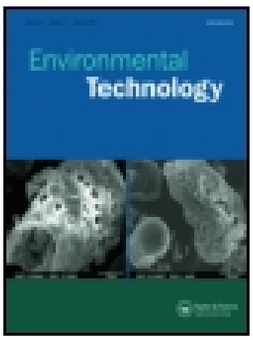

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Effect of source-separated urine storage on estrogenic activity detected using bioluminescent yeast *Saccharomyces cerevisiae*

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ABSTRACT

The objective was to demonstrate that a microbial whole cell biosensor, bioluminescent yeast, *Saccharomyces cerevisiae* (BMAEREluc/ERα) can be applied to detect overall estrogenic activity from fresh and stored human urine. The use of source-separated urine in agriculture removes a human originated estrogen source from wastewater influents, subsequently enabling nutrient recycling. Estrogenic activity in urine should be diminished prior to urine usage in agriculture in order to prevent its migration to soil. A storage period of 6 months is required for hygienic reasons; therefore, estrogenic activity monitoring is of interest. The method measured cumulative female hormone-like activity. Calibration curves were prepared for estrone, 17β-estradiol, 17α- ethinylestradiol and estriol. Estrogen concentrations of 0.29–29,640 μg L^{-1} were detectable while limit of detection corresponded to 0.28–35 μg L^{-1} of estrogens. The yeast sensor responded well to fresh and stored urine and gave high signals corresponding to 0.38–3,804 μg L^{-1} of estrogens in different urine samples. Estrogenic activity decreased during storage, but was still higher than in fresh urine implying insufficient storage length. The biosensor was suitable for monitoring hormonal activity in urine and can be used in screening anthropogenic estrogen-like compounds interacting with the receptor.

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Bioluminescence; estrogen; estrogenic activity; *Saccharomyces cerevisiae*; urine

1. Introduction

Natural and synthetic estrogens and their analogues have the ability to disrupt natural chemical processes in the wildlife, for example, by causing fish feminization.[1] Estrogens are excreted via human metabolism into urine mainly as conjugates (sulfate or glucuronide groups attached), which travel via the sewage to the wastewater treatment plants (WWTPs) as extensively diluted and mixed together with other types of wastewaters. Bacterial metabolism and enzyme activity can transform these conjugates back to their active forms in the sewers (before wastewaters enter the WWTP), [2,3] causing the distribution of estrogens into the receiving waters.[4] The concentrations in the WWTP effluents vary depending on the type of estrogen, from few to tens of ng L^{-1}.[3,5] Wastewaters arriving at WWTPs have been recognized as one of the main pathways via which endocrine disruptors, including estrogens, enter the environment. The degradation of estrogens in WWTPs is not yet fully understood.[6] The need for additional studies and cost-effective alternatives for the removal and degradation of estrogens from effluents has been noted.[7] The reduction of estrogen discharges to WWTPs could minimize their concentrations in effluents to inactive levels (concentration so low that no effect on environment can be detected;[8]). The main compound of interest regarding the environmental effects of estrogens has been synthetic 17α-ethinylestradiol, used in contraceptives, but also natural estradiol, estriol and estrone are of interest. Estrogen presence in WWTP effluents is primarily of domestic origin.[4] In addition, since estrogens may migrate into and in soils [9] from wastewater products used as fertilizers, measuring the concentration of estrogens prior to application is important. Thus, the monitoring of estrogenic activity of source-separated urine is of interest.

Separate collection of urine from other household wastewaters (source separation) is an environmentally feasible way to remove the source of human originated estrogens [4] from wastewater influents as well as to recycle the nutrients (N, P, K and micronutrients) which otherwise would burden the WWTP,[10] thus reducing the treatment requirements. Urine comprises approximately 1% of the wastewaters arriving at municipal WWTPs, but contains in liquid form 60% to 90% of N, P and K a person ingests,[11] as well as many micronutrients. Thus, source-separated urine is a good alternative fertilizer, which adds major key nutrients into soil (e.g.

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phosphorus [12]). Source separation promotes decentralized sanitation concepts which have received more attention [13] as the need for sustainable fertilizer sources has been recognized, and source separation is practiced, for example, in Sweden,[14] Germany [15] and Australia.[16] Source-separated urine should be stored for 6 months to achieve the required hygienic level regarding pathogens [17] and afterwards it can be used in fertilization (for discussion on using human excreta on plant production, see Ref. [18]). During storage, urine typically undergoes ureolysis where urea is transformed to ammonia and consequently pH is raised greater than nine, leading to pathogen inactivation. Application of sheep urine has been shown to enhance the leaching of radiolabelled estrogens in soils compared with application with distilled water.[19] In addition, estrogens have been shown to de-conjugate in soil.[20] If estrogens are not degraded already during storage, they can cause harmful environmental effects when leached in ground waters. Thus, estrogens should be removed and/or degraded from urine before fertilizer applications to mitigate their effect on the environment.

In order to use source-separated urine in crop fertilization, the use of a biosensor becomes a convenient method for overall estrogenic activity detection to demonstrate that the estrogenicity has diminished from the urine. In this study, a genetically modified yeast *Saccharomyces cerevisiae* was used.

