Developing a yeast-based assay protocol to monitor total oestrogenic activity induced by 17β-oestradiol in activated sludge supernatants from batch experiments

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Abstract

A yeast-based assay protocol developed for detecting oestrogenic activity in activated sludge (AS) supernatant is described. The protocol used *Saccharomyces cerevisiae* construct RMY/ER-ERE with human oestrogen receptor (ERα) and *lacZ* reporter genes, and was developed by modifying existing assays for use with AS samples from batch experiments. The method was able to detect total oestrogenic activity (without prior extraction) in supernatants of AS spiked with 17β-oestradiol (E2) with a detection limit of 0.03 ngE2-equivalent/ℓ and an overall quantification limit of 100 ngE2-equivalent/ℓ. Mean E2-induced oestrogenic activity recoveries of >56% were obtained from the spiked samples.

Keywords: activated sludge, oestradiol, oestrogenic activity, suspended solids, *Saccharomyces cerevisiae*, yeast assay

Introduction

Environmental oestrogens are contaminants that can mimic the biological activities of the female hormone oestrogen in the endocrine system, acting as endocrine disrupters. Several substances are reported to have oestrogen-like activity including steroid hormones, synthetic oestrogens (xenoestrogens), environmental pollutants and phyto-oestrogens (plant oestrogens) (Arnold et al., 1996; Routledge and Sumpter, 1996; Coldham et al., 1997; Körner et al., 2000; Miyamoto et al., 2003). Effects on the reproductive system due to exposure to environmental oestrogens have been observed including endocrine disruption in fish (Harries et al., 1997; Harries et al., 1999; Christiansen et al., 2002) and other potential effects include male reproductive disorders (Ganmaa et al., 2001).

Natural steroid oestrogens like 17β-oestradiol (E2), oestrone (E1) and oestriol (E3) are produced by the mammalian body's endocrine system. They are excreted in small amounts (µg/d) in the bodily wastes both in their active form and as less active - but more soluble - conjugated metabolites (sulphates and glucuronides) (D'Ascenzo et al., 2003). Synthetic oestrogens like 17 α-ethinyloestradiol (EE2), a principal ingredient in birth control pills (Ternes et al., 1999), and mestranol (ME2) are used in medicine. Substances excreted in bodily wastes eventually appear in the wastewater stream, entering treatment plants at ng/ℓ concentrations (Johnson et al., 2000). Other environmental oestrogens include phenolic compounds which may be present in wastewater treatment plants and effluents in concentrations of high µg/ℓ (Giger et al., 1987; Ahel et al., 1994), up to 2 to 3 orders of magnitude higher than steroid oestrogens. Elimination of oestrogens within the treatment plant is therefore an important objective.

Yeast-based oestrogen receptor-transcription activation (ERTA) assays like the yeast oestrogen screen (YES) and the recombinant cell bioassay (RCBA) have shown a potential for use in screening for oestrogens, detecting and measuring oestrogenic activity and oestrogenic potency in samples of various origin (Routledge and Sumpter, 1996; Coldham et al., 1997; Aerni et al., 2004; Rutishauser et al., 2004). Oestrogen binds to the oestrogen receptor (ER) in yeast cells exposed to it. The estrogen-ER complex binds to oestrogen response elements (EREs) on the reporter plasmid initiating the transcription of *lacZ* gene mRNA, leading to production of β-galactosidase. This enzyme allows the cells to use lactose and lactose-analogs as a substrate. A chromogenic substrate like *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) is hydrolysed to galactose and yellow ortho-nitrophenol (ONP). The colour intensity is proportional to the concentration of ONPG hydrolysed and is related to the enzyme activity expressed and, in turn, to the exposure of yeast to oestrogen-like activity. ERTAs are sensitive to E2 and its metabolites, and oestrogen mimics (Coldham et al., 1997) and therefore measure total oestrogenic activity in a sample (Tanaka et al., 2001; Nakada et al., 2004; Servos et al., 2005). An ERTA response is then the total effect of a sample containing both E2 and its metabolites which bind to the oestrogen receptor with less affinity. Studies of mixtures of oestrogenic compounds are growing (Kortenkamp and Altenburger, 1999; Payne et al., 2000; Rajapakse et al., 2002; Silva et al., 2002) and while the complexity of interactions between E2, its metabolites, and the oestrogen receptor in a mixture is unknown at this stage, their overall effect can be estimated by the ERTA response. The ability to measure this important parameter readily can assist in monitoring the effectiveness of total oestrogenic activity removal in AS treatment systems.

This paper presents the development of a yeast-based assay protocol capable of detecting and quantifying relative total oestrogenic activity in unextracted activated sludge (AS) super-

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natant, and crude extracts of AS solids. The total oestrogenic activity was defined as the total response of the oestrogen-sensitive yeast construct RMY/ER-ERE to oestrogen-like substances in a sample to which the yeast was exposed over a 20 h incubation period. The response would include any oestrogen-like substances capable of binding to the oestrogen receptor and inducing transcriptional activation, including the metabolites of the spiked 17β-oestradiol. The degree of binding and any competitive or additive effects were beyond the scope of the study.

This development is a first step towards a routine monitoring tool for use in wastewater treatment plants. The assay is based on the RCBA developed by Klein et al. (1994) and has been used for screening of oestrogenic compounds in aqueous samples (Coldham et al., 1997) and quantification of oestrogens in human plasma (Klein et al., 1994) and bovine plasma (Burdge et al., 1998). The assay was modified to allow its use to detect oestrogenic activity in AS supernatant with small sample volumes and a 20 h incubation time. Quantification of oestrogenic activity in the unextracted supernatant samples was made using assay E2 standards prepared in AS feed to provide a similar matrix as the assayed samples, and allowing the yeast to act as a biomonitor of the oestrogenic effect in the samples.

Materials and methods

Yeast construct information and cell culture

The *Saccharomyces cerevisiae* construct RMY/ER-ERE (*his3 leu2-3, 112 trp1-1 ura3-52* / hER-TRP1-2μ- [pG/ER(G)], ERE-CYC1-LacZ-URA3-2μ [pUCΔSS-ERE], HIS3-CEN/ARS [pRS423]) was a free gift from Dr Didier Picard (University of Geneva, Switzerland). Parent strain RMY326 (MATa his3 leu2-3, 112 trp1-1 ura3-52) was transformed with pG/ER(G) a plasmid encoding the wild-type human oestrogen receptor (ERα), and pUCΔSS-ERE a reporter plasmid containing an oestrogen response element (Liu and Picard, 1998; Picard, 2001). The sensitivity and specificity of the yeast strain to oestrogen-like compounds has been evaluated by Pinto et al. (2004).

RMY/ER-ERE was cultured in 0.2 μm filter-sterilised selective medium (SM) without histidine, methionine, and tryptophan [20 g/ℓ glucose/dextrose (Fisher Scientific, USA), 6.7 g/ℓ yeast nitrogen base without amino acids (Difco, USA), 0.032 g/ℓ adenine sulphate (ICN Biomedical, USA), 5 g/ℓ casamino acids (Fisher Scientific, USA), 2% bacto-agar for agar plate (Fisher Scientific, USA)]. The CYC1 promoter reduces the chance of oestrogen-independent gene transcription (Joyeux et al., 1997), but a blank of growth medium was included in all assays since some researchers have found oestrogenic effects in growth media (Liu and Picard, 1998) and assay substrate (Vanderperren et al., 2001). MilliQ deionised water (Barnstead Nanopure system, USA) was used to prepare all aqueous solutions.

