

Yeast Estrogen Screen Assay as a Tool for Detecting Estrogenic Activity in Water Bodies

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Summary

The presence of endocrine-disrupting compounds in wastewater, surface water, ground-water and even drinking water has become a major concern worldwide, since they negatively affect wildlife and humans. Therefore, these substances should be effectively removed from effluents before they are discharged into surface water to prevent pollution of ground-water, which can be a source of drinking water. Furthermore, an efficient control of endocrine-disrupting compounds in wastewater based on biological and analytical techniques is required. In this study, a yeast estrogen screen (YES) bioassay has been introduced and optimized with the aim to assess potential estrogenic activity of waters. First, assay duration, concentration of added substrate to the assay medium and wavelength used to measure the absorbance of the substrate were estimated. Several compounds, such as 17- β -estradiol, 17- α -ethinylestradiol, bisphenol A, nonylphenol, genisteine, hydrocortisone, dieldrin, atrazine, methoxychlor, testosterone and progesterone were used to verify its specificity and sensitivity. The optimized YES assay was sensitive and responded specifically to the selected estrogenic and nonestrogenic compounds in aqueous samples. Potential estrogenicity of influent and effluent samples of two wastewater treatment plants was assessed after the samples had been concentrated by solid-phase extraction (SPE) procedure using Oasis[®] HLB cartridges and methanol as eluting solvent. Up to 90 % of relative estrogenic activity was detected in concentrated samples of influents to wastewater treatment plants and estrogenic activity was still present in the concentrated effluent samples. We found that the introduced YES assay is a suitable screening tool for monitoring the potential estrogenicity of effluents that are discharged into surface water.

Key words: endocrine-disrupting compounds, estrogenic activity, monitoring, solid-phase extraction, yeast estrogen screen assay, water

Introduction

Endocrine-disrupting compounds (EDCs) comprise a wide range of natural and synthetic compounds that exhibit a potential to elicit negative effects on endocrine systems of humans and wildlife. The group of EDCs in-

cludes natural and synthetic estrogen hormones, pharmaceuticals, pesticides, industrial chemicals, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCB), surfactants and plasticizers, and heavy metals (1–4). Natural and synthetic estrogens generally display much stronger estrogenic effects than phyto-

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and xenoestrogens. However, the concentrations of the latter compounds in the aquatic environment are usually several orders of magnitude higher than those of estrogens (4,5).

EDCs can be hormonally active at low concentrations, and recent studies have reported their presence in wastewater, surface water, groundwater and even drinking water worldwide at significantly higher concentrations, therefore these findings are a cause for concern (6,7). Human population is exposed to EDCs in food, water and environment. Recent reviews and scientific consensus statements find evidence of adverse reproductive outcomes, *i.e.* infertility, cancers, malformations, and effects on other endocrine systems from exposure to EDCs (5, 8,9). Therefore, efficient wastewater treatment technologies and control of effluent quality are very important steps in prevention of the pollution of drinking water with EDCs. Traditionally, quality control of effluents is mainly based on analytical measurements of target compounds such as EDCs. However, environmental samples are almost always complex mixtures of known and unknown chemicals, which makes complete identification and quantification of all present compounds, as well as by-products or metabolites, unfeasible and economically unacceptable. Furthermore, adverse biological effects caused by EDCs cannot be measured by chemical analyses. For this reason, a biological assay, such as *in vitro* yeast estrogen screen (YES) assay, is required for screening the estrogenic activity of environmental samples, *i.e.* effluents, wastewater and landfill leachates (3,4,7,10,11). An estrogenic activity identification and evaluation procedure by means of YES assay has been described in many papers (5,12–14).

Since the amounts of some EDCs in the environment are at trace levels, samples must undergo extraction and concentration procedures to reach the levels of detection (15). Several solid-phase extraction (SPE) procedures for extraction and concentration of wastewater have been described by authors such as Ballesteros *et al.* (16), Janex-Habibi *et al.* (17), Yang *et al.* (18), *etc.*

The aim of this study is to optimize the *in vitro* YES assay as a tool for detecting estrogenic activity of aqueous samples. For this purpose, some important parameters were investigated, such as time of exposure, absorbance maximum and concentration of chromogenic substrate chlorophenol red β -D-galactopyranoside (CPRG) added to the assay medium. Specificity of the assay was assessed by several known estrogenic compounds. For the detection of estrogenic activity in water samples, an SPE procedure was optimized for the extraction and concentration of estrogenic-active compounds from aqueous samples. Finally, the introduced YES bioassay was used to investigate the estrogenic activity of influents and effluents from two wastewater treatment plants (WWTPs) in Slovenia.

