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DETECTION OF ESTROGENIC POTENCY IN WASTEWATER AND SURFACE WATER WITH THREE IN VITRO BIOASSAYS

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Abstract—A study was performed to optimize sample preparation and application of three in vitro assays for measuring estrogenic potency in environmental extracts. The three assays applied were an estrogen receptor (ER)-binding assay and two reporter gene effect assays: a yeast estrogen screen (YES) and the ER-mediated chemically activated luciferase gene expression (ER-CALUX) assay. All assays were able to detect estrogenicity, but the amounts of material needed for the assays differed greatly between the three assays (ER-binding assay $\gg$ YES $>$ ER-CALUX). In addition, in the ER-binding assay, both agonists and antagonists give an estrogenic response, resulting in higher estradiol equivalency (EEQ) levels than both the ER-CALUX and the YES assay for the same samples. The EEQs found in wastewater treatment plants (WTPs) with the ER-CALUX assay were in the range of 4 to 440 and 0.11 to 59 pmol/L for influent and effluent, respectively. Water extracts from four large rivers had levels ranging from 0.25 to 1.72 pmol/L. Extracts from suspended matter and sludge contained estrogenic potency of 0.26 to 2.49 and 1.6 to 41 pmol EEQ/g dry weight, respectively. In WTPs, the average reduction of estrogenic potency in effluent compared to influent was 90 to 95% in municipal WTPs and about 50% in industrial WTPs. In influent, 30% of the ER-CALUX activity could not be explained by the calculated potencies based on chemical analysis of a number of known (xeno)estrogens; in effluent the unexplained fraction was 80%. These first results of analyzing estrogenic potency in WTP water and surface water in The Netherlands indicate that further studies are warranted to investigate the actual risks for aquatic systems.

Keywords—Estrogenic potency Reporter gene assay Endocrine disruptors Environmental levels

INTRODUCTION

The Health Council of The Netherlands recently screened about 80 groups of known pesticides and industrial compounds for potential sex-hormone–disrupting effects, and concluded that 34 groups of these compounds might pose a risk in The Netherlands [1]. These included polybromobiphenyls, polybrominated diphenylethers, alkylphenols, alkylphenol-ethoxylates, bisphenol-A, and, to a lesser degree, phthalates. Thus, hundreds of suspected estrogenic compounds, including natural and synthetic hormones, may be present in the environment.

For compounds with an identical mode of action such as estrogenic hormones and xenoestrogens that act through an estrogen receptor (ER), performance of individual risk assessments is problematic. First, problems might occur because both ER agonists and antagonists might be present in environmental samples. Second, chemical analysis of all compounds with potential estrogenic potency would be very costly and unknown estrogenic compounds, including metabolites, may still be present in environmental mixtures. Therefore, a simple sum of the estrogenic contribution of each known and analytically measurable component may overestimate or underestimate the actual cumulative estrogenic potency. This is especially a risk in mixtures where hormones are less dominant than in municipal wastewater treatment plant (WTP) samples. In such situations, a bioassay offers an integrated measure of the estrogenic potencies of environmental mixtures without knowing all relevant compounds beforehand. By comparing the measured estrogenic potencies with the calculated estrogenic potency based on the known estrogenic potency of the chemically analyzed individual compounds, estimation of the contribution of unidentified compounds to the estrogenic potency of the mixture is possible. Additional advantages of most in vitro tests are that they are relatively inexpensive, rapid, and do not require a large amounts of sample material.

A number of in vitro assays have been developed to screen substances for estrogenicity [2]. These assays include competitive ligand binding assays, cell proliferation assays, recombinant receptor–reporter assays, and yeast-based screens for estrogens. Each assay measures different aspects of the effect chain resulting in estrogenic effects but these assays also suffer from a number of drawbacks, as earlier tests using standards of environmental pollutants have shown [2]. These assays previously had not been used to quantify estrogenic potencies of complex environmental matrices. Therefore, we tested the applicability of the assays for environmental matrices. The E-screen with MCF7 breast cancer cells [3] was not used in this study. This assay is based on estrogen-depen-

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dent cell proliferation, and, also, compounds other than (anti-)estrogens have been reported to stimulate or inhibit cell growth [4], thus over- or underestimating the response. In addition, the incubation period of 8 d is not considered practical. The applied assays were an ER competitive ligand binding assay [5] and two reporter gene effect assays based on an estrogenic response: a yeast estrogen screen (YES) [6] and the ER-mediated chemically activated luciferase gene expression (ER-CALUX) assay [7]. Figure 1 schematically represents the mechanisms of (anti-)estrogenic responses of these three in vitro assays, illustrating the fact that each measures different aspects of the effect chain resulting in estrogenic effects. The first step, binding of a compound to the ER, is measured in the ER competitive ligand binding assay. Binding to the ER of both agonists and antagonists will give a positive response, and all compounds can reach the ER without having to pass a cell membrane. In cells the next step after binding of a (xeno)estrogen is activation of the receptor, dimerization and translocation of this complex to the nucleus, and binding to the estrogen-responsive element in the DNA [8]. The YES assay uses a yeast cell transformed with a human ER and a plasmid containing the estrogen-responsive element and the *LacZ* gene as a reporter gene coding for β-galactosidase. Activation of the receptor results in increased red coloring of the assay medium. This assay is a measure of agonistic action. The two known antagonists, tamoxifen and ICI 182,780, are not active in the YES assay [9] and transport of some relatively large or lipophilic molecules through the yeast cell membrane may be impaired [9]. In the ER-CALUX assay, reporter gene expression also is a measure of the ER-mediated cascade of events resulting in activation of genes. The T47D human breast adenocarcinoma cells with endogenous estrogen receptor were stably transfected with an estrogen-responsive luciferase reporter gene containing three estrogen-responsive elements. Both hydrophilic and lipophilic compounds can pass the cell membrane and antiestrogenic potency can be detected as well [9]. In all three assays, the only (in the YES assay) or most important ER is ER-α, so no important difference is to be expected between assays on this point.