Representative yeast colonies were each incubated in 3 mℓ of SM liquid medium for 20 h. The growing cultures were made up to 30% v/v of glycerol (Fisher Scientific, USA) and 2 mℓ aliquots of stock culture were stored at -80°C. An inoculum of 10 μℓ of stock culture was streaked onto an SM agar plate and incubated at 30°C for 48 h to produce viable yeast colonies for use in the assay. Cells from the culture of one colony in SM broth were always harvested at T = 20 h (late-exponential phase). The spent medium was discarded and the cells resuspended in fresh SM to give a target optical density of 1.5 at 630 nm (all absorbances using Perkin Elmer UV/VIS Spectrometer Lambda 10, USA) corresponding to a viable plate cell count of $3 \cdot 10^{10}$ cells/mℓ.

Preparation of 17 β-oestradiol (E2) standards

10^{-2} M E2 standard solution was made by dissolving 136.2 mg of E2 (Calbiochem, USA) in 50 mℓ of absolute denatured ethanol (EtOH; Fisher Scientific, USA). A range of stock standards from 10^{-3} M to 10^{-11} M was obtained by serial dilutions of the 10^{-2} M stock (1:10) in absolute denatured ethanol. Diluting each stock 1:100 in sterile activated sludge feed (Ng, 2001) produced assay standards ranging from 10^{-4} M to 10^{-13} M which were stored at 4°C. The feed was used to avoid alcohol toxicity to growing yeast cultures and to provide the same liquid matrix as the samples taken from the AS batch reactors.

Glassware and plasticware preparation

To prevent organic matter contamination, all glassware was washed thoroughly (soaked overnight in Alconox or Fisherbrand Sparkleen detergent dissolved in hot tap-water, washed well, rinsed several times with tap-water, once with distilled water and once with isopropyl alcohol, before being air-dried and individually capped with, or wrapped in, aluminium foil for baking). The glassware was baked (≥400°C) in a muffle oven (Fisher Scientific, USA) for at least 3h, cooled and stored under a biohood (Forma Scientific, USA) until used. The use of plasticware was minimised to disposable pipette tips rinsed in isopropyl alcohol followed by milliQ water before use to avoid contamination by phthalates (used as plasticisers) which are suspected endocrine disrupters and oestrogen-mimics, and to avoid false positive results as the yeast assay has been found to be sensitive to phthalates (Coldham et al., 1997). With this treatment, control experiments did not show any significant differences between assays in glass and plastic, but the use of plasticware was avoided. For the batch experiments 250 mℓ glass conical flasks were prepared as outlined for glassware. Continuously mixed experiments used teflon magnetic stirbars prepared as outlined for plasticware.

Optimising assay duration

ONP production curves were linear during the first 6 min of the assay so that the concentration of ONP formed and yellow colour development could be related using the Lambert-Beer Law (results not shown). Based on this finding excess substrate (ONPG) was used in the assay, and all assays were terminated after 6 min so that oestrogen-induced enzyme activity was maximum and could be compared across assays and directly related to E2 concentration.

Development of the E2 standard curve

A volume of 250 μℓ of each standard in AS feed was added to 750 μℓ of SM with 50 μM $CuSO_4$ (VWR Scientific, USA) and an inoculum of 200 μℓ yeast cell suspension to give a final volume of 1.2 mℓ (0.2% EtOH), and the mixture incubated at 30°C for 20 h. Then cells were collected by centrifugation (all centrifugation at 3 600 r/min [2140 x g] for 10 min), the supernatant discarded, and the cells re-suspended in Z-buffer [(Klein et al., 1994): 60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 50 mM β-mercaptoethanol (ICN Biomedical, USA), 1 mM $MgSO_4.7H_2O$ (all other reagents from EM Science, USA)]. Cell suspension (100 μℓ) was added to 100 μℓ ONPG in Z-buffer, 40 μℓ chloroform, and 40 μℓ SDS in a 30°C water bath and incubated for 6 min. The reaction was stopped by adding 1 mℓ of 1 M sodium carbonate (Na_2CO_3) solution, cell debris removed by centrifugation, and the absorbance of the super-

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natant measured at 420 nm. All standards were assayed in triplicate.

Assay application to samples from batch experiments with activated sludge (AS)

AS source

The AS used for the batch experiments was obtained from laboratory bench-scale AS reactors at the University of California, Berkeley (California, USA), run by H-Y Ng who also provided the AS feed recipe (Ng, 2001). Prior to use the sludge settled overnight at 4°C, the supernatant was decanted and an equal amount of fresh AS feed added. The sludge was then continuously mixed overnight to equilibrate at room temperature, before being distributed into flasks for the batch experiments.

Estimation of liquid-solid partitioning of oestrogenic activity and spiked dose recovery

In continuously mixed batch experiments, AS was spiked with different concentrations of E2. Samples (2 mℓ) were collected before adding E2 and within 60s after addition, then every 5 to 10 min for 1h. Immediately after collection, the samples were centrifuged to separate the solid and aqueous phases. Centrifugation was preferred to micro-filtration to eliminate unknown effects of filtration (Jensen and Schäfer, 2001; Holbrook et al., 2002). Since only soluble oestrogens can enter the yeast cell during incubation, the cell wall should act as a filter for oestrogens associated with any remaining colloidal sludge solids. The supernatant was decanted into another tube and oestrogenic activity evaluated using 250 µℓ of supernatant in the assay. Non-mixed controls were included to study the importance of mixing on reduction of oestrogenic activity.

The AS solid phase was immediately treated with 200 µℓ of chloroform – to inactivate the microorganisms and to dissolve and preferentially concentrate hydrophobic oestrogens from the solids (x10). An aliquot of 100 µℓ of the chloroform-dissolved oestrogen was transferred to a clean tube and the chloroform evaporated at 40°C. The E2 coating the tubes was re-dissolved in 250 µℓ of SM for analysis in the assay. All samples were assayed in triplicate in the same manner descibed for the standards. A set of standards diluted in the same AS feed used to maintain the AS batch experiments was assayed with each group of samples. A clean blank tube and chloroform-only control tube were included to check for contamination.

Total oestrogenic activity determination

Absorbance readings from the samples were converted into enzyme activities and then E2-equivalent concentrations as follows. β-galactosidase activity is given by:

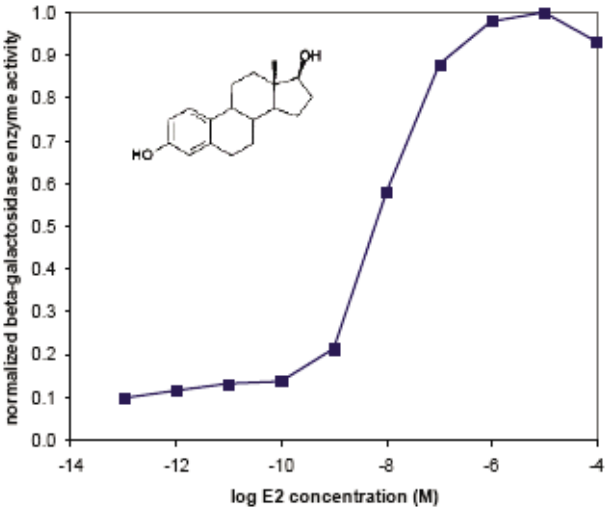

Figure 1

Typical dose-response of yeast assay when exposed to E2 standards dissolved in activated sludge feed solution. Inset is the chemical structure of E2. The beta-galactosidase activity was normalised both by yeast culture optical density and by maximum induced enzyme activity measured to enable comparison of dose-responses across assays.

$$Activity = \frac{A_{420} V_{TOT}}{\varepsilon L T V_{YWS} A_{630}} \quad [\mu mol/m\ell\text{-}min]$$

where (for each reaction tube):