Materials and Methods

Sample preparation

Compounds 17- β -estradiol (E2), 17- α -ethinylestradiol (EE2), bisphenol A (BPA), nonylphenol (NP), genistein (G), hydrocortisone (HC), dieldrin (D), atrazine (A), methoxychlor (M), testosterone (T) and progesterone (P)

were purchased from Sigma-Aldrich (Munich, Germany). Samples for testing specificity and sensitivity of YES assay were prepared in 1 mL of 99 % ethanol (Fluka, Sigma-Aldrich). Working mass concentrations of compounds were 27.2 μ g/L for E2; 29.6 μ g/L for EE2; 0.2 mg/L for BPA and G; 93.7 μ g/L for NP; 36.2 μ g/L for HC; 10 g/L for D, A and M; 28.8 μ g/L for T and 31.4 μ g/L for P.

For the solid-phase extraction (SPE) procedure, aqueous solutions of E2 (272, 27.2 and 2.72 μ g/L), EE2 (296, 29.6 and 2.96 μ g/L), BPA (0.2, 0.1 and 0.05 g/L) and G (0.2 g/L) were prepared in ultrapure water. Stock concentration of chlorophenol red β -D-galactopyranoside (CPRG) substrate was 10 mg/mL.

YES assay procedure

A recombinant yeast strain *Saccharomyces cerevisiae* BJ1991 used in this experiment was kindly provided by Professor John P. Sumpter (Genetics Department of Glaxo Wellcome, Stevenage, UK). Yeast hosts an integrated gene coding for human estrogen receptor (hER) in its genome and expression plasmids carrying the reporter gene *lac-Z* (encoding the enzyme β -galactosidase). Following the activation of *lac-Z* gene in the presence of estrogenic-active compounds, β -galactosidase degrades CPRG substrate (13).

Preparation of medium components

All the ingredients were purchased from Sigma-Aldrich. Minimal medium and growth medium were prepared following Routledge and Sumpter (13). The growth medium was inoculated with 0.25 mL of the concentrated stock yeast and incubated at 28 °C for approx. 24 h on an orbital shaker (150 rpm) until $A_{620\text{ nm}}=1.0$ was reached. The assay medium was prepared by adding 200 μ L of the chromogenic substrate CPRG ($\gamma=10$ mg/mL) to 50 mL of fresh growth medium and seeded with 2 mL of yeast from a 24-hour yeast culture. Serial dilutions of compounds were prepared in ethanol and 10- μ L aliquots of these solutions were transferred to 96-well flat-bottom microtiter plates under sterile conditions. After the ethanol dried, the yeast cells in the assay medium were added to each hole on the microtiter plate. Then, microtiter plates were incubated at 34 °C for 48–52 h. The absorbance of each sample was measured on the microtiter plate reader PowerWave XS (BioTek, Winooski, VT, USA). On each microtiter plate positive, negative and blank controls were used. Since 17- β -estradiol (E2) is the main natural human estrogen, it was used as a positive control. On the other hand, testosterone (T) and progesterone (P) are natural human hormones without the ability of binding to the human estrogen receptor; therefore, they were used as a negative control. As a blank control (BC), yeasts exposed to the growth medium with CPRG substrate were used in order to detect whether yeasts themselves, without exposure to estrogenic-active compounds, could degrade CPRG.

YES assay optimization

The YES assay was optimized for incubation time (by the absorbance measurements at different time intervals), concentration of the CPRG substrate in the assay medium (10, 20, 40, 60 and 80 μ g/mL) and the maximal absorption wavelength for degraded CPRG substrate

(scan in a range from 500 to 600 nm), by using compounds E2, EE2 and BPA.

