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

E Wozei* and SW Hermanowicz

Department of Civil and Environmental Engineering, 629 Davis Hall, University of California at Berkeley, Berkeley, CA, 94720

Abstract

Batch experiments were carried out with activated sludge from laboratory reactors and a full-scale treatment plant spiked with 17β-oestradiol (E2). An oestrogen-sensitive yeast-based assay protocol, described in detail in a related publication, was used to measure reduction of E2-induced total oestrogenic activity from the sludge supernatant over a 15 d period after which the sludge was re-spiked to check for possible enhancement of reduction by pre-exposed sludge during an additional 15 d period. The reduction was generally improved by increasing sludge solids concentrations and by continuous mixing. For a 100 ngE2/ℓ spike there was >40% reduction of oestrogenic activity within 15 d, which improved to >70% by pre-exposing the sludge. The oestrogenic activity produced by a dose of 100 µgE2/ℓ was readily removed by most sludges within 15 d. However, re-spiking the activated sludge with the same E2 concentration caused some sludges to lose reduction capacity.

Keywords: activated sludge, 17β-oestradiol, oestrogen, oestrogen receptor, oestrogenic activity, suspended solids, wastewater treatment, yeast assay

Introduction

Environmental oestrogens are environmental contaminants that can mimic the biological activities of the female hormone oestrogen in the endocrine system, i.e. they act as endocrine disrupters. Several substances are reported to have oestrogen-like activity. These include steroid hormones, synthetic oestrogens (xeno-oestrogens), environmental pollutants and phyto-oestrogens (plant oestrogens) (Arnold et al., 1996; Routledge and Sumpter, 1996; Coldham et al., 1997; Hu et al., 2000; Körner et al., 2000).

Some of these environmental oestrogens share physical and chemical characteristics which make them able to behave like, or induce a hormonal response similar to 17β-oestradiol (E2). These characteristics include their chemical structure, water solubility, and affinity for organic matter. Others do not share any structural resemblance, but can still induce a similar biological response (Zacharewski, 1997). This paper limits the definition of environmental oestrogens to those substances that can bind to and activate the oestrogen receptor (ER), and will refer to them as oestrogenic compounds or oestrogens. Primary emphasis is placed on the steroid oestrogen E2 found in domestic wastewater treatment.

Estrogens in wastewater treatment plants

Where there is a conventional wastewater collection and treatment system, some oestrogens will eventually pass through a wastewater treatment plant before being discharged to the environment (Ternes et al., 1999b). The oestrogenic input to rivers by treatment plant effluent can be quite significant. Körner et al. (2000) found E2-equivalent concentrations of between 2.5 and 25 ng/ℓ in effluent from municipal wastewater plants in southern Germany. Effluents may have up to 50 ng/ℓ of E2 alone (Desbrow et al., 1998; Belfroid et al., 1999; Layton et al., 2000) and total oestrogenic activity up to 150 ngE2-equivalent/ℓ (Körner et al., 2000; Tilton et al., 2002; Pawlowski et al., 2003; Pawlowski et al., 2004). Typical effluents have less than 20 ngE2/ℓ. While many receiving water studies have been carried out on river and lake water, there is now concern that ocean effluent outfalls may be a source of oestrogens to coastal marine environments (Atkinson et al., 2003). Solids disposal may also be a source of oestrogen to the environment from treatment plants as the hydrophobic oestrogens are expected to associate with the organic solids in treatment processes (Johnson et al., 1999). Sludge E2 concentrations of up to 4.3 ng/ℓ were measured by Murk et al. (2002).

Oestrogen reduction and organic solids in wastewater treatment

Steroid oestrogens have low water solubility – generally in the low mg/ℓ range (Tabak et al., 1981; *Merck Index*, 1996) – but at typical ng/ℓ concentrations found in wastewaters they will be in solution in samples to be analysed. However, some quantities of oestrogens will also be associated with solids and must be accounted for. Domestic wastewater typically has between 80 to 290 mg/ℓ of total organic carbon (TOC) and some treatment processes like the activated sludge (AS) system have up to 10 g/ℓ of organic solids (Tchobanoglous et al., 1991). Steroid oestrogens have relatively high K_{ow} values with calculated and experimental values of log K_{ow} reported in a range of 2.45 to 4.15 (Hansch et al., 1995; Hu et al., 2000; Lai et al., 2000). Their implied hydrophobicity means that some of the dissolved oestrogenic compounds may partition out of the water phase onto the

* To whom all correspondence should be addressed.
Current address: Lawrence Berkeley National Laboratory, Mail Stop 70A-3317, 1 Cyclotron Road, Berkeley, CA, 94720
☎ 1 510 486 7094; fax: 1 510 486 7152;
e-mail: EWozei@LBL.gov
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organic solids in the treatment plant processes, a phenomenon which could be exploited for reduction of oestrogens in the treatment plant.

Huang and Sedlak (2001) estimated that with an octanol-water partition coefficient (K_{ow}) of approximately 15 000 (corresponding to log K_{ow} = 4.2), up to 70% of E2 may be associated with organic solids for an AS system with a volatile suspended solids (VSS) concentration of 2 g/ℓ. Holbrook et al. (2003a; 2004) suggested that a substantial portion of the aqueous E2 and 17α-ethinyloestradiol (EE2) concentrations (up to 60%) may be associated with colloidal organic material, and that colloidal organic carbon may play a role in the fate and transport of E2 and EE2 in the activated sludge treatment process. This may be reflected in the removals in batch experiments with AS of up to 100% (Ternes et al., 1999a). Since other estrogenic compounds have similar or higher partition coefficients, it is possible that the reduction of total estrogenic activity with AS is at least 70% since AS systems are typically operated with mixed liquor VSS of 2 g/ℓ or more. Also, from reported removals, it appears that lower E2 concentrations are removed more readily, i.e. in shorter time periods (Ternes et al., 1999a; Ternes et al., 1999b; Murk et al., 2002; Onda et al., 2003). This could mean that removal of the ng/ℓ levels of steroid oestrogens entering treatment plants with AS systems is feasible during typical AS system hydraulic detention times of 4 to 8 h (Tchobanoglous et al., 1991). However, since E2 is metabolised to estrone (E1) during the treatment process, measurement of both substances – and less potent metabolites – is required to quantify overall reduction in oestrogenic activity. Spiked experiments with river water suggest that complete reduction of oestrogenic activity would take at least as long as the removal of E1 (Jurgens et al., 2002).