- A_{420} = absorbance of ONP product measured at 420 nm
- A_{630} = absorbance (optical density) of yeast cell suspension measured at 630 nm
- V_{TOT} = volume of stopped reaction mixture [mℓ]
- V_{YWS} = volume of yeast cell suspension used [mℓ]
- ε = molar extinction coefficient [4.5 mℓ/µmol-cm (Becerra et al., 2001)]
- L = cuvette path length [1 cm]
- T = time between addition of yeast culture to ONPG and stopping with 1 M Na_2CO_3 [6 min]

The activities from an assay of the standards were plotted against E2 concentration as shown in a typical example of a standard curve in Fig. 1. Experimental samples were assayed in the same way as the standards. Fresh standards were prepared for each fresh AS feed batch and a set of standards was assayed with each

TABLE 1
Summary characterisation of yeast assay standard dose-response and the respective blank controls

	Induced beta-gal activity (µmol/mℓ-min)[1]	AR[1]	E2 standard concentration (M)	E2 standard concentration	Number of observations
Yeast culture [2]	0.016±0.011	0.102±0.063	n.a.	n.a.	12
SM blank[3]	0.019±0.011	0.110±0.065	n.a.	n.a.	11
EtOH blank	0.029±0.013	0.154±0.062	n.a.	n.a.	4
Maximum	0.147±0.038	0.962±0.046	10^{-5}	2.7 mg/ℓ	29
Minimum	0.023±0.010	0.166±0.058	10^{-13}	0.027 ng/ℓ	13

[1] activity ratio value represents [mean] ± [standard deviation from the mean]
[2] Yeast culture before $CuSO_4$ addition
[3] Yeast culture after addition of $CuSO_4$
n.a. = not applicable

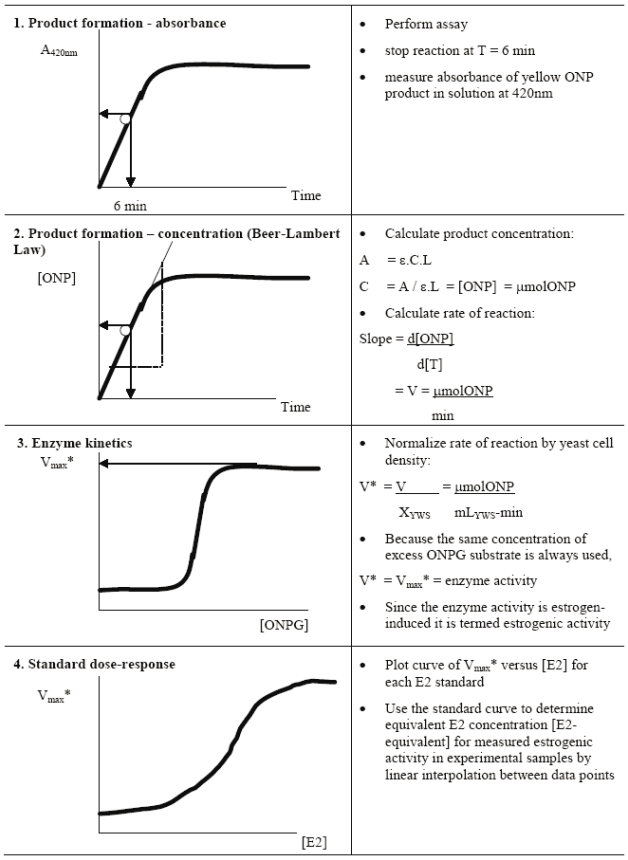

Figure 2
A summary of the steps followed in developing the E2 standard dose-response curve for use in estimating sample E2-equivalent concentrations

experimental sample set.

Normalisation by yeast cell density allowed for variations in culture growth rates across yeast colonies and across E2 standard concentrations, and expressing activities as a ratio of the maximum activity developed in the standards was used to compare results across assays. This was termed the 'activity ratio' (AR) = [measured activity in given sample] : [maximum activity measured in assay of standards with the same yeast culture]. Controls with no E2 (EtOH blank) and no EtOH (SM media blank) were routinely assayed to maintain accuracy. Assays were also carried out on the yeast culture to check inherent oestrogen receptor induction levels (see Table 1). Activity of the vehicle blank was subtracted from each sample activity. E2-equivalent concentrations were then obtained from the AR by point-by-point interpolation on the standard curve. The steps in this process are summarised in Fig. 2.

Estimating the proportion of total oestrogenic activity contributed by E2 alone

Samples from a continuously mixed AS batch experiment (1.53 gTSS/ℓ; 1.19 gVSS/ℓ) with 0 to 100 µgE2/ℓ were analysed for E2 concentration using a GC-MS/MS protocol developed by the Sedlak research group at the University of California, Berkeley (Huang and Sedlak, 2001; Kolodziej et al., 2003). Briefly, the unextracted supernatant samples were prepared according to protocol: derivatised, evaporated to dryness, re-dissolved in isooctane then refrigerated until analyses were carried out by

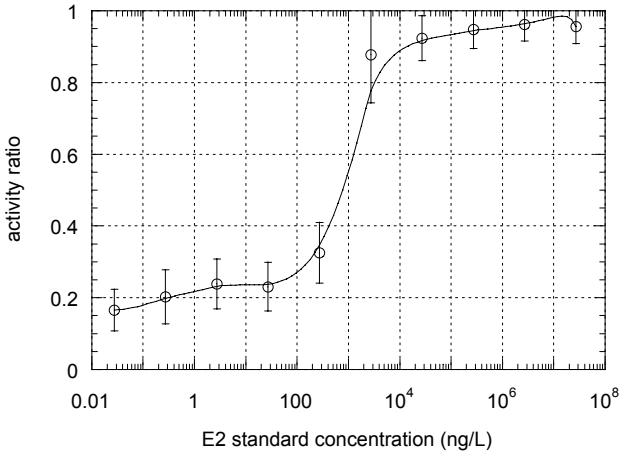

Figure 3
Overall standard dose-response curve compiled from assays of standards. Error bars denote standard deviation (n = 7 to 30). The smooth curve-fit is for visualisation purposes only.

GC-MS/MS. Duplicate samples were analysed for total oestrogenic activity using the yeast assay protocol.

Based on observations from reported studies, it was expected that less than or equal to 100% of the total oestrogenic activity detected in the assay would be due to E2 (Tanaka et al., 2001), and that the E2 concentration in the supernatant samples would reduce as rapidly as the E2 was metabolised by AS microorganisms (Ternes et al., 1999) and associated with solids (Jensen and Schäfer, 2001) while the total oestrogenic activity would decrease slower (Lee and Liu, 2002) since the metabolites are also oestrogenic, albeit less potent.

Results and discussion

E2 standard dose-response

The standard dose-responses for the assays carried out during the experiment duration were compiled into an overall standard dose-response curve shown in Fig. 3. The maximum E2-induced β-galactosidase activity overall was 0.147±0.038 µmol/min-mℓ, corresponding to an activity ratio (AR) of 0.962±0.046 for an E2 concentration of 10^{-5} M (2.7 mgE2/ℓ).

