SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 15, No. 3, pp. 241–248, 1996

Printed in the USA
0730-7268/96 $6.00 + .00

ESTROGENIC ACTIVITY OF SURFACTANTS AND SOME OF THEIR DEGRADATION PRODUCTS ASSESSED USING A RECOMBINANT YEAST SCREEN

EDWIN J. ROUTLEDGE* and JOHN P. SUMPTER
Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex UB8 3PH, UK

(*Received* 20 *March* 1995; *Accepted* 21 *July* 1995)

Abstract—An estrogen-inducible screen was developed in yeast (*Saccharomyces cerevisiae*) in order to assess whether surfactants and their major degradation products are estrogenic. The DNA sequence of the human estrogen receptor (hER) was integrated into the yeast genome, which also contained expression plasmids carrying estrogen-responsive sequences (ERE) controlling the expression of the reporter gene *lac-Z* (encoding the enzyme β-galactosidase). Thus, in the presence of estrogens, β-galactosidase is synthesized and secreted into the medium, where it causes a color change from yellow to red. This recombinant strain was used to determine whether representatives of major surfactant classes and some of their principal degradation products possess estrogenic activity. The results were compared to the effects of the main natural estrogen 17β-estradiol. None of the parent surfactants tested possessed estrogenic activity. However, one class of surfactants, the alkylphenol polyethoxylates, degrade to persistent metabolites that were weakly estrogenic. Another group of degradation products, the sulfophenyl carboxylates, which are derived from the biodegradation of linear alkylbenzene sulfonates, do not appear to possess estrogenic activity.

Keywords—Surfactants Alkylphenol Estrogen Yeast

INTRODUCTION

An increasing incidence of some cancers [1], damage to reproductive systems, and developmental problems, found in both humans and wildlife, has caused concern about the ubiquity of environmental contaminants that inadvertently mimic the biological activities of the female hormone estrogen [2]. A very wide range of chemicals, both natural and manmade, have now been found to be weakly estrogenic [3–6]. These include natural substances such as phytoestrogens and mycoestrogens, certain pesticides and herbicides, some polychlorinated biphenyls (PCB), certain combustion pollutants, plasticizers, and breakdown products of surfactants. The majority of these xenoestrogens bear little structural resemblance to natural estrogens. Therefore, it is presently impossible to predict whether a chemical is estrogenic based on chemical structure alone. Thus, the ability of a substance to act as an estrogen is usually discovered by accident, as illustrated by the recent realization that the alkylphenol nonylphenol [7] and bisphenol-A [8], both released from laboratory plasticware, are estrogenic.

Surfactants are a class of chemicals detectable in many surface waters. The discovery that nonylphenol was weakly estrogenic to mammals has led to concern regarding the environmental safety of a major class of nonionic surfactants, the alkylphenol polyethoxylates (APnEO, $n = 6–40$). During sewage treatment, APnEO are biodegraded initially via shortening of the hydrophilic chain, forming increasingly lipophilic and persistent metabolites, including short-chain alkylphenol ethoxylates and their carboxylic acid derivatives, and finally into alkylphenols such as nonylphenol and octylphenol [9–11]. These metabolites are widely reported to be present in sewage effluent and river water of Europe and the U.S. (see Jobling and Sumpter [6] and references therein), suggesting that aquatic organisms are being exposed to these estrogenic chemicals.

Alkylphenol polyethoxylates are just one class of surfactants used in very large amounts worldwide. We have screened representatives of all the major groups of surfactants and their primary degradation products (when known and available) for estrogenicity using a recently developed recombinant estrogen yeast screen. We discuss our findings in relation to known environmental concentrations, bioaccumulation data, and persistence in the environment of these estrogenic degradation products of some surfactants.

MATERIALS AND METHODS

The recombinant yeast estrogen screen

A recombinant yeast strain was developed in the Genetics Department at Glaxo for use in a test to identify compounds that can interact with the human estrogen receptor (hER). Yeast cells do not normally contain an estrogen receptor; therefore, the DNA sequence of the hER was stably integrated into the main chromosome of the yeast. The yeast cells also contain expression plasmids carrying the reporter gene *lac-Z* (encoding the enzyme β-galactosidase), which is used to measure the receptors' activity.

In this system, the hER is expressed in a form capable of binding to estrogen-responsive sequences (ERE). These sequences were situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the estrogen-occupied receptor interacts with transcription factors and other transcriptional components [12] to modulate gene transcription. This causes expression of the reporter gene *lac-Z* and the enzyme produced (β-galactosidase) is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm (Fig. 1).

The yeast strain was stored at −20°C as a 10× concentrated stock culture in 0.5-ml aliquots in 2-ml sterile cryogenic am-

* To whom correspondence may be addressed.

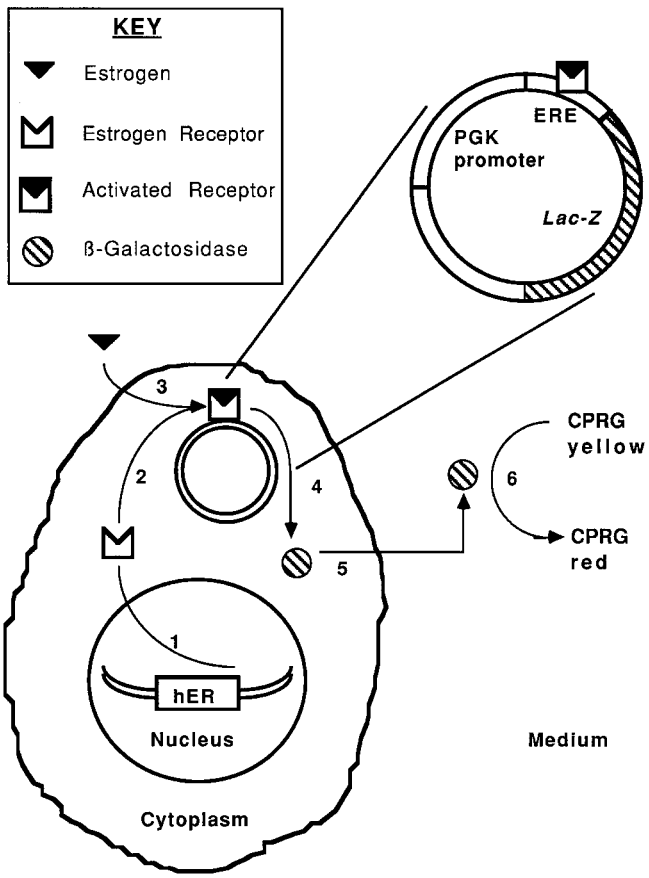

Fig. 1. Schematic of the estrogen-inducible expression system in yeast. The human estrogen receptor gene is integrated into the main genome and is expressed (1) in a form capable of binding to estrogen response elements (ERE) within a hybrid promoter on the expression plasmid (2). Activation of the receptor (3), by binding of ligand, causes expression of the reporter gene *Lac-Z* (4) which produces the enzyme β-galactosidase. This enzyme is secreted into the medium (5) and metabolizes the chromogenic substrate CPRG (normally yellow) into a red product (6), which can be measured by absorbance.

poules (Sterilin). To produce the stock, 10 cultures (each with a final volume of 50 ml) were grown to an optical density of 1.0 (A_{640}) and transferred to sterile 50-ml graduated centrifuge tubes. The cultures were centrifuged at 4°C for 10 min at approximately 2,000 *g*. The supernatant was decanted and the cells resuspended in 50 ml fresh, single-strength minimal medium (MMA-UT) with 15% glycerol. The samples were stored for a maximum of 4 months before being replaced by a new stock.

