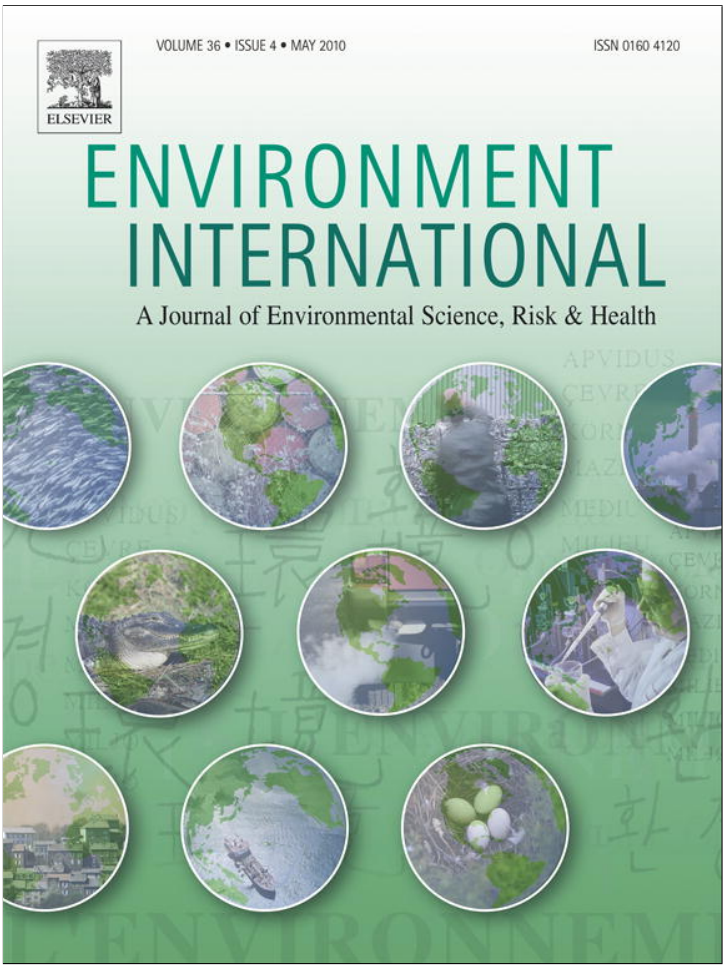

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Evaluation of the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool in environmental samples

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ABSTRACT

This paper presents a study evaluating the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool for monitoring of the chemical status of water bodies in support of the Water Framework Directive (WFD). Three different recombinant yeast-based assays were evaluated; the Yeast Estrogen Screen (YES), the Recombinant Yeast Assay (RYA) and the Rikilt Estrogen bioAssay (REA), of which the YES assay was employed by two different laboratories. No significant difference between the performance of neither the different laboratories, nor the different yeast-assays was observed.

Six batches of eleven samples each were analysed one week apart by the four participating laboratories and the robustness, repeatability and reproducibility of the participating yeast-based assays were evaluated.

The setup included a correlation between bioassay results and results from chemical target analysis, which gave valuable information in the evaluation of the assays' performance. A good agreement was found between chemical and bioassay results, showing that the yeast-based assays can give valuable information in WFD work. However, the low sensitivity of the assays towards alkylphenols needs to be significantly improved if they are to be used for monitoring of these compounds. The study further led to suggestions on ways to improve traceability and quality assurance of the yeast-based assays.

1. Introduction

The key objectives of the EU Water Framework Directive (WFD) are the prevention of any further deterioration of water bodies, and the protection and enhancement of the status (chemical and biological) of aquatic ecosystems and associated wetlands. In order to successfully implement the WFD, reliable and comparable chemical monitoring data are needed. A way to obtain these data is to use biological methods that utilise endpoints relevant for priority pollutants defined in the WFD. Biological methods can contribute to a highly valuable monitoring approach; some bioassays can provide quantitative data (i.e. concentration) of priority pollutants (Farre et al., 2006; Farre et al., 2007) and other toxicity/ecotoxicity characterisation of the combined effect of a group of compounds in the sample (Legler et al., 2002; Murk et al., 2002).

A toxicological endpoint of high importance in the environment is endocrine disruption. Endocrine disruption is an especially harmful effect arising from chemical pollution. It occurs when a chemical accumulates in organisms (including humans) and affects their endocrine system. Given the multiple functions of hormones in the body, this alteration can lead to various adverse effects, including hermaphroditism in fish (Rodgers-Gray et al., 2001) and reproductive deficiencies in humans (Andersen et al., 2000).

Previous studies have indicated that the use of yeast-based assays may be a highly reliable methodology for a first level screening to assess surface water quality in terms of estrogenic activity (Pawlowski et al., 2004; Reddy and Brownawell, 2005; Schmitt et al., 2005).

Currently, yeast-based bioassays for the determination of estrogenic activity are mainly used for research purposes within environmental monitoring (Cespedes et al., 2005; Thomas et al., 2004; Tollefsen et al., 2007). However, a different approach which could be included in routine monitoring is to screen unknown environmental samples for their total estrogenic activity without knowledge of the presence of individual compounds. In this approach, the idea is to use the assay as a pre-screening method before chemical analysis, thus it

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should basically give yes/no responses as to whether there are particular groups of contaminants present or not.

The samples with a positive response can then be further analysed by chromatographic techniques to identify and quantify the active compounds. The purpose of this type of screening is to perform a fast and cheap elimination of samples having low estrogenic activities and thus allow for a more in-depth analysis of the samples that give a clear response in the bioassay. In this paper we have evaluated three different recombinant yeast-based assays for their suitability as pre-screening tools before chemical analysis.

2. Experimental

2.1. Samples

To test the performance of the bioassays in a wide range of possible compositions of environmental samples, the following samples were prepared; Estradiol (I) (Standard solution of 17β-estradiol (10 nM)), Estradiol (II) (Standard solution of 17β-estradiol (15 nM)), MeOH (Blank sample), genistein (Standard solution of genistein (100 μM)), NP (I) (Standard solution of nonylphenol (50 μM)), NP (II) (Standard solution of nonylphenol (100 μM)), Sample (I) (Effluent waters of a WWTP (Martorel, Catalonia, Spain)), Sample (II) (Sample (I) spiked with 17β-estradiol to result in an added concentration of 10 nM), Sample (III) (Sample (I) spiked with nonylphenol to result in an added concentration of 50 μM), Sample (IV) (Sample (I) spiked with 17β-estradiol and nonylphenol to result in added concentrations of 10 nM and 50 μM respectively) and Sample (V) (Sample (I) spiked with 17β-estradiol to result in an added concentration of 15 nM). Both standards and samples were supplied in 100% methanol.