Some studies, however, have disputed the importance of organic solids in oestrogen and oestrogenic activity reduction. For example, a study by Fürhacker et al. (1999) using ^{3}H radiolabelled E2 found that for raw wastewater with 128 mg/ℓ suspended solids and 101 mg/ℓ total organic carbon content, 86% of the radioactivity remained in the liquid phase after filtration. This was taken to mean that very little of the oestrogen would associate with solids. Körner et al. (2000) estimated that of the 90% reduction of oestrogenic activity in a German wastewater plant with an AS system (effluent BOD_5 = 1 to 12 mg/ℓ), only about 3% could be accounted for in the dried sludge samples. It was concluded that the rest was removed by biodegradation. However, calculations by Johnson et al. (1999) estimated that after 22 h equilibration with organic flocs with an equilibrium distribution coefficient of 3 360 ℓ/kg, at least 92% of influent E2 is expected to adsorb even without degradation. And a study by Lee and Liu (2002) found that in aerobic batch experiments, while activated sludge supernatant (total suspended solids (TSS) concentration <1 gTSS/ℓ) could remove over 80% of a spiked dose of 200 μgE2/ℓ by almost quantitative conversion to its metabolite E1 within 24 h, the degradation of E1 was much slower requiring 14 d for >90% removal. This would mean that although the primary source of oestrogenic activity is rapidly removed, total oestrogenic activity (measured by bioassays like the yeast assay) would require over 2 weeks for complete reduction.

In another study, Jensen and Schäfer (2001) found that increasing activated sludge concentration from about 2 to 8 gTSS/ℓ did improve E1 reduction over 24 h of contact, although the percentage reduction remained the same for concentrations ranging from 1 to 500 ngE1/ℓ. At 8 gTSS/ℓ, the maximum reduction was about 30% at all concentrations. Slow reduction of E1 is expected; however, Jensen and Schäfer (2001) attributed all the removal to adsorption of solids although they did not report sludge sterility, which would mean that some microbial reduction cannot be entirely ruled out.

Metabolism of oestrogens in wastewater treatment

Layton et al. (2000) found that for the treatment plants they sampled the micro-organisms from the municipal treatment plants adapted and became more efficient in the removal of oestrogen than those from industrial treatment plants. Yu et al. (2005) used a quantitative molecular fingerprinting assay to show that after five successive weekly sub-culturing enrichments, oestrogen-degrading consortia of micro-organisms enriched from activated sludge showed enhanced degradation ability with a reduction in the time required for complete degradation of 1 mg/ℓ of EE2, E1 and E2 from 7 to 3 d, with changes in microbial diversity and abundance. This confirms the findings of an early study by Tabak and Bunch (1970) which showed that 'subculture enrichment … had a significant effect in enhancing the selection of micro-organisms in the total microbial population capable of cleaving and biodegrading the steroid molecule'. This could mean that wastewater management methods that actively encourage oestrogen removal could develop biological solids populations, which have good removal efficiencies, by selective sub-culturing or solids recycling.

If AS micro-organisms are able to metabolise oestrogens during the treatment process retention time, then microbial metabolism may be an additional important removal mechanism. A better understanding of how the micro-organisms already present in wastewater treatment process reactors metabolise oestrogen may allow process engineers to optimise the system for oestrogen removal. Wastewater micro-organisms such as *Escherichia coli* (*E. coli*) bacteria have existing metabolic pathways and enzymes that they may be able to use in oestrogen metabolism and removal *(Kyoto Encyclopedia of Genes and Genomes)*. The β-glucuronidase and arylsulphatase enzymes produced by the *E. coli* present in wastewater may be responsible for de-conjugation of conjugated oestrogens in the sewers before the water reaches the treatment plant (Ralovich et al., 1991; Kertesz, 1999; D'Ascenzo et al., 2003). Batch studies on male and female urine sample extracts showed that *E. coli* can convert oestrogenic metabolites in urine to biologically active oestrogens (Legler et al., 2002).

In this study, oestrogenic activity reduction was expected to be both due to adsorption of the hydrophobic oestrogens to organic solids, and the microbial reduction by live micro-organisms that make up AS. The effects of continuous mixing and absence of AS solids were investigated by including suitable controls. Re-spiking of sludges which had been pre-exposed to E2 was also carried out to determine whether there was any benefit in E2-induced oestrogenic activity reduction by acclimating activated sludges prior to their use in treatment.

Study objective

The objective of the study was to determine and better understand the role of activated sludge solids, and the trends in removal of E2-induced oestrogenic activity with activated sludge using a promising yeast-based assay protocol developed previously. The yeast-based assay allows rapid determination of the relative oestrogenic activity of the supernatant of E2-spiked AS samples without the requirement of sample extraction.

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Materials and methods

Yeast strains and culture technique

Parent strain RMY326 and its recombinant construct RMY/ER-ERE of the budding yeast *Saccharomyces cerevisiae* were a free gift from Dr Didier Picard (University of Geneva, Switzerland). The 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 (hER), and pUCΔSS-ERE a reporter plasmid containing an oestrogen response element to give the 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]). The construct expresses the wild-type human oestrogen receptor α (hERα) and contains an ER reporter gene (Liu and Picard, 1998; Picard, 2001).

The construct was selectively cultured using a selective medium (SM) without the amino acids histidine (His), methionine (Met) and tryptophan (Trp). Selective media broth/agar (Klein et al., 1994) contained: 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) in nanopure water (Barnstead nanopure system, USA). The medium was filter sterilised using an 0.2 μm pore-size syringe filter paper (Cole-Parmer, USA). The medium was prepared and stored under refrigeration as a 10X solution and diluted as necessary using nanopure water.

Selectivity of the medium was tested by attempting to grow the parent strain RMY326 on selective medium. No colonies were observed in 2 separate trials on SM agar plates and 1 trial in SM broth over 7 d periods. However, the parent strain was able to grow on general media agar containing: 10 g/ℓ yeast extract (Fisher Scientific, USA), 10 g/ℓ dextrose (Fisher Scientific, USA), and 15 g/ℓ bacto-agar in distilled water (Fisher Scientific, USA) and YPD (yeast peptone dextrose) broth containing: 10 g/ℓ yeast extract (Fisher Scientific, USA), 20 g/ℓ bacto-peptone (Difco, USA), and 20 g/ℓ dextrose (Fisher Scientific, USA) in nanopure water – confirming that in this study, the selective medium only allows growth of the yeast construct.