Yeast cell biosensors have been regarded as a viable alternative for first-stage screening of endocrine disrupting compounds since they are easy and fairly inexpensive to perform [21]; they have high specificity to hormones due to the lack of endogenous steroid receptors,[22,23] and can be grown on media devoid of steroids.[24] Yeast cells are more resistant to environmental contaminants compared with mammalian cells, which make them advantageous for use in measuring complex environmental samples.[21] Many applications have utilized an extra-chromosomal reporter system with β-galactosidase as a substrate-based reporter protein (yeast estrogen screen, YES [25]), human breast cancer estrogen-sensitive MCF-7 cells in E-SCREEN assay [26] and estrogen-receptor mediated chemically activated luciferase gene expression (ER-CALUX assay [27]). Androgens have been detected from urine using yeast with a modified androgen receptor.[22,23,28] The application of yeast estrogen (and androgen) bioassay to calf urine using green fluorescent protein required extensive pre-treatment steps involving solid phase extraction and the use of organic solvents.[29,30] However, mainly the aforementioned studies have used a human androgen-receptor (male hormone) based strain, whereas this paper focuses on the human estrogen-receptor strain.

To our knowledge, no information is available on using a bioluminescent yeast biosensor to monitor the estrogenic activity of source-separated human urine. The objective of this study was to demonstrate that a bioluminescent yeast cell-based biosensor can be applied as a rapid and simple screening tool in the overall estrogenic activity detection from source-separated urine with and without storage without laborious pre-treatments. The bioavailability of the estrogens was studied by adding β-glucuronidase to the samples, as estrogens are mainly excreted as conjugates. The aim was to use the biosensor as a screening tool for studying estrogenic presence in the samples in order to gain knowledge on the safety of urine in agricultural use, as well as to demonstrate the suitability of the biosensor in urine and environmental sample monitoring as a fast, stand-alone analytical tool.

2. Materials and methods

2.1. Chemicals and materials

Yeast nitrogen base w/o amino acids was purchased from Difco, agar-agar from Merck, and D-glucose, adenine, L-tryptofan, L-leucine, L-histidine and D-luciferin were obtained from Sigma-Aldrich. Estrone (E_1, ≥99%), 17β-estradiol (E_2, ≥98%), 17α-ethinylestradiol (EE_2, ≥98%) and estriol (E_3, ≥97%) were purchased from Sigma and used as standards for estrogenic activity measurements. Sterile Octavia-water was provided by Fresenius Kabi Ag (Germany). MilliQ-water (18.2 mΩ cm conductivity, Millipore) was used in media preparation. Urease (jack bean urease, lyophilized 5 U mg^{-1}, *EC* 3.5.1.5) was obtained from Merck and ionic strength adjusting (ISA) solution was purchased from Thermo-Scientific. *S. cerevisiae* strain BMAEREluc/ERα with modified estrogen receptor alpha (ERα) was developed by Leskinen et al. [21]. Liquid synthetic dextrose medium (SD-medium), AHL-medium (containing essential amino acids adenine, L-histidine, L-leucine aka AHL-medium) and AHL-agar were prepared according to Välimaa et al. [31].

2.2. Yeast cultivation

S. cerevisiae BMAEREluc/ERα was cultivated in AHL-agar. It was grown for 48 h at 30°C and subsequently one colony was transferred into 5 mL of liquid medium (two replicates) which was incubated at 30°C overnight and 300 rpm shaking. Un-inoculated pure AHL-medium was used as control. For bioluminescence and hormonal activity assays, yeast cells were grown to an optical

density (OD_{600}) of 3.0 and subsequently the culture was diluted with fresh AHL-medium to OD_{600} of 0.8 and used in the assay.

2.3. Hormonal activity assay

Bioluminescence assay was performed according to the method described by Leskinen et al. [21]. The method was tested for each estrogen (stock solutions of 10 mM in EtOH of E_1, E_2, EE_2 and E_3) by analyzing concentrations from 0.027 ng L^{-1} to 29.6 mg L^{-1} (in 10% EtOH), in order to create standard curves. The analyses were done in triplicate with 10% EtOH as a reference blank.

A standard curve was constructed by pipetting 10 µL aliquots of hormones into white 96-well plates (Thermo Electron Corporation). As blank, 10 µL 10% EtOH was used. Aliquots of 90 µL of the diluted yeast culture (BMAEREluc/ERα) were then added into the wells containing the samples. The plates were incubated at 30°C for 2.5 h at 300 rpm. After incubation, 100 µL aliquots of sterile filtered 1 mM D-luciferin in 0.1 M Na-citrate buffer (pH 5.0) were pipetted into the wells and bioluminescence was measured immediately using Victor^2 Wallac 1420 Multilabel counter (PerkinElmer Life Sciences, Inc.).

2.4. Characterization of the matrix

The major constituents of fresh and stored human urine (as indicators of storage; described in more detail below) were determined in order to characterize the urine matrix. pH and conductivity of both types of urine samples were measured with Orion SA 720 pH-meter and WTW LF95, respectively.

The sum of ammonium (NH_4^+) and ammonia (NH_3) was determined with an ammonia selective, gas-sensitive membrane electrode (Thermo-Orion), which measures the NH_3 concentration. ISA solution was added to the samples and standards to convert ammonium to ammonia. Urea concentration was determined by measuring ammonia from the sample, then adding the urease enzyme and afterwards letting the sample stand for ca. sixteen hours in room temperature, while urease catalyzed urea decomposition to ammonia. Subsequently, ammonia was measured and the difference between the two ammonia concentrations equalled the amount of urea in the sample.

