Comparison of Short-Term Estrogenicity Tests for Identification of Hormone-Disrupting Chemicals

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The aim of this study was to compare results obtained by eight different short-term assays of estrogenlike actions of chemicals conducted in 10 different laboratories in five countries. Twenty chemicals were selected to represent direct-acting estrogens, compounds with estrogenic metabolites, estrogenic antagonists, and a known cytotoxic agent. Also included in the test panel were 17β-estradiol as a positive control and ethanol as solvent control. The test compounds were coded before distribution. Test methods included direct binding to the estrogen receptor (ER), proliferation of MCF-7 cells, transient reporter gene expression in MCF-7 cells, reporter gene expression in yeast strains stably transfected with the human ER and an estrogen-responsive reporter gene, and vitellogenin production in juvenile rainbow trout. 17β-Estradiol, 17α-ethynyl estradiol, and diethylstilbestrol induced a strong estrogenic response in all test systems. Colchicine caused cytotoxicity only. Bisphenol A induced an estrogenic response in all assays. The results obtained for the remaining test compounds—tamoxifen, ICI 182.780, testosterone, bisphenol A dimethacrylate, 4-*n*-octylphenol, 4-*n*-nonylphenol, nonylphenol dodecylethoxylate, butylbenzylphthalate, dibutylphthalate, methoxychlor, *o,p'*-DDT, *p,p'*-DDE, endosulfan, chlomequat chloride, and ethanol—varied among the assays. The results demonstrate that careful standardization is necessary to obtain a reasonable degree of reproducibility. Also, similar methods vary in their sensitivity to estrogenic compounds. Thus, short-term tests are useful for screening purposes, but the methods must be further validated by additional interlaboratory and interassay comparisons to document the reliability of the methods. — *Environ Health Perspect* 107(Suppl 1):89–108 (1999). *http://ehpnet1.niehs.nih.gov/docs/1999/Suppl-1/89-108andersen/abstract.html*

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Several man-made chemicals that are widely distributed in the environment may have the potential to mimic estrogens or otherwise disrupt the endocrine system (*1–4*). Reliable short-term methods are needed to identify such chemicals, to characterize and control the environmental load, and to evaluate human exposures. Attempts to develop these methods must confront the problem that the biologic fate and the toxicology of the substances are poorly understood.

Estrogenic chemicals identified to date include some organochlorine pesticides, such as *o,p'*-DDT and methoxychlor, and industrial chemicals and byproducts, including some polychlorinated biphenyl congeners, alkyl phenols, phthalates, and bisphenol A (*5–8*). The chemical structure of these chemicals varies substantially, which makes it difficult to predict their estrogenicity solely on a structural basis.

Estrogenicity was first defined as a physiologic response to a compound that induced estrus *in vivo*. An *in vivo* rodent uterine bioassay was developed in which an estrogen-induced uterotropic response was estimated as an increase in uterine tissue mass in ovariectomized or immature rodents (*9*). This assay is often regarded as a gold standard. However, although it has been widely used for many years, the assay has still not been fully standardized (*10*), and even when the same protocol is used, the results sometimes vary between different laboratories (*11,12*). Finally, different tissues may respond differently to estrogenic chemicals. A well-known example is tamoxifen, an antiestrogen in breast tissue but a uterotropic estrogenic agonist in uterine tissue (*13*). More broadly based *in vivo* assays should aim at detecting the response in different tissues, thereby becoming more sensitive and informative.

The advantages of the rodent uterotropic assay and other *in vivo* assays are that they take into consideration the effects of metabolism, plasma–protein binding, and pharmacokinetics. These methods can also detect estrogenic responses due to altered metabolism of exogenous or endogenous estrogens as caused by, for example, some hepatotoxic chemicals such as carbon tetrachloride (*14*). However, *in vivo* methods are in general expensive and time-consuming,

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Abbreviations used: APE, alkylphenol polyethoxylate; BBP, benzylbutylphthalate; BSA, bovine serum albumin; cat, chloramphenicol acetyltransferase; CD, charcoal dextran; DBP, dibutylphthalate; DCC, dextran-coated charcoal; DES, diethylstilbestrol; DME, Dulbecco's modification of Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERE, estrogen-responsive element; FBS, fetal bovine serum; GST, glutathione *S*-transferase; hER, human estrogen receptor; IC_{50}, concentration that inhibits 50%; LBA, ligand-binding assay; LBD, ligand-binding domain; NP, nonylphenol; NPE, nonylphenol polyethoxylate; NP12EO, nonylphenol dodecylethoxylate; ONPG, *o*-nitrophenyl-D-galactopyranoside; OP, octylphenol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RBA, relative binding affinity; RP, relative potency; rtER, rainbow trout estrogen receptor; RT–PCR, reverse transcriptase–polymerase chain reaction; SDS, sodium dodecyl sulfate; SHBG, sex-hormone binding globulin; SRB, sulforhodamine-B; TCA, trichloracetic acid; tk, thymidine kinase.

and a variety of short-term assays are therefore being applied to identify estrogenic chemicals and to determine the relative potencies for hormonal responses. Some of these assays may be suitable for screening large numbers of chemicals and contaminated media, such as water and food, and may therefore be useful tools for prioritizing chemicals for more extensive studies *in vivo*.

However, most *in vitro* assays estimate primarily the intrinsic estrogenic activity of the chemicals as reflected by their binding to and activation of the estrogen receptor (ER) without taking into account factors that may affect their activity in the intact organism. These factors include: binding affinity of the chemical to proteins (especially sex-hormone binding globulin [SHBG] and albumin), ability to enter target cells, degradation time in the organism, and the concentration of endogenous estrogen.

Most circulating endogenous estrogen (> 99%) is bound to plasma proteins and only a minor fraction is able to penetrate into the cells and activate the ER. Estrogenic chemicals such as *o,p'*-DDT, octylphenol (*15*), bisphenol A (*16*), and the potent synthetic estrogen diethylstilbestrol (DES) (*15,17*) have a much lower affinity for these proteins than does 17β-estradiol. Hence, the major part of these chemicals in the blood would be available for activation of the cellular ER. The relative potency of the chemicals compared to estradiol may therefore be underestimated by *in vitro* tests conducted without addition of plasma proteins, as has recently been demonstrated for bisphenol A (*16*).

The biodegradation of estrogenic chemicals may influence the estrogenic response, as a long degradation time could enhance the response. Some environmental chemicals are very lipophilic and resistant to metabolism and may therefore accumulate in organisms and reach concentrations sufficient to produce estrogenic responses *in vivo*. Hence, although the environmental estrogens characterized to date are considerably less potent than estradiol (1/50th to 1/10000th) based on ER binding affinities or effects seen in cell cultures (*5,18*), low concentrations may still be detrimental to the reproductive success of exposed organisms. For example, feminization and decreased reproduction in gulls was apparently elicited by *o,p'*-DDT in eggs at concentrations as low as 5 mg/kg, which is similar to concentrations reported in the environment (*19*).