Preparation of medium components

All the ingredients, unless stated otherwise, were purchased from Sigma Chemical Company Ltd. (Dorset, England) and were research grade biochemicals suitable for cell culture.

Minimal medium (pH 7.1) was prepared by adding 13.61 g KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH pellets (Aldrich, Dorset, England), 0.2 g $MgSO_4$, 1 ml $Fe_2(SO_4)_3$ solution (40 mg/50 ml H_2O; purchased from Aldrich), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 L of purite double-distilled water. Aliquots of 45 ml were dispensed into 250-ml flasks, sterilized at 121°C for 10 min, and stored at room temperature.

The vitamin solution was prepared by adding 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol (Aldrich, Dorset, UK), and 20 ml of biotin solution (2 mg/100 ml H_2O) to 180 ml double-distilled water. The solution was then filter sterilized through 0.2-μm pore size Whatman PURADISC filters, and 10-ml aliquots were stored at 4°C in sterile glass bottles. A 20% w/v solution of D-(+)-glucose was sterilized in 20-ml aliquots at 121°C for 10 min and stored at room temperature. A stock solution of 4 mg/ml L-aspartic acid was sterilized in 20-ml aliquots at 121°C for 10 min and stored at room temperature. A stock solution of 24 mg/ml L-threonine was sterilized in 5-ml aliquots at 121°C for 10 min and stored at 4°C. A 20 mM copper (II) sulfate solution (Aldrich, Dorset, UK) was prepared and filter sterilized through 0.2-μm pore size Whatman PURADISC filters. The solution was stored at room temperature in a sterile glass bottle. A 10-mg/L stock solution of CPRG (Boehringer Mannheim, East Sussex, UK) was made in sterile distilled water and stored at 4°C in a sterile glass bottle.

Growth medium was prepared by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 μl copper (II) sulfate solution to 45 ml single strength minimal medium in a sterile conical flask. The growth medium was then inoculated with 0.25 ml of the concentrated stock yeast and incubated at 28°C for approximately 24 h on an orbital shaker (250 rpm with a 50-mm throw) until an absorbance at 640 nm of 1.0 was reached.

The assay medium was prepared by adding 0.5 ml of the chromogenic substrate CPRG to 50 ml fresh growth medium. The medium was seeded with 2 ml yeast from a 24-h yeast culture with an absorbance at 640 nm of 1.0 prior to use.

Compounds tested

The specificity of the screen was determined using steroids purchased from Sigma (Dorset, UK). Stock solutions (1 μM) were made in ethanol, and all serial dilutions were carried out in ethanol in order to achieve final concentrations of 5×10^{-8} to 2.5×10^{-11} M in the microtiter plate wells.

The ability of the test to identify environmental estrogens was assessed by examining the activity of known environmental estrogens, i.e., genistein (Sigma, Dorset, UK), bisphenol-A (Aldrich, Dorset, UK), and op′-DDT (MTM, Lancashire, UK).

Representative chemicals of all the major classes of surfactants (Table 1) were assessed for estrogenic activity. Additionally, biodegradation products were chosen based on their persistence in the aquatic environment, knowledge of what products are produced when the surfactant is biodegraded, and commercial availability. Abbreviations adopted in this paper for the alkylphenolic compounds are those used in Ahel et al. [13].

The nonylphenol polyethoxylate Igepal CO-720 (NP12EO) and 4-nonylphenoxycarboxylic acid (NP1EC) were purchased from Aldrich (Dorset, UK). 4-Nonylphenol (4-NP) was purchased from MTM (Lancashire, UK), 4-*tert*-octylphenol (4-OP) 4-nonylphenoldiethoxylate (NP2EO), ''Synperonic'' NP2 ether carboxylate (NP2EC), and ''Synperonic'' A3 (an alcohol ethoxylate containing a certain amount of branching in the hydrophobe and manufactured from petrochemical fatty alcohol by reaction with 3 mol of ethylene oxide) were gifts from ICI (Cleveland, UK). Genapol C-100 (a C12–C14 alcohol ethoxylate with a linear hydrophobe manufactured by reaction of the alcohol derived from coconut oil with 10 mol of ethylene oxide) was supplied by Unilever (Wirral, UK) but was produced by the German company Hoechst AG. The commercial LAS preparation from Petrelab 550, produced by the Spanish manufacturer PETRESA (via HF catalyst), was supplied by Unilever (Wirral, UK). The C10–C13 LAS preparation (19.9%

Table 1.Surfactants and biodegradation productes tested

Class	Surfactant group	Representative tested	Biodegradation products tested
Anionic	Linear alkylbenzene sulfonates (LAS)	Petrolab 550	SPC
		Sirene	
	Branched alkylbenzene sulfonate	ABS	[a]
	Alcohol sulfates (AS)	Sodium *n*-octyl sulfate	[a]
		Sodium *n*-nonyl sulfate	[a]
	Alcohol ether sulfates	Dobanol	[a]
	Secondary alkane sulfonates (SAS)	SAS	[a]
	Others	Linear-α-olefin sulfonate	[a]
		Sodium lignosulfonate	[a]
Nonionic	Alcohol ethoxylates (AE)	Synperonic A3	[a]
		Genapol C100	[a]
	Alkylphenol ethoxylates (APE)	NP12EO	4-OP
			4-NP
			NP1EC
			NP2EC
			NP2EO
Cationic	Tallow amine derivatives	Di(hydrogenated tallow) dimethyl ammonium chloride	[a]
	Benzalkonium chloride	C12-C14-alkyldimethylbenzyl ammonium chloride	[a]
Amphoteric	Betaines	Coconut amido betaine	[a]
		Cocobetaine	[a]
	Imidazolines	*N-b*-hydroxyethyl oleyl imidazoline	[a]

[a] Indicates unknown or not commercially available.