Ion chromatography (Dionex DX120) was used to determine the anions of interest (PO_4^{3-}, Cl^-, SO_4^{2-}, NO_3^-, NO_2^-) from the urine. The separating column was AS9-HC (Dionex) equipped with pre-column AG9-HC (Dionex). The eluent used was a buffer prepared of 12 mM Na_2CO_3 and 5 mM $NaHCO_3$. Urine was diluted with MilliQ water (1:100 v/v). The diluted urine was filtered (nylon 0.45 µm, VWR). Further analysis steps were made according to standard SFS-EN ISO 10304-1:en.[32]

For the determination of cations, an atomic adsorption spectrophotometer was used. Na, K, Mg and Ca were analyzed from urine with AA200 analyzer (PerkinElmer) using air acetylene flame. Before analysis, urine samples were filtered (0.45 µm nylon, VWR). The filtered samples were diluted at ratios from 1:50 to 1:5000 with MilliQ-water using acid washed glassware. The dilution for each sample depended on the cation and nature of urine. A 0.18 M CsCl + $LaCl_3$ solution (Merck) was added to the samples of which Ca was analyzed to stop the formation of substances that inhibit Ca atomization. The analysis was done according to Finnish Standard Association (SFS) standards 3018 [33] and 3044.[34]

Determination of dissolved organic carbon (DOC) was conducted according to SFS-EN standard 1484.[35] Urine samples were diluted with MilliQ-water and filtered through 0.45 µm prewashed syringe filter (nylon, VWR), after which the samples were analyzed with a TOC-5000 Analyzer (Shimadzu).

2.5. Detection of estrogenic activity in urine

For hormonal activity testing, triplicate urine samples collected from a volunteer female taking oral contraceptives, a female not using synthetic hormones, and male urine (two volunteers), child urine (both a boy and a girl), mixed urine (a combination of the previous) and stored urine (mixed, 1 or 5 months in polypropylene containers, in dark at room temperature) were used. The collected urine samples were not spiked with estrogens, but analyzed as such. The time points of 1 and 5 months were selected in estrogenic activity testing to obtain an understanding about the kinetics of hormone-like activity and degradation. As blank, 10 µL 10% EtOH and 10% EtOH in urine were used. Aliquots of 10 µL of samples were pipetted in triplicate without any pre-treatment into a white 96-well plate and analysis performed as described earlier. Analysis was repeated thrice for each urine sample. Concentrations of 0.027, 2.72, 272.4 and 27,238 µg L^{-1} of E_2 were spiked in fresh and stored urine to test the storage effect, and the recovery was determined against the E_2-standard in 10% EtOH. Adaptations of the sensor for a different matrix were not needed,[21] emphasizing the fact that the yeast biosensor can be used without any pre-treatment of the sample as a stand-alone tool in monitoring estrogenic activity changes during urine storage.

The bioavailability of the estrogens was tested either by heat treating samples at 80°C for 30 min (which is a normal procedure to hydrolyze compounds) or by

hydrolyzing them with β-glucuronidase enzyme (β-D-glucuronoside glucuronosohydrolase from *Escherichia coli* K12, *EC* 3.2.1.31, Fisher Scientific) at 46°C for 45 min to break the conjugated bonds. β-Glucuronidase interacts with the glucuronide group attached to the main molecule and breaks the bond, thus releasing the original estrogen in its bioactive form. Before hydrolysis with β-glucuronidase, pH of the samples was adjusted to 6.0–6.5 with 0.1 M NaOH or 0.1–1.0 M HCl. As a reference, the same samples were incubated at 46°C for 45 min without β-glucuronidase addition. The samples were heated in water baths at the specific temperatures, left to cool to room temperature and consecutively analyzed as previously described.

The impact of urine (fresh and stored) filtration on biosensor performance was not tested, as filtration/sterilization is not feasible in practice on a large scale and thus not considered feasible for applicability as a screening tool to assess urine safety in agricultural use. Filtration would be time-consuming and the costs would be high, as well as all the bacteria responsible for estrogenic degradation would be removed.

2.6. Analysis of obtained data

Light emission was measured as relative light units (RLU). By normalizing the RLU values of the samples with the background bioluminescence of the sensors, the data obtained from different experiments and/or luminometers can be compared.[21] The induction of the sensor by the sample was calculated as fold induction (FI): $FI = SL_S/SL_B$, where SL_S is the average bioluminescence of the yeast sensor (measured from three replicates) and SL_B is the bioluminescence of blank solvent (at least four replicates). FI is a dimensionless unit. FI in *y*-axis was plotted against $\log_{10}$ of the estrogen concentration. The limit of detection (LOD) describes the lowest measurable concentration and/or signal of the method. The minimum response considered as positive (FI_{LOD}) was determined according to Long and Winefordner [36]: $FI_{LOD} = 2(X_B + 3SD)/X_B$, where X_B is the average background bioluminescence value of the sensor (four 10% EtOH blanks included in each assay) and SD is the standard deviation. The LOD was subsequently determined from the standard curve using FI_{LOD}.

