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Determination of estrogenic potential in waste water without sample extraction

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HIGHLIGHTS

- A modified ER-Calux® (NE-(ER-Calux®)) does not need pre-extraction of raw water samples.
- NE-(ER-Calux®) enables determination of estrogenic potential of raw water samples.
- The sensitivities of NE-(ER-Calux®) and conventional ER-Calux® assay are comparable.
- NE-(ER-Calux®) assay is recommended as a screening assay in multi sample studies.

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ABSTRACT

This study describes the modification of the ER-Calux® assay for testing water samples without sample extraction (NE-(ER-Calux®) assay). The results are compared to those obtained with ER-Calux® assay and a theoretical estrogenic potential obtained by GC–MSD. For spiked tap and waste water samples there was no statistical difference between estrogenic potentials obtained by the three methods. Application of NE-(ER-Calux®) to "real" influent and effluents from municipal waste water treatment plants and receiving surface waters found that the NE-(ER-Calux®) assay gave higher values compared to ER-Calux® assay and GC–MSD. This is explained by the presence of water soluble endocrine agonists that are usually removed during extraction. Intraday dynamics of the estrogenic potential of a WWTP influent and effluent revealed an increase in the estrogenic potential of the influent from 12.9 ng(EEQ)/L in the morning to a peak value of 40.0 ng(EEQ)/L in the afternoon. The estrogenic potential of the effluent was <LOD (<0.68 ng(EEQ)/L). The overall reduction in estrogenic potential was 92–98%. Daytime estrogenic potential values varied significantly.

1. Introduction

Naturally excreted steroid estrogens are ubiquitously present in waste water and other environmental samples [1–3]. They have the highest estrogenic activity among endocrine disrupting compounds and account for the majority of estrogenic potential in municipal waste water [4–6]. Concentrations of steroid estrogens in WWTP effluent are typically in the low ng/L range [1], but this is still sufficient to affect the endocrine system of living organisms [7,8].

To detect such low concentrations of estrogens sensitive methods are necessary. Gas or liquid chromatography coupled with mass spectrometry are the techniques of choice for quantitative chemical analyses [1,3,9] while total estrogenic potential of the sample is obtained by biological *in vivo* or *in vitro* assays [2,7]. Several cell-based *in vitro* bioassays like MELN [10–13], MVLN [14], E-Screen [13,15,16], ER-Calux® assay [13,17–19] and MMV-Luc [20] have been developed for environmental samples. Alternatively, recombinant yeast based assays are available [2]. These are easier to use, but they lack complex estrogenic interactions [21,21], are less sensitive than mammalian cell-based assays and are unable to detect anti-estrogenic compounds [19].

Low concentrations of estrogens also mean that extraction from complex environmental matrices and pre-concentration of analytes is essential for both detection and quantification with the bio-assays. Liquid–liquid or solid phase extraction (SPE) with

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additional clean-up is generally applied for chemical analysis and bioassays [2,22] and only a few published studies describe the determination of estrogenic potential without extensive sample preparation and extraction. Of these the majority employ a recombinant yeast assay [6,22,23] and just one applies a mammalian cell-based assay (MELN cells) without presenting any results [10].

The aim of this study was to modify the ER-Calux® assay for the determination of estrogenic potential of water samples without extensive sample handling and extraction of analytes. The modified method that we named NE-(ER-Calux®) assay, was tested with tap and waste water samples spiked with steroid estrogens, and compared to conventional ER-Calux® assay and chemical analysis with gas chromatography–mass selective detection (GC–MSD). The modified method was then applied for investigating estrogenic potential of "real" environmental samples and for studying intraday dynamics of estrogenic potential in influent and effluent samples of WWTP.

2. Materials and methods

2.1. Standards, chemicals, growth media

Standards estrone (E1; min 99%), 17β-estradiol (E2; min 98%), 17α-ethinylestradiol (EE2; min 98% (HPLC)), estriol (E3; min 99%), a deuterated internal standard (bisphenol A)-d_{16} (98 atom% D) were purchased from Sigma (Steinheim, Germany). Standards were prepared freshly in ethyl acetate, for ER-Calux® assay calibration curve and in methanol for spiking water samples used for analyses.