Because estrogenic chemicals compete with endogenous estrogens for binding to ERs, the concentration of endogenous estrogen may influence the estrogenic effects of the chemicals, and a chemical that on its own shows a weak estrogenicity might therefore potentially act as an antiestrogen *in vivo*. In addition, humans and wildlife are exposed to several potential estrogenic chemicals simultaneously. Addition of two or more weak estrogens may induce an additive effect, as demonstrated in MCF-7 cells (*5*) and by induction of vitellogenin in male fish (*20*).

To obtain a reliable screening system for estrogenicity, it is necessary to validate the short-term assays and compare the results to those obtained in different types of *in vivo* studies. Further development of the short-term assay systems may be needed, and several assays may need to be combined in a screening panel. As an important step in this process, it is necessary to compare and validate those short-term assays already developed and used by different laboratories. Therefore, a comparison study of short-term tests for estrogenic activity of chemicals was initiated. Previous studies have compared the results obtained in different assay systems using the same chemicals within the same laboratory (*7,21–23*). In this study, 10 laboratories agreed to participate and test a panel of 20 chemicals without knowing the identity of the chemicals prior to testing.

The short-term methods were chosen to represent different types of response. Binding assays measure the specific binding to the ER but provide no information on activation of the receptor. The proliferation assay in MCF-7 cells (E-SCREEN) measures a cellular response known to be induced by estrogens but reveals no direct information of the mechanism involved. The reporter gene expression assays detect activation of the ER leading to binding of an estrogen-responsive element (ERE) and expression of a reporter gene. Several reporter gene assays were included, i.e., a mammalian cell type (MCF-7) transiently transfected with ERE and a reporter gene and different yeast assays stably transfected with ER and ERE-linked reporter genes. Finally, an *in vivo* assay using induction of vitellogenin in juvenile rainbow trout was included because vitellogenin induction in juvenile or male fish is reportedly a very sensitive biologic marker for estrogenicity (*20*). Several of the assays (e.g., the E-SCREEN and the yeast assays) have been widely used in an attempt to identify estrogenic chemicals (*5,8,24–28*).

The test chemicals (Table 1; Figure 1) were selected to represent documented direct-acting potent estrogens (17β-estradiol, 17α-ethynyl estradiol, DES), a complete antiestrogen (ICI 182.780), a partial antiestrogen (tamoxifen), a potent androgen (testosterone), and environmental pollutants reported to be estrogenic in different *in vitro* assays as well as *in vivo* by inducing a uterotropic response in rodents. The responses were induced either directly [i.e., octylphenol (OP) (*15,27,29–31*), nonylphenol (NP) (*12,23,27,31–35*), and *o,p'*-DDT (*5,21,27,36–39*)] or after

Table 1. Test chemicals.

Compound (CAS no.)	Supplier (Code no.)	Purity, %	Test no.
17β-Estradiol (50-28-2)	Sigma (E 1132)	99.4	A
17β-Ethynyl estradiol (57-63-6)	Sigma (E 0882)	99.1	B
Diethylstilbestrol (56-53-1)	Sigma (D 4628)	99	C
Tamoxifen (10540-29-1)	Aldrich (28,161-1)	96	D
ICI 182.780 (129453-61-8)[a]	Zeneca	99.3	E
Testosterone (58-22-0)	Sigma (T 1268)	100	F
Bisphenol A (80-05-7)	Aldrich (23,965-8)	99	G
Bisphenol A-dimethacrylate (3253-39-2)	Aldrich (15,632-9)	99.7	H
4-*n*-Octylphenol (1806-26-4)	Ehrenstorfer (C 157120)	99.4	I
4-*n*-Nonylphenol (25154-52-3)	Ehrenstorfer (C 156300)	99.9	J
Nonylphenol dodecylethoxylate	Promochem	?	K
Benzylbutylphthalate (85-68-7)	Riedel-de Häen (36927)	97	L
Dibutylphthalate (84-74-2)	Riedel-de Häen (36736)	98	M
Methoxychlor (72-43-5)	Ehrenstorfer (C 150600)	98.4	N
o,p'-DDT (789-02-6)	Ehrenstorfer (C120810)	99.8	O
p,p'-DDE (72-55-9)	Ehrenstorfer (C 120410)	99.7	P
Endosulfan (mixed isomers)[b] (115-29-7)	Ehrenstorfer (C 131200)	99	Q
Chlormequat chloride (999-81-5)	Ehrenstorfer (C113400)	97	R
Colchicine (64-86-8)	Sigma (C 3915)	95	S
Ethanol	BDH (15338 SE)	99.8	T

?, unknown. [a]Gift from Zeneca Pharmaceuticals. [b]75% α-isomer and 24% β-isomer.