To evaluate the average estrogen concentration in urine samples, an average estrogen calibration curve was constructed based on four estrogens and their average molecular weight (281.8 g mol^{-1}). Although in some cases the results were below FI_{LOD}, an estimation of the estrogen concentration has yet been made. Of course, it has to be kept in mind that the real urine samples possibly contained other estrogenic compounds which also induce a signal and thus the calculated estrogen concentrations represent the concentration of all estrogen-like compounds in the sample. For FI values higher than the average estrogenic response curve, the calibration curve of EE_2 was used (biosensor gave the best signal, as described later).

3. Results and discussion

The goal of this study was to investigate the applicability of the bioluminescent yeast cell-based biosensor as a fast, stand-alone analytical screening tool in estrogenic activity monitoring in urine. In the present study, the urine samples required no pre-treatment (e.g. filtration, extraction or centrifugation). The method could be applied to urine as such.

3.1. Characteristics of the matrix

Source-separated urine contains amino acids, solids, salinity and inorganic and organic constituents (urea, creatinine, acids, etc.).[37] The measured characteristics of human urine are presented in Table 1. Fresh urine usually has pH ranging from 5.5 to 6.5, as described in this study. Urine contains several grams per liter of DOC (7.3 g L^{-1}) and urea (4.9 g L^{-1}), a compound which transforms into ammonia during storage due to bacterial ureases.[38] Urine contains macronutrients (N, P and K) in relatively large quantities (g L^{-1}), whereas micronutrients up to tens of mg L^{-1} (Table 1). Therefore, removing urine from wastewater streams not only reduces the organic load arriving at WWTPs, but also enables nutrient recycling. The collected urine can be used as a liquid fertilizer, while WWTP effluent is too diluted in nutrients and contains more pathogens due to the presence of fecal matter. The composition of urine changes during storage: organic matter degrades,

Table 1. Characteristics of the urine matrix.

Urine composition (unit)	Fresh	Stored
pH	5.8	9.4
Conductivity (mS cm^{-1})	9.1	25.9
DOC (g L^{-1})	7.29	2.81
$NH_4^+ + NH_3$ -N (g L^{-1})	0.57	8.67
Urea (g L^{-1})	4.95	1.05
Anions		
NO_3^- (g L^{-1})	0.16	n.d.
SO_4^{2-} (g L^{-1})	1.61	1.28
PO_4^{2-} (g L^{-1})	2.86	1.18
F^- (mg L^{-1})	91.6	55.8
Cl^- (g L^{-1})	2.67	2.40
Cations		
Na (g L^{-1})	0.83	1.01
K (g L^{-1})	0.52	1.17
Mg (mg L^{-1})	76.0	n.d.
Ca (mg L^{-1})	88.0	3.1

Note: n.d. = not detected.

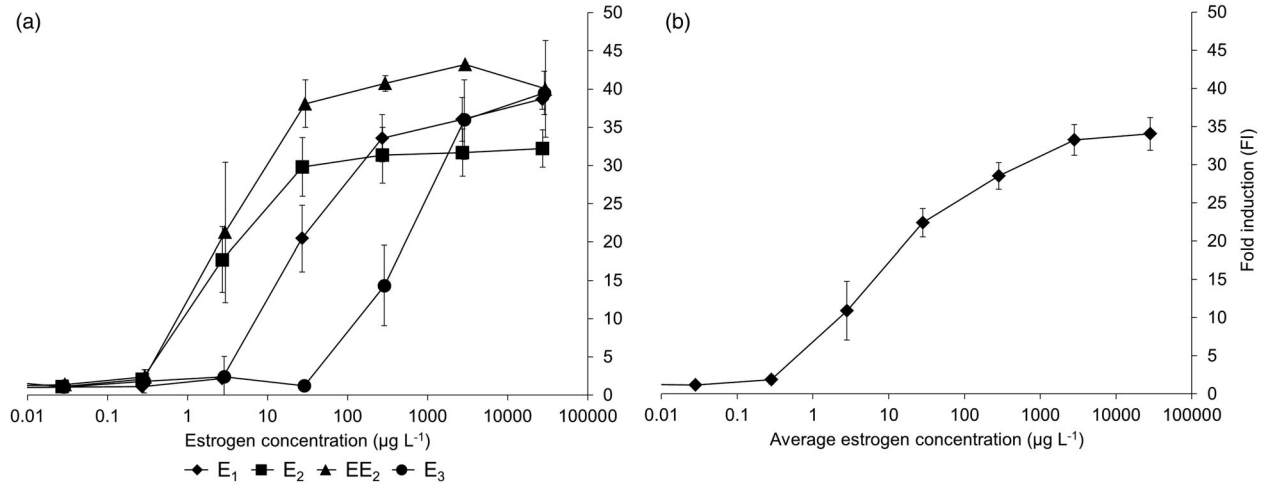

Figure 1. (a) Standard curves for estrone (E_1), 17β-estradiol (E_2), 17α-ethinylestradiol (EE_2) and estriol (E_3). (b) A standard curve calculated as the average of four estrogens from Figure 1(a) for the estimation of estrogenic concentration. The error bars represent the standard errors from three measurements. Estrogenic concentration determination: $FI < FI_{LOD}$: estimated from average estrogen curve; $FI > FI_{LOD}$: calculated from average estrogen curve; FI > average estrogen curve FI: calculated from EE_2 standard curve.

