Detecting estrogenic activity in water samples with estrogen-sensitive yeast cells using spectrophotometry and fluorescence microscopy

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Abstract

Environmental estrogens are environmental contaminants that can mimic the biological activities of the female hormone estrogen in the endocrine system, i.e. they act as endocrine disrupters. Several substances are reported to have estrogen-like activity or estrogenic activity. These include steroid hormones, synthetic estrogens (xenoestrogens), environmental pollutants and phytoestrogens (plant estrogens).

Using the chromogenic substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) we show that an estrogen-sensitive yeast strain RMY/ER-ERE, with human estrogen receptor (hERα) gene and the *lacZ* gene which encodes the enzyme β-galactosidase, is able to detect estrogenic activity in water samples over a wide range of spiked concentrations of the hormonal estrogen 17β-estradiol (E2). *Ortho*-nitrophenol (ONP), the yellow product of this assay can be detected using spectrophotometry but requires cell lysis to release the enzyme and allow product formation.

We improved this aspect in a fluorogenic assay by using fluorescein di-β-D-

galactopyranoside (FDG) as a substrate. The product was visualized using fluorescence microscopy without the need to kill, fix or lyse the cells. We show that in live yeast cells, the uptake of E2 and the subsequent production of β-galactosidase enzyme occur quite rapidly, with maximum enzyme-catalyzed fluorescent product formation evident after about 30 minutes of exposure to E2. The fluorogenic assay was applied to a selection of estrogenic compounds and the Synchrotron-based Fourier transform infrared (SR-FTIR) spectra of the cells obtained to better understand the yeast whole cell response to the compounds. The fluorogenic assay is most sensitive to E2, but the SR-FTIR spectra suggest that the cells respond to all the estrogenic compounds tested even when no fluorescent response was detected. These findings are promising and may shorten the duration of environmental water screening and monitoring regimes using yeast-based estrogen assays, and the development of biosensors for environmental estrogens designed to complement quantification methods.

Key words: estrogenic activity, estrogen, yeast, assay, fluorogenic, chromogenic, EDC, FTIR

Introduction

Endocrine disrupting compounds (EDCs) and estrogenic activity

EDCs are chemicals which affect the body's endocrine system by behaving like hormones, enhancing or inhibiting the body's response to hormonal induction. For a good overview see Filali-Meknassi et al. (2004). EDCs which mimic the effect of the natural hormonal estrogen 17 β-estradiol are termed estrogenic. This paper is limited to study of the estrogenic EDCs and the estrogenic activity their presence imparts on water. Several natural sources of estrogenic compounds include naturally produced steroid hormones (Shore and Shemesh, 2003) and soy-based foods (Dwyer et al., 1994). There is growing concern about additional souces of

estrogenic EDCs to the environment including personal care products and pharmaceuticals (McDonnell et al., 2002; Ingerslev et al., 2003).

Estrogens in wastewater treatment

A great potential exists for the removal of estrogenic compounds in wastewater treatment since the wastewater streams are combined and collected in wastewater treatment plants, providing a location where removal can be attained before effluent discharge back to the environment. For example, estrogens are hydrophobic and preferentially sorb out of the water phase onto organic solids (Clara et al., 2004), so that treatment processes like the activated sludge system which incorporate biological organic solids are able to remove a significant portion of the estrogens (D'Ascenzo et al., 2003; Onda et al., 2003). The present concern is that, even with this reduction, some estrogenic compounds persist and are found in treatment plant effluents at ng/L to low µg/L levels (Rutishauser et al., 2004; Servos et al., 2005). Even these low concentrations could still be physiologically important (Jobling and Sumpter, 1993; Welshons et al., 2003) if they are not moderated by additional treatment, or by dilution and sorption in the receiving water. In addition, the wastwater treatment process may convert some compounds with low estrogenic activity like alkyphenolethoxylates into more potent alkylphenol products (Johnson and Sumpter, 2001).

Estrogens in drinking water sources and supplies

Receiving waters to which wastewater treatment plant effluents are discharged may also be a source of drinking water supply, which gives additional impetus for removing as much estrogenic activity as possible before effluent discharge. Estrogens and estrogenic activity have

been found in aquatic systems including coastal waters (Atkinson et al., 2003; Burgess, 2003), river water and drinking water treatment plants (Fawell et al., 2001; Rodriguez-Mozaz et al., 2004). A comprehensive report on EDCs in drinking water in Europe expresses further concern about the possible effects of estrogenic and other EDCs in drinking water (Wenzel et al., 2003).