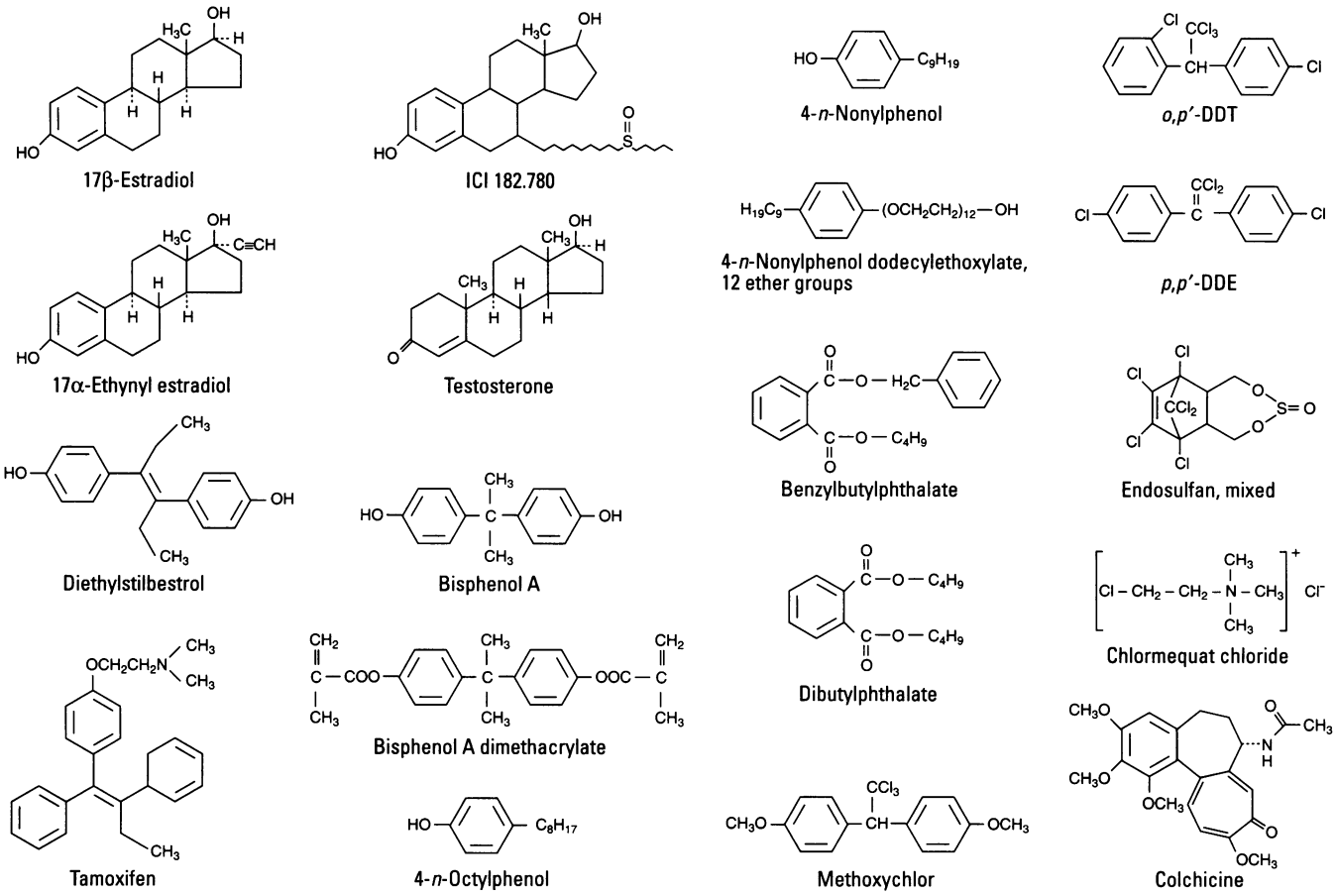

Figure 1. Chemical structure of the test compounds.

metabolism [i.e., methoxychlor (*5,12,40*)]. Other test compounds have been reported estrogenic *in vitro* but not uterotropic *in vivo*: benzylbutylphthalate (BBP), dibutylphthalate (DPB) (*6,23*), and endosulfan (*5,41,42*), and two of the test compounds have been reported estrogenic *in vitro*, but no information on uterotropic responses is available: bisphenol A (*8,27,28*) and bisphenol A–dimethacrylate (*8*). In addition, an antiandrogen [*p,p'*-DDE (*43*)], a nonylphenolpolyethoxylate (nonylphenol dodecylethoxylate [NP12EO]) that degrades to NP (*31*), a chlorinated nonaromatic compound (chlormequat chloride), a chemical with known cytotoxic effect (colchicine), and ethanol as solvent control were included.

Materials and Methods

Chemicals

The chemicals were obtained from Sigma Chemical Company (St. Louis, MO), Ehrenstorfer (Augsburg, Germany), Riedel-de Häen (Seelze, Germany), Aldrich (Steinheim, Germany), Promochem, (Wesel, Germany), or Zeneca Pharmaceuticals (Cheshire, UK), as listed in Table 1. The purity of the chemicals stated in the table was reported by the supplier.

Identical test panels consisting of 18 vials each containing approximately 2 ml stock solutions of 10 mmole/liter of the test substances were set up. All test compounds were dissolved in pure ethanol from BDH (Poole, UK). In addition, the test panel included one vial containing 2-ml 10 μmol/liter 17β-estradiol used as positive control and one vial containing 2 ml pure ethanol as solvent control. These test panels were used for all assays except induction of vitellogenin production in rainbow trout because this assay required much more of the test compounds. The same batches of chemicals were used for this assay. The chemicals were weighed, coded, and transferred to brown glass vials (10.0 ml, La-Pha-Pack, Langerwerde, Germany) before delivery to laboratory 9. The chemicals were dissolved in laboratory 9. Details of this assay are given below and in "Results."

All test compounds were weighed and dissolved in ethanol and transferred to brown glass vials (4.0 ml, La-Pha-Pack) with screw caps of butyl gummy with Teflon foliation (Brown Chromatography, Würzburg, Germany). DES, 17α-ethynyl estradiol, and 17β-estradiol were handled separately after all the other test compounds were weighed and dissolved. As the last step, ethanol for the solvent controls was transferred directly to the test vials. Because the solvent controls were prepared after the strongly active estrogens, the risk of cross-contamination was maximized, although rigorous efforts were made to limit this risk. All test vials were placed at –20°C until their distribution to the participating laboratories. During transport the test vials were kept on dry ice. After arrival the test panel was kept at –20°C until the analyses were performed. All participating institutions were informed that the test panel contained hazardous toxic chemicals and should be handled accordingly. The laboratories and methods are summarized in Table 2.

Table 2. Laboratories and methods included in the study.

Laboratory name (laboratory no.)	Test method
Department of Environmental Medicine, Odense University, Odense, Denmark (1)	E-SCREEN, proliferation of MCF-7 cells
Department of Anatomy and Cellular Biology, Tufts University, Boston, Massachusetts (2)	E-SCREEN, proliferation of MCF-7 cells
Laboratory of Medical Investigation, University of Granada, Granada, Spain (3)	E-SCREEN, proliferation of MCF-7 cells
Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UK (4)	Recombinant yeast (Saccharomyces) estrogen screen expressing hER
Laboratorie D'Endocrinologie Moleculaire de la Reproduction, Campus de Beaulieu, Rennes, Cedex, France (5)	Recombinant yeast (Saccharomyces) estrogen screen expressing rtER
Department of Growth and Reproduction, The National University Hospital, Copenhagen, Denmark (6)	Direct competitive estrogen *in vitro* binding assay, recombinant hER Recombinant yeast (Saccharomyces) estrogen screen expressing hER
Department of Environmental Medicine, University of Århus, Århus, Denmark (7)	Transient gene expression assay in MCF-7 cells
Novo Nordisk A/S, Måløv, Denmark (8)	*In vitro* ER binding assay, rabbit uterine tissue
Department of Biology, Odense University, Odense, Denmark (9)	Vitellogenin production in juvenile rainbow trouts *in vivo*
Environmental Endocrinology Laboratory, Tulane University, New Orleans, Louisiana (10)	Recombinant yeast (DY159) estrogen screen expressing hER

Direct Competitive Estrogen* in Vitro *Binding Assay (Laboratory 6) Based on Recombinant Human Estrogen Receptor Isolated by Reverse Transcriptase–Polymerase Chain Reaction from MCF-7 Cells. The complete coding region of the ER (amino acids 2 to 595) was prepared by reverse transcriptase–polymerase chain reaction (RT–PCR) from cDNA prepared from total RNA from MCF-7 cells. cDNA was synthesized from 1-µg total RNA using 0.5-µg T14V primer (V corresponds to A, C, or G) as described in Ausubel et al. (*44*). One microliter of the cDNA sample was used for PCR using 5 µ native Pfu-enzyme (Stratagene, La Jolla, CA) including 30 pmol of each primer:

5′-primer: 5′-*CGGGGATCC*ACCATGACCCTCCACACCAAAG-3′;

3′-primer: 5′-*GAGGAATTCC*GACTGTGGCAGGGAAACCCTC-3′.