Methanol, ethyl acetate "Baker ultra resi-analysed®" grade were purchased from J.T. Baker (Deventer, the Netherlands). Pyridine (max 0.01% H_2O) was purchased from Merck (Darmstadt, Germany). The derivatising agent N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA; derivatisation grade), was purchased from Sigma.

For ER-Calux® assay, media Gibco® D-MEM/F-12 with GlutaMAX™ (with phenol red), Gibco® D-MEM/F-12 with L-glutamine (without phenol red) and Stripped FBS (foetal bovine serum) were purchased from Invitrogen (Paisley, UK). EDTA, non-essential amino acids (MEM 100×), and penicillin/streptomycin were purchased from Sigma. FBS (foetal bovine serum) and PBS (Phosphate Buffered Saline) were purchased from PAA (Pasching, Austria) while Difco trypsin was obtained from Becton Dickinsen (Heidelberg, Germany).

2.2. Sampling and sample handling

Sampling was performed for three different purposes as follows. Spiked tap and waste water effluent samples were used for optimisation purposes, while waste water treatment plant (WWTP) influent and effluent samples as well as surface waters were used to study "real" samples. Hourly samples were collected at one WWTP to measure intraday variations of estrogenic potential in influent and effluent samples.

2.2.1. Spiked samples

Tap water from our laboratory and grab waste water effluent sample from WWTP2 (Table 1) were spiked with estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and estriol (E3) at environmentally relevant concentrations (0–40 ng/L) at levels that were chosen randomly within this range (see Supplementary material: Table S1). After spiking, samples were homogenised by shaking at 300 rpm for 30 min. For the NE-(ER-Calux®) assay, 10 mL of each sample was stored at −20 °C, while 200 mL was immediately used for SPE (see Section 2.3). Extracts of the samples were analysed by ER-Calux® assay and GC–MSD, while un-extracted samples were analysed by NE-(ER-Calux®) assay.

2.2.2. "Real" waste water and surface water samples

Grab samples (250 mL, glass bottles) of WWTP influent and effluent samples and surface river water samples (upstream and downstream of the effluent site) were collected from seven different WWTPs (Table 1). Samples at each WWTP were collected on four consecutive weeks (two WWTPs per week), on Monday morning. In order to assure the same sample storage and preparation time, effluent and river samples were taken without considering hydraulic retention time. Similar to spiked samples, 10 mL of the sample was stored at −20 °C for NE-(ER-Calux®) assay and 200 mL was used for the extraction and analysis by ER-Calux® assay and GC–MSD. To avoid sample degradation, the extraction was performed within 4 h after the sampling.

Table 1
Main characteristics of waste water treatment plants involved in the study.

Name	Treatment	Capacity (PU)		Flow/year (m^3)	Mean influent COD (BOD) (mg/L)	Mean effluent COD (BOD) (mg/L)	HRT (h)	SRT (days)	Estimated surface water flow (m^3/s)
		Design	Actual						
WWTP1	Biofiltration with P and N removal	50,000	45,000	6,161,222	400 (196)	39.7 (8.2)	2.5	NA[a]	2
WWTP2	Activated sludge, nitrification; no P removal	200,000	143,623	7,303,085	576 (66)	294 (15)	18	15–32	7
WWTP3	Activated sludge; nitrification; no P removal	360,000	420,000	29,928,900	590 (312)	43 (<10)	19	8	20
WWTP4	Activated sludge; nitrification; no P removal	100,000	85,000	5,500,000	800 (300)	80 (15)	22	20	110
WWTP5	Activated sludge, anoxic zones, P removal	250,000	160,000	10,000,000	740 (400)	26 (6)	29	12	20
WWTP6	Activated sludge, no P removal	68,000	110,805	4,795,963	1013 (506)	128 (28)	22	4–10	0.5
WWTP7	Activated sludge, nitrification, P removal	70,000	85,000	8,486,259	429 (214)	17 (4)	22.5	20–24	35

[a] Sludge age as determined for suspended biomass is not relevant in water treatment with biofiltration.