Assay overall limit of detection

Table 1 shows that the assay was able to differentiate between activity ratios of the lowest E2 standard dose (0.027 ng/ℓ, 10^{-13} M) and the SM blank (no E2; with $CuSO_4$) [$p < 0.05$, $\alpha = 0.05$, one-tail t-test]. There was no statistically significant difference between the SM blank and the EtOH blank [$p > 0.05$, $\alpha = 0.05$, one-tail t-test]. The overall detection limit of the assay was therefore considered as 0.03 ngE2/ℓ which compares well with yeast-based oestrogenic activity detection limits reported in the literature. Reported limits of detection for E2 in yeast-based assays include 0.01 ng/ℓ (YES on river water extract (Fawell et al., 2001)); 0.02 ng/ℓ (RCBA on blood plasma (Klein et al., 1994)); 0.3 ng/ℓ (YES on lake water extract (Aerni et al., 2004)); 0.5 to 0.9 ng/ℓ (YES on wastewater effluent (Aerni et al., 2004)); and 15 ng/ℓ (YES on raw river water (Fawell et al., 2001)). For comparison, reported limits of detection for chemical analysis range from 0.1 to 1.2 ng/ℓ (GC-MS/MS on surface water and effluent (Belfroid et al., 1999)).

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Assay overall limit of quantification

The overall method limit of quantification was 100 ngE2/ℓ [defined as (mean + 2 standard deviations) of the lowest point on the calibration dose-responses from all experiments] and is a compilation of up to 30 different standard dose-responses over a 2-year period. The overall inter-assay variation was 5 to 15% for $>10^{-8}$ M E2 (2720 ng/ℓ) and 26 to 37% for $<10^{-9}$ M E2 (272 ng/ℓ) (reflected in the standard deviation bars on Fig. 3). To our knowledge, most standard dose-response data sets reported in the yeast-based assay literature on environmental samples at the time of writing were smaller, so there was limited basis for comparison of inter-assay repeatability. The RCBA carried out by Burdge et al. (1998) on bovine plasma extracts showed mean inter-assay variation of up to 64.7% in samples with low E2 close to the assay detection limit (7 assays, 8 replicates per assay). Standards were assayed with each set of samples collected so that the total oestrogenic activity for each set of samples was based on an assay of standards prepared with the same batch of AS feed and exposed to the same yeast culture.

Three standard curves assayed on the same day using the same batch of AS feed were used to determine intra-assay variation during protocol development (3 assays, 3 replicates per assay). Intra-assay variation was 1 to 6% for $>10^{-8}$ M E2 (2720 ng/ℓ) and 7 to 14% for $<10^{-9}$ M E2 (272 ng/ℓ).

Activated sludge feed controls [no AS; no E2]

Control experiments of 100 mℓ AS feed without sludge or E2 were included in the batch experiments. The feed controls were sampled up to 10 times over a 15d period to account for oestrogenic activity of the sludge feed used in that particular experiment. The induced oestrogenic activity in the assays of samples was 0.048±0.018 µmol/mℓ-min (activity ratio = 0.197±0.160; n = 6 sets) for mixed experiments and 0.042±0.015 µmol/mℓ-min (activity ratio = 0.101±0.126; n = 3 sets) for non-mixed experiments. It was not possible to pinpoint the source of observed variation in feed oestrogenic activity, but it is possible that components of the AS feed recipe were responsible. To counter this feed-effect, the E2 standards for each batch experiment were prepared using the same feed used in that particular experiment.

Activated sludge feed dosed with E2 [no AS; with E2]

To study the possibility of sludge-free reduction of E2-induced oestrogenic activity in the batch experiments, flasks with AS feed dosed with E2 were included. Any oestrogenic activity reduction in these controls could be attributed to abiotic processes such as sorption to flask walls and feed components, and abiotic degradation. Three 15 d sets each of sludge-free sterile AS feed controls were spiked with E2 doses of 100 ng/ℓ, 100 µg/ℓ and 20 mg/ℓ, and a summary of the results is presented in Table 2. The variation in activities was highest for the 20 mgE2/ℓ dose which is partly un-dissolved at the start of the experiment. This high dose was included primarily so that comparisons could be made with older research data from oestrogen removal studies in the literature (Tabak and Bunch, 1970; Tabak et al., 1981).

Mean recoveries over the 15 d period were >58%. The lower recovery of the 100 ng/ℓ spiked dose which is close to the overall limit of quantification could also have been due to the presence of colloidal solids in the AS feed ingredients which could sorb oestrogen. While abiotic reduction of oestrogenic activity could have been studied using sterile water or a medium free of organics, the use of sterile AS feed was preferred in order to maintain matrix consistency. A study by Andersen et al. (2004) of abiotic reduction of 500 ng/ℓ E2 using a sterile mineral feed also found that there was little tendency for abiotic removal of E2 over a 4 d experiment period.

Activated sludge controls [with AS; no E2]

The background oestrogenic activity of the sludge used for the experiments is an important factor, especially at low concentrations because the yeast assay is quite sensitive to oestrogen-like substances. Batch experiments of 100 mℓ AS controls without E2 were dosed with an equivalent amount of EtOH vehicle used to dissolve the E2 in the standards used for E2 spikes (always less than 1% by volume). These controls did not show high oestrogenic activity over the duration of the experiment (activity ratio less than 0.2).

The continuously mixed experiments with fed activated sludge but no E2 dose induced an activity of 0.028±0.015 µmol/mℓ-min (activity ratio = 0.051±0.109; n = 10 sets) while the static experiments present lower background activities (0.028±0.009 µmol/mℓ-min; activity ratio = 0.009±0.096; n = 5 sets). When normalised by sludge total suspended solids (TSS) concentration the activity ratios decrease slightly to 0.029±0.085 (n = 8 sets) for mixed experiments and 0.028±0.018 (n = 3 sets) for static experiments.

The presence of sludge appears to reduce the background activity which is detected in the feed-only controls suggesting either possible interference by colloidal solids on detection of oestrogenic activity by the yeast assay or preferential association of any oestrogenic compounds in the feed with the solids removed by centrifugation. The association of oestrogenic compounds with particulate solids has been observed in several studies (Jensen and Schäfer, 2001; Schäfer et al., 2002). Since oestrogenic compounds are hydrophobic, they may preferentially associate with organic AS solids present, reducing their liquid-phase concentration and making the compounds less available to yeast in the assay. The liquid-solid partitioning during the first hour after dosing was estimated by collecting AS

TABLE 2
Oestrogenic activity in sludge-free AS feed controls dosed with E2

	Continuously mixed experiments			Non-mixed experiments	
	100 ngE2/ℓ	100 µgE2/ℓ	20 mgE2/ℓ	100 µgE2/ℓ	20 mgE2/ℓ
Beta-gal activity (µmol/mℓ-min)	0.079±0.029	0.145±0.019	0.168±0.043	0.156±0.016	0.099±0.036
Measured activity ratio	0.151±0.111	0.742±0.258	0.863±0.468	0.950±0.107	0.795±0.309
Expected activity ratio (E2 standard curve)	0.259±0.073	0.931±0.060	0.958±0.047	0.931±0.060	0.958±0.047
15-day mean recovery	58%	80%	90%	102%	83%

TABLE 3
Recovery calculations for continuously mixed batch experiments

E2 spiked dose	AR_{STD}	(AR_0-AR_{-1})	AR^*_{-1}	AR^*_0	AR^*_{60}	Mean reduction of oestrogenic activity after 60 min*	Mean recovery of oestrogenic activity
100 ng/ℓ	0.255±0.092	0.192±0.124	0.320±0.011 **0.107±0.006**	0.341±0.015 **0.152±0.025**	0.275±0.014 **0.193±0.103**	19% **(-27%)**	56±36%
100 µg/ℓ	0.892±0.080	0.760±0.113	1.209±0.055 **0.542±0.033**	1.245±0.205 **0.540±0.011**	1.290±0.149 **0.452±0.027**	(-4%) **16%**	78±12%
20 mg/ℓ	0.960±0.048	0.632±0.057	1.110±0.165 **0.589±0.088**	0.857±0.119 **0.682±0.010**	0.746±0.007 **0.554±0.052**	13% **19%**	63±6%