The CYC1 promoter reduces the chance of oestrogen-independent gene transcription (Joyeux et al., 1997), but a medium blank was included in all assays since some researchers have found media (Liu and Picard, 1998) and substrate (Vanderperren et al., 2001) oestrogenic effects in some assays.

17 β-estradiol (E2) standards preparation

A 10 mM (10^{-2} M) solution of 17 β-estradiol (E2) standard (Calbiochem, USA) was made by dissolving 136.2 mg of E2 in 50 mℓ of absolute denatured ethanol (Fisher Scientific, USA). A range of stock standards from 10^{-3} M to 10^{-11} M was then made up by serial dilution of the 10^{-2} M stock standard (1:10) in absolute ethanol. Working standards ranging from 10^{-4} M to 10^{-13} M were made by diluting each stock standard 1:100 in filter-sterilised AS feed solution.

Assay protocol outline

Briefly, 250 μℓ of working standard was incubated with 200 μℓ of yeast culture and 750 μℓ of SM for 20 h at 30°C. The yeast cells were collected by centrifugation, the supernatant discarded, and the cells re-suspended in 2 mℓ Z-buffer and the optical density read at 630 nm. 100 μℓ of cell suspension was added to a reaction tube containing excess *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) chromogenic substrate, SDS and chloroform at 30°C. The mixture was incubated for 6 min and the reaction stopped with 1 mℓ of 1 M sodium carbonate (Na_2CO_3). The reaction mixture was centrifuged to separate cell debris, and the absorbance of the supernatant containing yellow *o*-nitrophenol (ONP) product was read at 420 nm. All optical density absorbance readings were taken using a spectrophotometer with a 1 cm path length quartz cuvette (Perkin Elmer UV/VIS Spectrometer Lambda 10, USA).

AS supernatant samples were assayed in the same way as the working standards, with 250 μℓ of sample replacing the 250 μℓ of standard in the assay. Each group of samples was assayed with a set of standards dissolved in the same AS feed used to maintain the batch AS experiments, and with the same yeast culture.

Calculation of E2-induced oestrogenic activity

Absorbance readings from the samples were converted into enzyme activities and then E2-equivalent concentrations as follows (Hermanowicz and Wozei, 2002). β-galactosidase activity is given by:

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

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 activity induced by each standard was normalised by the maximum induced activity to give a ratio termed the activity ratio (AR) which was defined as the ratio of the measured activity in a given sample to the maximum activity measured in assay of standards with the same yeast culture, i.e.:

AR = [measured activity in given sample] : [maximum activity measured in assay of standards with the same yeast culture]

The normalised activities from an assay of the standards were plotted against E2 concentration to give a standard curve. Experimental samples were assayed in the same way as the standards and normalised to obtain the sample AR. Sample E2-equivalent concentrations could then be estimated by point-by-point interpolation on the standard curve. Fresh standards were prepared for each fresh AS feed batch and a set of standards was assayed with each experimental sample set.

Activated sludge (AS) sources

The sludge used for the experiments was obtained from laboratory bench-scale AS reactors (December 2001 – September 2002) at the University of California, Berkeley (California,

USA). Sludge samples from laboratory bench-scale sequencing batch reactors (SBRs) (SBR #1, July 2002) and (SBR #2, December 2001) were also obtained for laboratory-scale reactor comparison experiments. In addition, grab samples of mixed liquor and waste AS (WAS) from a full-scale domestic wastewater treatment plant (WTP, California, USA, July 2002) was used for comparison experiments. Prior to use the sludge was settled overnight at 4°C, the supernatant decanted and an equal amount of fresh AS feed added. The sludge was then continuously mixed overnight at room temperature, before being distributed into flasks for the batch experiments. The different sludge samples were from reactors operated under different process conditions with different feed types. For the purposes of this study the total and volatile suspended solids (TSS and VSS) concentrations as defined in *Standard Methods* (1998) were the primary attribute used to characterise the sludge. No attempt was made to identify differences in microbial populations.

Batch activated sludge experiments oestrogenic activity reduction trends

Batch experiments were set up with 100 mℓ of AS in 250 mℓ baked glass flasks. The AS experiments were spiked with E2 standards to give final E2 concentrations of 20 mg/ℓ, 100 µg/ℓ and 100 ng/ℓ. In one set of flasks the contents were mixed continuously (mixed test) while the other was allowed to stand (non-mixed test). Controls with no E2, and no sludge respectively were included. The un-mixed reduction test used was similar to that described in an early study of oestrogen removal by Tabak and Bunch (1970). 250 mℓ glass conical flasks were used for each test set. Into the flasks was added AS feed (Ng, 2001), pre-settled AS, and estradiol in ethanol or ethanol only, according to the matrix given in Table 1. The amount of AS used depended on the target suspended solids concentration for the given experiments. Target solids concentrations were grouped as: <1 to 1 gTSS/ℓ, 1 to 2 gTSS/ℓ, and 2 to 4 gTSS/ℓ. Actual TSS and VSS ranges were: 0.16 to 0.93 gTSS/ℓ (0.16 to 0.73 gVSS/ℓ), 1.01 to 1.53 gTSS/ℓ (0.79 to 1.19 gVSS/ℓ), and 2.42 to 3.58 gTSS/ℓ (1.74 to 2.82 gVSS/ℓ).

Tests were carried out for E2 spikes of 20 mgE2/ℓ, 100 µgE2/ℓ and 100 ngE2/ℓ. These concentrations represent a very large range. As mentioned previously, Tabak et al. (1981) estimated the water solubility of 17β-estradiol at about 13 mg/ℓ in removal studies with a 20 mgE2/ℓ spike. A test concentration of 20 mg/ℓ therefore represents a situation in which solubility of the oestrogen is a limiting factor as some of the oestrogen will remain in suspension in the aqueous phase. This E2 concentration was included in order to compare findings with those of early studies.

Typical E2 concentrations in raw and treated wastewater are up to 20 ng/ℓ, and up to 50 ng/ℓ have been measured (Desbrow et al., 1998; Belfroid et al., 1999; Layton et al., 2000). The test concentration of 100 ng/ℓ was chosen to represent the highest possible typical concentration in water influent to the AS system, assuming that no E2 is removed in prior treatment processes. To attain the desired final dose, the appropriate standard stock could be used to keep the spike volume to less than 1% of the total. Flasks with no E2 (0 mgE2/ℓ) and no sludge (0 gVSS/ℓ) were included as controls.

