Italy, 2004) and Kuch and Ballschmiter (River water, South Germany, 2001) analysed similar concentrations. In winter, dissolved estrone is diluted by higher discharge in the river containing larger amounts of soil water and groundwater. The seasonality observed is mainly resulting from simple dilution curves (Figure 4). Arian et al. (2008) highlight similar results; lower concentrations of hormones were observed in watersheds where more dilution occurs and during the wet season with higher amounts of unpolluted groundwater or soil water. According to Williams et al. (2003) river water estrone concentration declined along river stretches at rates that were in excess of that due to corresponding dilution. In addition, other factors have been identified as possible explanations, for example the role of air temperature, UV radiation (Feng et al., 2005) or the production of estrogens by vegetation.

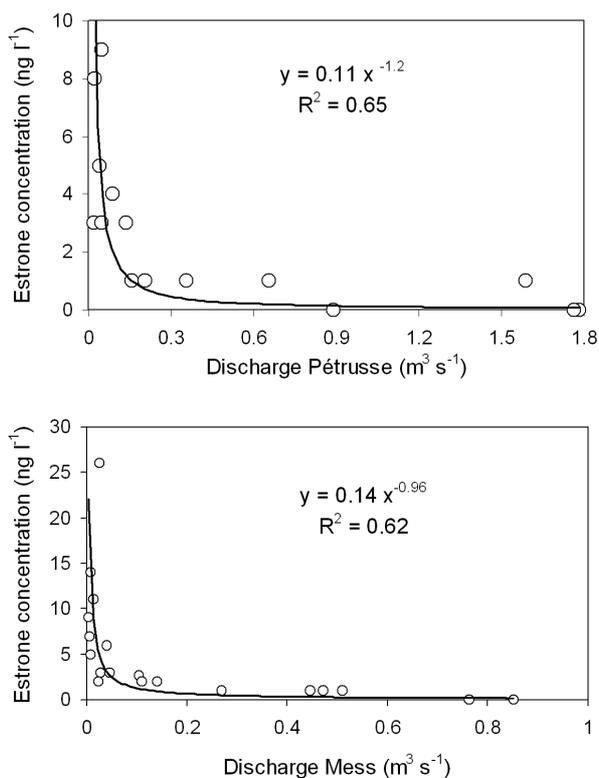


Figure 4. Concentrations of dissolved estrone measured in the Mess (sampling point 6) and Pétrusse (sampling point 5) versus river discharge during sampling

3.2 Comparison of Target Chemical Analysis to Biological Detection by YES assay

The measured values for steroids were transformed to corresponding estrogenic activities. This has been realised by combining the relative potency of each chemical with the reference, β -estradiol (E2). Therefore the calculated activity is expressed as a “concentration in nanogram per liter, equivalent to E2”. This method of calculating the activity does take into account the affinity of the steroid for the corresponding hormone receptor, which is 40 % for E1 and 90 % for EE2 compared to E2 (= 100 %) as we determined with our yeast assay in previous VITO studies (Van den Belt et al., 2004). The results in Table 2 show similar ranges compared to the results of Yeast analysis (Figure 2).

Table 2. The calculated estrogenic activity (ng l-1 E2 equivalents) based on the equation $1 * E2 + 0.4 * E1 + 0.9 * EE2$, using data from chemical analysis (this study)

Sampling date	Sampling locations					
	WWTP Roedgen Point 2	WWTP Reckange Point 1	WWTP Schifflingen Point 3	Mess Point 6	Mess 2 Point 4	Pétrusse Point 5
	December 1st 2009	3.6	2.4	0	0	0.4
December 8th 2009	4.8	2.4	0	0	0	0
January 5th 2010	3.6	2	0	0.4	0.8	0.4
January 19th 2010	2.4	13	2	0	0.4	0
March 23rd 2010	0.4	6.8	19.5	0.8	0.8	0.4
April 7th 2010	0.8	3.2	0.8	1.2	1.2	0.4
April 13th 2010	9.2	1.6	6.4	1.2	13.8	1.6
May 4th 2010	10.8	3.2	11.2	3.6	4.4	6.6
May 10th 2010	10.8	4.8	1.2	2.8	5.4	1.2
June 8th 2010	18.8	2.4	2	11.2	10.4	2
June 29th 2010	17.6	1.6	0.8	5.6	5.6	3.2

The binding and biological activation of the estrogen hormone receptor is likely one of the major mechanisms of endocrine disruption induced by numerous environmental chemicals. Thus steroids, but also other industrial chemicals, such as some pesticides, alkyl phenols, parabens, have the potency to bind and activate the hormone receptor. In the current study only a few natural and synthetic steroids were measured (results not shown) and only these could be included for calculation of estrogenic activity in order to compare with results obtained by the yeast assay. However, the advantage of these biological assays is that it can be considered as a “group parameter” because it detects all the chemicals in the sample extract that interfere with the estrogen hormone receptor.