It was chosen to spike with estradiol because it is the reference compound of the yeast-based assays. Estrone and its conjugate estrone sulphate are generally detected more frequently and at higher concentrations in the environment (Kuster et al., 2008; López de Alda et al., 2002; Rodriguez-Mozaz et al., 2004). However, because the difference in estrogenic potency between these compounds is rather constant independent of the assay employed, the reference compound was used to ensure the detection of matrix effects. The concentrations 10 and 15 μg/l were chosen because they correspond to 1.1 and 1.6 ng/l in the water sample before extraction which is close to and just above the concentration where estrogenic effect is observed (>1 ng/l) (Farre et al., 2002; Sole et al., 2002).

Before sending the samples to the participating laboratories they were coded by randomisation, with different randomisation for the 6 batches. As a measure to enhance traceability and quality assurance, each laboratory was provided with a standard solution of Estradiol, with known concentration (40 nM). Standard solutions of nonylphenol (400 μM) and genistein (400 μM) were also sent to each laboratory to achieve an improved estimation of their estrogenic potential.

2.2. Sample preparation of the WWTP water sample

Effluent water from a wastewater treatment plant, WWTP (Martorell, Catalonia, Spain) was filtered through 1 μm, followed by 0.45 μm membrane filters. This was followed by solid-phase extraction with LiChrolut RP-18 (500 mg) cartridges. The cartridges were conditioned with 7 ml acetonitrile, followed by 5 ml methanol and 5 ml HPLC-grade water. 200 ml of sample was passed through the cartridges and they were washed with 5 ml HPLC-grade water. The cartridges were dried and eluted with 2×5 ml of acetonitrile. The eluates were evaporated to dryness under nitrogen and the samples were restored in 0.5 ml of methanol (Cespedes et al., 2004). All sample fractions were pooled to insure a homogenous and identical sample for each participating laboratory.

2.3. Study design

The four participating laboratories were provided with all samples at the same time. The laboratories were not provided with a specific protocol or strain of yeast. They were asked to use the protocol that they use routinely in their laboratory.

The laboratories were asked to analyse the 6 batches 1 week apart or make sure that the batches were analysed with different cultures of yeast. This was done to ensure that all components of the intra-laboratory variation were included in the design. The results were to be reported as molar concentration (e.g. nmol/l, nM) (in the received sample) equivalent to 17β-estradiol (EEQ).

2.4. Recombinant yeast-based assays

2.4.1. Yeast Estrogen Screen (YES)

Laboratories 1 and 3 used the Yeast Estrogen Screen (YES), a yeast-based (*Saccharomyces cerevisiae*) screen developed by Glaxo-Wellcome, plc (Stevenage, Herts, UK). In this system, the human estrogen receptor (hER-α) has been integrated into the yeast genome, together with expression plasmids carrying estrogen responsive elements (EREs) which control the expression of the reporter gene Lac-Z. In the presence of chemicals with estrogenic activity (which bind to and activate the receptor), β-galactosidase is synthesised and secreted into the assay medium in which the yeast is grown. The β-galactosidase then breaks down the chromogenic substrate chorophenol red β-galactopyranoside (CPRG). CPRG is initially yellow but breaks down into a red product, the concentration of which can be read by absorbance spectrophotometry.

The assay was performed using the method of Routledge and Sumpter (1996), with only minor modifications as described below. Briefly; supplied blind samples (100 μl) were diluted to 500 μl, since 200 μl of sample per assay is used in this method (500 μl allows for the possibility of repeats).

Samples (50 μl) were serially diluted in 96 well microtiter plates (NUNC™, Roskilde, Denmark) and allowed to evaporate to dryness at room temperature. An assay medium, which consisted of the chromogenic substrate and a growth medium which had been inoculated with yeast cells, was added to the plate. The plate was then covered using an adhesive plate seal and incubated for 3 days at 32 °C. On the third day any change in colour of the chromogenic substrate was read colourimetrically using a UV–Vis plate-reader (Laboratory 1: PerkinElmer Victor^3 1420 multilabel counter. laboratory 3: Bio-Tek Instruments Inc.) at an absorbance of 540 nm for colour and 620 nm for turbidity. A dilution series of 17β-estradiol (E2), as a positive control and estrogenic standard, together with a solvent blank, were assayed alongside each batch of samples.

Dose–response curves were determined for each plate and E2 Equivalent concentrations in the samples calculated from the resulting equation. A significant response is described as one whose value is more than three times the standard deviation of the solvent control.

Laboratory 1 determined the level of detection (LOD) for 17ß-estradiol to be 2.5 nM. For laboratory 3, the limit of detection is described as being 3 times the standard deviation of the solvent control value for each plate. For this reason it varies from plate to plate. For the plates used in this study, the limit of detection ranged from 0.31 nM to 2.7 nM with an average of 1.09 nM.

2.4.2. Recombinant Yeast Assay (RYA)

Laboratory 2 used a Recombinant Yeast Assay (RYA). Briefly; yeast strain BY4741 (MATa *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) (EUROSCARF, Frankfurt, Germany) was transformed with plasmids pH5HE0 and pVitBX2 (Garcia-Reyero et al., 2001).

Expression plasmid pH5HE0 contains the human estrogen hormone receptor HE0 (Green and Chambon, 1991) cloned into the

constitutive yeast expression vector pAAH5 (Schneider and Guarente 1991). The reporter plasmid pVITB2x contains two copies of the pseudo-palindromic estrogen responsive element ERE2 from *X. laevis* vitellogenin B1gene (5′-AGTCACTGTGACC-3′) inserted into the unique *Kpn*I site of pSFLΔ-178K (Garcia-Reyero et al., 2004).

Transformed clones were first grown in 2 ml of rich media (YPD) o/n at 30 °C. Then, they were grown overnight in minimal medium (6.7 g/l yeast nitrogen base without amino acids, DIFCO, Basel, Switzerland; 20 g/l glucose, supplemented with 0.1 g/l of prototrophic markers as required). The final culture was adjusted to an optical density (OD) of 0.1 and split into 75 µl in the first row and 50 µl in the other wells of a siliconized 96-well polypropylene microtiter plate (NUNC™, Roskilde, Denmark).

