Rapid water sample screening for estrogenic activity using live yeast cells

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Endocrine disrupting compounds (EDCs) are substances that influence the endocrine system in living organisms. They include natural hormones, medicinal drugs, chemical compounds in industry, and pesticides in agriculture. The substances may be present in water and wastewater, soil and sediments, or airborne.

Our research is focused on the estrogenic EDCs, i.e. substances which mimic the natural hormone estrogen produced by the body, and their occurrence and fate in the context of wastewater management. The primary contributors to estrogenic activity wastewater are 17β-estradiol, estrone, and 17α-ethinylestradiol. We are interested in readily detecting and quantifying this estrogenic activity as a first step in the daily management and reduction of estrogenic EDCs in wastewater before discharge into the environment with effluent and biosolids.

We report progress on a fluorescence assay for the presence of estrogenic activity in water samples using a living estrogen-sensitive yeast cell strain, and on a study of responses of living yeast cells to estrogen and alkylphenol exposure using synchrotron radiation-based Fourier Transform infrared spectromicroscopy (SR-FTIR). The fluorescent response of the yeast allows for rapid sample screening, and the SR-FTIR infrared spectrum is a measure of the overall *in vivo* yeast biochemical response to the sample.

Key words: EDC, estrogenic activity, yeast assay, fluorescence, FTIR

Estrogenic endocrine disrupting compounds (e-EDCs) are ubiquitous in the environment (Campbell et al., 2006; Shappell, 2006). The largest sources of relatively concentrated e-EDCs appear to be places where wastewater and waste solids are collected and/or treated like wastewater treatment plants, manure amended soils, and animal waste lagoons (Kolodziej et al., 2004; Shore et al., 1993; Shore & Shemesh, 2003). Potential mobility of e-EDCs increases when they are present in wastewater samples, when treatment plant effluent is discharged into a water body, and biosolids are applied to land. The presence of e-EDCs in the environment is a potential source

to the food chain. Detection and quantification of e-EDCs in the water samples is important as a first step in the daily management and reduction of estrogenic EDCs in wastewater before eventual discharge into the environment with effluent and biosolids.

MATERIALS AND METHODS

Fluorescence microscopy. Samples from three wastewater treatment plants (denoted WTP1, WTP2 and WTP3), and representative e-EDC compounds at 10^{-6} M concentration – see **Table 1**, were applied to living estrogen-sensitive yeast cells for 30 min in a fluorescence assay as described previously (Wozei et al., 2006a; Wozei et al., 2006b). The fluorescence intensity of 100 µL aliquots of cells from the assay was determined after transfer to a 96-well plate using a TECAN SpectraFluor Plus microplate reader (TECAN, Austria) with excitation and emission wavelengths of 485 nm and 535 nm respectively. Direct counts of the proportion of cells exhibiting a positive response (green fluorescence), was also done from images captured using an epifluorescence microscope with a camera (Wozei et al., 2006a).

Synchrotron radiation-based Fourier Transform infrared spectromicroscopy (SR-FTIR). Aliquots of the control and exposed yeast cells were used in synchrotron radiation-based Fourier Transform infrared spectromicroscopy (SR-FTIR). The mid-infrared (mid-IR) reflectance-absorbance spectra (in the 650 – 4000 cm^{-1} wavenumber region) were collected from yeast cells immobilized on gold-coated glass slides following the protocol described in Wozei et al. (2006b).

The mid-IR region is rich in information from the vibrational modes of biologically important macromolecules such as lipids, proteins and nucleic acids in intact cells (Mourant et al., 2003; Naumann, 2001; Yu & Irudayaraj, 2005), and we have used SR-FTIR to study living cells with no discernible detrimental effects (Holman et al., 2002; Holman et al., 2000; Holman & Martin, 2006).

RESULTS AND DISCUSSION

Detection of estrogenic activity in water samples. Yeast cells exposed to samples with estrogenic activity exhibited fluorescence. Two methods were used to determine fluorescence: intensity measurements and direct counts of fluorescent cells (**Table 1**). The fluorescence intensity was also divided by the measured light scattering at a wavelength of 590 nm to account for differences in the well cell density.