Estrogenic activity

For calculating estrogenic activity (EA), the absorbance measurements (A) at 575 and 620 nm were carried out, and EA was expressed as the activity of enzyme β -galactosidase. Equation adopted by Fent *et al.* (12) reads:

$$\beta\text{-galactosidase activity (EA)} = \frac{A_{575 \text{ nm}(\text{sample})} - (A_{620 \text{ nm}(\text{sample})} - A_{620 \text{ nm}(\text{blank})})}{\Delta} \quad /1/$$

where $A_{575 \text{ nm}(\text{sample})}$ represents the absorbance of the sample measured at 575 nm, $A_{620 \text{ nm}(\text{sample})}$ represents the absorbance of the sample measured at 620 nm, while $A_{620 \text{ nm}(\text{blank})}$ represents the turbidity of yeast in the assay medium.

Solid-phase extraction procedure (SPE procedure)

Optimization of the SPE procedure involved testing of two different SPE cartridges Supelclean™ ENVI 18-SPE (Supelco, Sigma-Aldrich, St. Louis, MO, USA) and Oasis® HLB SPE (Waters Milford, MA, USA), and eluting solvents methanol and ethyl acetate. Conditioning of cartridges was performed with 4 mL of methanol or ethyl acetate, followed by 4 mL of distilled water. After loading the samples, the cartridges were washed with 5 % methanol or 5 % ethyl acetate. During these steps, vacuum was maintained. Then the cartridges were dried under a gentle stream of nitrogen (N_2) and compounds were eluted with 4 mL of methanol or ethyl acetate and collected in test tubes. Eluted samples in test tubes were exposed to the gentle stream of N_2 to concentrate the samples to the volume of 1 mL and stored at -20°C before further use. Recovery of compounds E2, EE2, BPA and G was calculated according to the following equation:

$$\text{Recovery} = (\text{EA}_{\text{SPE sample}} / \text{EA}_{\text{sample}}) \cdot 100 \quad /2/$$

Estrogenic activity ($\text{EA}_{\text{sample}}$) and estrogenic activity after SPE procedure ($\text{EA}_{\text{SPE sample}}$) of the samples were calculated using Eq. 1. In order to test for significant differences between recoveries obtained for both cartridges and eluting solvents, Student's *t*-test ($p < 0.05$) was used.

Wastewater samples

Influent and effluent grab samples from two wastewater treatment plants (WWTPs), WWTP1 and WWTP2, were collected in 5-litre plastic bottles. Wastewater marked as WW1 was collected from the wastewater treatment plant where municipal and industrial wastewaters flow in (WWTP1), whereas wastewater labelled WW2 was collected from the municipal wastewater treatment plant (WWTP2). Collected wastewater samples were processed immediately upon arrival. Pretreatment of samples (250 mL) involved centrifugation at 5000 rpm for 10 min and filtration through the 1.2- and 0.3- μm membrane filters (16). This was followed by the SPE procedure, where methanol and ethyl acetate were used as conditioning and eluting solvents on the same SPE cartridge (ethyl acetate followed methanol). Extracted and concentrated samples were stored at -20°C before they were used in YES assay.

The estrogenic activity of wastewater samples was expressed as the activity of β -galactosidase calculated by using Eq. 1 and the relative estrogenic activity (REA/%) was calculated using the following equation, adopted by Cajthaml *et al.* (19):

$$\text{REA} = (\text{EA}_{\text{sample}} - \text{EA}_{\text{blank}}) / \Delta \cdot 100 \quad /3/$$

where $\text{EA}_{\text{sample}}$ is estrogenic activity of the sample calculated using Eq. 1, EA_{blank} is the average of blank control estrogenic activity calculated using Eq. 1 and Δ is an interval of estrogenicity with the initial point at the highest EA_{E2} value and final point in EA_{blank} .

Enzymatic deconjugation

For activation of conjugated estrogens, deconjugation step has to be performed. According to Mouatassim-Souali *et al.* (20), the wastewater extract from SPE procedure was evaporated to dryness under N_2 gas. Then, the samples were redissolved in 1 mL of 0.1 M acetic acid buffer (pH=5) containing β -glucuronidase with minimum 2000 units of glucuronidase and 150 units of sulphatase activity (HP-2 from *Helix pomatia* (Sigma-Aldrich)). After 24 h of incubation at 40°C , the reaction of deconjugation was terminated by the addition of acidified water (pH=3). The incubation mixture was then extracted on preconditioned Oasis HLB® SPE cartridge as described previously. Estrogenic activity and relative estrogenic activity were calculated using Eqs. 1 and 3.