Positive controls were made by adding 17ß-estradiol at a final concentration of 10 nM. Moreover, we included a toxicity control by adding 10 nM of 17ß-estradiol to a sample with a dilution factor of 1:30.

Plates were incubated for 6 h at 30 °C under mild shaking. After incubation, 50 µl of Y-PER™ (PIERCE™, Rockford, IL, USA) was added to each well and further incubated at 30 °C for 30 min. Afterwards, 50 µl of assay buffer was added to the lysed cells. The assay buffer was prepared by mixing 100 ml Z-buffer, 1 ml Triton X-100 (Sigma), 1 ml SDS 10%, 70 µl 2-mercaptoethanol (Fluka) and 21 mg of 4-methylumbelliferyl ß-D-Galactoside (Sigma). Z-Buffer is a mix of: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$, pH 7.0. After brief centrifugation, plates were read in a Victor^3 Wallac spectrofluorometer (Perkin Elmer Inc., Wellesley, MA, USA), at 355 nm excitation and 460 nm emission wavelengths. Fluorescence was recorded for 15–20 min (one measurement per min); ß-galactosidase activity values were calculated as rates of the increment of arbitrary fluorescence units with time, using standard linear regression methods. Results were reported as nM concentration in the received sample equivalent to 17ß-estradiol (EEQ). Laboratory 2 reported a concentration of 0.034 nM 17ß-estradiol as the level of detection (LOD) for 17ß-estradiol (Noguerol et al., 2006).

2.4.3. Rikilt Estrogen bioAssay (REA)

Laboratory 4 used the Rikilt Estrogen bioAssay (REA).The modified yeast strain used was a gift from T. Bovee (RIKILT, Institute of Food Safety, Wageningen University and Research Center, The Netherlands) and the culturing conditions were according to Bovee et al. (2004). The bioassay was performed as described in Bovee et al. (2004) with some small modifications described as follows. The medium where the yeast was grown overnight consisted of yeast nitrogen base (YNB) that has neither amino acids nor ammonium sulphate (1.7 g/l, Difco), D-glucose (20 g/l, Merck), and ammonium sulphate (5 g/l, Merck) supplemented with L-leucine (120 mg/l). Aliquots of the bioassay medium consisting of growth medium adjusted to an OD of 0.1 were treated with 17ß-estradiol standards and samples in order to keep the ratio standard/medium and sample/medium at 0.5. The carrier solvent for the 17ß-estradiol standards was methanol and the standard curve ranged from 5×10^{-12} to 2×10^{-9} M. 96 well polypropylene microtiter plates (NUNC™, Roskilde, Denmark) were incubated at 30 °C, 225 rpm for 15 h or until reaching the maximal response in the 17ß-estradiol standard curve. Solvent blanks with bioassay medium were also included. Fluorescence was measured at 535 nm (excitation 485 nm) in a luminescence multi-well plate-reader (LumiCount, SPECTRA Fluor). The fluorescence signals were corrected with the blank medium values obtained at the measurement time. Limit of detection (LOD) was reported as ≤0.1 nM 17ß-estradiol.

2.5. Chemical analysis

2.5.1. Materials and standards

Water, acetonitrile and methanol were all HPLC grade from Merck (Darmstadt, Germany). LiChrolut RP-18 cartridges (500 mg, 6 ml) for the solid-phase extraction were also from Merck (Darmstadt, Germany). Standards of octylphenol (4-tert-OP) and nonylphenol (4-NP) (98%) were from Aldrich (Milwaukee, WI, USA). Nonylphenol with 1 ethoxylate unit (NP_1EO), nonylphenol with 2 ethoxylate units (NP_2EO) and nonylphenol with one carboxylate unit (NP_1EC) were synthesised according to a method described elsewhere (Diaz et al., 2002). Pure standards of natural and synthetic estrogens were purchased from Sigma-Aldrich (Steinheim, Germany).

2.5.2. LC–MS system

For the chemical characterisation, the samples were analysed by LC–MS, to determine the concentrations of: nonylphenol (NP), octylphenol (OP), nonylphenol ethoxylates (NP_xEO) and carboxylates (NP_xEC) along with other detergents. The HPLC system consisted of a HP 1100 autosampler with 100 µl loop and a HP 1090 A LC binary pump, both from Hewlett-Packard (Palo Alto, CA, USA). The column used was a C_{18}, particle size: 5 µm, dimensions: 125 mm × 2 mm i.d. (Purospher STAR RP-18). The column was protected by a guard column (4 × 4 mm) of the same packing material from Merck. The detection system was an HP 1100 LC-mass-selective detector (MSD) used with an electro spray ionisation (ESI) interphase.

The separation was performed under gradient conditions, using methanol and water as the mobile phase. Injection volume was 20 µl for all samples and standards. NP, OP, and NP_xEC were analysed using negative ionisation (NI). NP_xEO were analysed using positive ionisation (PI). The ESI-MS parameters (NI/PI) were as follows: drying gas flow: 12/11 l/min, drying gas temperature: 375/325 °C, nebulizer pressure: 55/50 psi, capillary voltage: 4500/4000 V, fragmentation voltage: 90/60 V. All ions were measured in selected ion monitoring (SIM) mode. The analytical method applied has been validated and described in further detail elsewhere (Gonzalez et al., 2004).

2.5.3. LC–MS/MS system

Samples were further analysed by LC–MS/MS (QqQ), to determine the concentrations of: estriol (E3), estradiol (E2), ethynyl estradiol (EE), estrone (E1), diethylstilbestrol (DES) and genistein (Gen). The HPLC system consisted of a Waters Alliance 2690 LC pump equipped with an autosampler (Waters, Milford, MA). The column used was a C_{18}, particle size: 5 µm, dimensions: 125 mm × 2 mm i.d. (Purospher STAR RP-18). The column was protected by a guard column (4 × 4 mm) of the same packing material from Merck. The detection system used was a Quattro LC triple-quadrupole mass spectrometer from Micromass (Manchester, UK). MS/MS detection was performed with selected reaction monitoring (SRM) using ESI in NI mode. Two SRM transitions were monitored for each compound. The more abundant was used for quantification and the second for confirmation. The MS parameters were as follows: capillary voltage: 3 kV, source temperature: 150 °C, desolvation temperature: 350 °C, extractor voltage: 4 V, RF lens: 0.2 V, flow of nebulizing gas: 60 l/h, flow of desolvation gas: 550 l/h (both nitrogen). Collision gas (argon) pressure: 2.49×10^{-6} bar. The analytical method applied has been validated and described in further detail elsewhere (Diaz-Cruz et al., 2003; Rodriguez-Mozaz et al., 2004).