Yeast cells detect estrogenic activity in water samples. The yeast cells exposed to water samples containing estrogenic compounds are able to cleave the FDG substrate molecule to produce fluorescein and exhibit whole cell fluorescence. The cells responded to the presence of estrogenic activity in both water spiked with known estrogenic compounds, and real samples from wastewater treatment plants within 30 min of exposure.

For all samples except those from WTP2, the total fluorescence intensity per unit cell density was 30% or more than that for the positive E2 control, even when the number of fluorescent cells was small. The fluorescence assay therefore shows great potential for detection of estrogenic activity in real samples for screening purposes.

TABLE 1. Fluorescence induction in living yeast cells exposed to water samples.

		Fluorescence induction intensity relative to positive E2 control		Cell counts relative to positive E2 control
Sample		**Total intensity**	**Intensity per unit cell density**	**Fluorescent cells**
Estrone	E1	-	++	--
17β-estradiol	E2	1	1	1
Estriol	E3	-	-	--
17α-ethinylestradiol	EE2	-	-	--
Nonylphenol	NP	-	-	--
4-octylphenol	4-OP	-	++	--
4-*tert*-octylphenol	4-TOP	-	-	--
Treatment plant #1	WTP1_1	+	-	--
	WTP1_2	-	+	--
	WTP1_3	+	+	--
	WTP1_4	++	+	--
	WTP1_5	+	+	--
	WTP1_6	-	--	--
	WTP1_7	-	--	--
Treatment plant #2	WTP2_1	--	--	--
	WTP2_2	--	--	--
	WTP2_3	--	--	--
	WTP2_4	--	--	--
	WTP2_5	--	--	--
	WTP2_6	--	--	--
	WTP2_7	--	--	--
Treatment plant #3	WTP3_1	+	--	--
	WTP3_2	++	-	--
	WTP3_3	-	--	--
	WTP3_4	+	-	--
	WTP3_5	++	-	--

--	<0.5	1	1.0	+	>1.0 to <1.5
-	>0.5 to <1.0			++	>1.5

All standards are at 10^{-6} M concentration. WTP samples are numbered from 1 to 5 or 7. A higher number indicates more treatment.

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Mid-IR spectra of exposed yeast cells. After the mid-IR spectra were collected for

cells exposed to each sample, the spectra were averaged and absorbance intensities were expressed as a ratio of the protein Amide II peak intensity (approx. 1550 cm^{-1}) to enable comparison of results across samples. The peak areas of the selected regions bands were then obtained. The bands were chosen to compare global changes in nucleic acids, amide proteins and membrane lipids. Exposure to estrogenic activity was expected to change absorbance intensity of nucleic acids if the sample was in any way toxic to the cells, possibly leading to nucleic acid modification. An overall increase in proteins was expected for cells responding to external stress by turning on gene expression and synthesizing proteins as part of coping mechanisms (Gasch et al., 2000; Melin et al., 2001; Posas et al., 2000). External chemical stressors may also affect the total membrane lipids if their internalization is undesirable and membrane density is increased to slow internalization. Membrane lipid unsaturation is a precursor for susceptibility to stress (Avery et al., 1996; Steels et al., 1994).

Figure 1 shows the results obtained from the mid-IR spectra of yeast cells exposed to water samples from the three wastewater treatment plants. Exposure to the e-EDC standards resulted in an increased absorbance in all region bands from 15% (E1) to greater than 150% (EE2) when compared to the unexposed control (CTRL). The exception was a 4% reduction in the nucleic acids band for cells exposed to NP.

In general, samples from WTP1 and WTP2 which induced higher fluorescence intensities also exhibited larger differences in the mid-IR spectra of exposed cells as evident in the larger peak areas in **Figure 1**. The fluorescence assay is primarily sensitive to E2. The yeast cells exposed to EE2, E3 and 4-OP respond with little fluorescence (**Table 1**), but the mid-IR spectra indicate the possibility that other stress signaling and response pathways are in effect. These were considered to be outside the scope of this study.