Results

YES assay procedure optimization

Absorbance of yeast culture suitable for use in the assay was at $A_{620 \text{ nm}} = 1$. This was reached between 18 and 24 h of incubation at 28°C on an orbital shaker at 140 rpm. Furthermore, the YES assay optimization showed that yeast should be exposed to the tested samples between 48 and 58 h in order to obtain sigmoidal curves (Fig. 1). When the YES assay is prolonged to 72 h, the response curve has different shape (Fig. 1). The CPRG degradation product might acquire estrogenic activity and

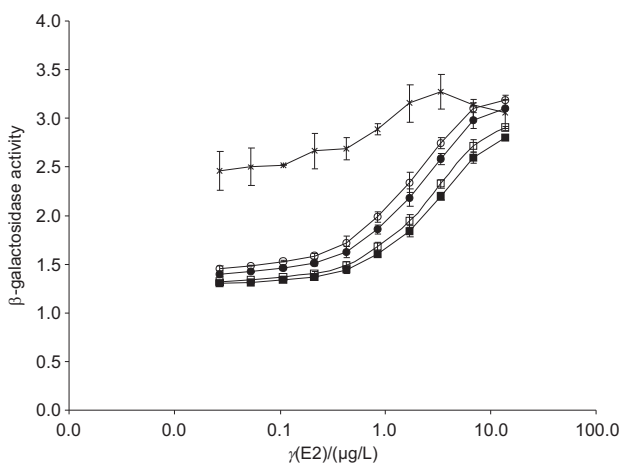


Fig. 1. Optimization of incubation time using standard E2: (■) 48 h, (□) 51 h, (●) 55 h, (○) 58 h, (*) 72 h. Arithmetic means and standard deviation ($N=2$) of activity of β -galactosidase are presented

therefore false positive results could be obtained, which was also confirmed by Vanderperren *et al.* (21).

Scan of visible light absorption in a wavelength range from 500 to 600 nm showed the highest obtained absorbance at the wavelength of 575 nm (data not shown). According to Routledge and Sumpter (13), 300 μL of CPRG (60 $\mu\text{g}/\text{mL}$) should be added to the assay medium. However, our results indicated that smaller volume of CPRG such as 200 μL (40 $\mu\text{g}/\text{mL}$) also gives satisfactory results (Fig. 2).

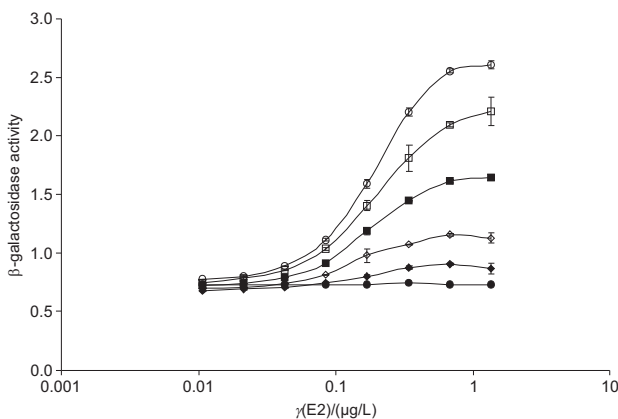


Fig. 2. Optimization of CPRG concentration (in $\mu\text{g}/\text{mL}$) in the assay medium: (O) 80, (□) 60, (■) 40, (◇) 20, (◆) 10 and (●) BC (blank control). Arithmetic means and standard deviation ($N=2$) of activity of β -galactosidase are presented

Specificity and sensitivity of YES assay

Specificity and sensitivity of YES assay were studied using compounds E2, EE2, BPA, NP, G, HC, M, D, A, T and P (Fig. 3 and Table 1). The obtained results show that E2, EE2, BPA, NP, G and M possess estrogenic activity, whereas other tested compounds do not bind to the estrogen receptor (Fig. 3). Sensitivity of the tested compounds is presented according to Kuruto-Niwa *et al.* (22) as concentrations at which the lowest, 50 % of the highest and the highest estrogenic effect are observed (Table 1).