Nucleotides in italics were added to facilitate cloning. The cycle conditions on a GeneAmp PCR System 9600 (Perkin-Elmer Corp., Norwalk, CT) were 96°C for 3 min, followed by 40 cycles of 96°C for 30 sec, 68°C for 1 min, 74°C for 3 min, and finally, 74°C for 8 min. The ligand-binding domain (LBD) (amino acids 282 to 595) was prepared from the complete coding region DNA fragment by PCR as described previously, except that the 5′-primer was exchanged with 5′-GAAGGATCCTCTGCTGGAGACATGAG-3′ and only 15 cycles were performed using an annealing temperature of 52°C. The resulting DNA fragments were digested with restriction enzymes *Bam*HI and *Eco*RI and cloned directionally into pGEX GTH (*45*), allowing ^{32}P-end labeling of the Est–LBD. The insert in the purified plasmid DNA was sequenced on an ALFexpress sequenator (Amersham-Pharmacia-Bioteck, Uppsala, Sweden) using the ThermoSequenase (Amersham-Pharmacia-Bioteck) and CY5 fluorescence labeled primers. The recombinant proteins were purified as glutathione *S*-transferase (GST) fusion proteins from *Escherichia coli* lysates, using the bulk GST purification module as described by the manufacturer (Amersham-Pharmacia-Bioteck). To normalize the amount of fusion protein used in each assay, an aliquot of the Sepharose–GST–LBD (about 5% of total amount used) was ^{32}P labeled with [γ^{32}P]adenosine triphosphate (Amersham-Pharmacia-Bioteck) and 2.5-µ bovine heart muscle kinase (Sigma Chemical) as described by the manufacturer (Amersham-Pharmacia-Bioteck) and then mixed 1:20 (vol:vol) with the unlabeled Sepharose-GST–LBD, resulting in approximately 200 cpm/10 µl.

In the binding assay, 10 µl aliquots of Sepharose–GST–LBD mixed with [^{32}P]-labeled Sepharose–GST–LBD were transferred to Eppendorf tubes (Eppendorf–Netheler–Hinz GmbH, Hamburg, Germany) and 1×phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.3) was added to a final volume of 48 µl. All the chemicals were diluted in 96% ethanol to the desired concentrations and mixed 1:1 with ^{3}H-estradiol (10 µCi/ml). Two microliters of this mixture was added to the Sepharose–GST–LBD beads and incubated at room temperature for 45 min with slow agitation. The beads were collected by centrifugation and washed four times with 500-µl 1×PBS before they were resuspended in 50-µl 1×PBS and transferred to 5 ml Ultima Gold scintillation solution (Packard Instrument Co., Meriden, CT) and measured in a scintillation counter measuring both ^{3}H-estradiol and ^{32}P activity. The [^{32}P]-labeled Sepharose–GST–LBD was used to normalize for the amount of Sepharose–GST–LBD used in each assay. The binding of ^{3}H-estradiol to the Sepharose–GST–LBD fusion protein was plotted as a function of the actual chemical concentration used in the assay. The concentration that inhibits 50% (IC_{50}) was calculated as the chemical concentration reducing the ^{3}H-estradiol binding to 50% of maximal binding. Data represent mean of two independent experiments.

In Vitro *Estrogen Receptor Binding Assay (Laboratory 8) Based on Rabbit Uterine Tissue.* A classical ligand-binding assay (LBA) employing dextran-coated charcoal (DCC) to separate bound and free ligands was used as described in detail in EORTC (*46*) and Thorpe (*47*). Cytosol prepared from rabbit uterine tissue was the source of the ER-rich cytosol; rabbit muscle was the source of ER-poor cytosol. Fresh aliquots of cytosols were thawed on the day of analysis. Both cytosols were diluted with assay buffer [PB: 10 mM K_2HPO_4/KH_2PO_4, 1.5 mM K_2EDTA, 10 mM monothioglycerol, 10 mM $Na_2MoO_4 \cdot 2H_2O$, 10% glycerol (v/v); pH 7.5] to approximately 3 mg cytosol protein/ml. ER-rich cytosol was diluted with ER-poor cytosol to achieve approximately 20 to 25% maximal binding of 0.5 nM ^{3}H-17β-estradiol (Amersham-Pharmacia-Bioteck). Radioinert 17β-estradiol was obtained from Sigma Chemical.

The stock solutions of test compounds were further diluted by ethanol for analysis with PB. Aliquots of 10 µl test compounds were incubated with 20 µl ^{3}H-estradiol (assay concentration 0.5 nM) and 50 µl cytosol in microtiter plates for 18 to 20 hr at 4°C. For control samples as well as maximal binding samples, 10 µl PB was added

in lieu of test compound. To assess DCC background counts, 50 µl = 0.3% bovine serum albumin (BSA) in PB was added in lieu of cytosol. To terminate the binding reaction, 100 µl DCC slurry (0.5% activated charcoal [Sigma Chemical] and 0.005% dextran T_{70} [Amersham–Pharmacia–Bioteck] in PB) were added to each sample and incubated with continuous shaking for 15 min at 4°C. To separate bound and free titrated estradiol, microtiter plates (Microwell, Nunc, Roskilde, Denmark) were centrifuged for 10 min (800 × *g*) at 4°C. Aliquots of 100 µl were removed from each sample for scintillation counting using Optiflour scintillation liquid (Packard BioScience B.V, Groningen, The Netherlands). Standards and control samples were incubated in quadruplicate; test compounds were incubated in duplicate. The mean counts per minute in each sample was calculated, background (DCC) was subtracted, and percent of maximal ^{3}H-17β-estradiol binding was calculated. This value was plotted against the concentration of test compound incubated (expressed logarithmically). IC_{50} values were used to compare binding affinities.