Estrogens in eukaryotic cells

In the body, estrogens are transported through the blood, and unbound estrogens enter cells across the cell membrane. Inactive estrogen receptors (ERs) are associated with heat shock proteins in the cell cytoplasm. When estrogen binds to estrogen receptors the proteins dissociate and estrogen-ER monomers dimerize. The dimer is activated and undergoes a conformational change (Ing and O'Malley, 1995; Legler, 2002). Activated dimers can bind to estrogen response elements (ERE) of genes in the cell nucleus where transcription can then be initiated. New proteins formed may alter cell function and cell physiology.

Nuclear steroid receptors have been widely studied and their proposed mechanisms of action comprehensively reviewed (Nilsson and Gustafsson, 2000; DeFranco, 2002; Shapiro, 2003b). In estrogen-responsive mammalian tissues, the mechanisms of response *in vivo* and *in vitro* are believed to be similar since induction of similar proteins was observed on exposure to estrogens (Katzenellenbogen and Gorski, 1972). The hypothesized generalized pathway *in vivo*, termed the “activation pathway” (Shapiro, 2003a) is similar to the pathway proposed in yeast when the ER gene is introduced into the yeast genome, as the estrogen-induced signaling pathways are highly conserved in yeast and mammalian cells (Pham et al., 1992).

The effects of estrogens in the recombinant yeast cells used in yeast-based assays are similar to the known mechanism of estrogenic action in mammalian cells, but less complex. This

combination of similarity and relative simplicity makes the yeast-based assays promising in modeling the actual estrogenic activity that mammalian cells including their response to estrogenic exposure. The robustness of the yeast cells provides an additional advantage for their use as a biosensor (Baronian, 2004) and bioreporter of estrogenic activity.

The estrogen-responsive yeast RMY/ER-ERE

The estrogen-responsive yeast was a gift from Dr. D. Picard (University of Geneva, Switzerland). The construct's parent strain RMY326 (MATa his3 leu2-3, 112 trp1-1 ura3-52) was transformed with plasmids pUCΔSS-ERE and pG/ER(G) to give 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]) which expresses the wild-type human estrogen receptor α (hERα) and contains an ER reporter gene and estrogen response elements upstream of the *lacZ* gene for β-galactosidase (Liu and Picard, 1998).

Chromogenic assay

The assay was based on two previously developed assays (Klein et al., 1994; Coldham et al., 1997), and uses excess *ortho*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. ONPG is a lactose analogue that is cleaved in a reaction catalyzed by the enzyme β-galactosidase to give yellow *ortho*-nitrophenol (ONP) – see **Figure 1** for a schematic. The assay requires at least 20 h for incubation of yeast with 250 μL of test sample after which the cells are lysed and the lysate exposed to ONPG for 6 min. Yellow product formation (ONP) is observed visually and can be quantified using spectrophotometry. The dose-response is repeatable (**Figure 2**) and the assay showed early potential for application in environmental sample screening

(Hermanowicz and Wozei, 2002).

Fluorogenic assay

The assay uses fluorescein di-β-D-galactopyranoside (FDG) as substrate and includes propidium iodide dye for identification of damaged cells. Details of the assay development can be found in a previous publication (Wozei et al., 2006). Briefly, the assay requires 30 min yeast incubation with a 20 μL test sample after which the cells are incubated for 2 min with FDG. Cell response does not require cell lysis or fixing and a positive response is observed as fluorescence in whole live cells with fluorescence microscopy using a GFP-FITC filter for positive-response cell green fluorescence (**Figure 3**) and Rhodamine filter for damaged cell red fluorescence. The response can be quantified using ratio of positive cell response count to total cell count.

Detection of estrogenic EDCs with the fluorescence assay

Seven estrogenic EDC compounds were selected for preliminary study, and tested for cell response in the fluorescence assay: estrone (E1), 17 β-estradiol (E2), estriol (E3), 17 α-ethinylestradiol (EE2), nonylphenol (NP), 4-octylphenol (4-OP), and 4-*tert*-octylphenol (4-TOP). For the detailed assay protocol refer to (Wozei et al., 2006). All chemicals were tested at 10 μM concentration, and yeast cells were exposed to the test compounds for 30 min. Control cells (CTRL) were sham-exposed, i.e. they were exposed to the EDC-free phosphate buffered saline (PBS) medium used to deliver the test compounds and the same percentage per volume of ethyl alcohol used to dissolve the test compounds, and underwent the same process as the EDC-exposed cells.