Mg and Ca salts precipitate (e.g. as struvite) and the salt content of urine decreases as a result of precipitation. After storage, the pH increased from 5.7 to 9.0–9.5 and urea transformed into ammonia.

3.2. Hormonal activity assay

Results obtained from constructing standard curves for estrogens (E_1, E_2, EE_2, E_3) in 10% EtOH were as presented in Figure 1(a). All the estrogens had similar standard curves, with an exponential increase in the signal at a specific concentration range. Similarly to Salste et al., [39] who studied WWTP effluents with the same biosensor, the yeast biosensor produced a signal with respect to all of the studied estrogens in a concentration-dependent way, thus demonstrating the repeatability of the results produced by this particular yeast strain in a different laboratory and matrix. The yeast response to estrogens varied depending on the estrogen (Figure 1(a)) with repeatable results, and mainly the response was obtained with estrogen range from 0.29 to 29,640 µg L^{-1}. In order to estimate the overall estrogenic concentration, an average of four estrogens was taken (Figure 1(b)) to approximate concentrations in urine.

The highest response with the biosensor was obtained with the synthetic EE_2. The FI_{LOD} for the biosensor was 2.4, corresponding to approximately 2.8 µg L^{-1} E_1, 0.28 µg L^{-1} E_2, 0.29 µg L^{-1} EE_2 and 35 µg L^{-1} E_3. The LOD determined from the average concentration curve corresponded to 0.29 µg L^{-1} estrogens. A comparison of different yeast cell-based bioassays and their detection limits to the current bioassay is presented in Table 2. A detailed comparison of the biosensors is unequal as some of the studies have measured estrogen equivalents, while some have used the bioassay only to demonstrate estrogenic activity: in those cases concentrations have been measured with chromatographic methods. One must bear in mind that practically all of the previous biosensor analyses used sample enrichment before analysis if the matrix has been some other than a standard solution (e.g. EtOH). Overall, the LOD obtained in this study is practical in monitoring real urine samples and the analysis is relatively fast to perform. In addition, as chromatographic methods usually require sample preparation and clean-up before analysis, use of this biosensor is well justifiable in simple and rapid monitoring. Compared with the analysis using mass spectrometer, which normally has LOD of few ng L^{-1} (e.g. Ref. [4]), the obtained LODs are higher; but compared with conventional liquid chromatography in combination with urine sample pre-treatment which has LOD around 0.1–0.2 µg L^{-1}, the results are comparable.[40]

Table 3 shows the typical excretion amount of estrogens per day in urine. The average amount of urine produced is ~1.2 L person^{-1} d^{-1},[37] which dilutes the estrogen concentrations in collected urine. Common birth control pills contain approximately 30 µg of EE_2. When comparing the LOD of the yeast biosensor with these excretion concentrations, it is obvious that the sensitivity of the sensor is applicable in urine estrogenic activity monitoring, especially, as the sensor reacts to other estrogenic compounds as well.

Estrogenic concentrations spiked in fresh and 5-month stored urine were different from the standards (0.027, 2.72, 272.4 and 27,238 µg L^{-1}). The results are presented in Figure 2(a),(b). The spiked E_2 concentrations in

Table 2. Comparison of yeast cell-based bioassays.

Bioassay	Reporter protein	Matrix	Sample pre-treatment	Time needed for analysis	LOD (μg L^{-1})	Reference
S. cerevisiae BMAEREluc/ERα	Luciferase	Pure compound in 10% EtOH	Not needed	2.5 h Incubation	0.008 (E_2)[a]	Leskinen et al. [21]
S. cerevisiae (yEGFP)	Green fluorescent protein	Spiked calf urine	Solid phase extraction, de-conjugation	De-conjugation overnight, plate drying overnight, 24 h incubation	1.0 (E_2 and EE_2)[a]	Bovee et al. [29]
S. cerevisiae	β-galactosidase	Pure compound in EtOH	Not needed	Three-day incubation	0.0015 (E_2)	Routledge and Sumpter [28]
Yeast estrogen screen (YES)	β-galactosidase	River water	Solid phase extraction	Pre-treatment 120–150+ min, 3-day incubation	0.0001 estrogen equivalents (EE_2)	Vermeirssen et al. [46]
S. cerevisiae BMAEREluc/ERα	Luciferase	Wastewater effluent	Solid phase extraction	Solid phase extraction 20+ min, freeze drying 4.5 h, chromatographic fractionation 28 min, LC–MS/MS[b] 15 min 2.5 h incubation	LOD was not determined for yeast assay	Salste et al. [39]
S. cerevisiae BMAEREluc/ERα	Luciferase	Pure compounds in 10% EtOH	Not needed	2.5 h Incubation	2.8 (E_1), 0.28 (E_2), 0.29 (EE_2), 35 (E_3)	This study
		Human urine (real samples)	De-conjugation	De-conjugation 45 min, 2.5 h incubation	0.38-3,804 (measured overall estrogenic concentration)	This study

[a]Measured as nM, converted by the authors.
[b]LC–MS/MS = liquid chromatography–mass spectrometry.

fresh urine gave reasonable signals, while the FI values were lower than that in standards prepared in 10% EtOH (0.5–6.2 FI units). This might be explained by sample toxicity, which might have had an effect on the yeast, thus decreasing the bioluminescence. However, the reason for such spiked E_2 behavior is not crucial regarding the use of the biosensor to the suggested application. The spiked E_2 concentrations in stored urine had high signals, with recovery comparable to fresh urine, if standard error is taken into account. This could be due to the purity of the estrogens, as they were not glycosylated, thus making them more bioavailable. The urine blank induced quite high signal, which caused a low FI value in stored urine. A high urine blank signal in stored urine is explained by the release of estrogenic compounds in urine in addition to the presence of other estrogenic substances.