E-SCREEN Based on Proliferation of Human Breast Cancer Cells (MCF-7) (Laboratories 1, 2, and 3). This assay introduced by Soto et al. (*48*) is based on the estrogen-sensitive human breast cancer cell line MCF-7. These cells require the presence of estrogen to grow as tumors in a host. When MCF-7 cells are grown in a culture medium supplemented with nonestrogenic charcoal–dextran (CD)-stripped human serum, proliferation is prevented. When estrogen is added, the cells proliferate. This assay was performed in three different laboratories as described by Soto et al. (*48*) (laboratory 2) or slightly modified as described by Villalobos et al. (*49*) (laboratories 1 and 3). Briefly, stock cultures of MCF-7 BUS cells (passage 143 to 148), were grown in Dulbecco's modification of Eagle's medium (DME) supplemented with 5% fetal bovine serum (FBS) in an atmosphere of 5% CO_2/95% air under saturating humidity at 37°C. MCF-7 cells were trypsinized and plated in 12-well plates (Costar, Cambridge, MA) (laboratory 2) or 24-well plates (Limbro, McLean, VA) (laboratories 1 and 3) at initial concentrations of 10^4 cells per well. Cells were allowed to attach for 24 hr, then the seeding medium (5% FBS in DME [laboratories 2 and 3] or 10% FBS in DME [laboratory 1]) was replaced with the experimental medium (5% CD-treated FBS [laboratory 2] or 10% CD-treated human serum [laboratories 1 and 3] supplemented to phenol red-free DME). CD-treated FBS and CD-treated human serum were prepared as described by Soto et al. (*48*) based on plasma supplied from local blood banks. A range of concentrations of the test compounds was added to this medium. All chemicals were diluted to desired concentrations with DME immediately prior to use. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells. In laboratory 2 a cell-lysing solution (10% ethylhexadecyl-dimethylammonium bromide) (Eastman Kodak, Rochester, NY) in 0.5% Triton X-100, 2 mM $MgCl_2$, 12 mM NaCl, 5 mM phosphate buffer, pH 7.4) was added and the nuclei counted in a Model ZM Coulter Counter Apparatus (Coulter Electronics, Hialeah, FL). In laboratories 1 and 3 the cells were fixed and stained with sulforhodamine-B (SRB) as described by Brotons et al. (*24*) and Villalobos et al. (*49*). Briefly, cells were treated with cold 10% trichloracetic acid (TCA) and incubated at 4°C for 30 min, then washed five times with tap water and left to dry. TCA-fixed cells were stained for 10 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 20 min. Finally, aliquots were transferred to a 96-well plate and read in a Titertek Multiscan plate reader (Titertek Instruments, Inc., Huntsville, AL) at 492 nm (laboratory 3). In all three laboratories the mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. Data represent the mean and are pools of either one (laboratory 3), at least two (laboratory 1), or at least three (laboratory 2) independent experiments run in duplicate.

Transient Gene Expression Assay in MCF-7 Cells (Laboratory 7). MCF-7 cells obtained from the Breast Cancer Task Force Cell Culture Bank (Mason Research Institute, Worcester, MA) (passage 298 to 310) were propagated in DME without phenol red supplemented with 1% CD-treated FBS, 64 µg/ml Gentamicin (Garamycin, Schering-Plough, Madison, NJ), 2.5 mM glutamine, and 6 µg/liter, insulin and transfected as described by Jorgensen and Autrup (*50,51*) using a chimeric reporter construct containing one ERE in front of the thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) gene p*ERE-tk-cat* (*52*). Upon transfection the cells were treated for 48 hr with solvent (0.1% ethanol [96%, Merck, Darmstadt, Germany]), 10 nM 17β-estradiol (E 8875, Sigma Chemical), or the test chemicals. The test chemicals were tested at the highest nontoxic concentration as deduced by a nonradioactive cell proliferation/cytotoxicity assay (Promega, Madison, WI). CAT activities were normalized to transfection efficiency and protein content as described by Jorgensen and Autrup (*50,51*). Preliminary results indicated that the use of CD-treated human serum and CD-treated FBS give similar results. Data are expressed as the mean ± SD and are a pool of at least nine samples from three independent experiments.

Recombinant Yeast Estrogen Screen Using Human Estrogen Receptor (Laboratories 4 and 6). Yeast (Saccharomyces) stably transfected with the human estrogen receptor (hER) gene and expression plasmids carrying an ERE and the reporter gene *lacZ* encoding the enzyme β-galactosidase was used and the assay performed as described by Routledge and Sumpter (*27*). The test chemicals were serially diluted in absolute ethanol and 10 µl of each concentration was transferred to a 96-well optically flat-bottom microtiter plate (Titertek) and allowed to evaporate to dryness on the assay plate. Aliquots (200 µl) of medium containing recombinant yeast and the chromogenic substrate chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, East Sussex, UK) were then dispensed to each sample well. Details of preparation of medium components are discussed in Routledge and Sumpter (*27*). Each plate contained at least one row of blanks (assay medium only) as well as a standard curve for 17β-estradiol. In laboratory 4, absorbance at 540 nm was measured after 72-hr incubation using a Titertek Multiscan MCC/340 plate reader (Titertek). In laboratory 6, absorbance at 550 nm was measured after 72- and 93-hr incubation using an Anthos 2010 plate reader (Anthos Labtec Instruments, Salzburg, Austria). In laboratory 6, testing of the chemicals was repeated by adding the chemicals directly to the medium instead of evaporating them to dryness on the plate. This repetition was to investigate if this modification of the procedure had any effect on the response obtained. Data represent mean values from a single experiment carried out in duplicate.

Recombinant Yeast Estrogen Screen Using Human Estrogen Receptor and Yeast Strain DY150 (Laboratory 10). The yeast strain DY150 (MAT a ura 3-1 leu

2-3 112 trp 1-1 his 3-11 15 ade 2-1 can 1-100) contains the yeast expression plasmid containing hER (YEPKB1) and the estrogen-sensitive *LacZ* reporter plasmid (2ERE-LacZ). This strain was grown overnight at 30°C in synthetic medium, supplemented with uracil and tryptophan, in 2 ml cultures. The next day, 25 µl of the overnight culture was diluted into 975 µl fresh medium and grown overnight (18 hr) with 1 µl of the various stock solutions of test chemicals corresponding to a final concentration of 10 µM for the test chemicals and 10 nM for 17β-estradiol. For β-galactosidase assays, the yeast cells were collected by centrifugation, resuspended in 700 µl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol), and permeabilized by the addition of 6 µl $CHCl_3$ and 4 µl 0.1% sodium dodecyl sulfate (SDS) followed by vortexing for 25 sec. The reactions were equilibrated at 30°C for 10 min, then 160 µl *o*-nitrophenyl-D-galactopyranoside (ONPG) (4 mg/ml in Z-buffer) was added and the reactions returned to 30°C for between 5 and 60 min. The reactions were terminated by the addition of 400 µl 1 M $NaCO_3$, the cell debris was removed by centrifugation, and the absorbance at 420 nM measured (A420). The growth of the yeast strains was monitored by measuring the absorbance at 600 nM (A600). Miller units were determined using the following formula: [A420/(A600 of 1/10 dilution of cells × volume of culture × length of incubation)] × 1000. The data represent mean from three independent experiments with determinations in triplicates.

Recombinant Yeast Estrogen Screen Using Rainbow Trout Estrogen Receptor (Laboratory 5). Saccharomyces cerevisiae (strain BJ-ECZ) were stably transfected with the rainbow trout estrogen receptor (*rtER*) gene and an estrogen-responsive reporter gene containing two ERE linked to the yeast CYC1 promoter located upstream of the *E. coli* gene for β-galactosidase (*lacZ*) as described by Petit et al. (*53*). The cells were grown in liquid culture in the absence (negative control) or presence (positive control) of 10 nM 17β-estradiol or test chemicals at 10 µM (1:1000 dilution of the stock solutions directly in the yeast culture media) for 4 hr at 28°C. Cells were harvested, lysed, the cell density was determined at 600 nm, and the β-galactosidase activity was measured at 420 nm using ONPG as substrate. The data represent the mean from three replicates in a single experiment.