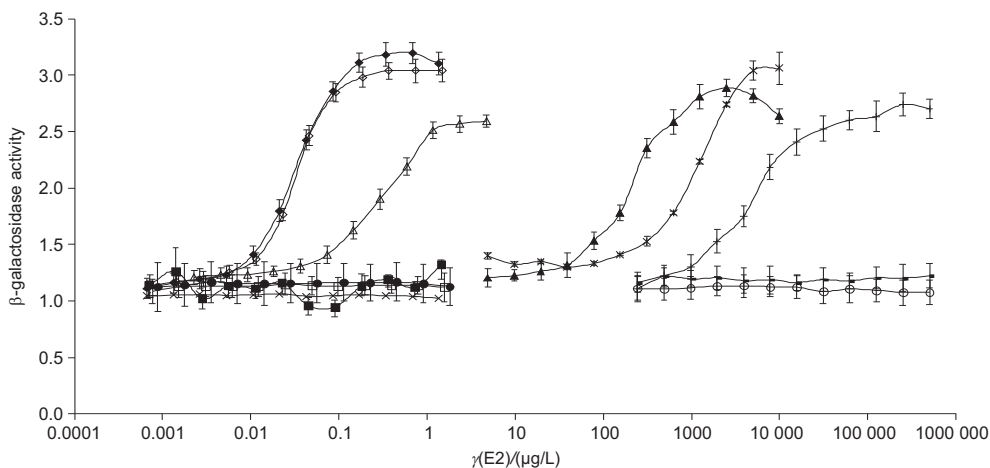


Fig. 3. Specificity of YES assay for: (◆) E2, (◇) EE2, (▲) BPA, (△) NP, (*) G, (●) HC, (+) M, (-) D, (○) A, (■) T, (□) P and (×) BC (blank control). Arithmetic means and standard deviation ($N=2$) of the activity of β -galactosidase are presented

Table 1. Sensitivity of YES assay for estrogenic-active compounds

Compound	$\gamma/(\mu\text{g}/\text{L})^a$	$\text{EC}_{50}/(\mu\text{g}/\text{L})^b$	$\gamma/(\mu\text{g}/\text{L})^c$
E2	0.015±0.002	0.044±0.004	0.34±0.03
EE2	0.015±0.002	0.042±0.004	0.37±0.04
bisphenol A	78±7	337±30	5000±100
nonylphenol	0.073±0.007	0.32±0.03	1.17±0.1
genisteine	313±30	1290±90	5000±150
methoxychlor	1953±200	11400±800	250000±2000

^aconcentration at which the lowest estrogenic effect was observed

^bconcentration at which 50 % of the highest estrogenic effect was observed

^cconcentration at which the highest estrogenic effect was observed

SPE procedure optimization

In general, the obtained recoveries for both cartridges were in the same range for all tested compounds (Table 2). The obtained recoveries using methanol and ethyl acetate on the Supelclean™ ENVI 18-SPE cartridge were similar. However, we observed some white turbidity of the ethyl acetate when we dropped it into holes of

Table 2. Recovery of E2, EE2, bisphenol A and genisteine using different SPE cartridges and eluting solvents

Compound	Recovery /%		Oasis® HLB Methanol
	Supelclean™ ENVI 18 Methanol	Supelclean™ ENVI 18 Ethyl acetate	
E2 ^a	99.3±0.7	95.2±4.8	99.3±0.7
EE2 ^b	100.8±1.0	83.3±16.7	91.0±9.0
bisphenol A ^c	97.4±2.6	95.5±4.5	91.4±8.6
genisteine ^c	100.6±0.6	90.4±9.6	83.0±17

^{a-c}concentrations of 13.6 $\mu\text{g}/\text{L}$ (E2), 14.8 $\mu\text{g}/\text{L}$ (EE2) and 10 mg/L (BPA and G), respectively

arithmetic means and standard deviation ($N=10-20$) are presented

the microtiter plate in the YES assay. It seems that ethyl acetate reacts with the plastic of the microtiter plate. For this reason, further experiments for the optimization of SPE procedure were conducted only with methanol. In the SPE procedure of wastewater samples, ethyl acetate in the vials was dried out under gentle stream of nitrogen and then replaced by methanol. Some additional concentrations of E2, EE2 and BPA were tested by using Oasis® HLB cartridges (Fig. 4). Results showed that the recovery rates are about 100 % at the highest tested concentrations, but they decreased at lower concentrations of E2, EE2 and BPA (Fig. 4).