Figure 4 shows a summary of the cell counts from the assay. The E2 response is about 15

times that of control cells. The EE2 response was much lower than expected since EE2 has been shown in yeast-based studies to have an equal or higher estrogenic potency relative to E2 (Coldham et al., 1997; Folmar et al., 2002; Rutishauser et al., 2004).

Synchrotron Radiation-based Fourier Transform Infrared (SR-FTIR) spectromicroscopy

To understand further the yeast cell response in the assay, SR-FTIR spectromicroscopy was performed on the yeast cells exposed to the selected EDCs in the fluorogenic assay. The experiments were carried out at the Lawrence Berkeley National Laboratory's Advance Light Source (ALS) Beamline 1.4.3 (For technical specifications refer to http://infrared.als.lbl.gov/index.html). The beamline light source provides a non-destructive high-brightness, high-resolution method that is used for monitoring real-time changes in the response of live cells to external stimuli (Holman et al., 1999; Holman et al., 2000a; Holman et al., 2000b; Holman et al., 2002) using the double-transmission technique. The infrared beam has a focused spot with a 10 μm diameter. A volume of 10 μL EDC-exposed or control yeast cells was spotted onto on gold-coated slides and allowed to attach. 128 co-added spectral scans were collected with a $4cm^{-1}$ data point resolution at a minimum of 13 discrete points of the attached cell monolayer. Each point corresponded to a 10 μm diameter beam spot potentially encompassing up to 25 yeast cells. The spectromicroscopy can detect rapid real-time response of the yeast cells to EDC exposure and establish the time-line of any structural and macromolecular changes.

Mean SR-FTIR spectra of the selected EDCs and peak area ratios of regions of interest are shown in **Figures 5** and **6** respectively. Preliminary analysis shows that EE2 has the largest overall effect on the yeast cells at the test concentration. These results are similar to studies in

fish that have shown that EE2 has a very high potency relative to E2 *in vivo* (Thorpe et al., 2003; Van den Belt et al., 2004) suggesting that the *in vivo* response to this compound is complex and of concern with respect to drinking water. Further studies are required as this compound has been measured – albeit in ng/L concentrations – in wastewater treatment plant effluents. In light of SR-FTIR results, a possible reason for low response to EE2 in the fluorogenic assay could be due to low β-galactosidase production, and this is being investigated.

As expected E1 has a smaller effect on the yeast cells than E2 (**Figure 6**), in other yeast-based studies E1 showed a potency up to about 40% that of E2 (Coldham et al., 1997; Andersen et al., 1999; Rutishauser et al., 2004) which is similar to its *in vivo* potency (Thorpe et al., 2003). The response to E3 was surprising since the potency of this compound was reported to be up to 30 times lower than E2 (Andersen et al., 1999; Rutishauser et al., 2004). It is possible that our yeast construct is more sensitive to E3, but further study will be carried out to confirm this response as the fluorogenic assay response was low.

Of the alkylphenols tested, the yeast cells responded to 4-OP more strongly (**Figure 6**). 4-OP has a slightly higher *in vitro* potency than NP (Coldham et al., 1997). 4-TOP has a potency lower than E2 (Coldham et al., 1997; Rehmann et al., 1999; Rutishauser et al., 2004), 4-OP and NP (Coldham et al., 1997) suggesting that yeast cells would respond to it less strongly, and this is confirmed by the weaker response in our preliminary results (**Figure 6**).

The SR-FTIR response is representative of the whole-cell response and has highlighted the complexity of the yeast cell response to EDCs that could be the focus of a further study.

Advantages of the fluorogenic assay

The assay requires a smaller sample volume than the chromogenic assay (10 μL vs. 250

µL) and total assay volumes do not exceed 2 mL per sample. In addition, shorter yeast incubation time with the sample is used (30 min vs. 20 h) and no cell lysis or cell fixing is required to detect the cell response as the live yeast cell response can be observed under a microscope. This means that the exposed cells can be used in several methods for results confirmation.