Vitellogenin Production in Juvenile Rainbow Trout* in Vivo *(Laboratory 9). Juvenile rainbow trout (46 to 174 g), kept in steel tanks at a photoperiod of 12 hr light:12 hr dark, were randomly assorted into experimental groups of six fish, anesthetized with 2-phenoxyethanol, and injected ip with a single dose (adjusted to 1 ml/kg) of the compounds to be tested. The compounds were dissolved in 50% ethanol, 99% ethanol, or peanut oil and injected in the concentrations shown in Figure 2. A preexposure blood sample was taken on day 0 and a final blood sample on day 9, and the vitellogenin level in the plasma was measured by a direct sandwich enzyme-linked immunosorbent assay (ELISA).

For the ELISA, purified vitellogenin for antibody production and standards was obtained from 17β-estradiol-treated rainbow trout. Protein for immunization of rabbits to raise polyclonal antibodies against vitellogenin was obtained by precipitation of trout serum with EDTA and $MgCl_2$ [modified from Wiley et al. (*54*)]. Vitellogenin used for standards and affinity columns was purified by gel filtration (Sephacryl S300HR, Amersham-Pharmacia-Bioteck) followed by anion exchange chromatography (DEAE Sephacel, Amersham-Pharmacia-Bioteck). Antibodies against vitellogenin were obtained from ammonium sulfate-precipitated rabbit antiserum after affinity chromatography on a CNBr-activated Sepharose 4B column (Amersham-Pharmacia-Bioteck) coupled with rainbow trout vitellogenin. The specificity of the antibodies was verified by Western blotting of trout plasma after native– and SDS–polyacrylamide gel electrophoresis (PAGE). The antibodies were used for constructing a direct sandwich ELISA. Microtiter plates were coated with specific antibodies and blocked with BSA (3%). Vitellogenin standards or samples were added followed by incubation with horseradish peroxidase-coupled antibodies. The color development after adding enzyme substrate (OPD) was monitored at 490 and 650 nm. The resulting assay had a linear range of 5 to 50 ng/ml and a detection limit of 500 ng/ml serum. The concentration of vitellogenin in serum at day 0 and day 9 was measured in samples from each fish and the difference in vitellogenin calculated. In cases where the vitellogenin levels were below the detection limit of the assay, the concentration of the sample was set to the detection limit of 0.5 µg/ml. The data express the mean increase in serum vitellogenin for the six fish in the experimental group.

Results

All test chemicals were tested in a blind fashion, and the identities of the chemicals were not revealed until all results had been reported to the project coordinator. All laboratories also tested their own positive control (17β-estradiol) and a hormone-free control. After reporting, all data were recalculated in reference to the positive control from the test panel to ensure comparability of the results. Some laboratories examined a range of concentrations; other laboratories chose to use only a single concentration of the test chemicals in accordance with their routine procedure for short-term tests.

For each *in vitro* assay, the response to a chemical was judged as fully estrogenic if the response was >75%, partially estrogenic if the response was 25 to 75%, weakly estrogenic if the response was 10 to 25%, and negative if the response was below 10% of the response induced by 17β-estradiol. In the *in vivo* assay in rainbow trout, the increase in vitellogenin production was steep upon stimulation with the three potent estrogens, 17β-estradiol, 17α-ethynyl estradiol, and DES. Because the maximal response of the individual test compounds in this assay is unknown and different single doses of the chemicals were used for testing, it is not possible to compare the responses directly or to judge the potencies of the chemicals as in the *in vitro* assays.

17β-Estradiol and 17α-ethynyl estradiol exhibited strong estrogenic activity in all assays (Figures 2–7 and Table 3). DES induced a partial estrogenic response in the reporter gene expression assay in MCF-7 cells and in the DY150 yeast assay but induced a full estrogenic response in all other assays. The relative binding affinities (RBA) of 17α-ethynyl estradiol and DES were higher than that for 17β-estradiol in both binding assays. In one laboratory using the MCF-7 cell proliferation assay, the response curve for 17β-estradiol showed a decrease at concentrations above 0.0001 µM, indicating a toxic response. The known cytotoxic compound colchicine induced no estrogenic response in any of the assays but was clearly toxic in most assays.

The antiestrogens tamoxifen and ICI 182.780 bind strongly to the recombinant hER from MCF-7 cells with affinities similar to 17β-estradiol. ICI 182.780 also binds strongly to ER from rabbit uterus, whereas the binding affinity of tamoxifen

Table 3. Relative binding affinities of the test chemicals in the two binding assays.

Test compound	IC_{50}, M recombinant hER	RBA[a] recombinant hER	IC_{50}, M rabbit uterus ER	RBA[a] rabbit uterus ER
17β-Estradiol	1.45×10^{-9}	1	2.0×10^{-11}	1
17β-Ethynyl estradiol	6.7×10^{-10}	2.2	2.0×10^{-12}	10
DES	2.5×10^{-10}	5.8	7.0×10^{-15}	2857
Tamoxifen	2.6×10^{-9}	0.6	1.2×10^{-7}	1.7×10^{-4}
ICI 182.780	3.6×10^{-9}	0.4	4.0×10^{-12}	5
Testosterone	$>2\times10^{-4}$	–	$>1.0\times10^{-5}$	–
Bisphenol A	1.1×10^{-5}	1.3×10^{-4}	1.6×10^{-6}	1.3×10^{-5}
Bisphenol A dimethacrylate	$>2\times10^{-4}$	–	4.3×10^{-6}	4.7×10^{-6}
4-*n*-OP	4×10^{-6}	3.6×10^{-4}	$>1.0\times10^{-5}$	–
4-*n*-NP	4.3×10^{-6}	3.4×10^{-4}	1.8×10^{-6}	1.1×10^{-5}
NP12EO	5.7×10^{-5}	2.5×10^{-5}	$>1.0\times10^{-5}$	–
BBP	1.2×10^{-5}	1.2×10^{-4}	$>1.0\times10^{-5}$	–
DBP	$>2\times10^{-4}$	–	$>1.0\times10^{-5}$	–
Methoxychlor	$>2\times10^{-4}$	–	6.5×10^{-6}	3.1×10^{-6}
o,p'-DDT	5×10^{-7}	2.9×10^{-3}	3.4×10^{-6}	5.9×10^{-6}
p,p'-DDE	1.6×10^{-5}	9.1×10^{-5}	$>1.0\times10^{-5}$	–
Endosulfan	1.3×10^{-5}	1.2×10^{-4}	$>1.0\times10^{-5}$	–
Chlormequat chloride	5.6×10^{-5}	2.6×10^{-5}	$>1.0\times10^{-5}$	–
Colchicine	$>2\times10^{-4}$	–	$>1.0\times10^{-5}$	–
Ethanol	NC	–	NC	–

Abbreviations: NC, no competition for the binding of 17β-estradiol. RBA, relative binding affinities. [a]RBA was calculated as the ratio between the binding affinity (IC_{50}) of 17β-estradiol and the binding affinity of the test compound to the ER in the two binding assays.