Leskinen et al. [21] have already proven that yeast estrogen strains have behaved and responded to hormones (two androgens, E_2) and xenoestrogens similarly with yeast estrogen screen. Relative estrogenic potencies determined with their assay seemed comparable to those determined using *in vitro* bioassays. Urine always contains estrogens (even in urine from males); thus, having urine blank is not possible. Even though long enough stored urine might be devoid of estrogens, the composition of the sample would be different and it might still contain estrogen-like compounds that induce a signal with the biosensor. The use of a different matrix as blank seems inappropriate, for example, as a solution of ammonia or water is not comparable with the composition of and the matrix effect caused by urine. However, urine blank is not needed since all FI values are corrected by dividing with the blank signal, and the aim of this study was not to determine exact concentrations but to monitor the overall change in estrogenic activity, in which the biosensor performed accordingly.

Table 3. Typical estrogen concentrations in human urine and their excretion.

Estrogen	Excretion in urine (μg d^{-1})	Excretion as glucuronides [47] (%)	Excretion as free form [47] (%)
Estrone (E_1)	0.54–54[a] 3.2–16.1[b]	85–89	1–3
Estradiol (E_2)	0.54–16.3[a] 4.6–269[b] 10.3[c]	90–95	1–3
Ethinylestradiol (EE_2)	7.1[b]	90–95	n.a.
Estriol (E_3)	1.4–43.2[a]	90–95	0–2

Note: n.a. = not available.
[a]U. Turpeinen, Helsinki University Central Hospital Laboratory, Helsinki, Finland, personal communication.
[b]Ref. [43], 269 μg E_2 d^{-1} for pregnant women.
[c]Average concentration in human urine [48].

3.3. Estrogen bioavailability

The bioavailability of the estrogens and the response of the biosensor are affected by the presence of estrogens as conjugates in urine (Table 3); thus, breaking up the conjugated bonds should improve the sensitivity of the sensor strain and provide knowledge on the magnitude of de-conjugation potential in soil.

Figure 3(a),(b) describes the signal given by the yeast biosensor in different urine samples without and after different treatments (Table 4). As previously described, time points of 1 and 5 months were selected in estrogenic activity testing due to the availability of samples. The overall recommended time for urine storage is 6 months,[17] but shorter periods are commonly used.

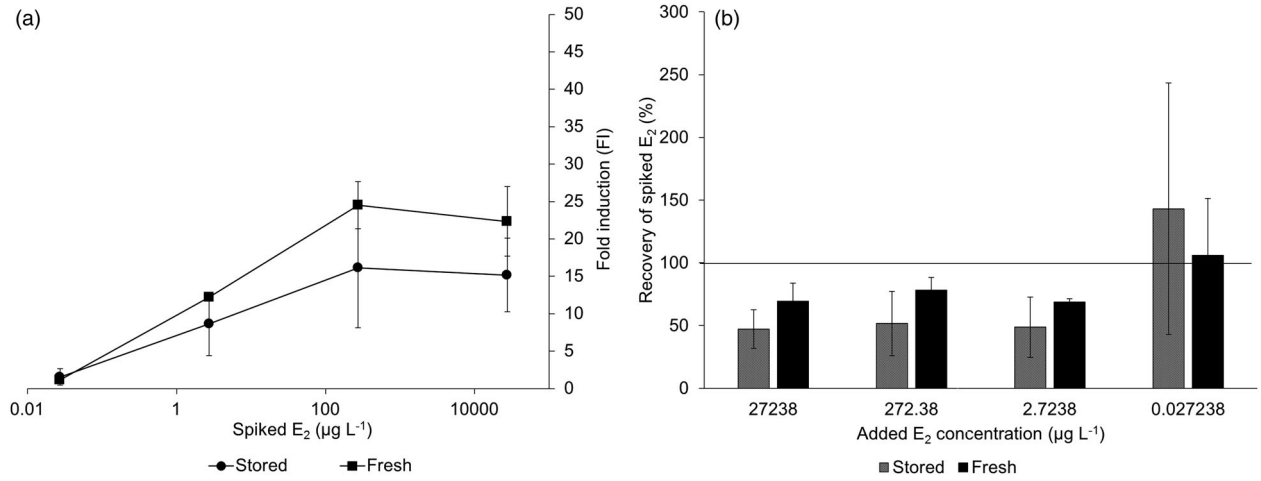

Figure 2. Comparison of the storage effect with spiked samples. Fresh urine was collected on the previous day. Urine (mixed) was stored for 5 months in plastic jars, at room temperature. Samples were spiked with four E_2 concentrations and analyzed. The FI values represent the mean of three replicates, with error bars showing standard error. (a) The higher FI values for fresh urine are explained by the lower bioluminescence of the blank. The blank stored urine had a higher background signal, thus decreasing the overall FI values. (b) Recovery of the added E_2 concentration compared with the E_2 standard in 10% EtOH. With fresh urine, the recovery was better.