2.5.4. Data-treatment

As a first step all results were evaluated graphically and from this it was evident that one laboratory reported values which were consistently lower than the remaining laboratories. Here, this is demonstrated numerically, using the measured responses to the sample containing 10 nM estradiol. Laboratory 1 measured an average response of 13.21 nM, laboratory 2; 2.41 nM, laboratory 3; 9.19 nM and laboratory 4; 8.50 nM. After careful examination of the data, especially the standards with estradiol, it was clear that the responses of laboratory 2 were consistently about a factor 4 below the remaining laboratories. The reason for this constant error should be investigated and corrected by the laboratory in question. After a careful study of possible causes the laboratory could confirm that the constant error

was due to a dilution error of the estradiol standard used as control. So, to assure consistency of the dataset for further data analysis, all measured values were multiplied by a correction factor found from the analysis of the standard solution of estradiol with known concentration.

2.5.5. Data-distribution and treatment of outliers

For the eleven samples in the six batches, the following parameters were calculated: the mean value ($\bar{x}$), the variance (σ^2) and the standard deviation (σ), along with the variation coefficients (%CV). As a test for possible outliers, a *Z*-score (AOAC et al., 1987) was calculated for each result. The *Z*-score can be calculated by the following equation:

$$Z = \frac{x_{\text{lab}} - \bar{x}}{\sigma}.$$

If the *Z*-score is over 3, the result is more than 3 standard deviations away from the mean value, which means that there is less than 1% chance that this result belongs to the normal distribution described by the remaining data. Because of this, results with a *Z*-score over 3 are discarded as outliers. If the *Z*-score is between 2 and 3, there is up to 5% chance that the result belongs to the normal distribution and in this case a Dixon test (Dean and Dixon 1951) is performed to determine if the result is an outlier or not.

This principle is demonstrated in Fig. 1, where the results of the sample with 15 nM Estradiol are plotted along with lines marking 2 standard deviations and 3 standard deviations. The figure shows that 2 results have a *Z*-score between 2 and 3. In those cases the data was subjected to the Dixon test.

3. Results and discussion

In Table 1 all results (given in nM) are displayed. Results in bold have been demonstrated as outliers by the *Z*-score or the Dixon test. The table shows that 10 values (3.8%) have been shown to be outliers and have been removed from further data treatment.

3.1. Intra- and inter-laboratory variation

Table 2 gives an overview of the statistical parameters, for each individual laboratory and all laboratories together. In general, intra-laboratory variation is in the range of 10–155% (%CV), where the highest variations were observed for samples in the lower concentration range. The variance of samples of higher estrogenicity was typically 10–50%.

An *F*-test (Ellison et al., 2009) revealed that the variation between laboratories is comparable to that within laboratories. Although there are significant differences between largest and smallest standard deviations, there is no clear pattern of one laboratory having consistently smaller or larger standard deviations than the remaining laboratories. There is, furthermore, no significant difference between the average intra-laboratory variations of the participating laboratories. Thus, we can conclude that there is no significant difference between the performance of the different laboratories, nor the different yeast-assays applied.

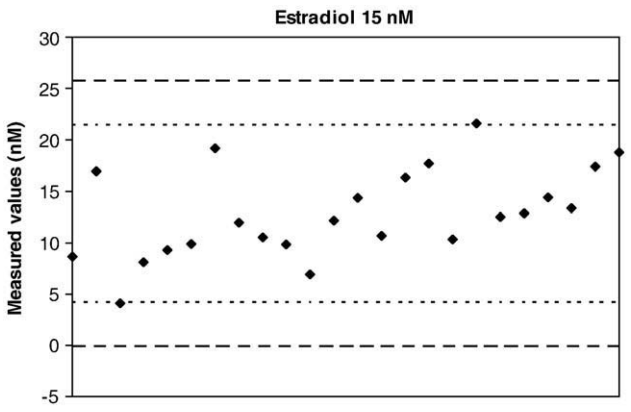

Fig. 1. Results for the standard solution of 15 nM estradiol, plotted with the value of ±2SD (dotted line) and ±3SD (solid line).

Table 1
Measured estrogenic activity (17β-estradiol equivalents, nM). Results marked in bold are considered to be outliers based on the *Z*-score or the Dixon test.

Batch/sample	Laboratory 1						Laboratory 2						Laboratory 3						Laboratory 4					
	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
Estradiol (I)	13.72	14.9	3.086	8.315	6.477	13.06	10.74	13.04	6.814	11.96	6.061	7.36	11.11	8.903	14.47	11.97	5.202	5.009	7.1	13.2	9.0	13.7	12.1	7.7
Estradiol (II)	8.677	16.95	4.115	8.117	9.311	9.907	19.16	11.98	10.54	9.867	6.946	12.2	14.33	10.71	16.32	17.66	10.36	21.58	12.5	12.9	14.4	13.3	17.4	18.8
MeOH	bld	bld	bld	bld	bld	bld	bld	bld	**0.124**	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld
Genistein	22.53	22.09	13.24	6.496	19.07	29.9	9.012	11.98	5.159	8.903	6.325	5.628	14.39	8.081	9.514	27.45	17.96	9.586	19.9	23.5	25.2	26.6	23.3	19.6
NP (I)	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	**1.036**	bld	bld	bld	**0.61**	bld	bld	bld	bld	bld	bld
NP (II)	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	**1.227**	bld	bld	**1.143**	bld	bld	bld	bld	bld	bld	bld	bld
Sample (I)	5.856	6.564	1.576	3.625	3.568	5.788	2.346	1.478	0.883	1.128	0.998	0.452	5.439	5.614	8.334	4.368	2.324	7.325	**10.4**	bld	7.0	bld	6.1	**12.2**
Sample (II)	20.64	17.44	5.078	8.46	7.062	9.202	7.877	5.379	9.876	6.564	3.426	4.894	13.91	10.18	23.95	11.8	11.09	19.14	10.9	17.1	13.4	16.2	16.8	17.4
Sample (III)	5.419	6.914	6.356	3.23	5.614	4.435	0.988	1.163	1.2	1.169	0.414	0.282	5.802	4.732	6.269	4.787	3.043	5.214	7.8	bld	8.4	6.6	9.4	2.0
Sample (IV)	16.64	**30.71**	7.405	6.568	10.71	11.04	5.679	3.862	7.09	6.749	4.668	2.955	15.6	18.62	17.58	12.62	6.365	17.89	14.3	13.6	13.2	14.2	14.6	17.4
Sample (V)	**26.4**	17.54	11.44	7.005	10.52	12.92	11.9	9.379	10.95	12.59	5.289	5.816	22	20.26	20.43	**29.49**	13.7	18.21	15.8	17.3	15.8	20	20.7	19.4

bld: below limit of detection.