active matter) from Sirene-113 (produced via $AlCl_3$ catalyst) and a range of sulfophenyl carboxylates (SPC) were supplied by EniChem Augusta Industriale (Milano, Italy). A mixture of 50 to 70 individual branched alkylbenzene sulfonates (ABS) manufactured by TCI (Tokyo) and a commercial mixture of C13–C17 secondary alkane sulfonates (SAS) manufactured by Hoechst AG were a gift from Jennifer Field (Oregon State University, Corvallis, OR, USA). The C12–C15 alcohol ether sulfate Dobanol 25-3S/70 (70% active matter), manufactured by Shell (UK/Netherlands), was supplied by Unilever (Wirral, UK). Sodium *n*-nonyl sulfate (99%) was purchased from MTM (Lancashire, UK). Sodium *n*-octyl sulfate, linear-α-olefin sulfonate, sodium lignosulfonate, di(hydrogenated tallow) dimethyl ammonium chloride, C12–C14-alkyldimethylbenzyl ammonium chloride, coconut amido betaine, cocobetaine, and *N-b*-hydroxyethyl oleyl imidazoline were all purchased from Greyhound ChemService (Merseyside, UK). Pure soap (formulated without perfume or colors) was purchased from the Sainsbury's branch at Uxbridge.

Many of the tested chemicals are hydrophobic and were dissolved in ethanol. Chemicals insoluble in ethanol (such as the SPC and sodium lignosulfonate) were dissolved in single strength minimal medium. Stock solutions (2 g/L) were prepared initially, after which serial dilutions were carried out to achieve final concentrations of 100 mg/L to 50 μg/L in the wells.

Assay procedure

Yeast assays were carried out within a type II laminar air flow cabinet to minimize aerosol formation. Chemicals were serially diluted and 10-μl aliquots of each concentration were then transferred to a 96-well optically flat bottom microtiter plate (Linbro/Titertek, ICN FLOW, Bucks, UK). Chemicals dissolved and diluted in absolute ethanol were allowed to evaporate to dryness on the assay plate. Chemicals that were insoluble in ethanol were dissolved and serially diluted in medium. Aliquots (200 μl) of the seeded assay medium (medium containing recombinant yeast) and the chromogenic substrate CPRG were then dispensed to each sample well using a Biohit Proline electronic multichanel pipettor (Jencons [Scientific] Limited, Bedfordshire, UK). Each plate contained at least one row of blanks (assay medium only), as well as a standard curve for 17β-estradiol (3,000 ng/L 17β-E2 to 1.5 ng/L). The plates were sealed with autoclave tape and shaken vigorously for 5 min on a titer plate shaker prior to incubation at 32°C in a naturally ventilated heating cabinet (WTB binder, BD-series). After 3 d incubation, the color development of the medium was checked periodically at an absorbance of 540 nm (using a Titertek Multiskan MCC/340 plate reader), to obtain data with the best contrast. After incubation, control wells appear light orange in color, due to background expression of β-galactosidase (β-gal), and turbid, due to growth of the yeast. Positive wells are indicated by a deep red color accompanied by turbid yeast growth. Clear wells (containing no growth) indicate lysis of the cells, and the color may vary.

RESULTS

Assay characteristics

The sensitivity and reproducibility of the estrogen screen were assessed by measuring the response of the yeast to triplicate dilutions of the natural estrogen 17β-E2 compared to triplicates containing only assay medium. The response of the yeast over this concentration range (3,072 ng/L to 1.5 ng/L) was highly reproducible, with a concentration of 3 ng/L 17β-estradiol producing a detectable increase in β-gal production (Fig. 2).

Specificity of the estrogen screen was assessed by the ability of a range of steroids and steroid metabolites, diluted from 5×10^{-8} M, to stimulate expression of β-gal in the yeast. The data presented in Figure 3a and 3b show that the strongest response was seen in the wells containing 17β-E2 (diluted from 1×10^{-8} M). The next most potent steroids were 17α-estradiol and 17β-E2 sulfate, which had potencies of approximately two

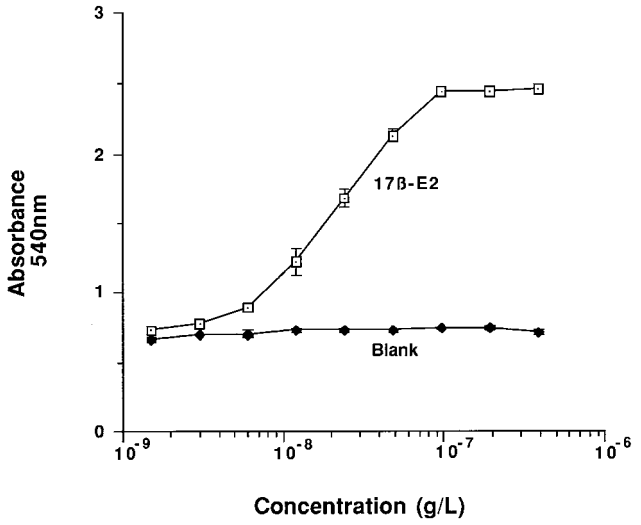

Fig. 2. Sensitivity and reproducibility of the estrogen screen. The graph shows the log concentration of 17β-estradiol (serially diluted from 3,072 ng/L to 1.5 ng/L) plotted against the absorbance of the medium after 3 d incubation. Values represent the mean ± SE (n = 3). In most cases the SEM were too small to illustrate.

and three orders of magnitude less than that of 17β-E2, respectively. Except for testosterone and cortisol, which induced a slight response at the highest concentration, the other steroids failed to induce β-gal production.

Assay response to known environmental estrogens

After assessing the response of the screen to natural hormones, we tested known estrogenic xenobiotics over a concentration range of 100 mg/L to 50 μg/L. Genistein (a phytoestrogen), bisphenol-A (a plasticizer), and two alkylphenols (NP and OP) were all estrogenic (Figs. 4, 5). Genistein was the most potent of these chemicals, and the two alkylphenols the least potent, with OP being about fourfold more potent than NP. The phytoestrogen genistein and the plasticizer bisphenol-A were about three orders of magnitude less potent than 17β-E2, while the pesticide op′-DDT produced a shallow gradient curve that failed to reach a maximum response over the concentration range tested, possibly due to a bioavailability effect caused by solubility problems and sorption to the plastic plates.