This paper describes a study performed to optimize sample preparation and storage and application of three in vitro assays for measuring estrogenic potency in environmental extracts. This work is the first time these bioassays were applied to quantify the estrogenic potency of extracts from influent, effluent, and sludge from municipal and industrial WTPs and from water and particulate matter from four large freshwater rivers. Concurrent analysis of the levels of relevant estrogenic hormones and several xenoestrogenic chemicals was carried out to enable comparison of the calculated estrogenic potencies with the bioassay responses in the various samples.

MATERIALS AND METHODS

Sample locations and treatment

Water samples were collected in the Rhine and Meuse rivers at two locations where both rivers respectively enter the Netherlands (Lobith and Eijsden), and two downstream estuaries where these rivers discharge in the North Sea (Maasluis and Haringvliet) in August and in November 1997. Water treatment plants were sampled in September and December 1997. At four biological WTPs for municipal (WTPs B, C, and D) and industrial wastewater (WTPs E and F), the influent, effluent, and sewage sludge were sampled, whereas wastewater (WW) and sewer sludge were sampled at three local sewer collection

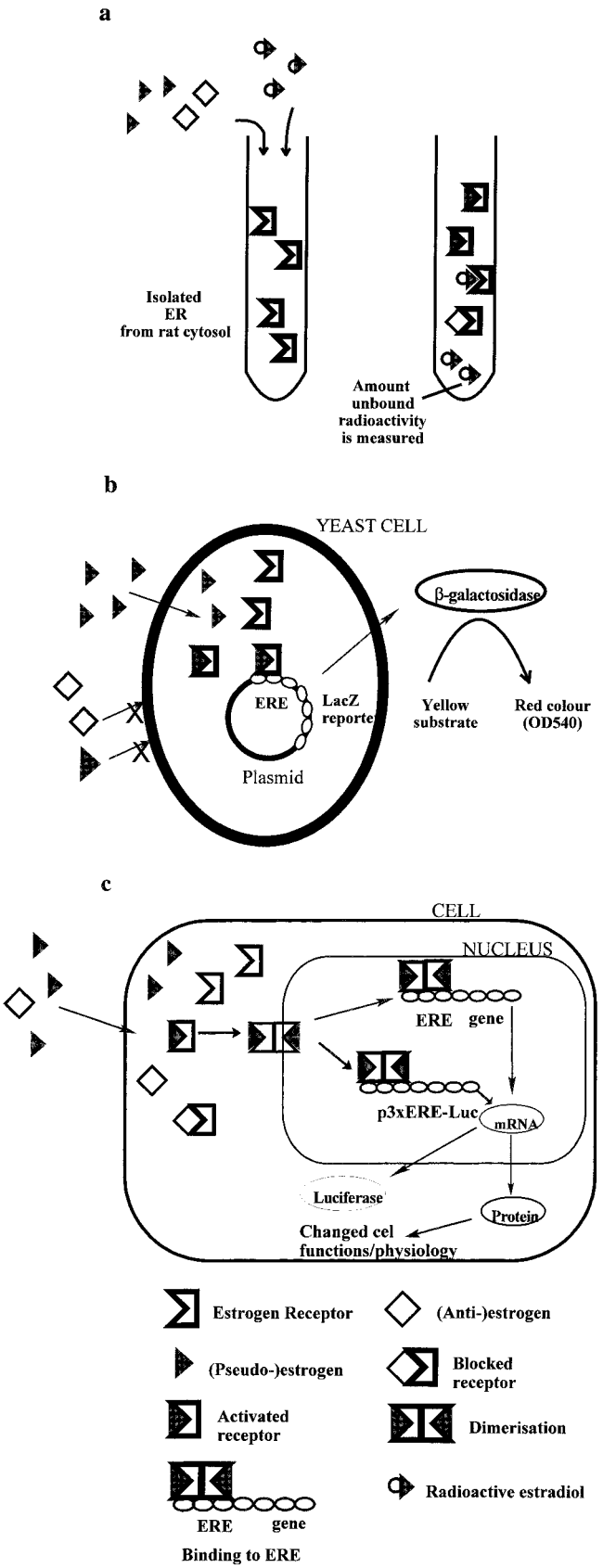

Fig. 1. Schematic representation of mechanisms of action of (anti-)estrogenic responses as measured in three in vitro bioassays. (**a**) Estrogen receptor (ER) competitive ligand binding assay [5], (**b**) a yeast estrogen screen assay in stably transformed yeast cells [6], and (**c**) ER-mediated chemically activated luciferase gene expression in stably transfected T47D human breast cancer cells [7]. ERE = estrogen-responsive element.

stations of a residential area (WW A) and two industrial areas (WWs G and H). The samples were collected every 30 min and combined over a 7-h period.