Limitations of the fluorogenic assay

At present the assay still has some limitations. The assay requires the use of an available epifluorescence microscope with the correct filters (GFP-FITC and Rhodamine) and a suitably trained microscopist. Quantification of a positive response requires visual cell counts which can be time-intensive. To-date this has been accomplished by taking digital images of the cells under bright field and fluorescent light using a digital microscope camera with image-capture software, and using these images to do the cell counts.

Toxic components of some samples may kill cells or inhibit their response since the sample is in direct contact with the yeast cells. Sample dilutions may be needed if sample toxicity is suspected, although this was not necessary in our study for the test compounds and concentration chosen. It should also be kept in mind that the 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 that the yeast cell membrane preferentially internalizes lipophilic compounds.

Summary and future steps

Preliminary results show that the estrogen-sensitive yeast based fluorescence assay, in

combination with other test methods, has a strong potential for environmental sample screening of estrogenic EDCs. We anticipate that with further validation and protocol development, the yeast-based assays can form the basis of the equivalent of Tier 1 water screening efforts (Borglin et al., 2005). Since small cell culture, reagent and sample volumes are required; it should be possible to further miniaturize the assays for high-throughput and field testing. The yeast cell ultrastructural and macromolecular responses to estrogenic EDCs and environmental samples are being studied using microscopy and SR-FTIR spectromicroscopy. Additional experiments with different concentrations of the selected EDCs are in progress, and data analysis from all test methods is ongoing.

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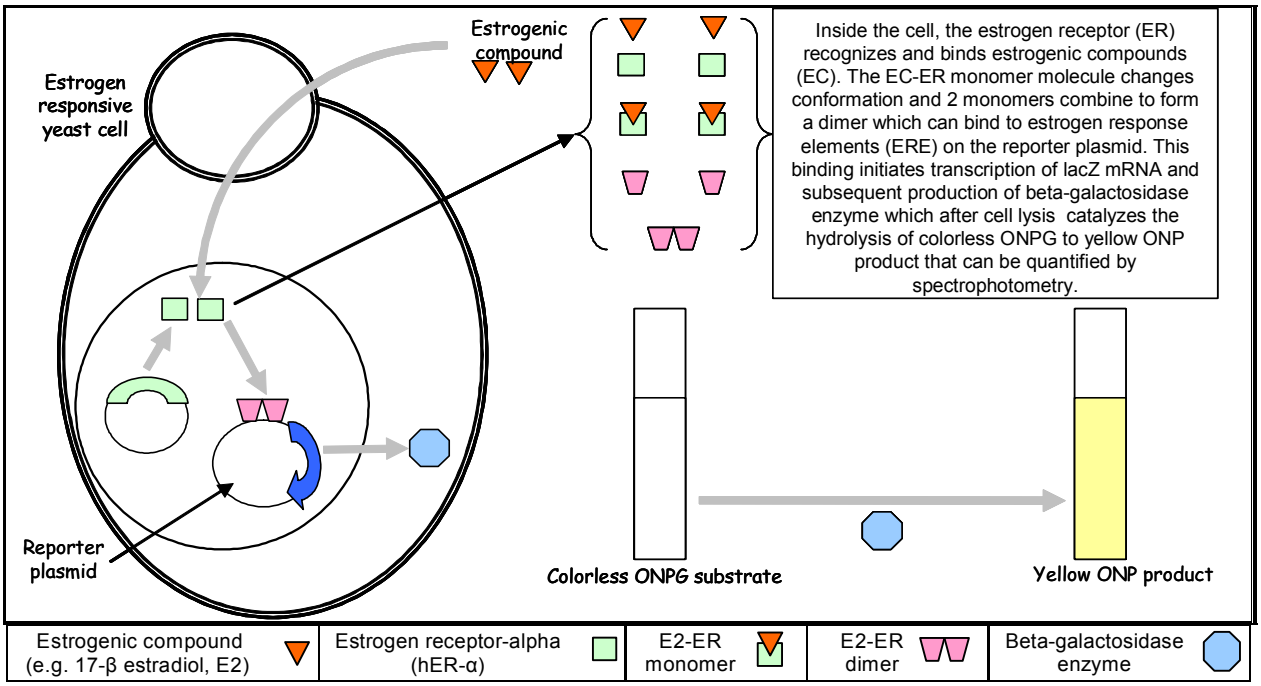


Figure 1: Schematic of the colorimetric assay using the chromogenic substrate ONPG to give a yellow product.