For the un-mixed experiments, the flasks were allowed to stand at the ambient room temperature (26.6±1.3 °C) for the duration of the experiment. Prior to sampling the flask contents were mixed by swirling for 15 s and then a 2 mℓ sample was collected using a flask-dedicated pipette tip rinsed in alcohol and nanopure water. Samples were collected up to 10 separate times over the 15 d test periods. The samples were stored in baked glass test tubes, covered with parafilm, and frozen until the assay. This was done so that each set of samples could be assayed using the same yeast culture used to assay the set of standards which were included to estimate the E2-equivalent concentrations. The entire 15 d set of samples were thawed overnight in the refrigerator (4°C) for the yeast assay the next day. Each sample was centrifuged at 3 600 r/min (2 140 x g) for 10 min to separate out the solids. 250 µℓ of supernatant was then used in the place of the standard as described in the outlined assay protocol.

TABLE 1
Design matrix for oestrogenic activity reduction batch-reactor tests

Flask number	I	II	III	IV
AS feed	Yes	Yes	Yes	Yes
AS	Yes	Yes	No	No
E2 in EtOH	Yes	No	No	Yes
EtOH	No	Yes	Yes	No
Description	Spiked AS experiment	AS control	AS feed control without E2	AS feed control with E2

Yes = present; No = absent

Results and discussion

AS feed controls – no sludge, no E2 control

15 d sets of control experiments with flasks containing AS feed only were included to account for any oestrogenic activity of the sludge feed. The induced oestrogenic activity in the assays of AS feed 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 un-mixed experiments.

AS feed dosed with E2 – no sludge control

To check if there was reduction of oestrogenic activity in samples without sludge, flasks with sterile AS feed were dosed with E2. Three 15 d sets each were carried out for E2 spiked doses of 100 ng/ℓ, 100 µg/ℓ and 20 mg/ℓ and a summary of results

TABLE 2
Oestrogenic activity in sludge-free controls spiked with E2

	100 ngE2/ℓ	100 µgE2/ℓ	20 mgE2/ℓ
Induced beta-gal activity (µmol/mℓ-min)	0.079±0.029	0.145±0.019	0.168±0.043
Activity ratio obtained	0.151±0.111	0.742±0.258	0.863±0.468
Expected activity ratio (E2 standard curve)	0.259±0.073	0.931±0.060	0.958±0.047
Mean recovery over 15 d period	58%	80%	90%

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is presented in Table 2. The variation in activities – measured as the standard deviation from the mean of the sample activities – is highest for the 20 mgE2/ℓ dose. This may be attributed to the fact that the dose level is above the known solubility and the E2 is partly un-dissolved at the start of the experiment.

It was important to define any abiotic reduction (due to chemical degradation, sorption to flask walls, etc.) in case it was a significant factor in the reduction of the oestrogenic activity. While other studies used sterile mineral medium (Andersen et al., 2004) to study abiotic reduction, in this study the AS feed was used so that matrix consistency was preserved. However, the presence of colloidal solids in the feed constituents may have affected the recoveries obtained at lower E2 concentrations since the mean recovery was found to be concentration-dependent (Table 2). The importance of colloidal solids in the biological treatment of wastewater containing oestrogens has been discussed by Holbrook et al. (2003a; 2004). Andersen et al. (2004) found that there was no clear tendency for reduction of E2 in the abiotic experiments, and obtained an approximately 75% recovery of the 500 ng/ℓ spike after a 96 h (4 d) experiment period.

AS with no dosed E2 added – no E2 control

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. Controls without E2 were dosed with an equivalent amount of EtOH vehicle used to dissolve the E2 in the standards (always less than 1% by volume). They did not show high oestrogenic activity over time (activity ratio less than 0.2).

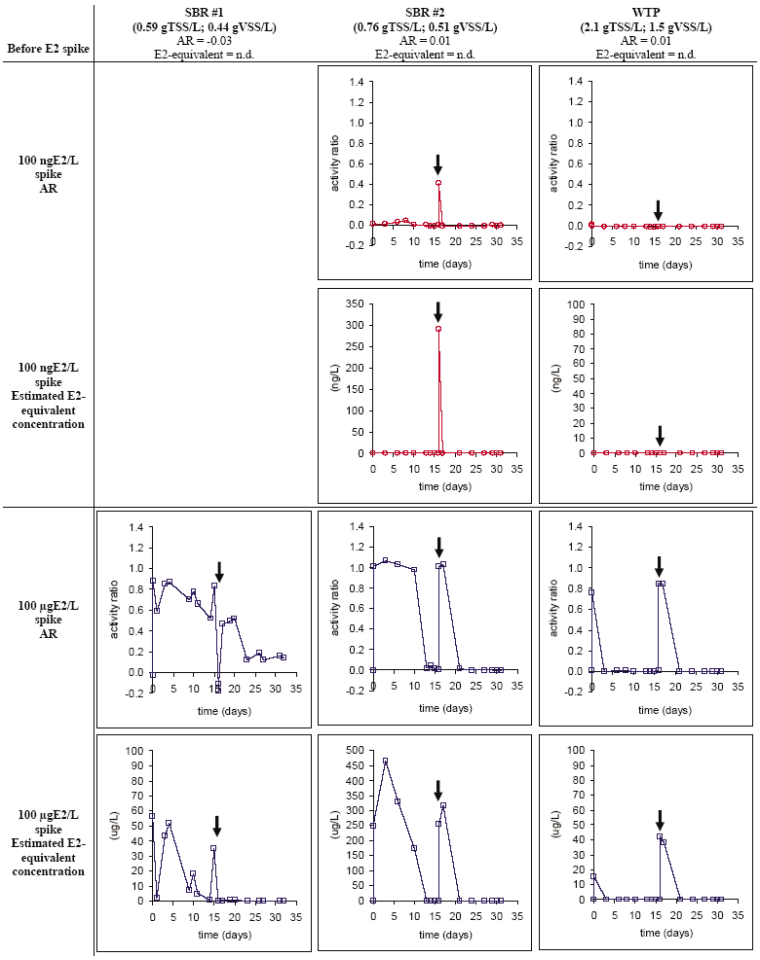

Figure 1

Reduction of total oestrogenic activity by activated sludges from laboratory-scale sequencing batch reactors (SBR #1 and SBR #2) and a full-scale wastewater treatment plant (WTP). The laboratory reactor activated sludges exhibit a lag time in oestrogenic activity reduction when spiked with 100 µg/ℓ. Arrows show re-spike with the same E2 dose as initial spike.