All samples were collected in prewashed glass bottles with a Teflon®-lined stopper, transported to the laboratory within 1 d and stored at 4°C for no more than 10 d. Water samples were extracted as described for hormones and bisphenol-A [10]. In short, water was filtered over 1.2- and 0.45-μM glass fiber filters (GF/C filters, Whatman, Clifton, NJ, USA,) and extracted with an SDB-XC disk (Varian, Walnut Creek, CA, USA). For surface water, 9-L samples were extracted with six SDB-XC disks. Compounds on the disks were eluted three times with 5 ml of methanol each time. For WTP samples (influent or effluent), 2- or 1-L samples were extracted with one disk per liter of filtered water. Control samples were made with 1 L of high-performance liquid chromatography water. For particulate matter and WTP sludge, representative suspension samples of 100 ml were freeze-dried. Of this dried material, about 1 g was extracted in an accelerated solvent extractor at high pressure (200 psi) at 100°C with dichloromethane:acetone (50:50, v/v). Control samples were made with silica. All extracts were dried at 60°C and taken up in dimethylsulfoxide (DMSO) or ethanol for exposure in the in vitro assays. Note that the extraction methods were based on validated methods for groups of known estrogenic chemicals. The recovery of the procedure for hormones is 88 to 98%. Although a broad spectrum of compounds will be extracted, indication of extraction efficiency for yet unknown compounds is impossible. This can only be done after tracking down these chemicals in a second stage of research by bioassay-directed fractionation and identification of active compounds.

ER-CALUX assay

The T47D human breast adenocarcinoma cells stably transfected with an estrogen-responsive luciferase reporter gene containing three estrogen-responsive elements (ER-CALUX cells) were cultured in a 1:1 mixture of Dulbeccos's modified Eagle's medium and Ham's F12 medium (Gibco, Invitrogen, Breda, The Netherlands) supplemented with sodium bicarbonate, nonessential amino acids, sodium pyruvate, and 7.5% fetal calf serum (Bodinco, Alkmaar, The Netherlands). The T47D cells were cultured at 37°C and 7.5% CO_2. For an assay the cells were plated in 96-well plates (Nunc, Life Technologies, Breda, The Netherlands) at a density of 5,000 cells per well in 0.1 ml of Dulbeccos's modified Eagle's medium and Ham's F12 medium without phenol red plus 5% dextran-coated charcoal–fetal calf serum (assay medium). After incubating for 24 h, cells were approximately 50% confluent. Assay medium was renewed and the cells were incubated another 24 h. Before exposure, 0.8 ml of assay medium in 48-well plates was mixed well with the test solution with a maximum solvent concentration of 0.2%. The medium on the cells was then renewed again and the cells were dosed in triplicate with 0.1 ml of assay medium per well containing chemicals. In addition to one 17β-estadiol (E_2) standard curve in triplicate per experiment, control wells, solvent control wells, and E_2 calibration points (1, 6, and 30 pM) were included in triplicate on each plate. Cells were dosed for 24 h before luciferase measurement. For this, medium was removed and the cells were lysed in 50 μl of Triton lysis buffer by gentle shaking at 4°C for a minimum of 1 h. A 25-μl sample of the cell lysate was then transferred to a black 96-well plate (Costar-Corning BV, Schiphol-Rijk, The Netherlands), 25 μl of luciferin substrate (Luclite, Packard Bioscience, Groningen, The Netherlands) was added, and the luciferase activity was assayed in a scintillation counter for 0.1 min per well.

YES assay

The YES assay was performed according to the method of Routledge and Sumpter [6]. Two hundred microliters of a YES yeast cell suspension with an optical density of 1 at 620 nm was transferred to each well of a 96-well plate (Costar). The yellow substrate chlorophenol red-βb-D-galactopyranoside was present in the growth medium. Each sample concentration was tested in triplicate by adding 4 μl of DMSO solution to each well. The plates are incubated at 32°C under humid conditions after shaking well. The plate was shaken well every day for 5 min to mix and disperse the growing cells. The optical density at 540 nm (OD540; for color) and the optical density at 620 nm (OD620; for turbidity or cell growth) were measured after 2 and 3 d. The background color without yeast was tested to be able to correct for strongly colored samples. One E_2 standard curve was included with each experiment, as well as a solvent control and E_2 calibration points (100 pM and 1,000 pM) on each plate. To correct for turbidity, the following equation was applied: YES response = OD540 − OD620. The OD620 (blank) was subtracted to correct for background color only when necessary.

ER competitive binding assay

The method for the ER-binding assay was based on the method of Schwartz and Skafar [5]. In short, rat uterus cytosol containing the ER was incubated for 3 h at 4°C with the standard or environmental extract of interest in different dilutions and a constant concentration of [^{3}H]-E_2 in Tris-ethylenediaminetetraacetic acid buffer. After incubation, the amount of unbound radioactivity was measured (Fig. 1a). The percentage of free radioactively labeled E_2 increased with increasing concentration of estrogenic compounds. Several dilutions of each environmental extract were tested in duplicate for the ER-binding assay, including dilutions that displaced [^{3}H]-E_2 for at least 80%.