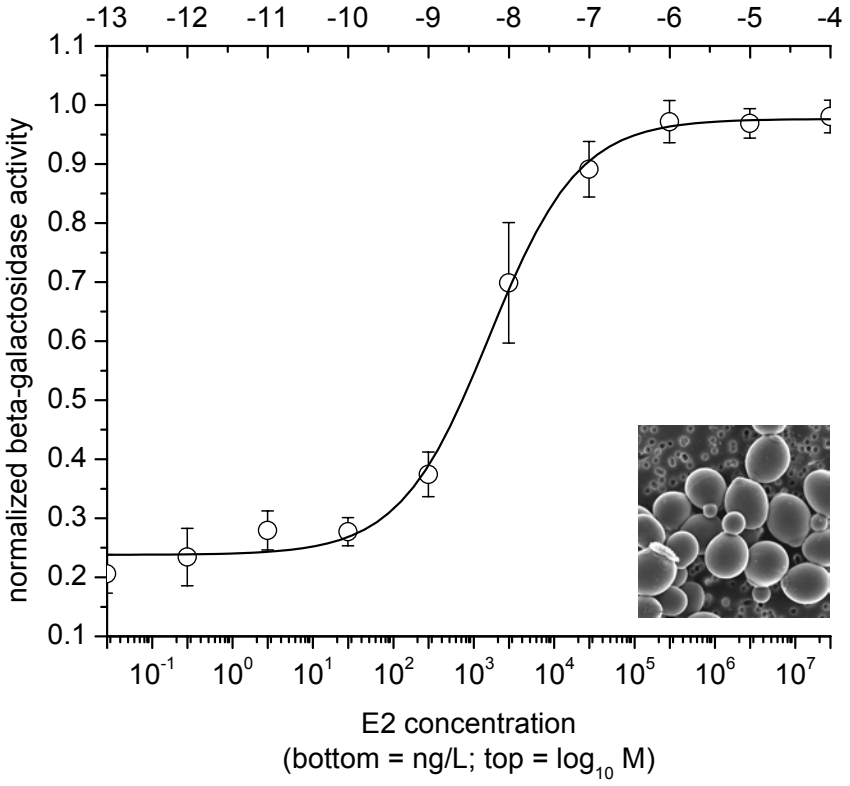


Figure 2: Dose response of RMY/ER-ERE to E2, the error bars show standard deviation (n = 5). The curve-fit is for visualization purposes only. Inset is a scanning electron micrograph (SEM) image showing the budding RMY/ER-ERE cells which typically have a 2 – 5 μm diameter.