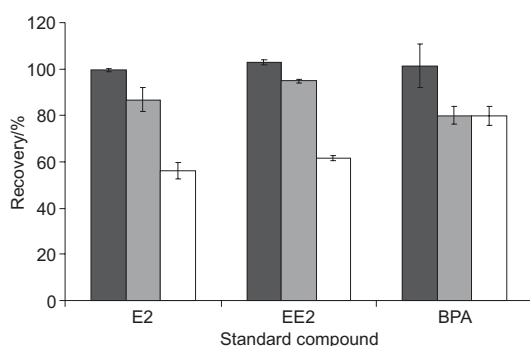


Fig. 4. Recovery of SPE procedure for E2, EE2 and BPA: (■) 13.6 µg/L of E2, 14.8 µg/L of EE2 and 10 mg/L of BPA, (■) 1.36 µg/L of E2, 1.48 µg/L of EE2 and 5 mg/L of BPA and (□) 0.136 µg/L of E2, 0.148 µg/L of EE2 and 2.5 mg/L of BPA. Arithmetic means and standard deviation ($N=16$) of recovery calculated from the activity of β -galactosidase are presented

Wastewater samples

Influent and effluent samples examined by YES assay indicated estrogenic activity as described in Tables 3 and 4. When yeast growth was inhibited for 50 % or more due to toxic compounds present in the tested samples, the REA was not calculated due to possible false results.

Influent concentrates of WW1 eluted by methanol (WW1-influent-MeOH) caused high inhibition (up to 90 %) of yeast growth and consequently REA was not determined (n.d.). When influent concentrates were eluted by ethyl acetate (WW1-influent-EtAc), up to 62 % of REA was determined before deconjugation, but after it the REA increased up to 93 %. Although effluent concentrates were less toxic than influents, similar REA was determined. However, REA stayed at the same level or even slightly decreased in the effluent concentrates after deconjugation; this could be explained by deconjugation of estrogens during treatment processes in WWTP and/or losses of EDCs due to repeating SPE procedures.

WW2 influent and effluent concentrates revealed lower toxicity manifested in growth inhibition of yeast and REA in comparison with WW1 influent and effluent samples (Table 4). No estrogenic activity was detected either in the influent concentrates (the concentrates with growth inhibition lower than 50 %) or in the effluent concentrates. However, after deconjugation step up to 46 and 48 % of REA was determined in influent and effluent concentrates, respectively.

Table 3. Relative estrogenic activity (REA) of WW1 influent and WW1 effluent

concentration factor	REA/%			
	Before deconjugation			
	WW1-influent-MeOH	WW1-influent-EtAc	WW1-effluent-MeOH	WW1-effluent-EtAc
12.5	n.d.	62.1	n.d.	57.1
6.3	n.d.	59.2	37.7	51.6
3.1	n.d.	47.8	32.8	34.7
1.6	n.d.	19.6	14.6	0
concentration factor	After deconjugation			
	WW1-influent-MeOH	WW1-influent-EtAc	WW1-effluent-MeOH	WW1-effluent-EtAc
	12.5	n.d.	93.4	n.d.
6.3	n.d.	60.6	32.4	14.8
3.1	n.d.	24.5	6.2	0
1.6	0	0	0	0

n.d.=not determined (growth inhibition was 50 % or more)

Table 4. Relative estrogenic activity (REA) of WW2 influent and WW2 effluent

concentration factor	REA/%			
	Before deconjugation			
	WW2-influent-MeOH	WW2-influent-EtAc	WW2-effluent-MeOH	WW2-effluent-EtAc
12.5	n.d.	0	0	0
6.3	n.d.	0	0	0
3.1	0	0	0	0
1.6	0	0	0	0
concentration factor	After deconjugation			
	WW2-influent-MeOH	WW2-influent-EtAc	WW2-effluent-MeOH	WW2-effluent-EtAc
	12.5	37.5	46.1	0
6.3	26.7	15.3	0	28.8
3.1	0	0	0	0
1.6	0	0	0	0

n.d.=not determined (growth inhibition was 50 % or more)