Only unconjugated forms of estrogens contributed to the ERα activity when urine was measured without treatment (first and second bar), and the results were repeatable. Thus, bioavailability testing was conducted to get the response for all estrogenic activities present in the sample (measuring the sum of free and glucuronidated forms). Bioavailability of estrogens is important as different soil microorganisms [41,42] may break down the conjugated bonds, thus releasing active hormones in the soil.

Heat treatment at 80°C for half an hour resulted in increased activities in urine from a female not taking contraceptives (FI value 18.5, 14 µg L^{-1}) and a female child (FI value 2.5, 0.35 µg L^{-1}) when compared with urine without heating (concentrations below average LOD, 0.05–0.29 µg L^{-1}). The addition of β-glucuronidase and incubation at 46°C for 45 min increased the FI values of all urine samples, ranging from FI 7.8 (1.4 µg L^{-1}) to FI 41.4 (10,200 µg L^{-1}, a female without contraceptives, determined using standard for EE_2), except for the

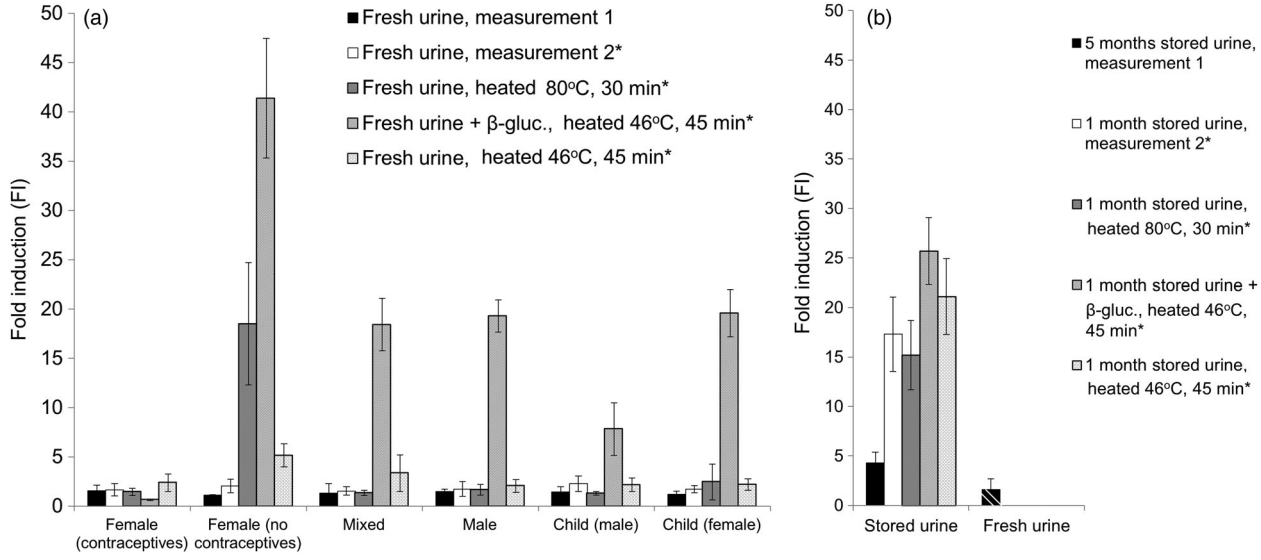

Figure 3. FI of different urine samples using the bioluminescent yeast biosensor. The bars represent the mean of three replicates, with error bars showing standard error. β-gluc. = β-glucuronidase. (a) FI values for the different fresh urine samples with and without different pre-treatments. (b) FI values of fresh and stored urine (at room temperature in plastic jars with a tight lid, 1 or 5 months) with and without different pre-treatments. * indicates that urine samples were collected and analyzed at the same time (measurement 2).

Table 4. Different pre-treatments conducted to urine samples presented in Figure 3.

Figure		Pre-treatment
3A: Fresh urine	Measurement 1: fresh urine only	None
	Measurement 2: fresh urine with different pre-treatments, indicated by*	None
		Heat treatment 80°C (30 min)
		Heat treatment 46°C (45 min)
		Heat treatment with β-glucuronidase addition 46°C (45 min)
3B: Stored urine	Measurement 1: Stored urine (5 months)	None
	Measurement 2: Stored urine (1 month) with different pre-treatments, indicated by*	None
		Heat treatment 80°C (30 min)
		Heat treatment 46°C (45 min)
		Heat treatment with β-glucuronidase addition 46°C (45 min)

female taking contraceptives (FI 0.6, <LOD). Mere incubation at 46°C did not affect the FI values as much as enzyme addition, except in the case of the female taking oral contraceptives and in stored urine. The high FI value for the female not taking contraceptives can be explained with the menstrual cycle and/or pregnancy: during different phases, the excreted estrogen concentrations can vary markedly.[43] The observed signals corresponded to estrogen concentrations of 0.27–2,964 μg L^{-1} (determined from the standard curves based on measured FI, Figure 1(a),(b)), the highest of which could be due to other estrogen-like compounds excreted in urine and binding to the ERα receptor.