Table 3 shows [mean] ± [standard deviation from mean] of 2 experiments, each time-point sample assayed in triplicate.
regular text = AR of sludge supernatant sample
bold text = AR of sludge solids sample
underlined text = AR of both supernatant and solids co-added

TABLE 4
Recovery calculations for non-mixed batch experiments

E2 spiked dose	AR_{STD}	(AR_0-AR_{-1})	AR^*_{-1}	AR^*_0	AR^*_{60}	Mean reduction of oestrogenic activity after 60 min*	Mean recovery of oestrogenic activity
100 ng/ℓ	0.255±0.092	0.475±0.596	0.307±0.025 **0.128±0.015**	0.272±0.045 **0.286±0.138**	0.243±0.020 **0.135±0.016**	11% **53%**	137±172%
100 µg/ℓ	0.892±0.080	0.660±0.135	1.332±0.068 **0.596±0.003**	1.026±0.231 **0.589±0.006**	1.182±0.196 **0.489±0.015**	(-15%) **17%**	68±14%

Table 4 shows [mean] ± [standard deviation from mean] of 2 experiments, each time-point sample assayed in triplicate.
regular text = AR of sludge supernatant sample
bold text = AR of sludge solids sample
underlined text = AR of both supernatant and solids co-added

samples from E2 spiked batch experiments every 5 to 10 min for 60 min. The partitioning and recovery of the E2-induced total oestrogenic activity was estimated as described in the following section.

Sludge sample extraction recovery estimates

For each experiment, samples were collected before E2 spike (AR_{-1}), within 60 s after spike (AR_0), and the spiked activity = (AR_0 - AR_{-1}) was compared with the expected activity of the respective spiked dose (100 ng/ℓ, 100 µg/ℓ and 20 mg/ℓ) for agreement. As mentioned previously, all standards were prepared as dilutions in the same AS feed used in the batch experiments. Tables 3 and 4 show the recoveries from samples collected during the first hour after dosing the batch experiment flasks. The flasks were used for longer-term total oestrogenic activity reduction monitoring. Non-mixed flasks were included to study the possible reduction of oestrogenic activity by unmixed settled AS, a situation which would occur in sedimentation tanks in a full-scale treatment plant.

Recoveries were calculated as:

$$\% \text{ recovery} = 100 \bullet (AR_0 - AR_{-1}) \div AR_{STD}$$

Reduction after 60 min of detected oestrogenic activity was calculated as:

$$\% \text{ reduction} = 100 \bullet (AR^*_{60} - AR^*_0) \div AR^*_0$$

where:

AR_{STD} = activity ratio of respective spiked dose in the assay of E2 standards
AR^*_0 = activity ratio of sample at the start of 60 min test
AR^*_{60} = activity ratio of sample at end of 60 min test.

All *AR* marked with an asterisk (*) were normalised by sludge TSS concentration to facilitate comparison of duplicate experiments. The recovery for each experiment was calculated separately before calculating the mean recoveries at each spiked dose.

The recoveries for the 100 ngE2/ℓ dose were variable, probably due to the closeness to the assay limit of quantification. These recoveries showed that the assay is very sensitive to low concentrations of oestrogenic activity, but that at these low concentrations more accurate quantification would require confirmation by another quantification technique.

For the 100 ngE2/ℓ spike the average reductions in the liquid phases of both mixed and un-mixed experiments were 19% and 11% respectively during the first 60 min of contact with AS. There was an increase in solid phase activity of the mixed experiments only (27%). Non-mixed experiments showed reduction of solid phase oestrogenic activity by 53%. This would imply that in the short term when the AS solids remained settled and unmixed, over time the oestrogenic activity in the sludge decreased while that in the liquid phase remained almost the same. A similar finding was reported by Holbrook et al. (2002) in wastewater treatment facilities with <100 ng/ℓ where the effluents contained

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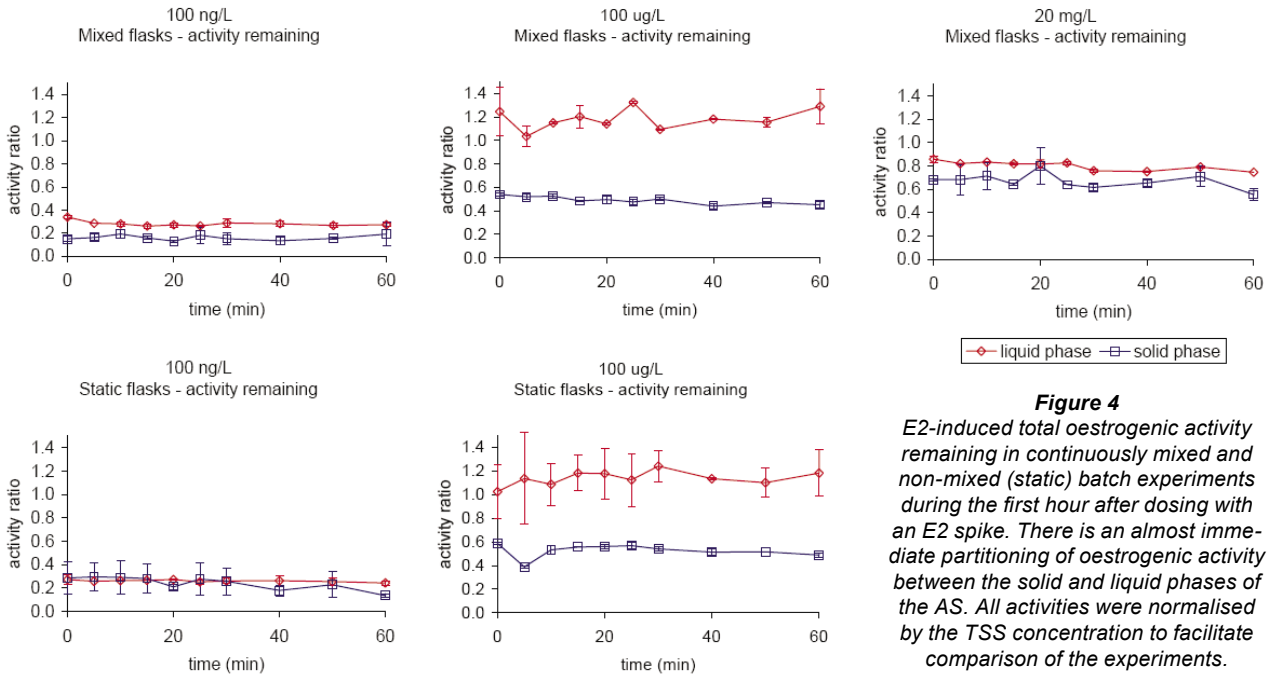

Figure 4
E2-induced total oestrogenic activity remaining in continuously mixed and non-mixed (static) batch experiments during the first hour after dosing with an E2 spike. There is an almost immediate partitioning of oestrogenic activity between the solid and liquid phases of the AS. All activities were normalised by the TSS concentration to facilitate comparison of the experiments.

more oestrogenic activity than the waste AS (WAS) and the activity associated with the liquid phase did not change much over time.