Estrogenic activity of surfactants

After having assessed the response of the screen to a variety of natural and synthetic estrogens, we then proceeded to test representatives of the different classes of surfactants and some of their primary degradation products, when available, for estrogenic activity. All the compounds were tested over a concentration range of 100 mg/L to 50 μg/L. Figure 6 shows the response of the yeast to a selection of nonionic surfactants and their primary degradation products. ''Synperonic'' A3 (AE3) and Genapol C100 did not stimulate β-gal production in the yeast and therefore possess no detectable estrogenic activity. The sudden drop in absorbance when AE3 was present at 10 mg/L was due to the surfactant lysing the yeast. The alkylphenolic compounds 4-OP, 4-NP, NP1EC, NP2EC, and NP2EO all produced a dose-dependent elevation in synthesis of β-gal in the yeast, but all were less potent than 17β-E2 (fold less potency given in parentheses): 4-OP (1.5×10^3), 4-NP (7×10^3), NP1EC and NP2EC (2.5×10^4), and NP2EO (5×10^5). This indicates that the presence of two oxyethylene groups markedly reduces estrogenic activity. The compound NP12EO did not show

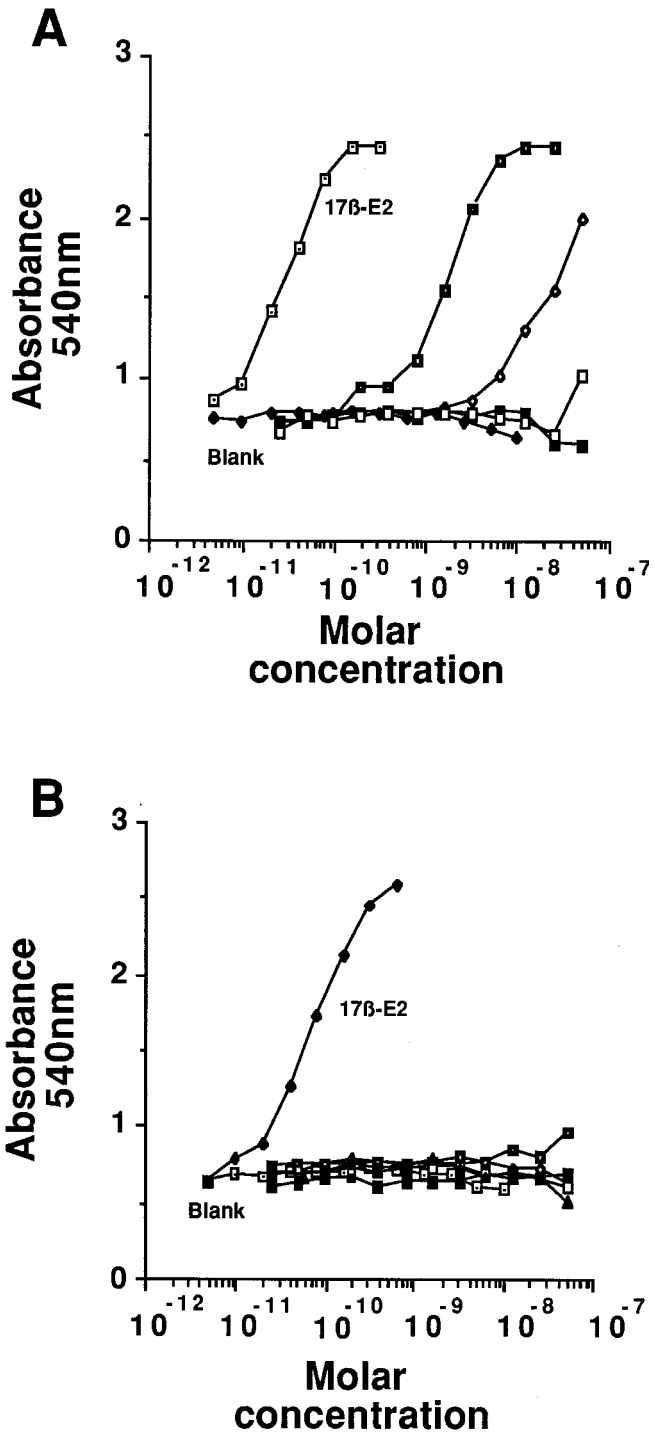

Fig. 3. Specificity of the estrogen screen for a range of steroids and steroid metabolites. The graphs depict the log concentration of 17β-estradiol (17β-E2), serially diluted from 10 nM, and other natural steroid hormones, serially diluted from 50 nM, plotted against the absorbance (A_{540}) of the medium after 3 d incubation. Maximal responses at the highest concentrations are not shown. (**A**) Estrogens and corticosteroids; 17β-E2 (⊡), 17α-E2 (■), E2-SO4 (◇), E2-Gluc (■), cortisol (□). (**B**) Androgens and progesterones; testosterone (■), progesterone (◇), DHT (■), 17α-P (□), 17α,20β-P (▲).

any observable activity, therefore alkylphenolic compounds with a high degree of ethoxylation are not estrogenic.

None of the cationic (Fig. 7) or anionic (Fig. 8) surfactants tested were estrogenic although some lysed the yeast at high

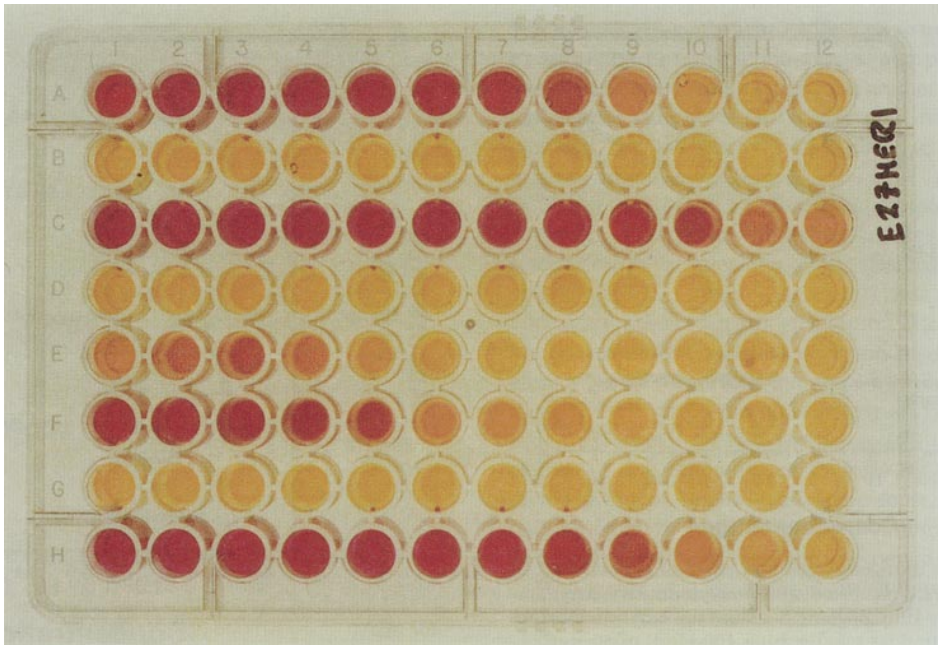

Fig. 4. Plate showing the response of the yeast screen to some known environmental estrogens. Bisphenol-A (row A) and genistein (row C) were serially diluted (left to right) from 50 mg/L. Nonylphenol (row E) and octylphenol (row F) were both serially diluted from 5 mg/L. 17β-Estradiol (row H) was diluted from 3,072 ng/L (1×10^{-8} M). Rows B, D, and G are blanks (no sample added) and show the background color of the medium.

concentrations. Neither SPC (Fig. 9) nor amphoteric surfactants (Fig. 10) were estrogenic.