Mixed experiments with fed AS 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 un-mixed 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 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 un-mixed experiments. The presence of sludge reduces the background activity detected in the feed alone, 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. Colloidal solids are important as they adsorb oestrogens (Holbrook et al., 2003a; Holbrook et al., 2004), a factor which may be further improved with a higher solids organic content (Lai et al., 2000) as would be expected in activated sludge. An early study by Scherrer et al. (1974) reported that 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, and the yeast cell membrane preferentially internalises lipophilic compounds, so it is possible that some oestrogenic compounds may not be detected by the assay, especially if the compounds are associated with colloidal solids.

Batch experiments with sludges from laboratory-scale sequencing batch reactors and a full-scale wastewater treatment plant

Figure 1 shows the results from the experiments with activated sludges from 3 different sources. Before E2 dosing, the total oestrogenic activity of the sludge supernatant was not distinguishable from the assay blank. The AS from the laboratory-scale sequencing batch reactors (SBR #1 and SBR #2) required a lag time of about 10 d to remove oestrogenic activity from the supernatant when initially spiked with 100 µgE2/ℓ, while that from the full-scale treatment plant (WTP) did not. A similar observation was made by Lee and Liu (2002) on experiments with sludge supernatant from an Ontario treatment plant. This may be due to the fact that all the laboratory reactors were fed with artificial wastewater with little or no oestrogenic activity added. While there was some variability in the feed used during the batch experiment (see section on sludge-free, E2-free controls), oestrogenic activity was defined by a mean activity ratio of less than 0.2. To counter this feed effect, all standards were prepared in the same AS feed that was used for a given batch

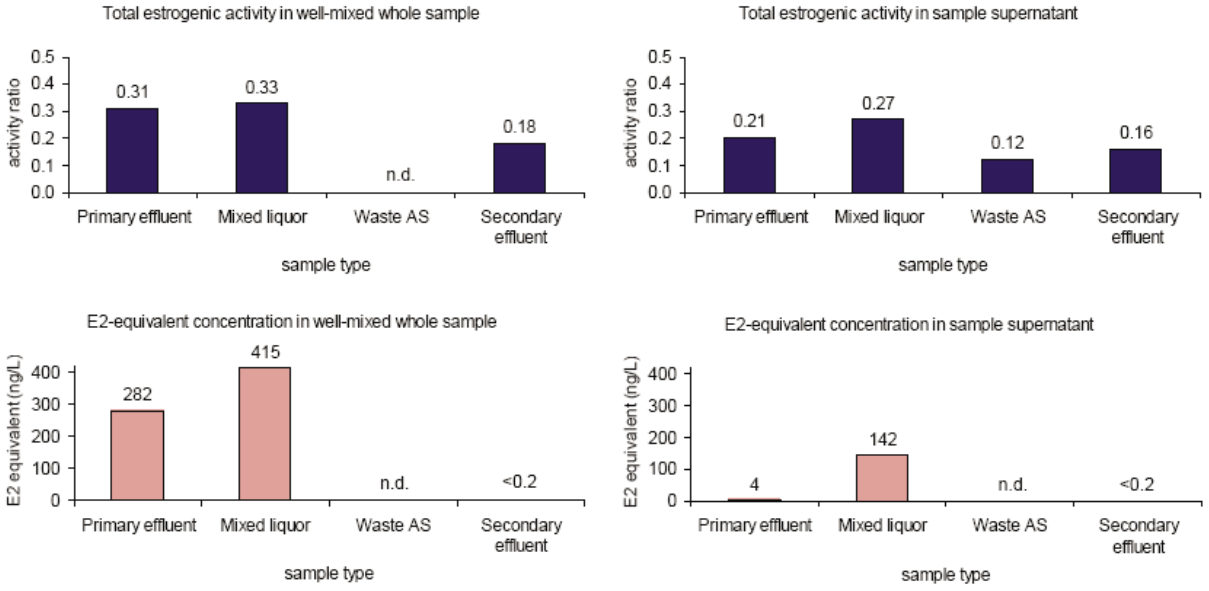

Figure 2

Total oestrogenic activity and estimated E2-equivalent concentrations in grab samples from different locations in the full-scale WTP activated sludge system. Note that the waste settled AS (5 gVSS/ℓ) exhibits lower activity than the effluent, and in the supernatant, suggesting that oestrogenic activity associated with suspended solids is less available for detection in the yeast assay. [n.d. = not distinguishable from the assay blank].

experiment, and in most cases a large amount of concentrated stock feed was prepared and diluted as necessary.

When re-spiked on Day 16 (see arrows in Fig. 1) the SBR sludges removed oestrogenic activity within a shorter time (within 5 d) – suggesting that the sludge had become acclimatised to the presence of a relatively high concentration oestrogen. The AS micro-organisms from both SBRs #1 and #2 had been selectively cultured for enhanced biological phosphorus removal (EBPR) over a number of years in a laboratory environment and had been fed with an artificial wastewater feed during that time (Harper, 2002; McMahon, 2002). On the other hand the sludge from the full-scale treatment plant – which is expected to receive domestic wastewater with fluctuating levels of natural and synthetic oestrogens – removed oestrogenic activity to background levels within 5 d from dosing both with and without acclimatisation.

It is not known at this time why the SBR #2 activated sludge sample induced an assay response equivalent to a dose up to 4 times higher than the initial spiked doses, but similar responses were seen with other samples in this study. In other studies it has been observed that when biological responses to are converted to E2-equivalent concentration, there may be a discrepancy due to the presence of compounds which may contribute to oestrogenic activity even at low concentrations (Onda et al., 2003; Pawlowski et al., 2003). Also it was observed that SBR#1 sludge did not remove total oestrogenic activity to non-detectable levels even after re-spiking. A similar residual response was observed in a study by Servos et al. (2005) where in aerobic batch experiments with a sewage sludge able to reduce E2 and E1 by > 95% in less than 24 h, traces of yeast assay response were detected even after 5 d (120 h).