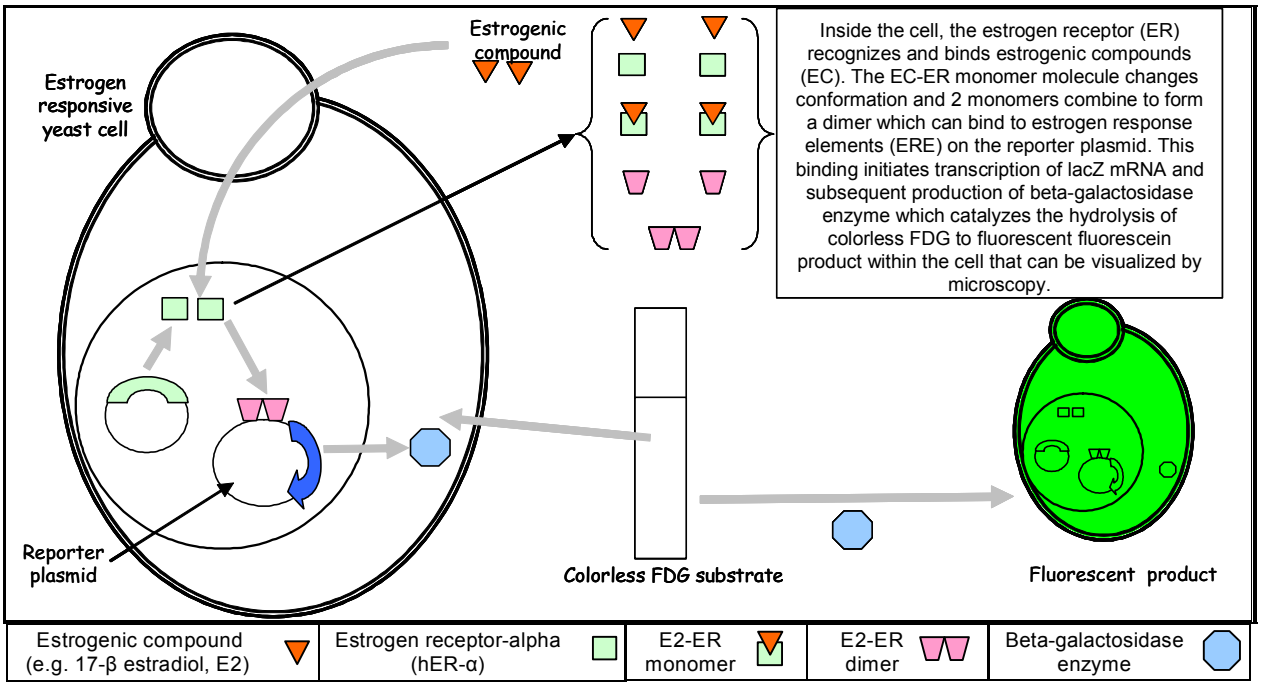


Figure 3: Schematic of the fluorescence assay which uses the fluorogenic substrate FDG which causes cell fluorescence. A fluorescent response can be observed within 30 min after cell exposure to an estrogenic compound.

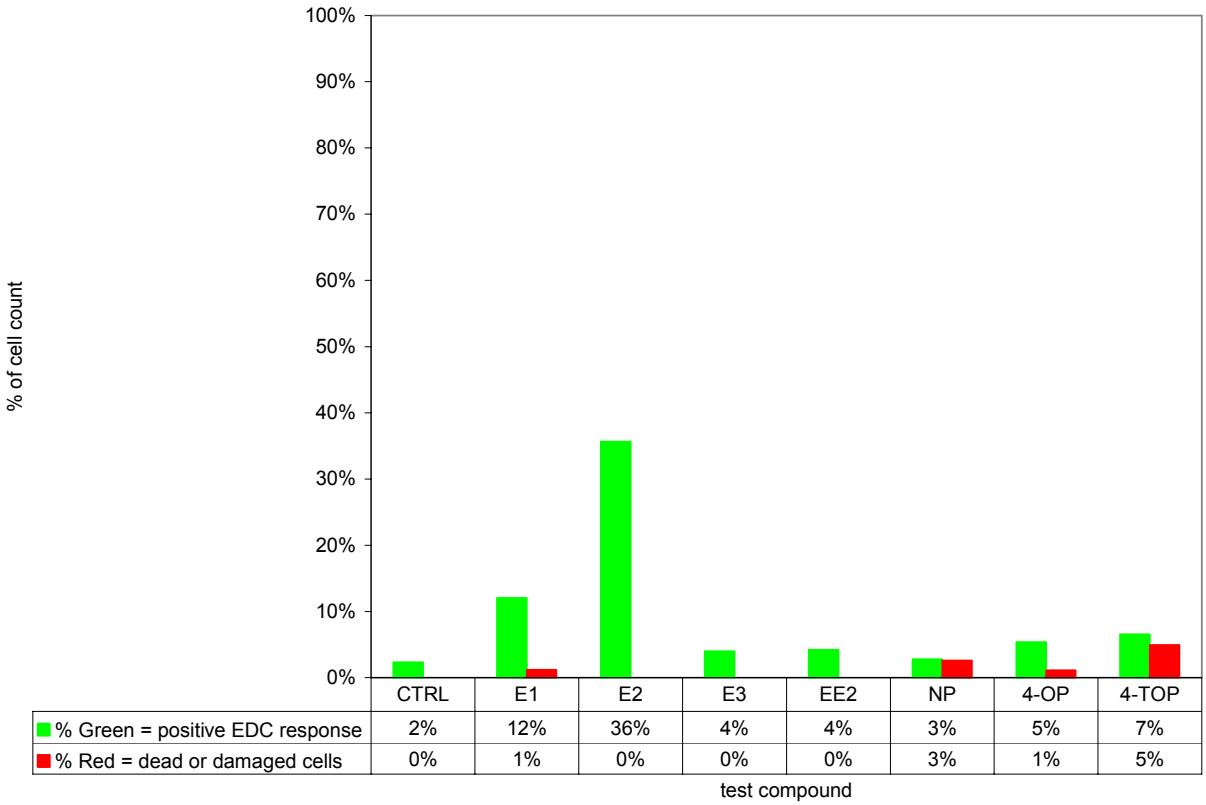


Figure 4: Cell counts from the fluorogenic assay with the substrate FDG. The positive response to E2 is about 12 times that of the control (CTRL).