DISCUSSION

A wide variety of assays have been developed to assess the estrogenic activity of a chemical. The classical assay, still in use, is based on stimulation of uterine growth in immature mice [14]. More recently, various in vitro assays have been developed, such as those based on stimulation of DNA synthesis and cell division in selected breast tumor cell lines, such as MCF-7 and ZR-75 cells [5,15] and those that rely on the expression of a reporter gene in transfected cells [15].

This paper describes an assay system in which the hER is expressed in yeast in a form capable of activating transcription of a promoter carrying ERE, in an estrogen-dependent manner. The basic molecular mechanisms of hormone-dependent transcription activation by the hER in mammalian cells have been shown to operate in yeast [16], indicating that the underlying regulatory mechanisms have been highly conserved. Yeast was used as the recipient organism because its rapid growth rate,

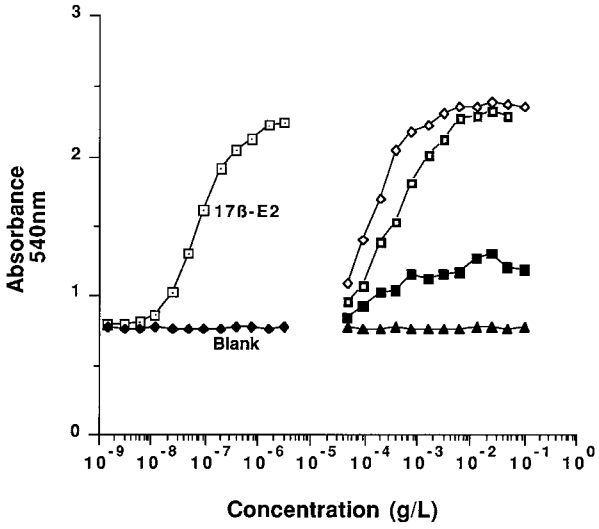

Fig. 5. Response of the estrogen screen to a range of established estrogenic compounds. The yeast cells were incubated with bisphenol-A (□), genistein (◇), or op′-DDT (■) at concentrations ranging from 100 mg/L to 50 μg/L.

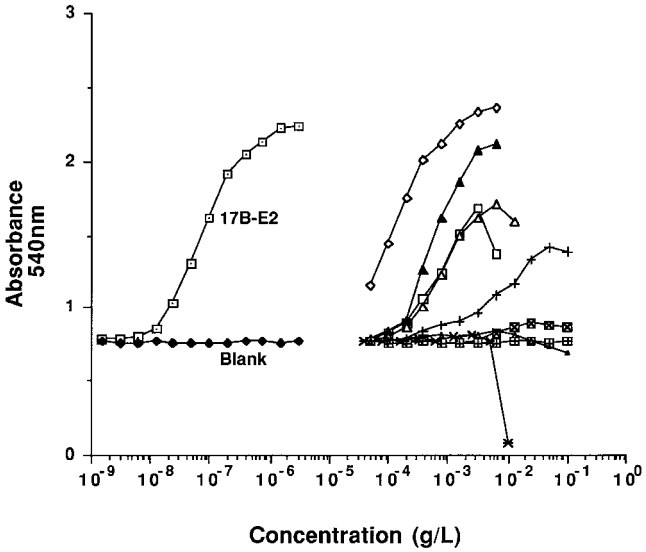

Fig. 6. Response of the estrogen screen to a range of nonionic surfactants and their biodegradation products. The yeast cells were incubated with an alkylphenol polyethoxylate (NP12EO; ×□) and the alcohol ethoxylates AE3 (∗) and Genapol 100 (▲). Alkylphenol polyethoxylate biodegradation products included two 4-nonylphenoxycarboxylic acid derivatives (NP1EC; □, and NP2EC; △), as well as 4-nonylphenoldiethoxylate (NP2EO; +), 4-nonylphenol (4-NP; ▲) and 4-octylphenol (4-OP; ◇), all at concentrations ranging from 100 mg/L to 50 μg/L. The sudden drop in absorbance seen with AE3 at a concentration of 10 mg/L was due to lysis.

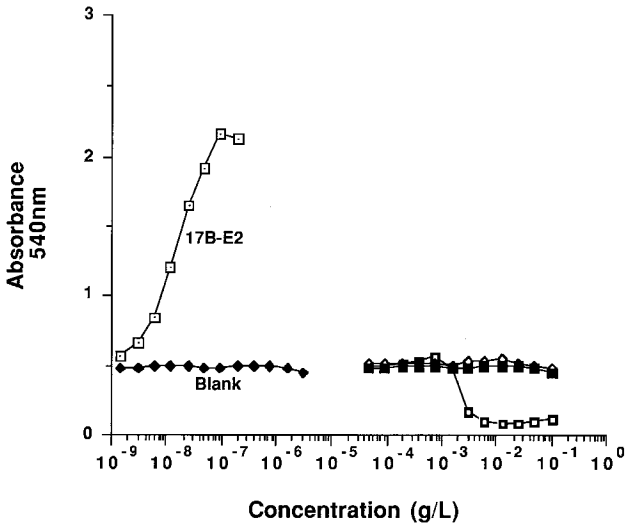

Fig. 7. Response of the estrogen screen to a range of cationic surfactants. The yeast cells were incubated with *n*-alkyldimethylbenzyl ammonium chloride (□) or dihydrogenated tallow dimethyl ammonium chloride (◇) at concentrations ranging from 100 mg/L to 50 μg/L.

sexual cycle, and availability of a range of plasmids and promoters have made *S. cerevisiae* (budding yeast) a powerful tool for molecular and cell biologists studying regulatory processes associated with mammalian cells [17].

Using this recombinant yeast strain, compounds were tested for their ability to directly stimulate the transcriptional activity of the hER. As yeast does not normally contain an estrogen receptor, this system overcomes concern regarding the effects of other gene targets, or the complex interplay that exists between the estrogen receptor and receptors for other steroids, peptide hormones, and growth factors that may be associated with systems relying on the activation of an endogenous estrogen receptor. However, in vitro tests represent only a part of the metabolic system present in entire animals and may give false positives and negatives. Chemicals identified as being estrogenic should therefore be tested in in vivo studies retrospectively.