Overall live-cell response. On the whole, the cells did not appear to be adversely affected by the 30 min exposure to the treatment plant samples during the experiment. This is an encouraging finding suggesting that the yeast cells can be used to detect estrogenic activity in a variety of real samples. However, the infrared spectra show apparent depression of overall responses by cells exposed to real samples when compared to the unexposed control indicating either the presence of toxicity or the reduced bioavailability of the e-EDCs in real samples. With the depressed response there was still a reduction in overall cell response between the first and last WTP sample of 18% for WTP1, and 19% for WTP3. The 1% reduction in response for WTP2 was much smaller by comparison.

Yeast estrogen assays accurately predict the total estrogenic activity in complex mixtures like wastewater effluents and their extracts (Rutishauser et al., 2004; Thorpe et al., 2006), so the complexity of the samples was not considered a problem. Since the cells responded to the E2 standard as expected, any toxins present in the real samples were probably non-estrogenic. In this study the original samples had visible turbidity and/or green coloring, and microscopic examination showed the presence of plankton.

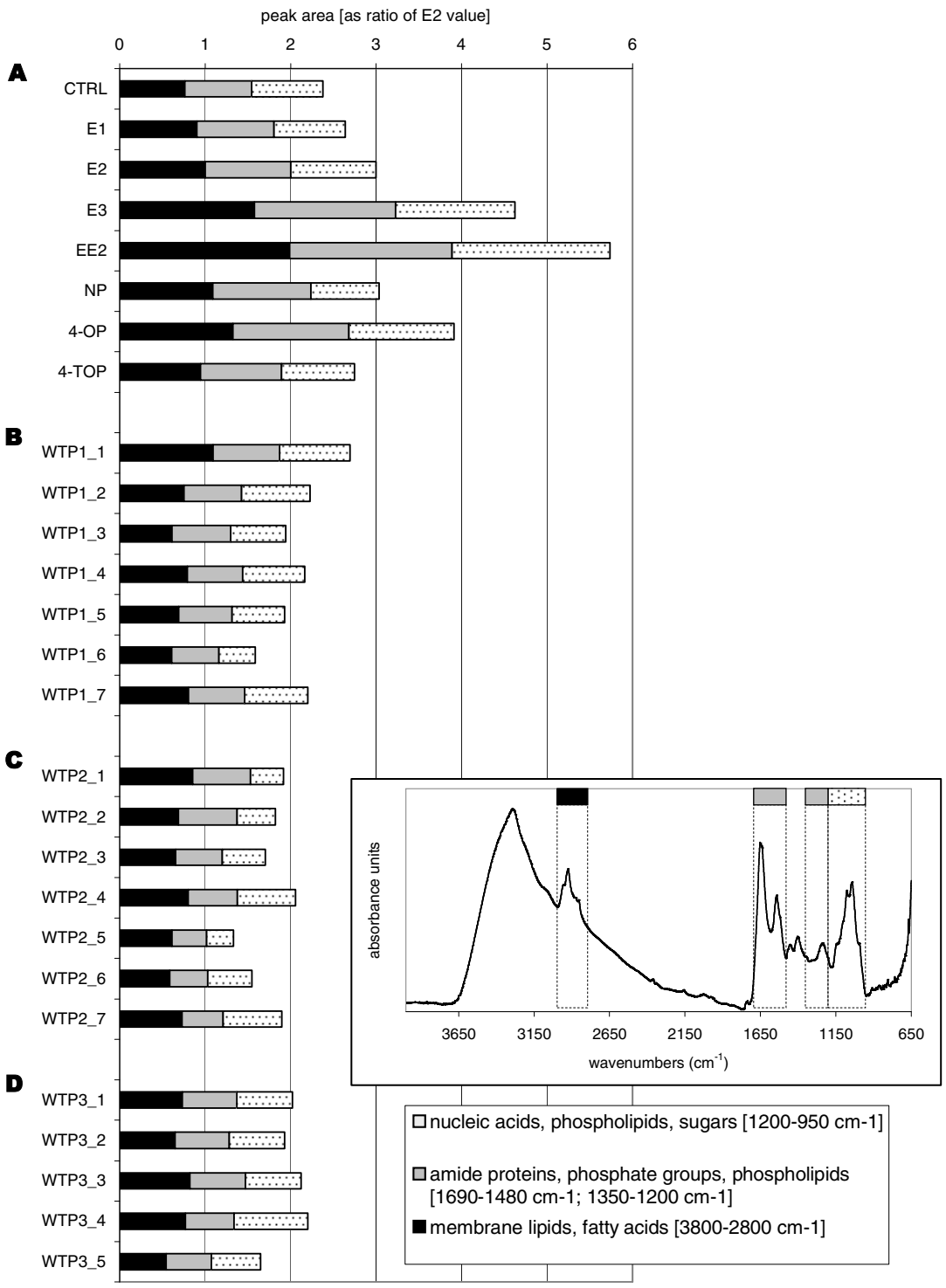

FIGURE 1. The peak areas of selected regions of the mid-IR spectra of yeast cells exposed to water samples with known and suspected estrogenic activity relative to E2. A typical spectrum is shown inset. **A**: Yeast cell response when exposed to known e-EDC standards at 10^{-6} M. **B – D**: Yeast cell response to real samples from wastewater treatment plants. Selected peak area regions are bounded by the defined wavenumbers. Measurements shown are means of a minimum of 5 (for WTP samples) or 20 (for standards) discrete spectral measurements. Each measurement comprises 128 co-added spectra.

Samples were pre-settled for these experiments, but the presence of organic colloids can potentially interfere with availability of estrogenically active compounds to the yeast in the assay (Holbrook et al., 2004; Holbrook et al., 2005). This factor must be taken into consideration during future field and lab sample screening efforts.