Comparison of both calculated and measured estrogenic activity showed similar results if the ranking from low to high values is considered. Based on Spearman rank a significant correlation between both approaches was seen ($n=66$, Spearman $R=0.8$, $p < 1.16E^{-15}$).

In general it is expected that the measured activity in the yeast assay, which includes all potential estrogenic active chemicals for receptor activation, is higher than the calculated activity based on LC/MS-MS measurements of only 3 compounds (E2, E1 and EE2). Our results showed indeed that for 59 of 66 samples (~90 %) the estrogenic activity derived from chemical measurements is lower than the one determined in the yeast assay, and only for ~10 % the estrogenic activity, derived from chemical measurements is higher than the one determined in the yeast assay.

Other publications that have compared hormone concentrations with the YES assays also concluded that there are significant differences between the two methods. Converting the responses of the assays to corresponding E2 equivalents, Saleste et al. (2007) found that the activity was about ten times higher than the activity in the original effluent sample. A possible explanation could be – according to the authors – the existence of chemicals in the sample that causes a kind of suppression in the response of the yeast assay. Fernandez et al. (2009) showed that the combination of LC-MS analysis with Yeast assays tests is an adequate approach for the determination of estrogenic endocrine-disrupting compounds within a WWTP and a useful technique for an identification of the

compounds or fractions that are responsible for realised estrogenic effects. They concluded that the application of a fractionation to evaluate the alkylphenols, hormones, and hormone conjugates responsible for the estrogenicity in influents and effluents of WWTPs has provided evidence that nonylphenol is the main compound causing estrogenicity.

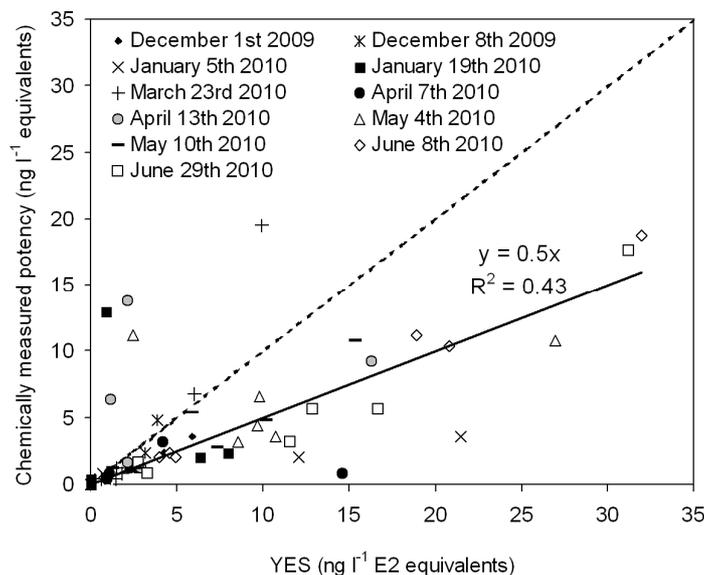


Figure 5. Comparison of results in the yeast test with calculated estrogenic potency derived from chemical measurements. The dashed line represents the 1:1 line

4. Conclusions

This study allowed illustrating the usefulness of the yeast bioassay to screen for the presence of chemicals with estrogenic activity. By comparison of the biological signal in the yeast assay, expressed as estrogen equivalents, with available chemical analysis for steroid hormones we only could obtain a similar ranking for the majority of sites with high or low estrogenic activity.

The estrogenic activity obtained with the biological assay is for 90 % of the samples higher than the values obtained with the chemical analysis. This is as expected, because the biological assay is able to detect all estrogenic active chemicals which bind to the estrogen receptor, which is likely more than the activity calculated from the chemical analysis of 3 target compounds (E2, E1 and EE2) with high potency. This indicates that measurements with a bioassay, which can be used as a “group parameter” of biological active chemicals, can be only used as a first screening tool of compounds with estrogenic activity. In this way as we demonstrated, it could be used to evaluate surface water quality, but also as a follow up for the performance of WWTP’s, or evaluate efficiency of purification technologies for certain hazardous chemicals. Moreover, the signal obtained with the biological assay is likely to be more relevant from a water quality perspective as it is “mechanism”-based and could have a higher predictive value when the possible ecological effects on natural populations are to be assessed.

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