The results show that the yeast screen is highly sensitive and has no incurred problems of variable background. The yeast can consistently detect 17β-estradiol at a concentration as low as 2 ng/L, which is five times lower than that of the MCF-7 cell system [5]. Animal bioassays using chicken, rat, or mouse are costly and laborious techniques that are useful for investigating the effects of metabolism and bioaccumulation of xenobiotics, but questions have been raised involving possible species differences in their hormonal activity. The proliferative effect of natural estrogens on the female genital tract (vaginal cornification) is a reliable indication of estrogenicity but is not suitable for large-scale screening.

The yeast-based screen reported here is performed in 96-well microtiter plates, allowing screening of multiple compounds over a wide range of doses, and results can be obtained in 3 to 4 d without the need for cell harvesting and counting. The color change can be clearly seen with the naked eye, allowing results to be qualitative or quantitative. The results show that this system is highly specific, reproducible, and rapid, facilitating its use as a routine assay for screening the estrogenic activity of chemicals.

A number of recombinant yeast-based estrogen assays have recently been developed for use in various research fields; these include studies into the mechanisms of hormone binding and

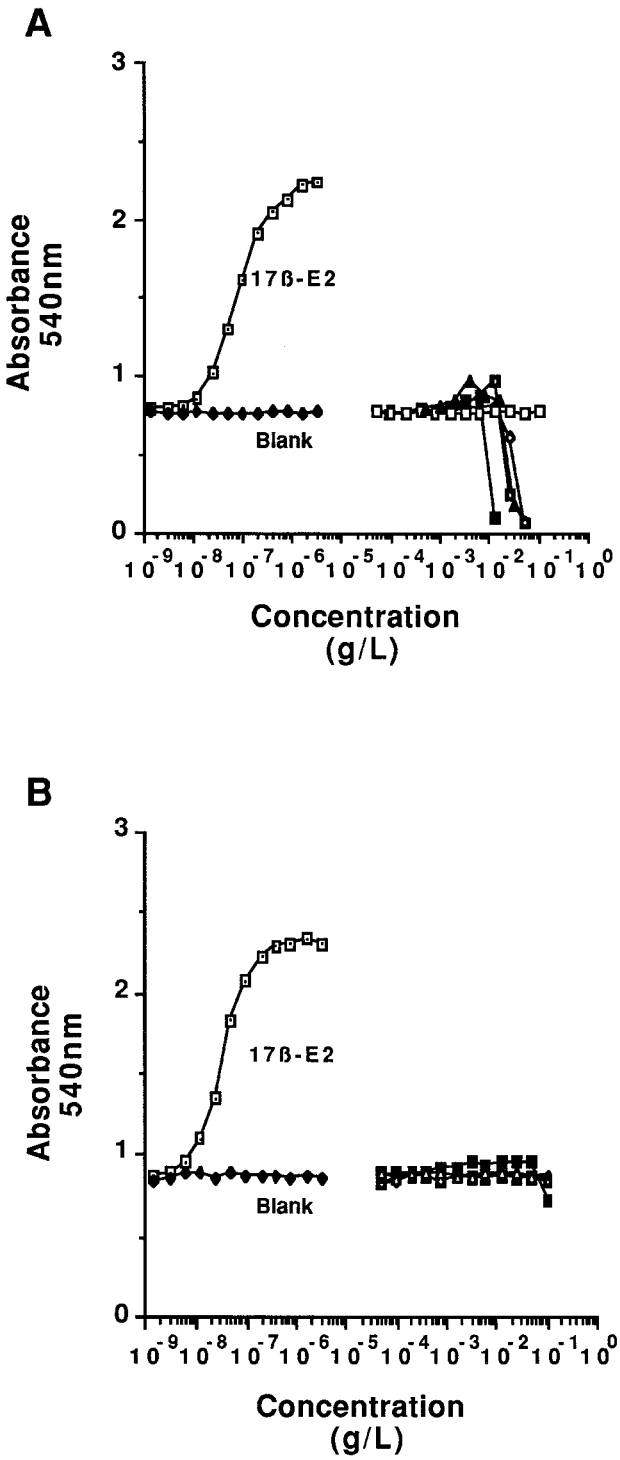

Fig. 8. Response of the estrogen screen to a range of anionic surfactants (100 mg/L to 50 μg/L). (**A**) A secondary alkane sulfonate (□), a branched alkylbenzene sulfonate (◇), a linear alkylbenzene sulfonate (LAS, e.g., Petrolab; ■ and Sirene; ▲). (**B**) Sodium lignosulfonate (▲), linear α-olefin sulfonate C14 (△), an alkyl ethoxylate sulfate (Dobanol ■), the alcohol sulfates sodium *n*-octyl sulfate (□) and sodium *n*-nonyl sulfate (◇), and soap (□).

transcriptional activation, using site-directed mutagenesis [12], as well the use of a screen to determine blood plasma levels of estradiol [18]. The recombinant yeast screen reported in Klein et al. also relies on the expression of β-gal but involves a more complicated and lengthy procedure [18].

There are a wide range of available surfactants used in many

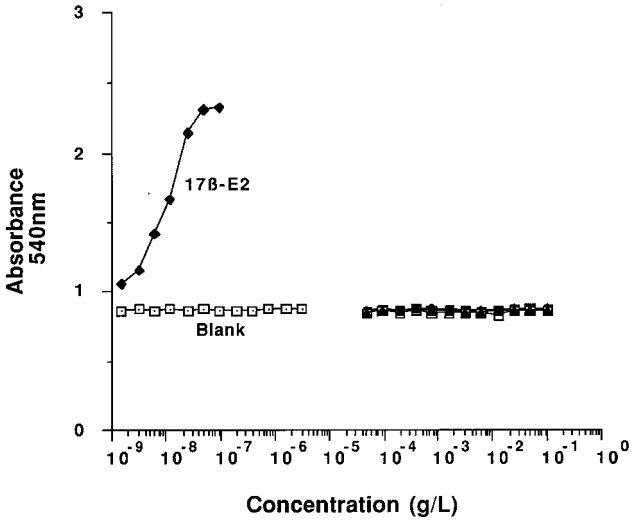

Fig. 9. Response of the estrogen screen to a range of sulfophenyl carboxylates (SPC), which are biotransformation products of LAS. The yeast cells were incubated with sulfophenyl acetic acid (◊), sulfophenyl propionic acid (■), sulfophenyl butyric acid (□), and sulfophenyl valeric acid (▲) at concentrations ranging from 100 mg/L to 50 μg/L.

different products. Each exhibits different combinations of cleaning (detergency), foaming, wetting, emulsifying, solubilizing, and dispersing properties. In the present study, we tested what we considered to be representatives of all the major groups of surfactants and, when available and known, their primary degradation products. None of the surfactants tested possessed estrogenic activity. In view of this, it appears that the parent surfactants themselves may provide no cause for concern regarding the effects of exposure to estrogenic chemicals. However, caution is warranted because presently we know little, if anything, about metabolism of these chemicals in vivo.