Estimated E2-equivalent concentrations of grab samples from a full-scale wastewater treatment plant

Grab samples from the full-scale activated sludge system (WTP) were assayed for total oestrogenic activity. The samples were collected on July 2002 in baked glass jars and aliquots stored frozen until analysis. The samples were thawed overnight at 4°C for the assay. Both well-mixed whole samples, and supernatant from centrifuged samples were assayed. There is an apparent reduction of total oestrogenic activity in the activated sludge system (see Fig. 2).

To estimate the E2-equivalent concentrations of the grab samples shown in Fig. 2, the activity of the assay blank [AR = 0.2] was subtracted from all sample activities to give the sample AR. Then using the sample AR, an estimate of the E2 concentration which would induce an equivalent activity was interpolated from the standard curve. Samples marked "n.d." induced an activity indistinguishable from the assay blank.

Effect of increasing sludge TSS and of pre-exposing sludge to oestrogen

Dose of 100 ngE2/ℓ: The mixed experiments gave variable results. At sludge concentrations of less than 1 gTSS/ℓ there were slightly improved oestrogenic activity reductions when the experiments were re-spiked with E2 on the 16th day of the experiments (see arrows on Fig. 3 showing re-spike). At 0.62 gTSS/ℓ (81% VS) reduction increased from 109% to 110% while at 0.86 gTSS/ℓ (77% VS) reduction increased from 42% to 46%. When TSS was increased, the total oestrogenic activity reductions changed as well. At 1.53 gTSS/ℓ (78% VS) reductions improved from 79% to 102% after re-spiking while at 1.98 gTSS/ℓ (77% VS), reduction decreased from 84% to 75%. With sludge concentrations above 2 gTSS/ℓ (2.36 gTSS/ℓ, 73% VS; 3.4 gTSS/ℓ, 92% VS; and 3.58 gTSS/ℓ, 75% VS) there was an overall increase in oestrogenic activity at the end of the experiment (Day 30/31) after re-spiking. Extending the experiment by an additional 5 d reduced the activity to background levels at Day 36/37.

In a full-scale AS treatment plant, this would mean that longer solids retention times (SRT) should enhance oestrogenic activity removal by allowing a longer contact time for the removal of compounds which contribute to oestrogenic activity. Andersen et al. (2004) for example predicted higher oestrogen removals in a Danish treatment plant with an SRT of approximately 35

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d compared with a treatment plant with a 20 to 25 d SRT. Holbrook et al. (2002; 2003b) reported higher specific equivalent estradiol activity (µg E2-equivalent/kg TSS) for facilities utilising secondary clarification when the SRT was 20 to 25 d compared with 8 to 12 d. A study by Svenson et al. (2003) on samples from several Swedish treatment plants showed that the plants with biological treatment (including an activated sludge process) and longer retention times had higher reductions of estradiol equivalents. At 100 ngE2/ℓ doses, the reduction was shown to be enhanced both by increasing VSS concentrations and continuous mixing. 100 ngE2/ℓ is close to the lower quantification limit of the assay and the data are more variable over time. E2-equivalent concentrations greater than 4 times the spiked dose were omitted since recovery experiments did not support their validity.

Dose of 100 µgE2/ℓ: Experiments spiked with 100 µgE2/ℓ (Fig. 4) showed >20% reduction of oestrogenic activity within 15 d on average. Re-spiking the sludge with an additional 100 µg/ℓ on the 16th day after the first set of experiments either resulted in additional reduction or an increase in total oes-

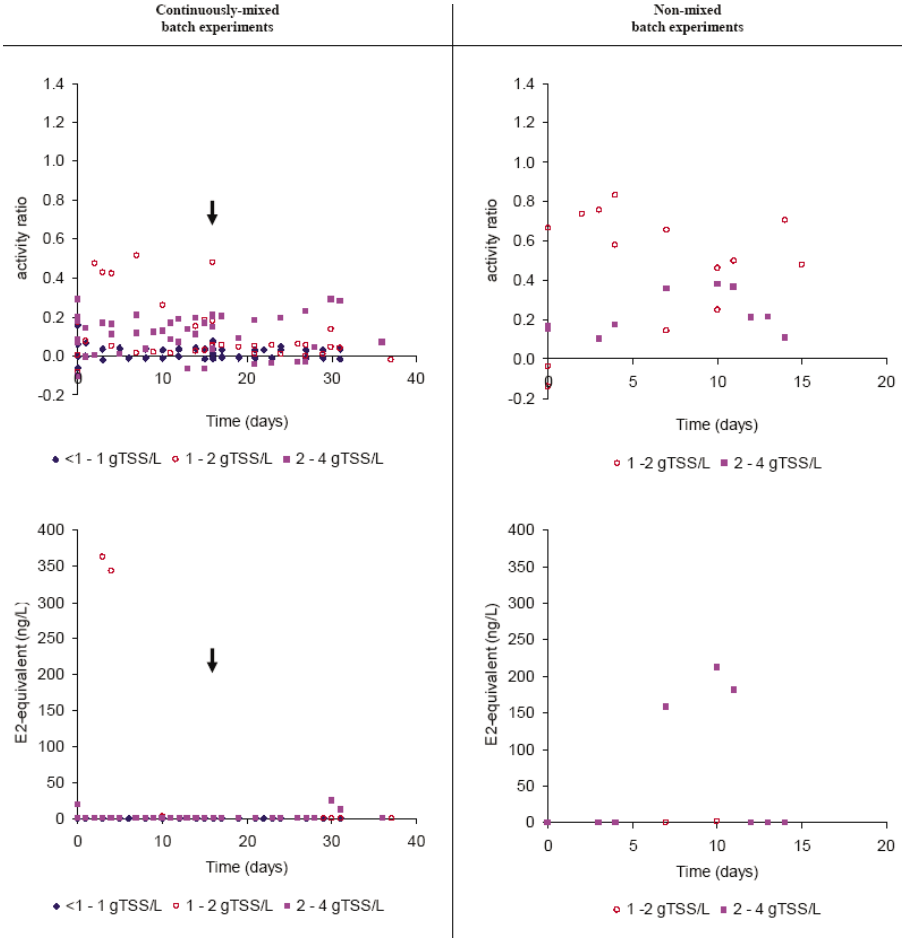

Figure 3 (top right)
Reduction of oestrogenic activity induced by a spiked dose of 100 ngE2/ℓ in all continuously mixed and unmixed batch experiments. There is >40% reduction of total oestrogenic activity detected from the sludge supernatant after 15 d. The percent reduction increases slightly when the experiments are re-spiked on Day 16. Arrows show re-spike.