Table 2
Statistical parameters for the results, values given in nM.

	All laboratories			Laboratory 1			Laboratory 2			Laboratory 6			Laboratory 7		
	$\bar{x}$	σ	%CV	$\bar{x}$	σ	%CV	$\bar{x}$	σ	%CV	$\bar{x}$	σ	%CV	$\bar{x}$	σ	%CV
Estradiol (I)	9.8	3.4	35.2	9.9	4.7	47.3	9.3	3.0	31.6	9.4	3.8	40.3	10.5	2.9	27.6
Estradiol (II)	12.8	4.3	33.6	9.5	4.2	43.9	11.8	4.1	34.6	15.2	4.3	28.3	14.9	2.6	17.4
MeOH	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Genistein	16.1	7.9	49.1	18.9	8.1	43.0	7.8	2.6	33.3	14.5	7.3	50.7	23.0	2.8	12.1
NP (I)	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	162.5	0.0	0.0	0.0
NP (II)	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.6	155.1	0.0	0.0	0.0
Sample (I)	3.7	2.7	72.5	4.5	1.9	42.1	1.2	0.6	53.3	5.6	2.1	38.3	4.7	4.6	97.3
Sample (II)	12	5.6	46.6	11.3	6.2	55.1	6.3	2.3	36.3	15.0	5.4	36.1	15.3	2.6	17.0
Sample (III)	4.2	2.8	66.4	5.3	1.3	25.0	0.9	0.4	47.5	5.0	1.1	22.5	5.7	3.8	67.1
Sample (IV)	11.3	5.1	44.8	10.5	4.0	37.9	5.2	1.6	31.6	14.0	4.8	34.0	14.5	1.5	10.3
Sample (V)	14.5	5.1	35.1	11.9	3.8	32.3	9.3	3.1	33.4	18.9	3.2	17.0	18.2	2.1	11.8

The part of the table dedicated to the treatment of all data shows standard deviations between 2.7 and 7.9 nM (inter-laboratory). The relative standard deviation (%CV) demonstrates how large the standard deviation is in relation to the average of values. The coefficients of variation lie between 33.6 and 72.47% (inter-laboratory). This variation is comparative to the inter-laboratory variation found in previous inter-laboratory studies including recombinant yeast-based assays (Andersen et al., 1999; Dhooge et al., 2006).

The yeast-based assays are biological assays and may as such, have a large inherent variation. However, with standard deviations this high, the confidence intervals would also become large. If we consider the sample with 10 nM estradiol with the pooled results of all laboratories, the result with 95% confidence interval (2 standard deviations) is 9.8 ± 6.8 nM. This means that almost 70% of the results reported as 10 nM estradiol equivalents (i.e. just above the biological critical limit) will be false positives; thus making the assays less powerful as a pre-screening tool for chemical analysis.

3.2. Chemical analysis

Chromatographic analysis (LC–MS and LC–MS/MS) was carried out on all samples. Table 3 shows levels of nonylphenols and estrogens in the water extracts (the samples that the participants received). In this paper we have presented concentrations in sample extracts throughout the text. However, to be able to compare the observed concentrations with environmental concentrations detected in other studies we have to take the concentration factor into consideration. The concentrations in the actual water samples are 400 times lower than the values stated in Table 3, due to the pre-concentration steps performed during the sample preparation. This means that the concentration of nonylphenol in the spiked sample would correspond to 28.2 µg/l in the effluent water sample and the concentrations of estrone and estradiol to 10.5 ng/l and 10.25 ng/l respectively, in the sample with the highest spike. The detected concentration levels of estrogens in the natural samples are comparable to concentrations found in previous studies of WWTP effluents (Murk et al., 2002; Pawlowski et al., 2003; Rutishauser et al., 2004). The concentration of nonylphenol in the spiked sample is higher than what is normally found in WWTPs. This is because the spiking level of nonylphenol was chosen so that nonylphenol would represent the accumulated estrogenic activity of the whole group of alkylphenols of which especially nonylphenol-carboxylates and nonylphenol-ethoxylates are found at elevated concentrations in the environment (Gonzalez et al., 2004).

3.3. Relative estrogenic activity

As the *in-vitro* estrogen assays express estrogenic activity as estradiol equivalents, a considerable number of studies have been published determining the estrogenic activity of a range of compounds relative to estradiol. There is a large variance between different estimations, e.g. the relative estrogenic activity (or estradiol equivalency factor (EEF)) of nonylphenol determined by yeast-based assays has been reported in the range of 7.2×10^{-7} to 4×10^{-4} (Brix and Barceló, 2009). The high variance may be partially due to the fact that the assays may use different estrogen receptors and different reporting elements, but is also due to variations between laboratories. This means that the EEFs are not universe determinations related only to 17β-estradiol, but also to the specific assay and to the laboratory using the assay.

Thus, in order to improve the correlation between measured and calculated results, the estrogenic activity of nonylphenol and genistein has been determined by the participating laboratories by analysing standard solutions. There was good correlation between determinations of EEF for genistein (lab. 1: 1.52×10^{-4}, lab. 2: 8.66×10^{-5}, lab. 3: 2.19×10^{-4} and lab. 4: 2.56×10^{-4}). However there was a larger variation in the determinations of EEF for nonylphenol; while there is a factor five difference between the values from laboratories 1 (1.2607×10^{-5}) and 3 (3.29984×10^{-6}), the setup in laboratories 2 and 4 did not detect any estrogenic activity of nonylphenol. The values determined by laboratories 1 and 3 are within the range found in the literature, however they are about a factor ten below the average of the literature values. This is possibly due to the fact that straight chained nonylphenol (n-NP) was used, which may have lower estrogenic activity than branched nonylphenol. The majority of papers do not specify if they have used straight or branched chain nonylphenol, but Rutishauser et al. (2004) determined the EEF of branched chain nonylphenol to be 2.5×10^{-4}, while Coldham et al. (1997) determined the EEF of n-NP to be 5×10^{-5}. Even though the difference in EEFs for nonylphenol between laboratories 1 and 3 (both using the YES protocol) may seem large, the EEF from laboratory 3 is included in the 95% confidence interval (2 standard deviations) of the value from laboratory 1.