Quantification of estrogenic potencies

For quantification of the estrogenic potency of an environmental extract, in both the ER-CALUX and the YES assays, a suitable response of the unknown mixture was interpolated in a dose–response curve of the standard compound E_2. The interpolation for the ER-CALUX assay was performed with the responses between the quantification limit (0.5 pM) and the median effective concentration (EC50; 6 pM) only, because quantification in this part of the standard curve is very reproducible [11]. Any value between the detection limit and the maximum value for E_2 was used for the YES assay. The ER-CALUX and YES extracts were tested in triplicate. At least three dilutions were tested, especially with extracts from matrices for which estrogenicity is yet unknown, to ascertain that quantification was not performed at a concentration with inhibition of the signal due to, for example, slight cytotoxicity. The estrogenic potency of the compound or environmental extract was expressed as estradiol equivalency (EEQ). SlideWrite® 4.0 (cumulative fit; Advance Graphics, Encinitas, CA, USA) was used for curve-fitting. The r^2 of the fit of the standard curve usually was above 0.98, but always was above 0.92. The concentration (or dilution) at which 50% of the [^{3}H]-E_2 was unbound (median inhibitory concentration [IC50]) was

Table 1. The molar-based estradiol equivalency factors (EEFs, estrogenic potency relative to estradiol) used for calculation of the estradiol equivalency (EEQ) of the mixture of chemically measured (xeno)estrogens[a]

Abbreviation	Compound	EEF, CALUX	EEF, YES	EEF, binding assay
E_2	17β-Estradiol	1	1	1
E_1	17α-Estradiol	0.016[b]	001	0.11
E_2-17α	Estrone	0.056[b]	0.1	0.07[c]
EE_2	17α-Ethinylestradiol	1.2[b]	1.2	0.8
bis-A	Bisfenol-A	7.8×10^{-6}[d]	1.0×10^{-5}	1.0×10^{-3}
DMP	Dimethylphtalate	1.1×10^{-5}[b]	1.0×10^{-6}[e]	0
DEP	Di-ethylphtalate	3.2×10^{-8}[b]	5.0×10^{-7}	5.0×10^{-7}[b]
DBP	Di-*n*-butylphtalate	1.8×10^{-8}[b]	1.0×10^{-7}[f]	> Solubility limit[g]
BBP	Butylbenzylphtalate	1.4×10^{-6}[b]	1.0×10^{-6}	> Solubility limit[g]
DEHP	Di(2-ethylhexyl)phtalate	0[b]	0	> Solubility limit[g]
DOP	Di-octylphtalate	0[b]	0	> Solubility limit[g]
NPE	Nonylphenol ethoxylates	3.8×10^{-6}[b]	4.0×10^{-6}	1.0×10^{-5}
OPE	Octylphenol ethoxylates	0[b]	40×10^{-6}	4.0×10^{-6}[h]
NP	4-Nonylphenol	2.3×10^{-5}[d]	5.7×10^{-4}	5.0×10^{-4}
OP	4-Octylphenol	1.4×10^{-6}[d]	1.0×10^{-5}[i]	5.0×10^{-5}[i]

[a] CALUX = chemically activated luciferase gene expression; YES = yeast estrogen screen.
[b] [9].
[c] [12].
[d] [7].
[e] Value was $< 10^{-6}$.
[f] Value was $< 10^{-7}$.
[g] Greater than solubility limit: effective concentration, 50% effect levels are much higher than the water solubility of the compound [13].
[h] Estradiol equivalency factor value of yeast estrogen screen assay.
[i] Value was $<\times 10^{5}$.

calculated with Graphpad Prism 2.0 (Graphpad Software, San Diego, CA, USA) for the ER-binding assay based on a dose–response curve with at least 80% of the [^{3}H]-E_2 displaced. Based on this and the IC50 of the E_2, the estrogenic equivalency of the sample could be calculated.

Calculation of the EEQ of a chemically determined mixture was based on all measured (xeno)estrogens with a known estradiol equivalency factor (EEF; Table 1) according to

$$[\text{compound 1}] \cdot \text{EEF 1} = \text{EEQ 1}$$

$$[\text{compound 2}] \cdot \text{EEF 2} = \text{EEQ 2}$$

$$\vdots$$

$$[\text{compound } x] \cdot \text{EEF } x = \text{EEQ } x$$

$$\text{EEQ (total)} = \text{EEQ 1} + \text{EEQ 2} + \cdots + \text{EEQ } x$$

The EEFs were on a molar basis because this is toxicologically more relevant than expressing concentrations on a weight basis. Because of the high limit of detection of the ER-binding assay the EC50 of more lipophilic compounds could not be reached because of the poor water solubility of these compounds.

Determination of best carrier and storage temperature

The best carrier of the two used most often and the best first stock concentration for the extracts was tested with the ER-CALUX assay. For this test, an extract of an influent sample was divided into two aliquots, one aliquot was dissolved in ethanol and the other was dissolved in DMSO, and the ER-CALUX signals were compared. Both stocks again were divided into three aliquots and kept at room (±22°C), refrigerator (4°C), and freezer (−20°C) temperatures. The signals were tested repeatedly over six weeks to test the stability of the estrogenic compounds in the extract. The same was done with a second influent sample dissolved in DMSO and an E_2 stock producing the EC50 response (6 pM).