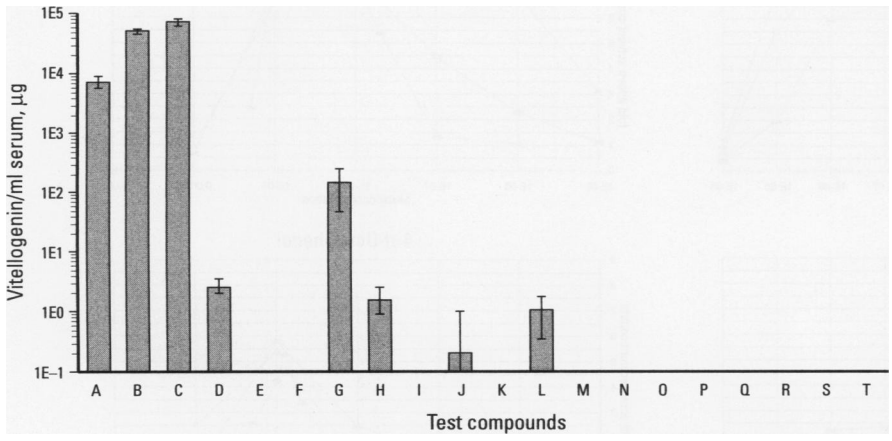

Figure 2. Increase in vitellogenin production in juvenile rainbow trout 9 days after a single intraperitoneal injection of the test compounds at the doses indicated. The increase in vitellogenin production was calculated as the serum concentration at day 9 minus the serum concentration at day 0. *A*, 17β-estradiol (0.5 mg/kg in 50% ethanol); *B*, 17α-Ethynyl estradiol (5 mg/kg in peanut oil); *C*, DES (5 mg/kg in 50% ethanol); *D*, tamoxifen (50 mg/kg in 99% ethanol); *E*, ICI 182.780 (50 mg/kg in 99% ethanol); *F*, testosterone (not investigated); *G*, bisphenol A (50 mg/kg in 50% ethanol); *H*, bisphenol A dimethacrylate (50 mg/kg in peanut oil); *I*, 4-*n*-octylphenol (50 mg/kg in peanut oil); *J*, 4-*n*-nonylphenol (50 mg/kg in peanut oil); *K*, nonylphenol dodecylethoxylate (50 mg/kg in 50% ethanol); *L*, benzylbutylphthalate (500 mg/kg in peanut oil); *M*, dibutylphthalate (500 mg/kg in peanut oil); *N*, methoxychlor (100 mg/kg in peanut oil); *O*, *o,p'*-DDT (50 mg/kg in peanut oil); *P*, *p,p'*-DDE (100 mg/kg in peanut oil); *Q*, endosulfan (5 mg/kg in peanut oil); *R*, chlormequat chloride (250 mg/kg in peanut oil); *S*, colchicine (1 mg/kg in peanut oil); *T*, ethanol (1 ml/kg).

for this receptor was only 1/6000th the binding affinity of 17β-estradiol (Table 3). In the proliferation assay in MCF-7 cells, tamoxifen induced an estrogenic response in one laboratory (77% of the maximum response induced by 17β-estradiol), a weak response in another laboratory (21% of the 17β-estradiol response), and no response in the third laboratory using this assay. ICI 182.780 induced no estrogenic activity in any of the three laboratories using the E-SCREEN assay (Figure 3). Reporter gene expression in MCF-7 cells was below the hormone-free control for both antiestrogens (Figure 5). In yeast, ICI 182.780 exhibited full estrogenic activity in all assays (Figures 4,7) except one (laboratory 10) using the yeast strain DY150 (Figure 5). Tamoxifen responded as a partial estrogenic agonist in all the yeast assays, though only weakly in yeast DY150. In Saccharomyces expressing the hER, a maximal response was observed at tamoxifen concentrations just below 1 µM, whereas the response at higher concentrations was decreased because of toxicity (Figure 4). Tamoxifen, but not ICI 182.780, induced vitellogenin production in rainbow trout. However, the level of vitellogenin produced was only 0.04% of the level induced by 17β-estradiol at 1% of the dose of tamoxifen (Figure 2).

Although no ER binding was registered in the binding assays (Table 3), testosterone exhibited full estrogenic activity in the proliferation as well as the reporter gene expression assays in MCF-7 cells (Figures 3,5). Maximal proliferation was induced at 1/1000th to 1/10000th the 17β-estradiol concentration inducing maximal response. Testosterone induced a weak reporter gene activity in Saccharomyces expressing the hER but only at concentrations above 10 µM (Figure 4), while no response was induced in any of the other yeast assays (Figures 6,7) in which only one concentration of 10 µM testosterone was tested. Testosterone was not investigated in the vitellogenin assay in rainbow trout because we were unable to obtain sufficient quantities of the chemical because of legal restrictions for importing steroid hormones.

In the binding assay using recombinant hER, *o,p'*-DDT was the most effective of the environmental chemicals tested in reducing the binding of [^{3}H]17β-estradiol; binding affinities were 1/350th of the binding affinity of 17β-estradiol (Table 3). The binding affinity of *p,p'*-DDE was considerably lower, i.e., approximately 1:10,000 compared to the binding affinity of 17β-estradiol. Also, 4-*n*-OP and 4-*n*-NP had similar affinities for the ER of approximately 1:3000 that of 17β-estradiol, whereas the ratio for bisphenol A, BBP, and endosulfan was about 1:8000. Both NP12EO and chlormequat chloride only barely reduced the binding of [^{3}H]17β-estradiol to hER with binding affinities of approximately 1/40,000th of the binding affinity of 17β-estradiol. Bisphenol A dimethacrylate, DBP, methoxychlor, and colchicine all had IC_{50} values above the limit of 200 µM for nonspecific binding.

The binding affinities of the environmental chemicals for the ER from rabbit uterus were in general lower than for the hER from MCF-7 cells, and only five