The partitioning of E2-induced oestrogenic activity out of the water phase is very rapid (Fig. 4). Confirming this observation, a study by Andersen et al. (2004) using a 400 ngE2/ℓ spike into a 1 g/ℓ sterile AS showed that the loss of oestrogen from the water phase is almost complete after the first 30 min of contact and only an additional 11% loss occurs after 6 h. With a sterile sludge, the loss by microbial metabolism and any desorption and resuspension to replenish metabolic reduction is not included so the sorption-only loss may be an overestimate of actual reduction potential of the sludge in the given time period. However, the observations made in this study agree with the finding by Andersen et al. (2004) that association with solids is reponsible for the initial reduction of spiked oestrogen doses from the water phase.

When spiked with 100 µg/ℓ of E2, there was an average decrease in solids–associated oestrogenic activity by about 16 to 17% whether the experiments were continuously mixed or non-mixed, and there was a corresponding increase in liquid phase activity at the end of the hour by 4% and 15% for the mixed and non-mixed experiments respectively. With 100 µgE2/ℓ there was less reduction of oestrogenic activity within 60 min of dosing, and twice as much activity was detected in the liquid phase than was associated with the solids (Fig. 4) suggesting that the solids, though able to remove total oestrogenic activity, may have a limited short-term ability to do so. At this concentration, higher oestrogenic reductions may require longer retention times than 60 min since there may be desorption of compounds contributing to oestrogenic activity even as they are metabolised. The complexity of the reduction of estrone may also have an impact on the effective total oestrogenic activity reduction (Onda et al., 2003; Servos et al., 2005). To our knowledge it is not yet clear whether the persistence of E1 has any effect on the metabolism of E2.

A 20 mgE2/ℓ spike induced liquid phase oestrogenic activities lower than the 100 µg/ℓ spike. This concentration exceeds the solubility of E2 by about 7 mg/ℓ, and reduction is expected to take a long time since liquid phase concentrations may be replenished by desorption and dissolving. An early study by Tabak and Bunch (1970) reported 100% reduction of 20 mg/ℓ E2 spikes by AS after 3 weeks. In our short study, a mean recovery of 63% of the spike-induced activity was obtained at this dose and at the end of 60 min there was some reduction of oestrogenic activity in both the liquid (13%) and solid (19%) phases showing that higher reductions would require longer contact times. To confirm this, longer-term studies (15 to 30 d) were planned and the findings will be compared with oestrogenic activity reductions from longer batch experiments and treatment plants with activated sludge systems e.g. (Andersen et al., 2003) and long solids retention times (Andersen et al., 2004).

Estimating the proportion of total oestrogenic activity contributed by E2 alone after a 100 µg/ℓ spiked dose

Both the yeast assay and the GC-MS/MS analysis did not detect oestrogenic activity or E2 in blank control (no-E2 dose) samples, i.e. there were no false positives in the assay. The GC-MS/MS detection limit of 0.3 µgE2/ℓ in this instance was higher than the 0.03 ngE2/ℓ detection limit of the yeast assay. Samples from experiments dosed with 100 µgE2/ℓ showed >95% reduction of detectable E2 within 1 min even though there is still oestrogenic activity. This suggests rapid conversion of E2 into oestrogenic metabolites (Fig. 5) which still impart oestrogenic activity. Servos et al. (2005) also reported >95% removals of E1 and E2 in aerobic batch reactors with 2:1 sewage sludge (raw sewage: return activated sludge) within 24 h, but noted that traces of E1 and yeast oestrogen assay response persisted even after 120h.

As expected, less than 100% of the total oestrogenic activity detected in the assay was due to E2 (Tanaka et al., 2001), and the E2 concentration in the supernatant samples reduced as rapidly as the E2 was metabolised by AS micro-organisms (Ternes et al., 1999) and associated with solids (Jensen and Schäfer, 2001) while the total oestrogenic activity decreased slower (Lee and Liu, 2002) since the metabolites are also oestrogenic, albeit less potent.

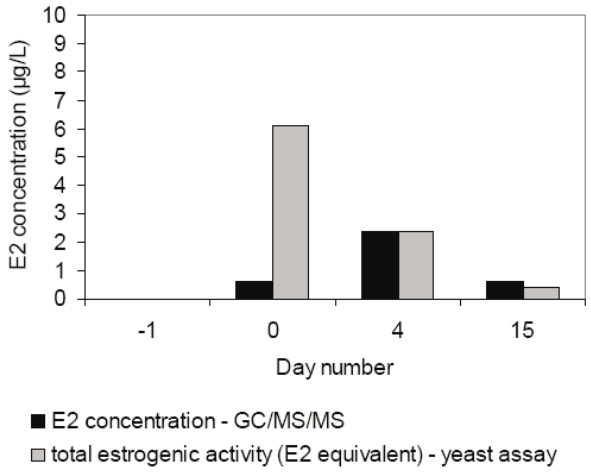

Figure 5

Sludge samples dosed with E2 show reduction of total oestrogenic activity with time. Within 1 min after spiking (Day 0 sample) very little E2 is detected in the sludge supernatant although the total oestrogenic activity increases to an equivalent of <10% of the dosed E2. This would mean that almost immediately, the E2 dose is converted to other forms of oestrogen (not detected by the GC/MS/MS in this instance) while about 90% of the total oestrogenic activity is removed from the liquid phase implying that the contributing compounds are either less potent or associated with the solids.

Advantages of the assay envisaged in application to routine treatment plant monitoring

The primary reason for developing the oestrogen-sensitive yeast-based assay protocol was to provide the basis for a potential tool for practical routine monitoring of total oestrogenic activity in wastewater treatment plants. The assay is a good detector of the presence or absence of oestrogenic activity and a fair technique for quantification of the relative oestrogenic activity (based on an assay of E2 standards) which can be further improved. If required, evaluation of quantification and identification of the components of the oestrogenic activity can be obtained by analytical methods. The yeast culture is relatively easy to maintain and the 20 h 30°C sample-yeast-contact incubation time and 48h protocol compares well with the 5 d BOD_5 test for example, in terms of practicality; 1 yeast colony is required for the assay of a set of 15 or more samples in triplicate. An additional advantage is the small volumes of samples and reagents needed and the potential for miniaturisation of the assay for high-throughput using 96-well plates. The assay protocol also uses equipment already available in many treatment plant quality monitoring labs, e.g. muffle oven – used for TSS and VSS; waterbath and incubator – used for total and faecal coliform counts and BOD determination; centrifuge; and spectrophotometer – used for colour and turbidity measurements, etc.

Assay limitations

While the yeast assay was sensitive to low concentrations of total oestrogenic activity, the E2-equivalent response was much higher and more variable than expected at concentrations close to the upper and lower limits of the dose-response curve. The amplification of the dosed response in some instances in the assay could be partly explained by the fact that the biological response of the yeast is complex and encompasses all oestrogen-like compounds able to bind to the oestrogen receptor. This binding may either enhance or inhibit the response depending on the quantities and combination of compounds present. The assay sensitivity should act as a good sample screening tool for total oestrogenic activity in activated sludge supernatants, and as an indicator of the relative amounts of oestrogenic activity when compared with an assay of E2 standard dilutions. However, the actual compounds which contribute to this activity, and their respective concentrations would require an additional combination of sample clean-up, extraction, concentration, fractionation, and chemical analytical techniques, e.g. (Ternes et al., 2002; Tashiro et al., 2004). Even then, the identities of all the compounds which contribute to the oestrogenic activity may be elusive (Tanaka et al., 2001; Servos et al., 2005). The isolated yeast cell wall may exclude compounds with an average hydrodynamic radius larger than 0.8 nm and average molecular weight greater than 620 g/mol (Scherrer et al., 1974), and the yeast cell membrane preferentially internalizes lipophilic compounds. Although De Nobel and Barnett (1991) noted that the walls of growing yeast cells can be permeable to much larger molecules, it is possible that in this study some oestrogenic compounds may not be detected.