3.4. Calculation of estrogenic activity of samples from chemical concentrations

It has previously been demonstrated that the estrogenic activity of the compounds analysed in the samples can be considered additive (Rutishauser et al., 2004). Thus, the estimated estrogenic activity (in molar units of estradiol) of a sample can be calculated from the following equation:

$$EEq = \sum_i EEF_i \times c_i$$

where: EEq: Estradiol equivalency quotient, EEF: estradiol equivalency factor and *c*: concentration in the sample.

In order to be able to compare results between laboratories we have used the average values of the EEFs for nonylphenol and genistein determined in this study. For the remaining compounds the average of the literature values has been used. On this basis, a rough estimation of the estrogenic activity was calculated, resulting in a total estrogenity of 5.57 nM (Sample I), 15.57 nM (Sample II), 5.79 nM (Sample III), 16.31 nM (Sample IV), 20.57 nM (Sample V), 0.22 nM (NP (I)) and 0.43 nM (NP (II)).

In Fig. 2 the results from all laboratories are plotted against the estimated estrogenic activity of the samples. The figure shows a good correlation between expected and measured values ($R^2 = 0.975$). The slope ($\alpha = 0.83$) is, however, slightly lower than optimal ($\alpha = 1.0$). Further, it can be observed that even though there is a good correlation, the standard deviations are quite large.

Table 3
Concentration of alkylphenols (in µM/mg/l) and estrogens (in nM/µg/l) in samples (I)–(V) determined by LC–MS and LC–MS/MS respectively. Concentrations are given in both mass and molar concentrations for comparison reasons.

	OP		NP		NP1EO		E2		E3		E1		EE	DES	Gen
	µM	mg/l	µM	mg/l	µM	mg/l	nM	µg/l	nM	µg/l	nM	µg/l	µg/l	µg/l	µg/l
Sample (I)	1.5	0.31	1.25	0.27	22.77	6.01	n.d.	n.d.	22.87	4.2	14.57	6.18	n.d.	n.d.	n.d.
Sample (II)	1.5	0.31	1.25	0.27	22.77	6.01	9.91	2.7	22.87	4.2	14.57	6.18	n.d.	n.d.	n.d.
Sample (III)	1.5	0.31	51.2	11.29	22.77	6.01	n.d.	n.d.	22.87	4.2	14.57	6.18	n.d.	n.d.	n.d.
Sample (IV)	1.5	0.31	51.2	11.29	22.77	6.01	9.91	2.7	22.87	4.2	14.57	6.18	n.d.	n.d.	n.d.
Sample (V)	1.5	0.31	1.25	0.27	22.77	6.01	15.05	4.1	22.87	4.2	14.57	6.18	n.d.	n.d.	n.d.

n.d.: not detected. Estriol (E3), estradiol (E2), ethynyl estradiol (EE), estrone (E1), diethylstilbestrol (DES) and genistein (Gen).

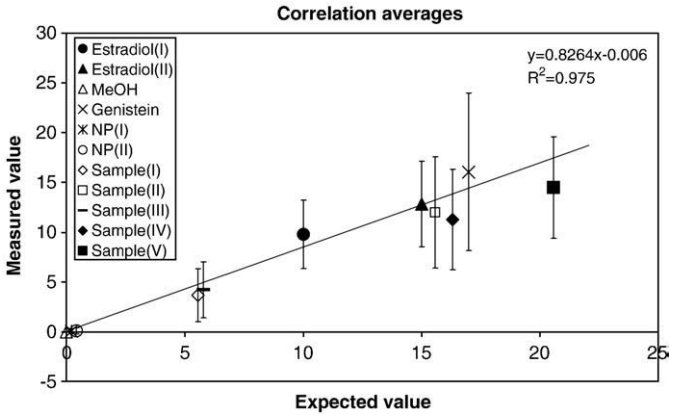

Fig. 2. Correlation of average measured values from each laboratory, including error bars (1SD) to describe the variation between laboratories.

3.5. Correlation to chemical concentrations, using individual EEFs

The correspondence between the bioassay results and the concentrations of the analysed chemicals was evaluated by performing correlation analysis between measured EEqs and those calculated using the EEFs obtained by each laboratory. These correlations are shown in Fig. 3.

The upper left corner of Fig. 3 shows the correlation for laboratory 1. If the point (15.2; 18.9—genistein in methanol) is disregarded, it can be seen that the correlation is probably not linear, indicating a masking effect by the matrix. It is recommended that this laboratory in future analyses should use standard addition with at least two spikes to be able to calculate back to the concentration in the sample without matrix effect.

The upper right corner of Fig. 3 shows the correlation for laboratory 2. The figure shows that even though there is a significant correlation between predicted and observed values, residuals from the fitting of a linear correlation are fairly large in the results from this laboratory.

The correlation for laboratory 3 is shown in the lower left corner of Fig. 3. If the point ((21.9; 14.5) genistein in methanol) is removed, the correlation yields a slope of 0.94 and a correlation coefficient (R^2) of 0.99, showing a very good dose–response relationship. The main difference between laboratories 1 and 3 is the level of experience; while laboratory 1 has relatively little experience with the YES assay, laboratory 3 has been using it routinely for years. It is likely that the better correlation for laboratory 3 is due to more experience in interpreting the results from the assay.

Finally, the correlation of laboratory 4 is shown in the lower right corner of Fig. 3. This laboratory shows an excellent correlation between expected and measured values.

3.6. Suitability of these bioassays as pre-screening tools in monitoring within the WFD

The European Union has recently established environmental quality standards (EQS) for a number of priority pollutants (European Parliament and Council, 2008). The standards specify both the annual average (AA) and maximum allowable concentration (MAC) in surface waters.