The relative potencies of the selected e-EDCs in yeast-based transcriptional activation assays are generally as follows: EE2≈E2>E1>E3>NP≈4-OP>4-TOP (Coldham et al., 1997; Rutishauser et al., 2004; Van den Belt et al., 2004), where ≈ means that some assays showed an equal or greater potency for one or the other compound. Based on the mid-IR results, in this study the live yeast cells responded overall as follows: EE2>E3>4-OP>E2≈NP>4-TOP≈E1 with an unexpectedly high response for E3 and low response for E1, even though these compounds induced fluorescence as expected. For all e-EDCs tested there was an increased absorbance in the membrane lipids/fatty acids, protein and nucleic acids/phospholipids bands when compared to the unexposed control indicating a whole-cell response to the e-EDC exposure. The exceptions were E1, NP and 4-OP which had peak areas of the nucleic acid bands similar to that of the unexposed control.

Preliminary conclusions. Alkylphenols and their metabolites are found at microgram per liter (µg/L) concentrations in WTP effluents (Aerni et al., 2004), but because of their low potency relative to E2, they are usually regarded as a small contributor to the overall effluent estrogenic activity. However, the mid-IR cellular response implies that even though these compounds did not exert an estrogenic effect, defined in this study by estrogen-induced fluorescence, they may affect the yeast cells in other undetermined ways. The same can also be said for the other e-EDCs selected, but the estrogen-sensitive yeast cell response to e-EDC exposure by eliciting fluorescence was adequate for the defined purposes of this study.

Fluorescence was detected in all samples with spiked e-EDCs. The 30 min fluorescence assay is most sensitive to E2, but the mid-IR spectra indicate that the exposed yeast cells respond to all the e-EDCs tested, even when a low fluorescent response was detected. When exposed to real samples the cells exhibited some, albeit low, fluorescence indicating low estrogenic activity. Further investigation will continue using dilutions of, and pre-concentrated, real samples. These findings show that the yeast-based fluorescence assay has strong potential for use in rapid environmental water screening and monitoring regimes designed to complement quantification methods for estrogenic activity. This rapid screening would be useful prior to estrogenic activity speciation and quantification using chromogenic assays and/or chemical analyses

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