Activated sludges have the potential for reduction of total oestrogenic activity from both the solid and liquid phases of the wastewater matrix. Understanding the factors influencing this potential in order to further maintain or improve existing treatment before effluent discharge is the growing focus of research studies (Baronti et al., 2000; Holbrook et al., 2002; Andersen et al., 2003; Svenson et al., 2003; Servos et al., 2005). The possibility of improved oestrogenic activity removal by increasing the contact time with AS was raised in this study, and there was a need to carry out longer-term experiments since to our knowledge there have been no reports of long-term (7 d or more) AS batch experiments since Tabak and Bunch (1970). Preliminary results showed that this hypothesis was feasible (Hermanowicz and Wozei, 2002) and recent studies confirm that the SRT is an important factor in the reduction of oestrogenic compounds and other micropollutants in wastewater treatment (Kreuzinger et al., 2004; Clara et al., 2005).

Longer-term studies (15 to 30 d) are being planned and the findings will be compared with oestrogenic activity reductions from treatment plants with activated sludge systems, e.g. Andersen et al. (2003) and long solids retention times, e.g. Andersen et al. (2004). The advantage of enhancing the oestrogenic activity reduction ability of the sludge by acclimatisation and enrichment of the sludge microorganisms also needs to be investigated. Longer-term batch experiments including AS enrichment studies by re-spiking E2 doses are planned for future studies. In addition, to verify its suitability for environmental monitoring, application of the yeast-based assay protocol to AS supernatant samples from different sources including a full-scale treatment plant with an AS system is planned.

Conclusions and recommendations

- Using the developed yeast-based assay protocol, it was possible to detect oestrogenic activity in un-extracted supernatants of AS spiked with 17β-oestradiol (E2) with a detection limit of 0.03 ngE2-equivalent/ℓ, and an overall quantification limit of 100 ngE2-equivalent/ℓ. The key to this detection was using a set of E2 standards dissolved in the same matrix as the AS solids, and running an assay of a set of samples and standards with the same yeast culture.

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- The assay had greatest quantitative sensitivity overall between 250 and 2 900 ngE2/ℓ. While this range is higher than found in nature, the sensitivity was suitable for the purpose of developing the assay, and for studying the partitioning and reduction of the oestrogenic activity induced by the chosen range of spiked E2 doses. The assay could be further sensitised by additional sample clean-up. Assay sensitivity is improved by assaying a set of standards with the same yeast culture as the assayed samples.
- Mean E2-induced oestrogenic activity recoveries of >56% were obtained in batch experiments in which E2 spikes were in contact with AS for up to 1 h, and trends in reduction of oestrogenic activity in samples from batch experiments could be monitored
- Solid-liquid phase partitioning of oestrogenic activity appears to occur immediately after E2 spiking of continuously mixed batch experiments. Total oestrogenic activity of AS supernatant reduces by less than 20% during the first hour of spiked E2 contact with AS implying that longer retention times may be needed to achieve higher reductions. Longer-term experiments to increase contact time should give an indication of the time required to reduce total oestrogenic activity to background levels.

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References

AERNI HR, KOBLER B, RUTISHAUSER BV, WETTSTEIN FE, FISCHER R, GIGER W, HUNGERBUHLER A, MARAZUELA MD, PETER A, SCHONENBERGER R, VOGELI AC, SUTER MJ and EGGEN RI (2004) Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. *Anal. Bioanal. Chem.* **378** (3) 688-696.

AHEL M, GIGER W and KOCH M (1994) Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment - I. Occurrence and transformation in sewage treatment. *Water Res.* **28** (5) 1131-1142.

ANDERSEN H, SIEGRIST H, HALLING-SORENSEN B and TERNES TA (2003) Fate of estrogens in a municipal sewage treatment plant. *Environ. Sci. Technol.* **37** (18) 4021-4026.

ANDERSEN HR, KJOLHOLT J, HANSEN M, STUER-LAURIDSEN F, BLICHER TD, INGERSLEV F and HALLING-SORENSEN B (2004) *Degradation of Estrogens in Sewage Treatment Processes. Environmental Project No. 899.* Danish Environmental Protection Agency, Danish Ministry of the Environment. 61 pp.

ARNOLD SF, ROBINSON MK, NOTIDES AC, GUILLETTE LJ, JR. and McLACHLAN JA (1996) A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. *Environ. Health Perspect.* **104** (5) 544-548.

BARONTI C, CURINI R, D'ASCENZO G, DI CORCIA A, GENTILI A and SAMPERI R (2000) Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ. Sci. Technol.* **34** (24) 5059-5066.

BECERRA M, DÍAZ PRADO S, GONZÁLEZ SISO MI and CERDÁN ME (2001) New secretory strategies for *Kluyveromyces lactis* ß-galactosidase. *Protein Eng.* **14** (5) 379-386.

BELFROID AC, VAN DER HORST A, VETHAAK AD, SCHÄFER AJ, RIJS GBJ, WEGENER J and COFINO WP (1999) Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci. Total Environ.* **225** (225) 101-108.

BURDGE GC, COLDHAM NG, DAVE M, SAUER MJ and BLEACH ECL (1998) Determination of estrogen concentrations in bovine plasma by a recombinant oestrogen receptor-reporter gene yeast bioassay. *Analyst* **123** 2585-2588.

CHRISTIANSEN LB, WINTHER-NIELSEN M and HELWEG C (2002) *Feminisation of Fish: The Effect of Estrogenic Compounds and their Fate in Sewage Treatment Plants and Nature. Environmental Project No. 729.* Danish Environmental Protection Agency. 184 pp.

CLARA M, KREUZINGER N, STRENN B, GANS O and KROISS H (2005) The solids retention time-a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. *Water Res.* **39** (1) 97-106.

COLDHAM NG, DAVE M, SIVAPATHASUNDARAM S, MCDONNELL DP, CONNOR C and SAUER MJ (1997) Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect.* **105** (7) 734-742.

D'ASCENZO G, DI CORCIA A, GENTILI A, MANCINI R, MASTROPASQUA R, NAZZARI M and SAMPERI R (2003) Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. *Sci. Total Environ.* **302** (1-3) 199-209.

DE NOBEL JG and BARNETT JA (1991) Passage of molecules through yeast cell walls: a brief essay-review. *Yeast* **7** (4) 313-323.

FAWELL JK, SHEAHAN D, JAMES HA, HURST M and SCOTT S (2001) Oestrogens and oestrogenic activity in raw and treated water in Severn Trent Water. *Water Res.* **35** (5) 1240-1244.

GANMAA D, WANG PY, QIN LQ, HOSHI K and SATO A (2001) Is milk responsible for male reproductive disorders? *Med. Hypotheses* **57** (4) 510-514.

GIGER W, AHEL M, KOCH M, LAUBSCHER HU, SCHAFFNER C and SCHNEIDER J (1987) Behaviour of alkylphenol polyethoxylate surfactants and of nitrilotriacetate in sewage treatment. *Water Sci. Technol.* **19** 449-460.

HARRIES JE, JANBAKHSH A, JOBLING S, MATTHIESSEN P, SUMPTER JP and TYLER CR (1999) Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environ. Toxicol. Chem.* **18** (5) 932-937.

HARRIES JE, SHEAHAN DA, JOBLING S, MATTHIESSEN P, NEALL M, SUMPTER JP, TAYLOR T and ZAMAN N (1997) Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Toxicol. Chem.* **16** (3) 534-542.

HERMANOWICZ SW and WOZEI E (2002) *Biodegradation of Estrogenic Compounds and its Enhancement in a Membrane Bioreactor. Technical Completion Report.* Project Number W-944. University of California Water Resources Center. 11 pp.