There are no steroid estrogens included in the list and of the alkylphenols, only nonylphenol and octylphenol are included. The maximum allowable concentration for nonylphenol is 2 µg/l or 9 nM. Converting this to estradiol equivalents, using the average of the estradiol equivalency factors from laboratories 1 and 3, results in an EEq of 0.07 pM. This number is far below the observed limits of detection for the yeast-based assays and it has to be concluded that for routine monitoring of compliance with the EQS for alkylphenols, liquid chromatography is the best option.

However, the aim of the water framework directive is the improvement of the status of aquatic ecosystems, thus, estrogenic activity needs to be considered and analysing alkylphenolic compounds will only explain a part of the estrogenic activity, as seen in this study. For the steroid estrogens we see a very good correlation with chemical data and comparable limits of detection.

In light of the above we propose using the bioassays to eliminate samples without biological response and only performing chemical analysis for steroid estrogens on the remaining samples. It is difficult to predict the financial consequences of using this practise, but there is no doubt that it would result in a large reduction in overall costs. If we estimate that a laboratory carries out 400 analyses per year and that the cost of the bioassays is approximately 8% of the cost of chemical analysis is per sample. Assuming that 5 repetitions are performed per sample and assessing that 350 samples will be negative; then the overall reduction of costs will be around 80%.

4. Conclusions

Based on the present study, it can be concluded that the tested bioassays show a good correlation with expected values from chemical analysis and no significant difference between the different laboratories, or type of yeast-assay. It is thus possible to use these tests as preliminary screenings of water samples for estrogenic activity. However, because of the low estrogenic activity of alkylphenols, the tested bioassays are currently not suitable for monitoring of compliance with the European environmental quality standards (EQS) for alkylphenol and octylphenol.

A fairly large variation is seen and future work should include the development of further suitability or quality control tests so that it is confirmed that the system is always in control. The importance of an external calibration with 17β-estradiol has been confirmed and

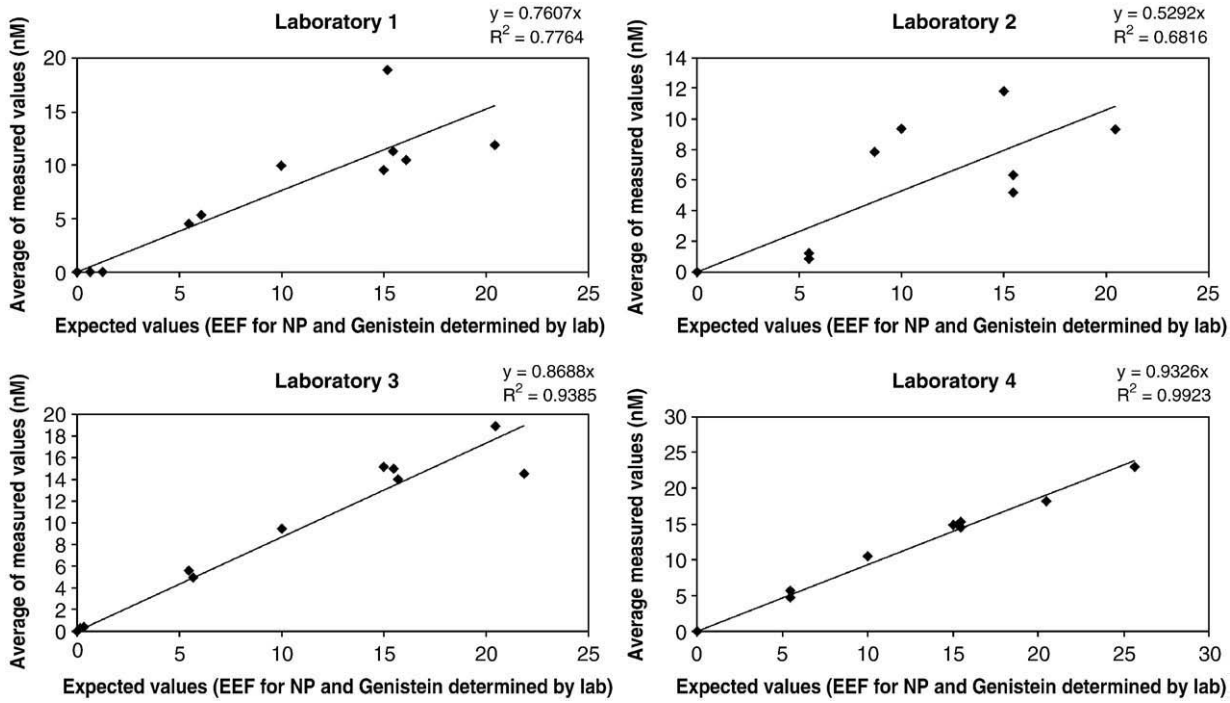

Fig. 3. Correlation between expected and measured values for the participating laboratories.

would ensure more (internationally) comparable results in future analyses. The optimal would be to use a certified reference material, but so far this does not exist. Furthermore, the study demonstrated the necessity of estimating estradiol equivalency factors (EEF) for all present compounds in each laboratory in order to obtain a good correlation with results from chemical analysis.

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References

Andersen AG, Jensen TK, Carlsen E, Jorgensen N, Andersson AM, Krarup T, et al. High frequency of sub-optimal semen quality in an unselected population of young men. Hum Reprod 2000;15(2):366–72.

Andersen HR, Andersson AM, Arnold SF, Autrup H, Barfoed M, Beresford NA, et al. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ Health Perspect 1999;107(SUPPL. 1):89-108.

AOAC, ISO, IUPAC. Z-scores. Canada: Food Inspection Directorate, Agriculture; 1987.

Bovee TFH, Helsdingen RJR, Koks PD, Kuiper HA, Hoogenboom R, Keijer J. Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein. Gene 2004;325:187–200.

Brix R, Barceló D. Chapter 5.3. Traceability and interlaboratory studies on yeast-based assays for the determination of estrogenicity. In: Brix R, Barceló D, editors. Rapid chemical and biological techniques for water monitoring. John Wiley & Sons, Ltd; 2009. p. 371–81.

Cespedes R, Lacorte S, Raldua D, Ginebreda A, Barcelo D, Pina B. Distribution of endocrine disruptors in the Llobregat River basin (Catalonia, NE Spain). Chemosphere 2005;61(11):1710–9.

Cespedes R, Petrovic M, Raldua D, Saura U, Pina B, Lacorte S, et al. Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC–ESI-MS. Anal Bioanal Chem 2004;378(3):697–708.