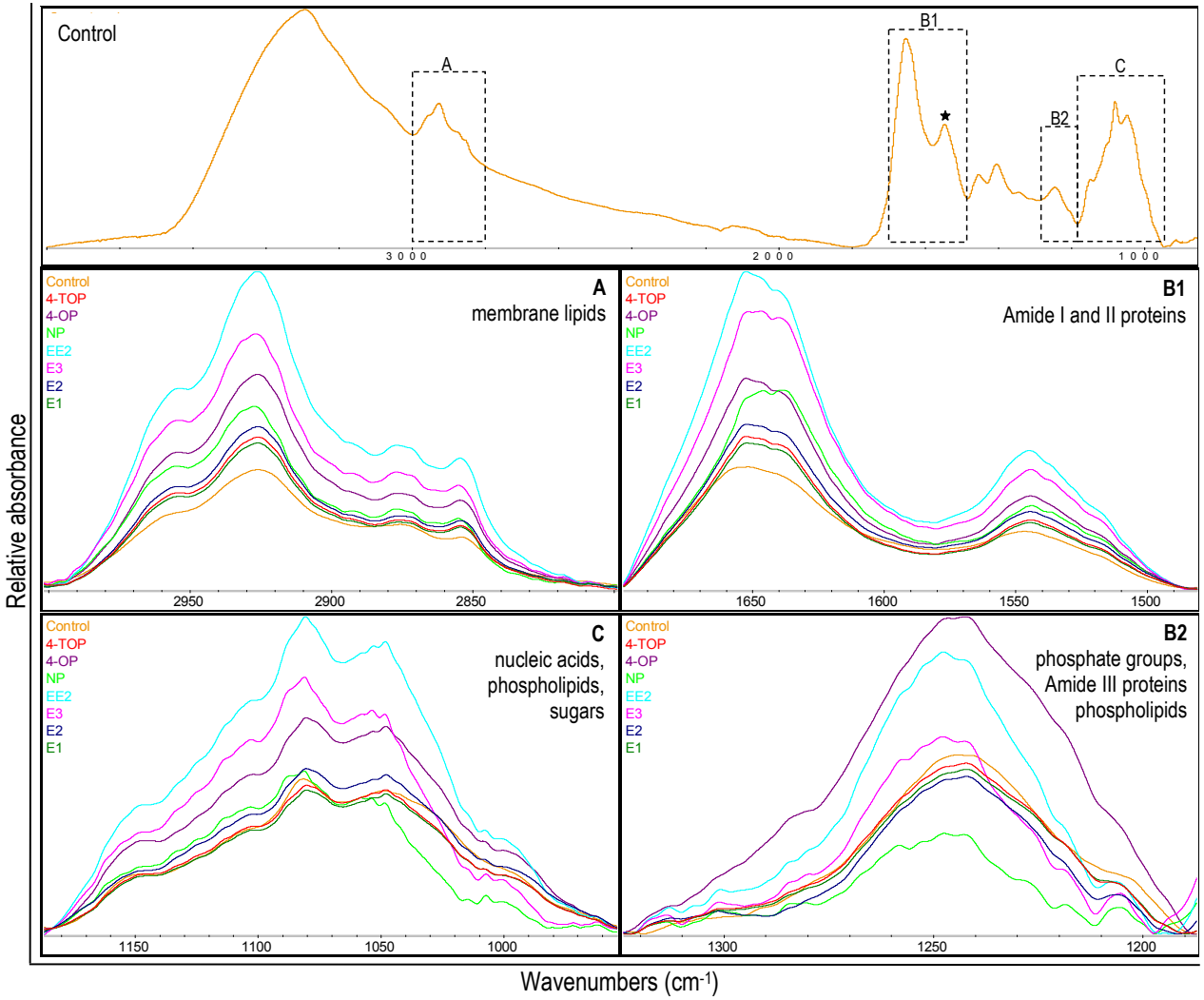
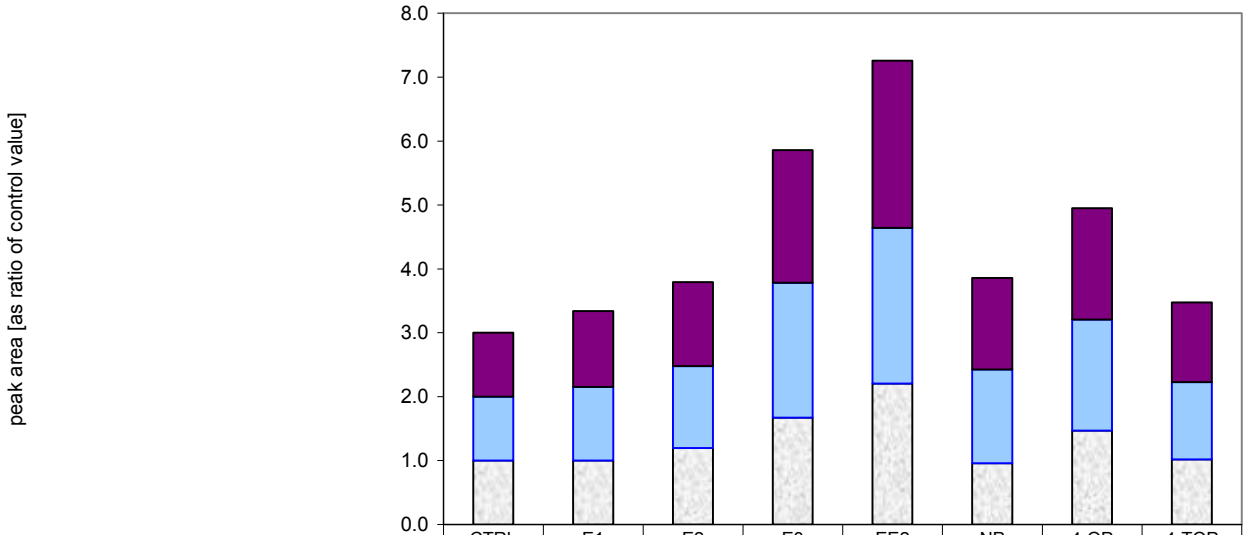