In the present study, only one group of chemicals tested proved to possess any estrogenic activity, the alkylphenols. Of these compounds, only the degradation products of the APnEO, which are environmentally persistent (discussed below), were weakly estrogenic (Fig. 6). These compounds have also proved

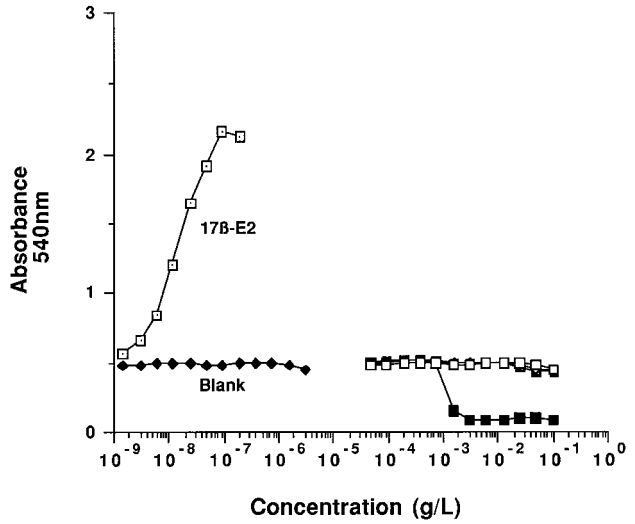

Fig. 10. Response of the estrogen screen to a range of amphoteric surfactants. The yeast cells were incubated with coconut amido betaine (□), cocobetaine (◊), or *N-b*-hydroxyethyl oleyl imidazoline (■) at concentrations ranging from 100 mg/L to 50 μg/L.

estrogenic in other studies, most notably a detailed study in which 4-OP, 4-NP, 4-NP1EC, and 4-NP2EO were all able to stimulate vitellogenin gene expression in trout hepatocytes, gene transcription in transfection assays, and proliferation of breast cancer cell lines [15]. In contrast, neither the parent linear alkylbenzene sulfonates (LAS) nor their biotransformation metabolites, the sulfophenyl carboxylates (SPC) [19], proved estrogenic in our assay (Fig. 9).

The results of the alkylphenolic compounds reported in this paper are consistent with other findings [5,6,15]. The yeast screen detects estrogenicity of alkylphenolic compounds at concentrations up to hundred times less than those needed to produce a response in hepatocyte culture. The findings of the yeast screen are more consistent with the results from the MCF-7 human breast cancer cell line used by White et al. [15], which showed 4-OP to be the most potent alkylphenol (active at 10 μg/L) and found NP1EC (active at 100 μg/L) to be approximately 10 times more potent than NP2EO. The potency of NP in the different screens varies the most, with concentrations ranging from 10 mg/L (hepatocyte culture) to 10 μg/L (yeast and MCF-7 systems) required to produce a response.

The purity of a chemical is very important for accurate interpretation of the results. The 17β-E2 3-sulfate tested (Fig. 3, ◊) was reported to be >99.0% pure (derived by thin-layer chromatography). Nevertheless, a 0.1% impurity of 17β-E2 within this sample would produce the activity we observed in the assay. The differences evident when comparing the relative potencies of alkylphenolic compounds in different assay systems may be due to variations in their composition, uptake, bioavailability, or metabolism in the different screens.

It is not known presently what particular structural features are necessary for estrogenic activity. Commercially available alkylphenolic compounds are a complex mixture of homologues, isomers, and oligomers. This suggests that there may be only one structurally active estrogenic form of a compound and may explain why some preparations of 4-*tert*-nonylphenolic compounds are more potent estrogens than others (unpublished observation). Separation of these mixtures into individual isomers and oligomers is necessary to answer this question. Despite this, there is a high degree of consistency in the results obtained from the different assay systems. All agree that OP is more potent than NP, and that as the EO chain length increases, the estrogenicity decreases, and that alkylphenols, alkylphenol ethoxylates with a low degree of ethoxylation, and their carboxylic acid derivatives are all active.

Alkylphenol polyethoxylates are nonionic surfactants consisting of a branched-chain alkylphenol, which has been reacted with ethylene oxide to produce a polyoxyethylene derivative. Although the full breakdown pathway for APnEO has yet to be determined, studies following the biotransformation of APnEO in primary effluents in sewage treatment works indicate that microbial metabolism normally starts by an attack on the ethoxylate chain, rather than on the ring or the hydrophobic chain [20]. The ethoxylate groups are systematically removed by ether cleavage or by terminal alcohol oxidation followed by cleavage of the resulting carboxylic acid. As the ethoxylate chain becomes shorter, the products become more resistant to biodegradation and increasingly lipophilic. The parent APnEO are transformed into persistent estrogenic short-chain APEO, APEC, and AP metabolites, which are then discharged into the aquatic environment via secondary effluents. The alkylphenols tend to partition into depositional phases of the rivers where they are found at concentrations significantly higher than those

in the water phase [11]. The production of alkylphenoxy carboxylic acids, by carboxylation of the terminal alcohol group, may be the most important process involved in the transformation of APnEO [20]. Ahel et al. found that up to 90% of APnEO were converted to APnEC after 23 days in shake cultures with APnEO as the sole carbon source [20]. The short-chain carboxylic acids are highly water soluble and have been reported to be present in drinking water [21].

Reported concentrations of alkylphenolic compounds in the aquatic environment are extremely variable and range from nanograms to milligrams/liter (see Clark et al. [21] for detailed information). The in vitro estrogen assay systems generally require higher concentrations of alkylphenolic compounds than those reported to be present in the environment to elicit an estrogenic response. These assays, however, measure short-term responses to estrogenic xenobiotics and do not reflect the real situation in the aquatic environment, where biota are probably chronically exposed, possibly throughout their lives. In such a situation, factors such as bioaccumulation become extremely important considerations.

Bioaccumulation of the lipophilic metabolites of APnEO has been observed in freshwater organisms [9,22]. Studies on the bioconcentration of NP in marine animals [23] have reported concentration factors from 100 times (wet tissue weight) in the shrimp (*Crangon crangon*) to 3,400 times in the mussel (*Mytilus edulis*). Particularly notable is the bioconcentration factor of approximately 7,000 times (dry weight) in the macrophytic algae (*Cladophora glomerata*), leading to levels of NP up to 38 mg/kg [9].