Coldham NG, Dave M, Sivapathasundaram S, McDonnell DP, Connor C, Sauer MJ. Evaluation of a recombinant yeast cell estrogen screening assay. Environ Health Perspect 1997;105(7):734–42.

Dean RB, Dixon WJ. Simplified statistics for small numbers of observations. Anal Chem 1951;23(4):636–8.

Dhooge W, Arijs K, D'Haese I, Stuyvaert S, Versonnen B, Janssen C, et al. Experimental parameters affecting sensitivity and specificity of a yeast assay for estrogenic compounds: results of an interlaboratory validation exercise. Anal Bioanal Chem 2006;386(5):1419–28.

Diaz-Cruz MS, de Alda MJL, Lopez R, Barcelo D. Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS). J. Mass Spectrom. 2003;38(9):917–23.

Diaz A, Ventura F, Galceran MT. Simultaneous determination of estrogenic short ethoxy chain nonylphenols and their acidic metabolites in water by an in-sample derivatization/solid-phase microextraction method. Anal Chem 2002;74(15):3869–76.

Ellison SLR, Barwick VJ, Farrant TJD. Practical statistics for the analytical scientist — a bench guide. Cambridge, UK: RSC Publishing; 2009.

European Parliament and Council (2008). Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council.

Farre M, Brix R, Kuster M, Rubio F, Goda Y, de Alda MJL, et al. Evaluation of commercial immunoassays for the detection of estrogens in water by comparison with high-performance liquid chromatography tandem mass spectrometry HPLC–MS/MS (QqQ). Anal Bioanal Chem 2006;385(6):1001–11.

Farre M, Kloter G, Petrovic M, Alonso MC, de Alda MJL, Barcelo D. Identification of toxic compounds in wastewater treatment plants during a field experiment. Anal Chim Acta 2002;456(1):19–30.

Farre M, Kuster M, Brix R, Rubio F, Alda M-J, Alda M-JLd, et al. Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography–tandem mass spectrometry, and ultra performance liquid chromatography-quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. J Chromatogr 2007;1160(1–2):166–75.

Garcia-Reyero N, Grau E, Castillo M, De Alda MJL, Barcelo D, Pina B. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. Environmental Toxicology and Chemistry 2001;20(6):1152–8.

Garcia-Reyero N, Requena V, Petrovic M, Fischer B, Hansen PD, Diaz A, et al. Estrogenic potential of halogenated derivatives of nonylphenol ethoxylates and carboxylates. Environmental Toxicology and Chemistry 2004;23(3):705–11.

Gonzalez S, Petrovic M, Barcelo D. Simultaneous extraction and fate of linear alkylbenzene sulfonates, coconut diethanol amides, nonylphenol ethoxylates and their degradation products in wastewater treatment plants, receiving coastal waters and sediments in the Catalonian area (NE Spain). J Chromatogr 2004;1052(1–2):111–20.

Green S, Chambon P. The oestrogen receptor: from perception to mechanism. London, UK: Academic; 1991.

Kuster M, López de Alda MJ, Hernando MD, Petrovic M, Martín-Alonso J, Barceló D. Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain). J Hydrol 2008;358(1–2):112–23.

Legler J, Dennekamp M, Vethaak AD, Brouwer A, Koeman JH, van der Burg B, et al. Detection of estrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays. Sci Total Environ 2002;293(1–3):69–83.

López de Alda MJ, Gil A, Paz E, Barcelo D. Occurrence and analysis of estrogens and progestogens in river sediments by liquid chromatography–electrospray-mass spectrometry. Analyst 2002;127(10):1299–304.

Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, et al. Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. Environmental Toxicology and Chemistry 2002;21(1):16–23.

Noguerol TN, Boronat S, Jarque S, Barcelo D, Pina B. Detection of hormone receptor ligands in yeast by fluorogenic methods. Talanta 2006;69(2):351–8.

Pawlowski S, Ternes T, Bonerz M, Kluczka T, van der Burg B, Nau H, et al. Combined in situ and *in vitro* assessment of the estrogenic activity of sewage and surface water samples. Toxicological Sciences 2003;75(1):57–65.

Pawlowski S, Ternes TA, Bonerz M, Rastall AC, Erdinger L, Braunbeck T. Estrogenicity of solid phase-extracted water samples from two municipal sewage treatment plant effluents and river Rhine water using the yeast estrogen screen. Toxicol In Vitro 2004;18(1):129–38.

Reddy S, Brownawell BJ. Analysis of estrogens in sediment from a sewage-impacted urban estuary using high-performance liquid chromatography/time-of-flight mass spectrometry. Environmental Toxicology and Chemistry 2005;24(5):1041–7.

Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, et al. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environmental Science & Technology 2001;35(3):462–70.

Rodriguez-Mozaz S, de Alda MJL, Barcelo D. Picogram per liter level determination of estrogens in natural waters and waterworks by a fully automated on-line solid-phase extraction-liquid chromatography–electrospray tandem mass spectrometry method. Anal Chem 2004;76(23):6998–7006.

Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry 1996;15(3):241–8.

Rutishauser BV, Pesonen M, Escher BI, Ackermann GE, Aerni HR, Suter MJF, et al. Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three *in vitro* assays and chemical analysis of steroids. Environmental Toxicology and Chemistry 2004;23(4):857–64.

Schmitt M, Gellert G, Lichtenberg-Frate H. The toxic potential of an industrial effluent determined with the *Saccharomyces cerevisiae*-based assay. Water Res 2005;39(14):3211–8.

Schneider JC, Guarente L. Vectors for expression of cloned genes in yeast: regulation, overproduction, and underproduction. Methods Enzymol 1991;194:373–88.

Sole M, Barcelo D, Porte C. Seasonal variation of plasmatic and hepatic vitellogenin and EROD activity in carp, *Cyprinus carpio*, in relation to sewage treatment plants. Aquat. Toxicol. 2002;60(3–4):233–48.

Thomas KV, Balaam J, Hurst M, Nedyalkova Z, Mekenyan O. Potency and characterization of estrogen-receptor agonists in United Kingdom estuarine sediments. Environmental Toxicology and Chemistry 2004;23(2):471–9.

Tollefsen KE, Harman C, Smith A, Thomas KV. Estrogen receptor (ER) agonists and androgen receptor (AR) antagonists in effluents from Norwegian North Sea oil production platforms. Mar Pollut Bull 2007;54(3):277–83.