To test whether the first stock could be too concentrated to allow full availability of the estrogenic compounds, an extract was divided in two portions and either dissolved in 10 μl of DMSO (stock 1) or dissolved in 1,000 μl of DMSO (stock 2). A second dilution (stock 1a), which theoretically has the same final dilution as stock 2, was made from stock 1. This was done by adding 4 μl of stock 1 to 396 μl of DMSO. Finally, 445 μl of DMSO was added to the remaining approximately 4.5 μl of stock 1 (stock 1b), which theoretically also has the same final dilution as stock 2 given full solubility of all compounds in the concentrated first stock 1. The ER-CALUX response of stocks 1a, 1b, and 2 was measured.

RESULTS AND DISCUSSION

Characteristics of the three bioassays after exposure to E_2 are shown in Figure 2 and Table 2. The maximum induction relative to solvent control was 5- to 14-fold for the YES assay

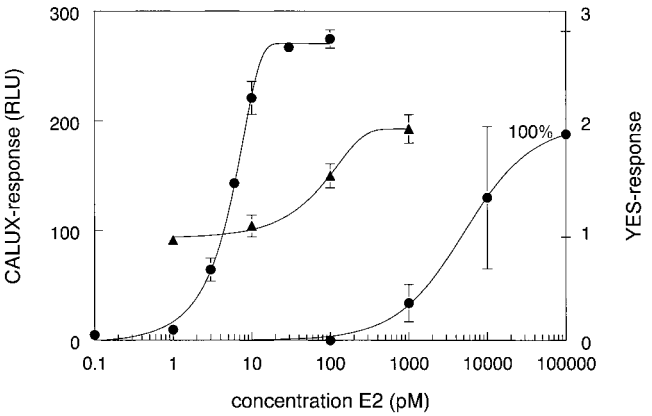

Fig. 2. Response of the three bioassays to 17β-estradiol (E_2). The estrogen receptor–mediated chemically activated luciferase gene expression (ER-CALUX) and yeast estrogen screen (YES) responses are from control value to maximum response, for the ER-binding assay from 0 to 100% competition (free [^{3}H]-E_2). RLU = relative light units.

Table 2. Response to 17β-estradiol and characteristics of the three in vitro assays and amount of material needed to determine the estrogenic potency with the three in vitro assays[a]

	ER-CALUX	YES	ER binding
Parameter			
Detection limit (pM)	0.5	10	1,000
EC50 (pM)	6	100	5,000
CV (%)	5–10	10–15	15–25
(Anti-)estrogens	<	−/≤	>
Material needed			
Surface water (ml)	6–30	60–250	±950
Particulate matter (g dry wt)	16–40	50–400	±1,500
WTP influent (ml)	0.2–2.5	4–100	±400
WTP effluent (ml)	0.9–9	10–100	±400
Sludge (g dry wt)	0.5–4	5–40	±150

[a] ER-CALUX = estrogen receptor–mediated chemically activated luciferase expression; YES = yeast estrogen screen; ER binding = estrogen receptor–binding assay; EC50 = 50% effect concentration; CV = coefficient of variation; WTP = wastewater treatment plant; < = decrease in signal; > = increase in signal; −/≤ = no or limited effect.

(at 1,000 pM) and 80- to 100-fold for the ER-CALUX assay (at 30 pM). The maximum effect in the ER-binding assay was reached at 10^5 pM. All three in vitro bioassays were able to detect estrogenic potencies in wastewater and surface water. However, the different characteristics of the assays, such as exposure volume and detection limit (Table 2), and the need to make a full dose–response curve (ER-binding assay), resulted in very different minimal amounts of material needed to determine the estrogenic potencies between the three assays (Table 2). Especially for matrices containing fewer EEQs (e.g., influent or rainwater) the ER-CALUX assay offered important advantages over the two other assays. The initial amounts of material needed for the assays could be reduced to the amounts mentioned in Table 2 after more knowledge was gathered on the actually occurring ranges of EEQ levels in the matrices of interest. In the ER-binding assay, antiestrogens gave a positive signal as well; sometimes they even were more potent in binding the ER than was E_2 (e.g., 4-hydroxytamoxifen, EEF = 1.75 [12]). A disadvantage of the the ER-binding assay is that higher concentrations are needed to achieve a significant reduction of [^{3}H]-E_2 bound to 100% of the ER in a mixture also including nonspecific binding compounds and antagonists, compared to the production of the reporter above background level. An additional disadvantage of the high concentrations needed in the ER-binding assay is that for lipophilic compounds and less potent xenoestrogens (e.g., phthalates), competitive binding occurs at concentrations that are high in comparison to their water solubilities [13].

Determination of best carrier and storage temperature

The signal obtained in the ER-CALUX assay with the same extract dissolved either in ethanol or in DMSO was identical (data not shown). However, ethanol stocks quickly lose a relatively large percentage of their small volume by evaporation at 4°C and room temperature. Even at −20°C, ethanol evaporates within months. Therefore, ethanol was considered less suitable for experiments where stocks must be stored for a longer period, and DMSO was used for the rest of the experiments. The three portions of the DMSO stocks kept at room temperature, 4°C, and −20°C were tested repeatedly over six weeks and a significant reduction ($p < 0.05$) in signal of 27% was observed at room temperature. The stocks kept at 4°C and −20°C did not differ significantly from the initial values over the six-week period, although some reduction was observed at 4°C (−11%; data not shown).