Discussion

In the present work, YES assay and SPE procedure were optimized for testing estrogenic activity of water samples. Firstly, the specificity of YES assay was tested for the selected chemicals. Specificity of YES assay is explained by the nature of the estrogen receptor, where ligand-binding site is larger and more flexible than the 17- β -estradiol molecule requires (22,23). This makes the estrogen receptor more vulnerable as a target for interference by many different compounds with analogy in

chemical structure, such as synthetic estrogen hormone (EE2), industrial chemicals (BPA, NP), phytoestrogen (genisteine) or pesticide (methoxychlor). On the other hand, homology does not play any role, since among the group of steroid hormones 17- β -estradiol (E2), hydrocortisone (HC), testosterone (T) and progesterone (P), by the common origin from cholesterol, only E2 caused estrogenic activity (with the ability of binding with the estrogen receptor). All chemicals showed an activation of the estrogen receptor in a concentration-dependent manner. The observed sensitivity of YES assay to natural and synthetic estrogens is in the same range (15 ng/L), whereas its sensitivity to nonylphenol (NP) is 5-times lower (73 ng/L), to bisphenol A (BPA) and genisteine (G) more than 5000 times lower (78 μ g/L for BPA and 312 μ g/L for G) and to methoxychlor (M) more than 100 000 times lower (2 mg/L). The presented results are comparable to the previously described results by Routledge and Sumpter (13), Daston *et al.* (25), Giesy *et al.* (26) and Ternes *et al.* (27).

In our case, the incubation time of YES assay was between 48 and 58 h, the absorbance of the degraded CPRG substrate was measured at the wavelength of 575 nm as it provides about 75 % higher response in comparison with the measurements at 540 nm, and the volume of CPRG substrate added to the assay medium was equal to 200 μ L (which corresponds to the concentration of CPRG in the assay medium equal to 40 μ g/mL). While Routledge and Sumpter (13) showed estrogenic activity as the absorbance of degraded CPRG substrate measured at 540 nm, in our work estrogenic activity was presented as the activity of β -galactosidase calculated by Eq. 1.

Introducing and optimizing the SPE procedure, Oasis[®] HLB sorbent cartridge and the use of methanol as eluting solvent were found to be the most optimal. Recovery of SPE procedure was above 60 % in all cases and is comparable to the previously reported data by Balasteros *et al.* (16) and Lopez de Alda and Barceló (28). The highest recovery (above 90 %) was obtained at the highest tested concentrations followed by the medium concentration (recovery above 80 %). As expected, the lowest recovery (above 60 %) was found at the lowest tested concentrations of the examined compounds E2, EE2 and BPA.

Concentrated samples of influent and effluent examined for estrogenic activity indicated up to 90 % of REA. In general, EA of influents was higher than the EA determined in effluents, which could be explained by the removal of estrogens and xenoestrogens during treatment processes in WWTPs (29–31). Furthermore, the EA of the tested samples after deconjugation step increased in most cases in comparison with the original samples, which means that the present estrogens were in inactive (conjugated) form and became active only after deconjugation. This was not the case for the effluent sample obtained from the WWTP1 (WW1-effluent-EtAc) as lower EA level was obtained when concentrates were eluted with ethyl acetate. This could be explained by possible elimination of estrogenic compounds from water samples due to repeated SPE procedures before and after deconjugation step.

Conclusions

The obtained results showed that the optimized YES assay was sensitive and specifically responded to the selected estrogenic and nonestrogenic compounds in water samples. The introduced YES assay was then applied to assess the estrogenicity of concentrated influent and effluent samples from two municipal WWTPs. Up to 90 % of relative estrogenic activity of influent and effluent samples was detected, which was probably due to the presence of natural and synthetic estrogen hormones and xenoestrogens from households. Lower estrogenic activity was found in effluent and influent samples of the WWTP2 (up to 48 %) in comparison with the samples obtained from the WWTP1 (up to 93 %). However, both WWTPs were partially successful in removing EDCs, since estrogenic activity was still present in most concentrated effluent samples.

We can conclude that the introduced YES assay is a suitable screening tool for monitoring the potential estrogenicity of effluents which are discharged into surface waters. Only effective removal of EDCs from wastewater using appropriate treatment processes could protect the aquatic environment reliably from pollution with these compounds and consequently prevent adverse effects on wildlife and humans.

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