Figure 5: Regions of mean SR-FTIR spectra of estrogen-sensitive yeast cells exposed to the selected EDCs at 10 µM concentration for 30 min. Region peak areas represent the overall response of the yeast to the EDCs. All spectral absorbances are expressed as a ratio of their protein Amide II peak absorbance (~1550 cm^{-1};★) to facilitate comparison. The spectral region baselines have been corrected for clarity of presentation only. EE2 has the largest observable effect on the yeast cells. Estrone (E1), 17 β-estradiol (E2), estriol (E3), 17 α-ethinylestradiol (EE2), nonylphenol (NP), 4-octylphenol (4-OP), and 4-*tert*-octylphenol (4-TOP).



	CTRL	E1	E2	E3	EE2	NP	4-OP	4-TOP
membrane lipids [A: 3000-2800 cm-1]	1.00	1.19	1.32	2.07	2.62	1.43	1.74	1.25
Amide proteins, phosphate groups, phospholipids [B: 1690-1480 cm-1; 1350-1200 cm-1]	1.00	1.15	1.28	2.11	2.43	1.47	1.74	1.21
nucleic acids, phospholipids, sugars [C: 1200-950 cm-1]	1.00	1.00	1.20	1.67	2.21	0.96	1.47	1.02

test compound

Figure 6: Peak area ratios of regions of the mean SR-FTIR spectra from **Figure 5** with respect to the unexposed control cells (CTRL). Three spectral regions were chosen to compare the overall effect of the different estrogenic EDCs on the live yeast cells. EE2 has the largest overall effect on the yeast cells. Estrone (E1), 17 β-estradiol (E2), estriol (E3), 17 α-ethinylestradiol (EE2), nonylphenol (NP), 4-octylphenol (4-OP), and 4-*tert*-octylphenol (4-TOP).