A large influence was found for the degree of concentration of the first stock from which the test solutions were to be made. Stock 1a and stock 2 of an influent extract theoretically had the same concentrations, but stock 1a was made from a first stock that was 100 times more concentrated. The EEQs measured in a dilution made from stock 1a were only 34% of the response measured in stock 2. As expected, stock 1b, diluted in the first tube to the same concentration as stock 2, gave a response of 95% of stock 2 (not significantly different). Therefore, it is important not to make the first stock too concentrated, because undissolved compounds may not be included in the dilutions made. In that case, the number of dilutions of the too-concentrated first stock that are tested does not make any difference. Making the first stock of an extract from a known matrix no more than 10 times more concentrated than needed for a successful measurement of relatively clean environmental samples is advisable.

Table 3. The ranges of estrogenic potencies measured in different matrices with the three in vitro bioassays[a]

Matrix	ER-CALUX	YES	ER binding
Surface water (pmol EEQ/L)	0.25–1.72	<dl—4	<dl–9
Particulate matter (pmol EEQ g dry wt)	0.26–2.49	<dl–1.06	<dl–302
WTP influent (pmol EEQ/L)	4–440	<dl–317	<dl–5,270
WTP effluent (pmol EEQ/L)	0.11–59	0.35–58	<dl–230
WTP sludge (pmol EEQ/g dry wt)	1.6–41	<dl–13[b]	360–2,000

[a] ER-CALUX = estrogen receptor–mediated chemically activated luciferase expression; YES = yeast estrogen screen; ER binding = estrogen receptor–binding assay; EEQ = estradiol equivalency, < dl = below detection limit; WTP = wastewater treatment plant.
[b] Excluding outliner of 2,750.

Estrogenic potencies in environmental matrices

The ranges in estrogenic potencies measured in several matrices with the three bioassays are given in Table 3. Although estrogenic potencies were measurable in all samples (with the most sensitive ER-CALUX assay), these differed by at least one order of a magnitude, depending on the matrix tested. More samples were below the limit of detection, increasing the risk of false negatives, with the ER-binding assay, and to a lesser extent with the YES-assay.

Interestingly, particulate matter and sludge also contain surprisingly high amounts of estrogenic compounds. Until now not much attention has been paid to the contribution of these materials to the total estrogenic potency of surface water or wastewater, although more lipophilic compounds also could reach biota via the food chain.

The estrogenic potencies determined with the YES and ER-CALUX assays correlated well both for water (surface water, influent, and effluent, $r = 0.82$, $n = 49$) and solid-phase samples (particulate matter and sewage sludge, $r = 0.93$, $n = 19$; Fig. 3). Measured levels typically are much higher with the ER-binding assay than in the other two assays (Tables 3 and 4). This is to be expected because both agonists and antagonists increase the EEQ and compounds that can not pass cell membranes or would otherwise be metabolized can still bind to the ER in the ER-binding assay. This, together with the larger

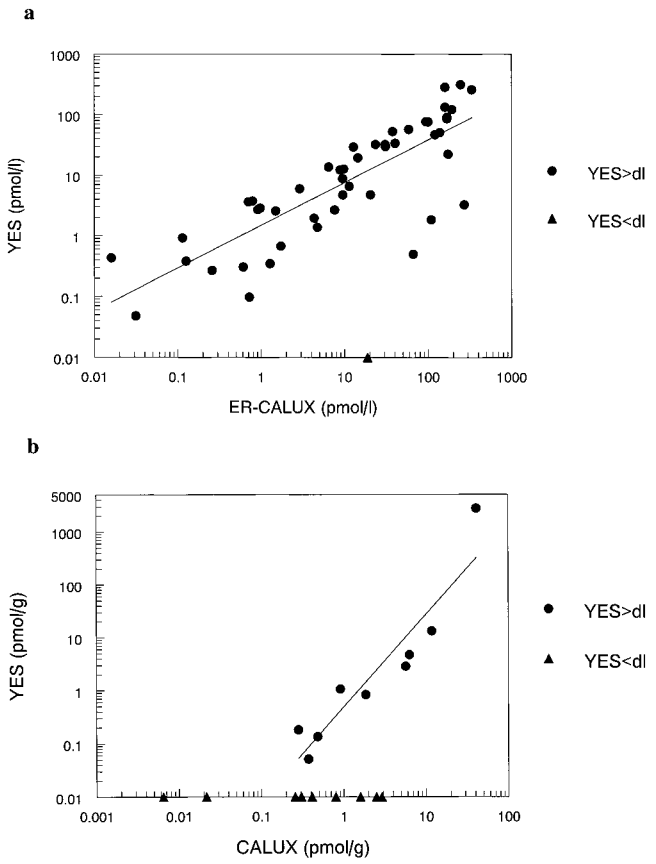

Fig. 3. Linear regression of estrogenic potency in environmental extracts as measured in the estrogen receptor–mediated chemically activated luciferase gene expression (ER-CALUX) and yeast estrogen screen (YES) assays. (**a**) Surface water, influent, and effluent (r = 0.82, n = 49, slope = 0.71). (**b**) Particulate matter and sewage sludge (r = 0.93, n = 19, slope = 1.7). ▲ = YES response was below detection limit (dl).