HOLBROOK RD, NOVAK JT, GRIZZARD TJ and LOVE NG (2002) Estrogen receptor agonist fate during wastewater and biosolids treatment processes: A mass balance analysis. *Environ. Sci. Technol.* **36** (21) 4533-4539.

HUANG C-H and SEDLAK DL (2001) Analysis of estrogenic hormones in municipal wastewater effluent and surface using enzyme-linked immunosorbent assay and gas chromatograph/tandem mass spectrometry. *Environ. Toxicol. Chem.* **20** (1) 133-139.

JENSEN RL and SCHÄFER AI (2001) Adsorption of estrone and 17 beta-estradiol by particulates - activated sludge, bentonite, hematite and cellulose. *Proc. Recent Advances in Water Recycling Technologies.* Brisbane Workshop, Brisbane, Australia. 93-102.

JOHNSON AC, BELFROID AC and DI CORCIA A (2000) Estimating steroid inputs into activated sludge treatment works and the observations on their removal from the effluent. *Sci. Total Environ.* **256** 163-173.

JOYEUX A, BALAGUER P, GERMAIN P, BOUSSIOUX AM, PONS M and NICOLAS JC (1997) Engineered cell lines as a tool for monitoring biological activity of hormone analogs. *Anal. Biochem.* **249** 119-130.

KLEIN KO, BARON J, COLLI MJ, MCDONNELL DP and CUTLER GB (1994) Estrogen levels in childhood determined by an ultrasensitive recombinant cell bioassay. *J. Clin. Invest.* **94** (6) 2475-2480.

KOLODZIEJ EP, GRAY JL and SEDLAK DL (2003) Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. *Environ. Toxicol. Chem.* **22** (11) 2622-2629.

KÖRNER W, BOLZ U, SUSSMUTH W, HILLER G, SCHULLER W, HANF V and HAGENMAIER H (2000) Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. *Chemosphere* **40** (9-11) 1131-1142.

KORTENKAMP A and ALTENBURGER R (1999) Approaches to assessing combination effects of oestrogenic environmental pollutants. *Sci. Total Environ.* **233** (1-3) 131-140.

KREUZINGER N, CLARA M, STRENN B and KROISS H (2004) Relevance of the sludge retention time (SRT) as design criteria for wastewater treatment plants for the removal of endocrine disruptors and pharmaceuticals from wastewater. *Water Sci. Technol.* **50** (5) 149-156.

LEE HB and LIU D (2002) Degradation of 17 beta-estradiol and its metabolites by sewage bacteria. *Water Air Soil Pollut.* **134** (1-4) 353-368.

LIU JW and PICARD D (1998) Bioactive steroids as contaminants of the common carbon source galactose. *FEMS Microbiol. Lett.* **159** (2) 167-171.

MIYAMOTO J, BURGER J, ASHBY J, KELCE W, KLEIN W, KORACH K, LAMB J and MATTHIESSEN P (eds.) (2003) *Special Topic Issue on the Implications of Endocrine Active Substances for Humans and Wildlife*. Scientific Committee on Problems of the Environment and International Union of Pure and Applied Chemistry. 1617-2615.

NAKADA N, NYUNOYA H, NAKAMURA M, HARA A, IGUCHI T and TAKADA H (2004) Identification of estrogenic compounds in wastewater effluent. *Environ. Toxicol. Chem.* **23** (12) 2807-2815.

NG H (2001) Personal communication - Activated sludge source and feed information. Currently at Division of Environmental Science and Engineering, National University of Singapore, Singapore.

ONDA K, NAKAMURA Y, TAKATOH C, MIYA A and KATSU Y (2003) The behavior of estrogenic substances in the biological treatment process of sewage. *Water Sci. Technol.* **47** (9) 109-116.

PAYNE J, RAJAPAKSE N, WILKINS M and KORTENKAMP A (2000) Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environ. Health Perspect.* **108** (10) 983-987.

PICARD D (2001) Personal communication - Yeast construct RMY/ER-ERE and parent strain RMY326. Department of Cell Biology, University of Geneva, Switzerland.

PINTO B, PICARD D and REALI D (2004) A recombinant yeast strain as a short term bioassay to assess estrogen-like activity of xenobiotics. *Ann Ig.* **16** (4) 579-585.

RAJAPAKSE N, SILVA E and KORTENKAMP A (2002) Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. *Environ. Health Perspect.* **110** (9) 917-921.

ROUTLEDGE EJ and SUMPTER JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **15** (3) 241-248.

RUTISHAUSER BV, PESONEN M, ESCHER BI, ACKERMANN GE, AERNI HR, SUTER MJF and EGGEN RIL (2004) Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three in vitro assays and chemical analysis of steroids. *Environ. Toxicol. Chem.* **23** (4) 857-864.

SCHÄFER AI, MASTRUP M and JENSEN RL (2002) Particle interactions and removal of trace contaminants from water and wastewaters. *Desalination* **147** (1-3 Special Issue SI) 243-250.

SCHERRER R, LOUDEN L and GERHARDT P (1974) Porosity of the yeast cell wall and membrane. *J. Bacteriol.* **118** (2) 534-540.

SERVOS MR, BENNIE DT, BURNISON BK, JURKOVIC A, MCINNIS R, NEHELI T, SCHNELL A, SETO P, SMYTH SA and TERNES TA (2005) Distribution of estrogens, 17beta-estradiol and estrone, in Canadian municipal wastewater treatment plants. *Sci. Total Environ.* **336** (1-3) 155-170.

SILVA E, RAJAPAKSE N and KORTENKAMP A (2002) Something from "nothing" - Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* **36** (8) 1751-1756.

SVENSON A, ALLARD AS and EK M (2003) Removal of estrogenicity in Swedish municipal sewage treatment plants. *Water Res.* **37** (18) 4433-4443.

TABAK HH, BLOOMHUFF RN and BUNCH RL (1981) Steroid hormones as water pollutants II. Studies on the persistence and stability of natural urinary and synthetic ovulation-inhibiting hormones in untreated and treated wastewaters. *Dev. Ind. Microbiol.* **22** 497-519.

TABAK HH and BUNCH RL (1970) Steroid hormones as water pollutants I. Metabolism of natural and synthetic ovulation-inhibiting hormones by microorganisms of activated sludge and primary settled sewage. *Dev. Ind. Microbiol.* **11** 367-376.

TANAKA H, YAKOU Y, TAKAHASHI A, HIGASHITANI T and KOMORI K (2001) Comparison between estrogenicities estimated from DNA recombinant yeast assay and from chemical analyses of endocrine disruptors during sewage treatment. *Water Sci. Technol.* **43** (2) 125-132.

TASHIRO Y, TAKAHIRA K and NAKANISHI Y (2004) Improved application of recombinant yeast assays on environmental samples by size exclusion chromatography. *J. Environ. Monit.* **6** (6) 546-551.

TERNES TA, ANDERSEN H, GILBERG D and BONERZ M (2002) Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. *Anal. Chem.* **74** (14) 3498-3504.

TERNES TA, KRECKEL P and MUELLER J (1999) Behaviour and occurrence of estrogens in municipal sewage treatment plants - II. Aerobic batch experiments with activated sludge. *Sci. Total Environ.* **225** (1-2) 91-99.

VANDERPERREN E, DEMARÉ W, BLUST R, COOREMAN K and BOSSIER P (2001) Oestrogenic activity of CPRG (chlorophenol red-b-D-galactopyranoside), a ß-galactosidase substrate commonly used in recombinant yeast oestrogenic assays. *Biomarkers* **6** (5) 375-380.

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