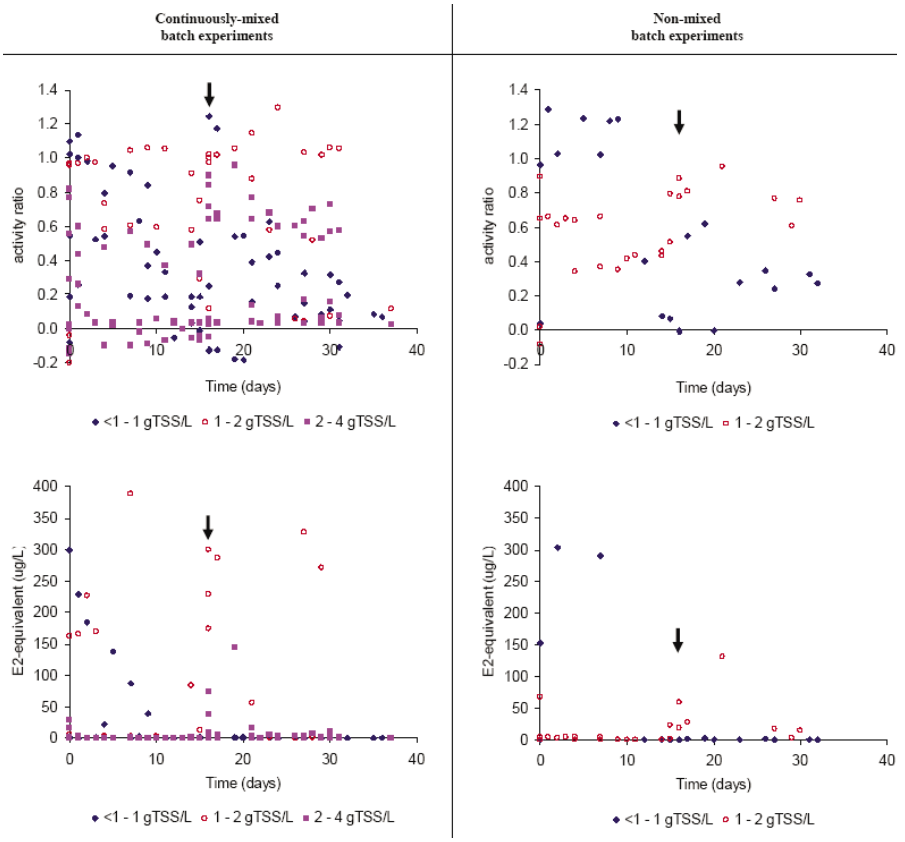

Figure 4 (bottom right)
Reduction of oestrogenic activity induced by a spiked dose of 100 µgE2/ℓ in all continuously mixed and unmixed batch experiments. The reduction of oestrogenic activity at this dose varied, ranging from sludges requiring 3 to 10 d of lag-time for reduction to background levels to some sludges removing only 20% of the dose-induced activity. Experiments with 1 gTSS/ℓ or less were able to reduce oestrogenic to background levels both with and without mixing. Some sludges lost their ability to reduce the oestrogenic activity after the re-spike (see arrows for re-spike), while others reduced the oestrogenic activity within a shorter time.

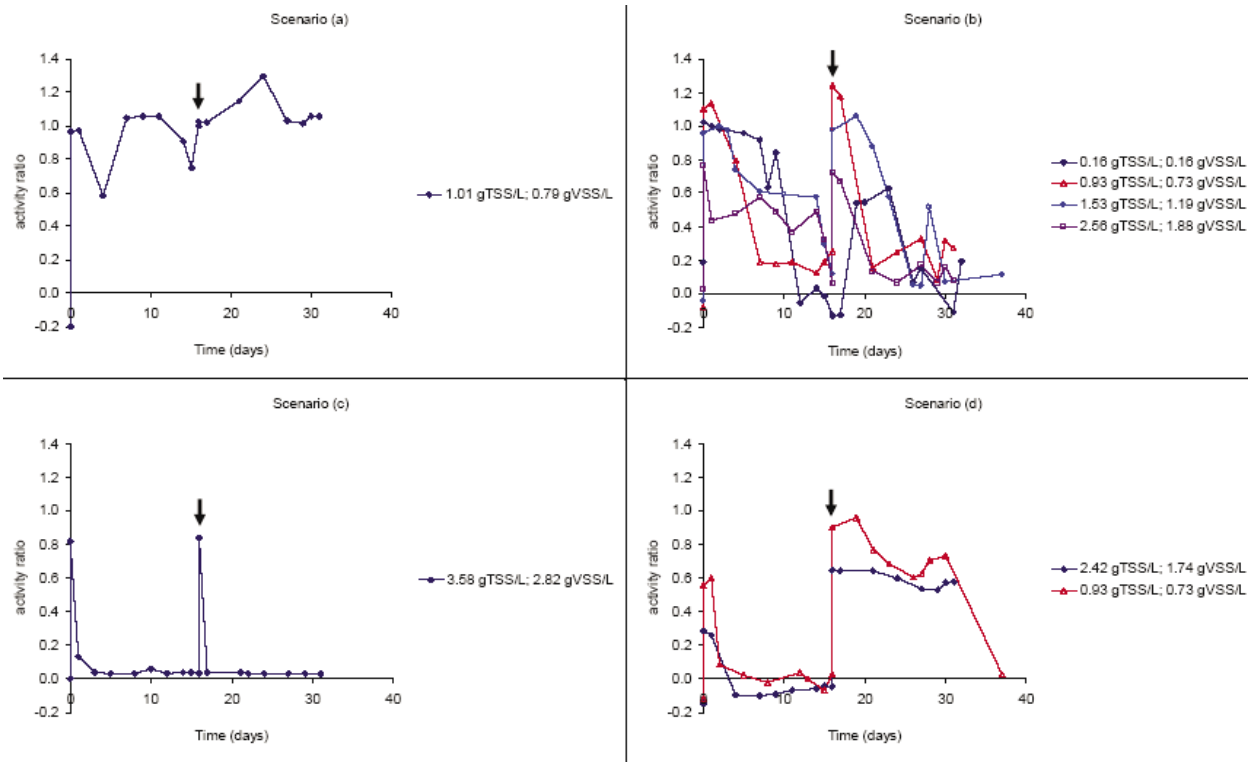

Figure 5

The four scenarios observed for reduction of total oestrogenic activity induced by a spiked dose of 100 µgE2/ℓ in continuously mixed batch experiments. Most of the reductions followed Scenario (b), i.e. there was a lag time before removal to background levels at the end of the first 15 d period, and the sludges required a shorter lag-time after the re-spike.

trogenic activity. It is not clear why there would be no reduction in some of the experiments after re-spiking as the lack of reduction appeared to have no relationship with the TSS or VSS concentrations. Further study is required to establish whether this phenomenon is due to loss of sorption capacity (saturation of available sites) or due to compromised microbial metabolism.