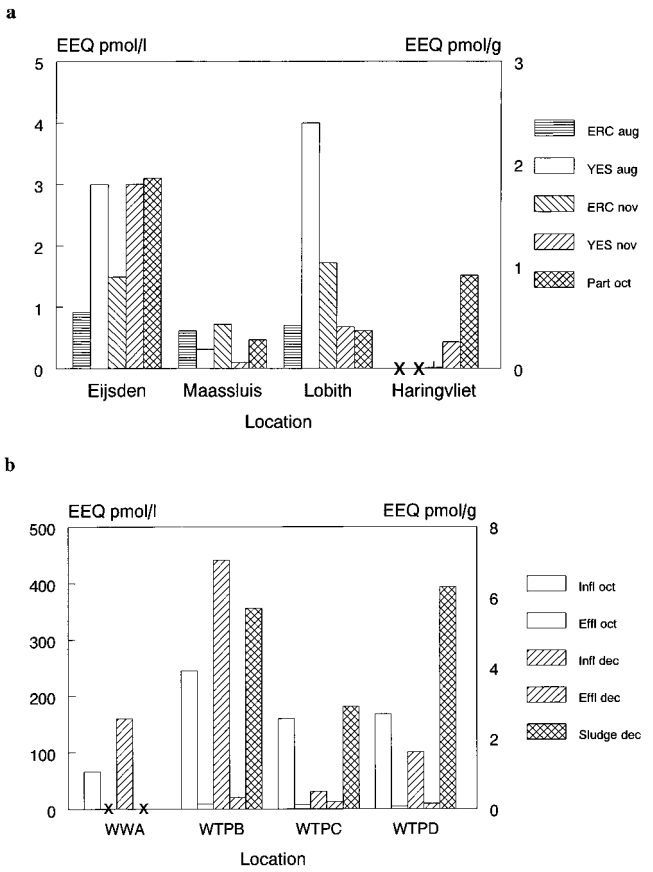

Fig. 4. Estrogenic equivalencies (EEQs) measured with the estrogen receptor–mediated chemically activated luciferase gene expression (ERC) and a yeast estrogen screen (YES) in (**a**) surface water and particulate matter and (**b**) household influent (Infl) and effluent (Effl) from wastewater treatment plants (WTPs) B, C, and D and wastewater (WW) A and sludge in October (oct) and December (dec) measured with the ERC. × = not measured or not relevant.

quantity of extract needed, is a disadvantage of the ER-binding assay. Although absolute levels differ substantially with both reporter gene assays and many levels are below the detection limit for the ER-binding assay, the responses do correlate with those of the ER-CALUX assay (water, r = 0.89, n = 23, 10 less than the detection limit; solid phase, r = 0.75, n = 8, 1 less than the detection limit).

Temporal and spatial fluctuations in estrogenic activities were observed for surface waters and WTPs (Fig. 4). In surface water downstream of the Rhine and Meuse rivers (Haringvliet and Maassluis), the EEQs were much lower than at Eijsden and, to a lesser degree, at Lobith, where the Meuse and Rhine rivers enter The Netherlands, respectively. This could simply be expained by dilution. However, in the sedimentation area of the Rhine River (Haringvliet), EEQ levels were relatively high in the sediment, although not as high as at Eijsden. Temporal fluctuations possibly can be explained by differences in rainfall and draining.

A substantial reduction is observed between estrogenic potency in effluent compared to influent in WTPs (Fig. 4b). The average reduction is about 75%, but this reduction is much higher (90–95%) in municipal WTPs than in industrial WTPs (about 50%; data not shown). This can be explained by the

Table 4. Average measured estrogenic equivalency (EEQ) in wastewater treatment plants by in vitro assay, compared with the EEQs calculated based on chemical analysis (percent of the activity explained with the chemical analysis). The contribution of estrogenic hormones to this explained percent is given in parentheses[a]

	Average EEQ (pmol/L)			% EEQ explained by chemical analysis (% of this due to hormones)		
Matrix	ER-CALUX	YES	ER binding	ER-CALUX	YES	ER binding
Influent	153 ± 123 (n = 15)	117 ± 113 (n = 15)	1,463 ± 1,330 (n = 8)	67 ± 46 (99 ± 3)	127 ± 136 (80 ± 11)	10 ± 10 (47 ± 36)
Effluent	9.5 ± 3.1 (n = 9)	11.5 ± 9 (n = 9)	142 ± 119 (n = 2)	21 ± 23 (78 ± 23)	51 ± 76 (49 ± 20)	10 ± 6 (21 ± 6)

[a] ER-CALUX = estrogen receptor–mediated chemically activated luciferase expression; YES = yeast estrogen screen; ER binding = estrogen receptor–binding assay; n = number of samples greater than the detection limit.