The discovery that some alkylphenolic compounds are estrogenic has led to increasing concern over their use in cleaning agents, paints, agrochemicals, emulsion polymers, and textile products [24]. Every year about 600,000 metric tonnes of APE are produced worldwide [25]; 18,000 metric tonnes of NPE (1992 data) are used annually in the United Kingdom alone. Of this quantity, 37% is lost to the aquatic environment [24]. Although much work has now been done to support the findings that alkylphenolic compounds are weakly estrogenic, little is presently known about the real impact these compounds have in the environment. It is not known which compounds get into aquatic organisms, whether they are present at concentrations high enough to have any effects and whether further structural changes, which might alter their activity, occur by metabolism. Availability and exposure to alkylphenolic degradation products may in part depend upon the life style of the organism; for example, whether it is free swimming or bottom dwelling. The physiological effects of exposure to alkylphenolic estrogens are still unclear. These questions need to be addressed, and their findings will help to assess the impact of human exposure to estrogenic xenobiotics.

Acknowledgement—We are extremely grateful to research scientists at Glaxo Group Research Ltd., particularly Ian Purvis, who developed the yeast screen that we have used. They also advised us on running the screen. We also thank the National Rivers Authority for funding (National R&D Project 490), and Hugh Thomas, Luciano Cavalli, and Jennifer Field for both supplying chemicals and commenting on earlier drafts of the paper.

REFERENCES

1. **Raloff, J.** 1994. EcoCancers. Do environmental factors underlie a breast cancer epidemic? *Science News* **1448**:10–13.
2. **Colborn, T.** and **C. Clement.** 1992. *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection,* Vol. 21—Advances in Modern Environmental Toxicology. Princeton Scientific, Princeton, NJ, USA.
3. **Bitman J.** and **H.C. Cecil.** 1970. Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agric. Food Chem.* **18**:1108–1112.
4. **Nelson J.A., R.F. Struck** and **R. James.** 1978. Estrogenic activities of chlorinated hydrocarbons. *J. Toxicol. Environ. Health.* **4**:325–339.
5. **Soto A.M., T.M. Lin, H. Justicia, R.M. Silvia** and **C. Sonnenschein.** 1992. An 'in culture' bioassay to assess the estrogenicity of xenobiotics (E-SCREEN). In T. Colborn and C. Clement, eds., *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection,* Vol. 21—Advances in Modern Environmental Toxicology. Princeton Scientific, Princeton, NJ, USA, pp. 295–309.
6. **Jobling, S.** and **J.P. Sumpter.** 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Onchorhynchus mykiss*) hepatocytes. *Aquat. Toxicol.* **27**:361–372.
7. **Soto, A.M., H. Justicia, J.W. Wray** and **C. Sonnenschein.** 1991. *p*-Nonylphenol: An estrogenic xenobiotic released from ''modified'' polystyrene. *Environ. Health. Perspect.* **92**:167–173.
8. **Krishnan, A.V., P. Stathis, S.F. Permuth, L. Tokes** and **D. Feldman.** 1993. Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**:2279–2286.
9. **Ahel, M.** and **W. Giger.** 1993. Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. *Chemosphere* **26**:1471–1478.
10. **Ahel, M., W. Giger** and **C. Schaffner.** 1994. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment—I. Occurrence and transformation in sewage treatment. *Water Res.* **28**:1131–1142.
11. **Ahel, M., W. Giger** and **C. Schaffner.** 1994. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment—II. Occurrence and transformation in rivers. *Water Res.* **28**:1143–1152.
12. **Katzenellenbogen, B.S., B. Bhardwaj, H. Fang, B. A. Ince, F. Pakdel, J.C. Reese, D. Schodin** and **C.K. Wrenn.** 1993. Hormone binding and transcription activation by estrogen receptors: Analyses using mammalian and yeast systems. *J. Steroid Biochem. Mol. Biol.* **47**:39–48.
13. **Ahel, M., W. Giger** and **C. Schaffner.** 1991. Environmental occurrence and behaviour of alkylphenol polyethoxylates and their degradation products in rivers and groundwaters. *Proceedings,* Seminar on Nonylphenolethoxylates (NPE) and Nonylphenol (NP), Saltsjobaden, Sweden, February 6–8, pp. 105–151.
14. **Lan, N.C.** and **B.S. Katzenellenbogen.** 1976. Temporal relationships between hormone receptor binding and biological responses in the uterus: Studies with short and long-acting derivatives of estriol. *Endocrinology* **98**:220–230.
15. **White, R., S. Jobling, S.A. Hoare, J.P. Sumpter** and **M.G. Parker.** 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135**:175–182.
16. **Metzger, D., J.H. White** and **P. Chambon.** 1988. The human oestrogen receptor functions in yeast. *Nature* **334**:31–36.
17. **Purvis, I.J., D. Chotai, C.W. Dykes, D.B Lubahn, F.S. French, E.M. Wilson** and **A.N. Hobden.** 1991. An androgen-inducible expression system for *Saccharomyces cerevisiae. Gene* **106**:35–42.
18. **Klein, K.O., J. Baron, M.J. Colli, D.P. McDonnell** and **G.B. Cutler, Jr.** 1994. Estrogen levels in childhood determined by an ultrasensitive recombinant cell bioassay. *J. Clin. Invest.* **94**:2475–2480.
19. **Argese, E., A. Marcomini, P. Miana, C. Bettiol** and **G. Perin.** 1994. Submitochondrial particle response to linear alkylbenzene sulfonates, nonylphenol polyethoxylates and their biodegradation derivatives. *Environ. Toxicol. Chem.* **13**:737–742.
20. **Ahel, M., D. Hrsak** and **W. Giger.** 1994. Aerobic transformation of short-chain alkylphenol polyethoxylates by mixed bacterial cultures. *Arch. Environ. Contam. Toxicol.* **26**:540–548.
21. **Clark, LB., R.T. Rosen, T.G. Hartman, J.B. Louis, I.H. Suffet, R.L. Lippincott** and **J.D. Rosen.** 1992. Determination of alkylphenol ethoxylates and their acetic acid derivatives in drinking water by particle beam liquid chromatography/mass spectrometry. *Int. J. Environ. Anal. Chem.* **147**:167–180.
22. **McLeese, D.W., V. Zitko, D.B. Sergeant, L. Burridge** and **C.D Metcalfe.** 1981. Lethality and accumulation of alkylphenols in aquatic fauna. *Chemosphere* **10**:723–730.
23. **Ekelund, R., A. Bergman, A. Granmo** and **M. Berggren.** 1990. Bioaccumulation of 4-nonylphenol in marine animals—A re-evaluation. *Environ. Pollut.* **64**:107–120.
24. **Consultants in Environmental Sciences.** 1993. Uses, fate and entry to the environment of nonylphenol ethoxylates. Final Report. U.K. Department of the Environment, Beckenham, Kent, UK.
25. **Cox, M.F.** 1993. Ethylene oxide-derived surfactants. *Proceedings,* 3rd World Conference on Detergents: Global Perspectives, Montreux, Switzerland, September 26–30, pp. 141–146.