In general, the oestrogenic activity induced in continuously mixed batch experiments by an E2 spike of 100 µg/ℓ followed four scenarios (Fig. 5):

a There was hardly any reduction of oestrogenic activity at the end of the 15 d or 30 d period (1.01 gTSS/ℓ)

b After an initial lag time there was some removal or removal to background levels by the end of the first 15 d period, and after the re-spike a shorter lag time was required to reduce oestrogenic activity to background levels (0.16, 0.93, 1.53, and 2.56 gTSS/ℓ)

c There was a reduction of oestrogenic activity to background levels within the first 3 d after E2 dosing both before and after the re-spike (3.58 gTSS/ℓ)

d There was a reduction of oestrogenic activity to background levels within the first 5 d after E2 dosing, but there was little or no reduction after the re-spike (2.42 and 0.93 gTSS/ℓ).

At 100 µgE2/ℓ dose there is an enhancement of oestrogenic activity reduction with AS present as control experiments without sludge showed little reduction of oestrogenic activity over time. Mixing the sludge also improves reduction at this dose for TSS concentrations above 1 gTSS/ℓ. Below 1 gTSS/ℓ sludges removed >70% of the induced oestrogenic activity before and after re-spiking, and with or without mixing. At higher TSS however, the unmixed sludges removed 20% of the induced oestrogenic activity after 15 d. The re-spiked experiment removed only 2% of the oestrogenic activity by Day 30, underscoring the fact that mixing is important for long-term oestrogenic activity reduction capacity.

Batch experiments by Lee and Liu (2002) showed that sludge supernatant (<1 gTSS/ℓ) degraded E2 and its metabolites very rapidly under aerobic conditions. Within 22 h after dosing 200 µgE2/ℓ they measured an almost quantitative oxidation of two-thirds of the E2 to E1. Although less than 10% of the dosed E2 was detectable after 48 h, the concentration of E1 increased by over 50% within 3 d and did not drop below 10% until more than 10 d had passed (Lee and Liu, 2002). This confirms the observations in this study that more than 10 to 14 d are required to remove total oestrogenic activity from sludge supernatant after spiked doses of 100 µgE2/ℓ or more. Any oestrogen removal monitoring regime would then need to follow the fate of both E2 and E1 simultaneously. This would be complicated in a full-scale treatment plant AS system which receives new oestrogen loads continuously, and in addition to sludge recycling has micro-organisms with enzymes to metabolise and de-conjugate oestrogenic compounds.

The complex and varied responses observed in this study may be attributed to the complex nature of the sample. While every attempt was made to replicate the experiments, it was not possible to duplicate the TSS and VSS concentrations exactly even with AS from the same source. The different batch experiments may therefore have different responses due to different TSS and VSS concentrations, different suspended solids compositions, different microbial communities, and a different predisposition for oestrogen reduction. It is expected that the behaviour of the oestrogenic activity – activated sludge relationship in a full-scale treatment plant would be just as complex if not more so.

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The response to, and reduction of, oestrogenic activity in activated sludge systems is then a combination of the types and quantities of oestrogenic compounds present, the suspended solids compositions and the microbial community make-up. In this study, the type and quantity of oestrogenic compounds present was defined by the spiked E2 dose and the total and volatile suspended solids concentrations were used as a surrogate for the suspended solids composition, including the organic solids and microbial community. However, in this complex mixture, the reduction of oestrogenic activity is primarily due to sorption of the hydrophobic oestrogens to the organic AS solids and metabolism by the consortia of micro-organisms that make up the AS. In order to differentiate the effects of microbial metabolism and sorption to solids on the reduction of total oestrogenic activity it was necessary to either remove the solids (as in the AS-free controls described in this study) or eliminate the microbial metabolism by sterilising the sludge.

The next step is then to apply the oestrogen-sensitive yeast assay to study the reduction of oestrogenic activity obtained in batch experiments with sterile sludge. Experiments with sterilised activated sludge are being planned and the results will be compared with findings from recent studies including studies by Jensen and Schäfer (2001) comparing the sorption of oestrogens to different particulate solids including activated sludge and cellulose, and by Clara et al. (2004) in which the sludge was inactivated using mercury (II) sulphate, and a comprehensive study of oestrogen sorption using washed, heat-sterilised sludge published by the Danish Environmental Protection Agency (Andersen et al., 2004).

Conclusions

- From the results of the batch experiments with activated sludges from laboratory reactors and a full-scale treatment plant, it was observed that the AS from the laboratory reactors required a lag time of days to remove E2-induced total oestrogenic activity when initially spiked with 100 µgE2/ℓ while the AS from the full-scale plant did not. The laboratory reactor sludges showed an improved oestrogenic activity reduction capability after re-spiking.
- At 100 ng E2/ℓ spiked dose, total oestrogenic activity reduction is enhanced both by increasing activated sludge TSS and VSS concentration and by continuous mixing.
- For the E2 doses in the study, many sludges required more than 10 to 14 d to remove total oestrogenic activity from AS supernatant to background levels due to the oestrogenic nature of the metabolites of E2.
- Acclimation of the AS to E2, by dosing and re-spiking, may improve E2-induced oestrogenic activity reduction in some cases. In general, increasing sludge TSS concentrations also improved oestrogenic activity reduction, but TSS concentrations between 1 and 2 gTSS/ℓ gave the most varied responses in the batch experiments.
- The relationship between oestrogenic activity and organic suspended solids is a complex one and merits further study.

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