Table 5. The ranges of concentrations of estrogenic hormones measured in different matrices in ng/L (the number of samples above the detection limit [dl] is given in parentheses). Data are from Belfroid et al. [20][a]

	n	17β-Estradiol	17α-Estradiol	Estron	17α-Ethinyl estradiol
Surface water	7	<dl–5.5 (4)	<dl–1.1 (4)	<dl–5.3 (4)	<dl–4.3 (3)
WTP influent	13	10–48 (13)	<dl–9 (9)	10–140 (13)	<dl–10 (8)
WTP effluent	10	<dl–12 (4)	<dl–5 (3)	<dl–47 (7)	<dl–8 (2)
Sludge	6	<dl–4.3 (1)	<dl (0)	<dl (0)	<dl (0)

[a] WTP = wastewater treatment plant.

relative lower contribution of hormones to the total EEQs in industrial influents (Table 4).

Suspended matter and sludge contain significant levels of EEQs (Table 3), but the responsible compounds remain to be identified. For example, candidates are the more hydrophobic phthalates and alkylphenols and pesticides and their metabolites (*o,p*-DDT, *o,p*-dichlorodiphenyldichloroethylene, dieldrin, endosulfan, and chlordane) and flame retardants (e.g., brominated diphenylethers), which have been shown to exert an estrogenic potency [7,14] and their accumulative effect could be large [9]. Many of these compounds are more or less exclusively sorbed to particulate matter. This is in accordance with Legler [9], who found relatively high EEQ levels in extracts of sediments with the ER-CALUX assay. Further studies with bioassay-directed fractionation of samples in combination with chemical analysis will be performed to determine as yet unknown responsible xenoestrogens, including phytoestrogens. In addition, whether all compounds of interest were obtained must be studied. However, by definition, assessment of whether other as yet unknown compounds are too hydrophilic or volatile to be included is very difficult with the current extraction methods.

Comparison with calculated EEQs

The bioanalysis results were compared with the calculated EEQs based on chemical analysis (Table 4) with the EEFs from Table 1. In WTPs, the bioassays yielded a higher EEQ than was calculated based on the chemical analysis. In influent, 70% of the ER-CALUX activity could be explained by chemical analysis, although, other than estrogenic hormones, only a few compounds were measured. This is because of the relatively high levels of the potent hormones. The explained fraction decreased to 20% in the effluent. This coincides with a lower contribution of hormones to the calculated EEQ in the effluent compared to in the influent (Table 4). Thus, the increase in unexplained estrogenic potency may be due to relative accumulation of persistent compounds or newly formed bioactive metabolites. The percent of explained potency in the influent was as low as in the effluent with the ER-binding assay. However, the number of samples above the detection limit was relatively low.

The calculated EEQs in the surface waters were 5 to 10 times higher than the measured EEQs. Only the hormones could contribute to these calculated EEQs, but because the measured levels were close to or below the limit of detection, these levels are relatively uncertain, especially compared to levels in the influent (Table 5). Because of the high potencies of hormones, this results in high uncertainty in the calculated EEQs. In addition, the calculated EEQs do not include interaction and antagonism between compounds, and compounds may be lost during evaporation of the extract at 60°C. This discrepancy needs to be studied further. Comparisons between calculated and measured toxic equivalents are troubled by the fact that extraction and cleanup procedures often differ, and that levels of individual compounds may be just above or below the limits of detection or quantification.

Possible consequences for wildlife

Adverse effect EEQ levels can best be derived from in vivo exposure experiments in flow-through systems, or static systems where the actual concentration was checked. In a 19-d exposure experiment in flow-through tanks, a biologically significant adverse effect on egg production EC50 was observed in fathead minnows at a concentration slightly less than 0.5 nM EEQ (120 ng E_2/L) [15]. Biomarkers for exposure to E_2 already were increased at lower concentrations. The lowest effect levels for vitellogenin production reported in the literature are 1.3 pM EEQ (0.3 ng E_2/L) in immature male rainbow trout after 28 weeks of dosing [16] and 35 pM EEQ (10 ng E_2/L) in male rainbow trout and roach exposed for three weeks in flow-through aquaria [17]. Jobling et al. [18] reported effects on vitellogenin production and testicular growth in sexually maturing male rainbow trout exposed during three weeks to a concentration of 7 pM EEQ (2 ng E_2/L). The EEQs measured with the ER-CALUX assay in surface water were close to or greater than 1 pM at Eijsden (0.9 and 1.5 pM) and Lobith (0.7 and 1.7 pM; see also Fig. 3a). This is close to the effect level for vitellogenin production, as mentioned above. However, this level is not an adverse effect as such, and even effluent EEQ levels never came close to the effect level of 0.5 nM EEQ. However, consider that in field situations exposure is life-long, and not only via the water phase. Moreover, in this study only four large river locations were sampled, whereas smaller surface waters may be under greater influence of local sources of contamination. Also, accumulation of more hydrophobic compounds via food and through contact with particulate matter and sediment will contribute to the total EEQ load. Therefore, the conclusion can be made that wild fish populations in The Netherlands also may be at risk of estrogenic compounds. Although the biological significance of increased vitellogenin production is still unclear, it is useful to study it as a biological exposure parameter that in combination with effect parameters such as changes of gonadal histology, can help to elucidate possible risks for local wild fish populations. Also, the newly developed in vivo bioassays with transgenic zebrafish [19] containing the same ER-responsive luciferase construct as the ER-CALUX assay could be very useful for studying the correlation between in vivo and in vitro studies and the exact target tissue and sensitive period. Because exposure levels possibly are higher in other water systems, further studies should focus particularly on smaller rivers and ditches.

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