Estrogen excretion for men [43] has been calculated to average 3.2 μg d^{-1}, whereas our results using heating and β-glucuronidase addition gave 15 μg L^{-1}, which adjusted to 1.2 L d^{-1} makes 18 μg d^{-1}; however, this estrogen concentration includes all estrogen-like compounds excreted in male urine. Clearly, the method's sensitivity was better with β-glucuronidase addition: the samples which earlier gave lower FI values now showed more clearly estrogenic activity. This was in accordance with the results published by Kaiser et al. [44] who tested their yeast biosensor with swine urine and noted that samples needed a pre-treatment before analysis to break the conjugated bonds (incubation with *Helix pomatia* solution), because estrogen conjugation reduced the method's sensitivity. The FI values were higher than the calculated LOD, except in the case of the female taking contraceptives, which could not be explained.

The effect of urine storage on estrogenic activity was as hypothesized. Storing urine affected the bioavailability of estrogens. After 1 month of storage, mixed urine gave a markedly higher signal (FI value 17, 9.5 μg L^{-1}) than fresh urine (FI value 1.4, 0.1 μg L^{-1}, <LOD). Urine stored for 5 months gave an FI value of 4.3 (6.2 μg L^{-1}), indicating that some biotransformation had occurred. This was expected since source-separated urine is never devoid of bacteria and as previously stated, filtration of urine on a large scale is not feasible due to economic reasons. The bacterial enzymatic (glucuronidases, estradiol dehydrogenases and sulfatases) and metabolic activity breaks the conjugated bonds, thus increasing the observed signal, which reduces over time due to estrogen degradation. After 5 months, the estrogenic concentration was still higher than in fresh urine (0.1 μg L^{-1}, <LOD), indicating the presence of estrogen-receptor binding compounds. According to the review by Yu et al. [7] some microorganisms isolated from the intestine can convert E_2 to E_1 and vice versa, or degrade E_1, E_2 and E_3; they can also co-metabolize EE_2 in the presence of other estrogens. Thus, further studies are required to monitor how the estrogenic activity changes and which enzymes are active during the 6-month storage period. Furthermore, it might be interesting to add certain microbial mixed communities known to degrade estrogens to the urine.

The advantage of biosensors in estrogenic activity monitoring is their capability to estimate the cumulative estrogenic effects of a variety of chemicals in an environmental sample [8] that correspond to the overall activity of all ERα-binding chemicals present.[45] Although the measurement of exact concentrations is informative, measuring the overall estrogenic activity can be more useful regarding practical applications, such as fertilizer use. The yeast used in this study gave a positive response to all major forms of estrogenic compounds and it could also be used in pre-screening anthropogenic estrogen-like compounds which interact with the receptor, such as bisphenol-A or parabens (e.g. Ref. [21]). To be more specific, the method applied in this experiment measures the cumulative concentration of endocrine disruptor compounds (EDCs), not just estrogens. Therefore, it is very difficult to make comparisons to other methods, as the molecular diversity of such EDCs is so vast that it is impossible to measure the accurate concentrations of a single compound. In addition to the before mentioned, it is possible that degradation products of EDCs are inducing a signal. As stated previously, the calculated estrogenic concentrations in this study were only estimates when calculation was based on average estrogen molecular mass.

Biosensors are also suitable for the development for onsite tests because of their high specificity. Since estrogens have been shown to migrate in soil, this screening tool could be used to test estrogenic activity in soil samples after extraction to liquid. This method may

also prove useful in a quick screening of endocrine-disrupting chemicals for the efficacy of municipal WWTPs, which has been previously performed, for example, by Desbrow et al. [4] using a different reporter gene as well as a selection of sample pre-treatment steps.

Contrary to the conventional chromatographic methods, the method has several advantages, including complementarity to traditional analytical chemistry analyses, a minimal sample pre-treatment need, the provision of analyte bioavailability information and simultaneous analysis of multiple samples. The bioluminescent yeast assay is easy to handle, robust, cost-efficient and needs no high-tech analysis equipment but can be used with cheap portable detectors.

4. Conclusions

To our knowledge, this is the first experiment where bioluminescent yeast biosensor *S. cerevisiae* with β-glucuronidase addition has been used to monitor estrogenic activity in source-separated urine. From the results obtained in this study, we concluded the following:

- Estrogenic activity present in urine after storage implied that the recommended length of urine storage may be insufficient in reducing the overall estrogenic activity to agriculturally 'safe' levels.
- Four estrogens (E_1, E_2, EE_2 and E_3) could be detected using the biosensor with an effective measuring range of 0.29–29,640 μg L^{-1} and LOD corresponding to 0.28–35 μg L^{-1} of estrogens.
- The yeast sensor produced a cumulative signal, which included both natural and synthetic estrogens and estrogen-like compounds.
- The addition of β-glucuronidase and heating for 45 min at 46°C enabled the assay to be used in monitoring the change in the estrogenic activity of source-separated urine.
- The yeast sensor can also be used in screening anthropogenic estrogen-like compounds which interact with the ERα receptor.
- The method has several advantages on conventional chromatographic techniques, for example, a minimal need for sample pre-treatment and the simultaneous analysis of multiple samples in a short three-hour analysis time.

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Disclosure statement

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