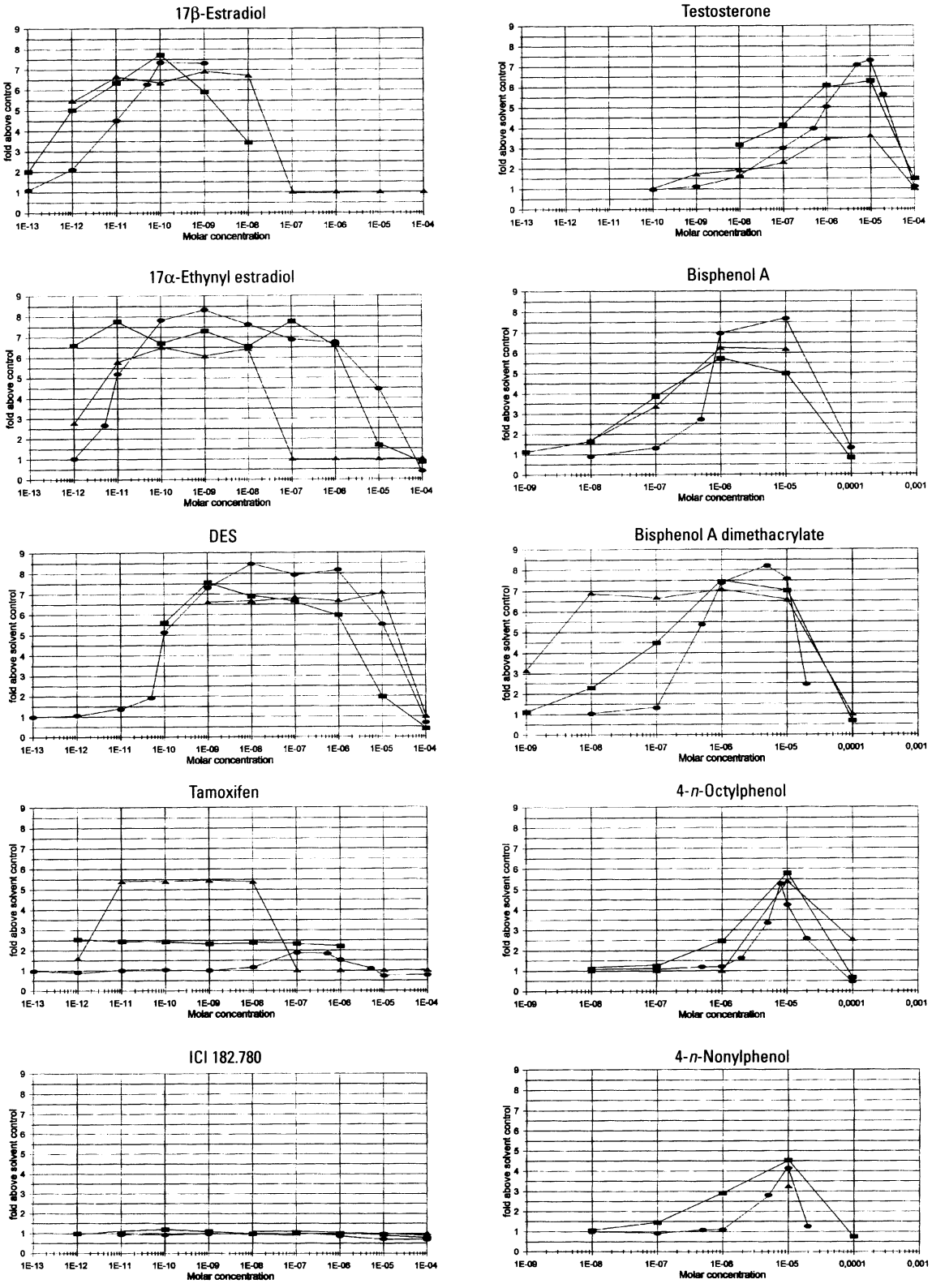

Figure 3. Effects of the test chemicals on proliferation of MCF-7 cells expressed as fold increase in cell number above hormone-free control as a function of the log concentration of the test compound. The test compounds were added to cells growing in medium supplemented with 10% CD-treated human serum (laboratory 1 and 3) or 5% CD-treated FBS (laboratory 2) and incubated for 6 days. Laboratory 1 (■), laboratory 2 (●), and laboratory 3 (▲). (*Continued on next page*)

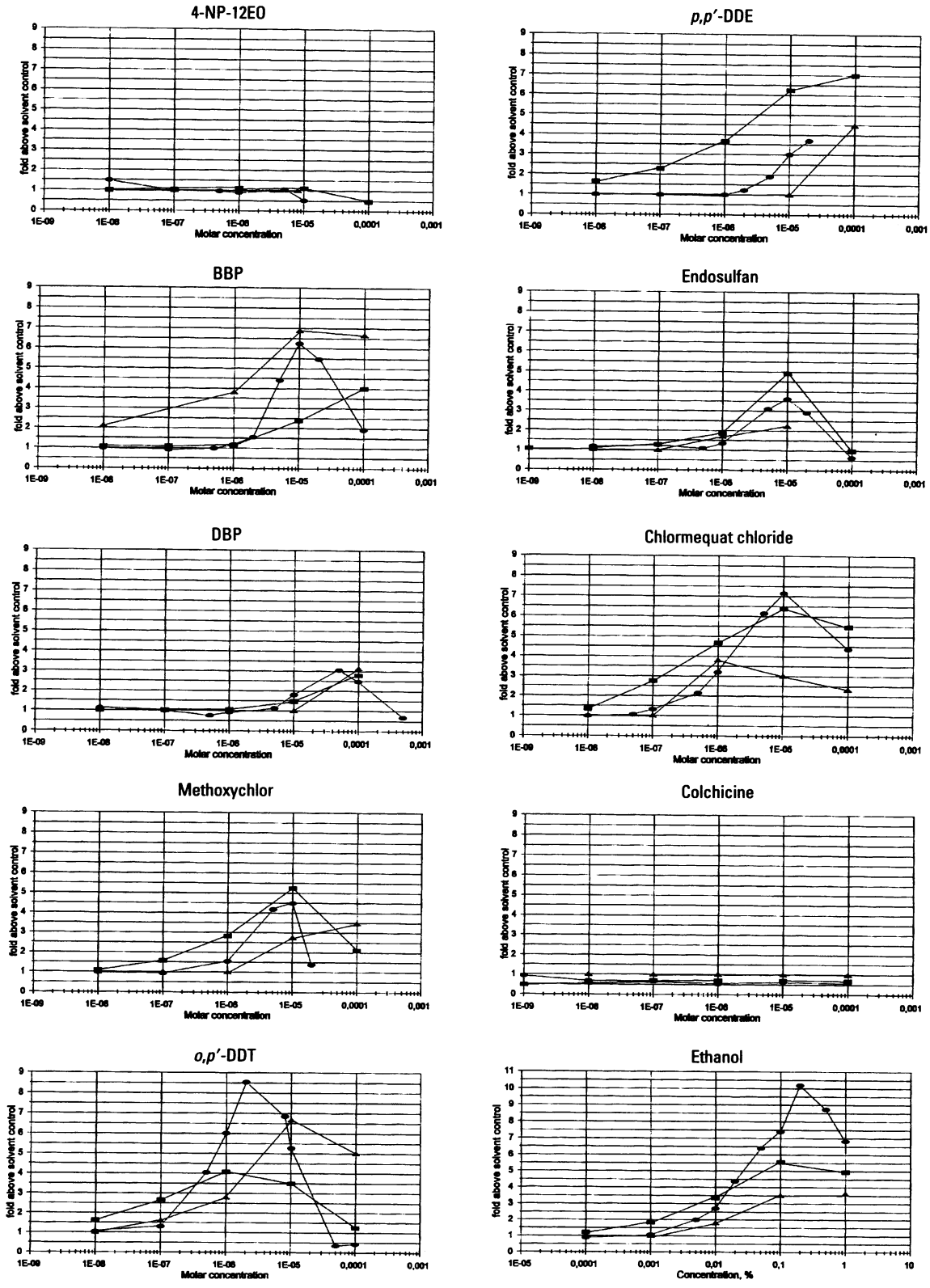

Figure 3. *Continued.*

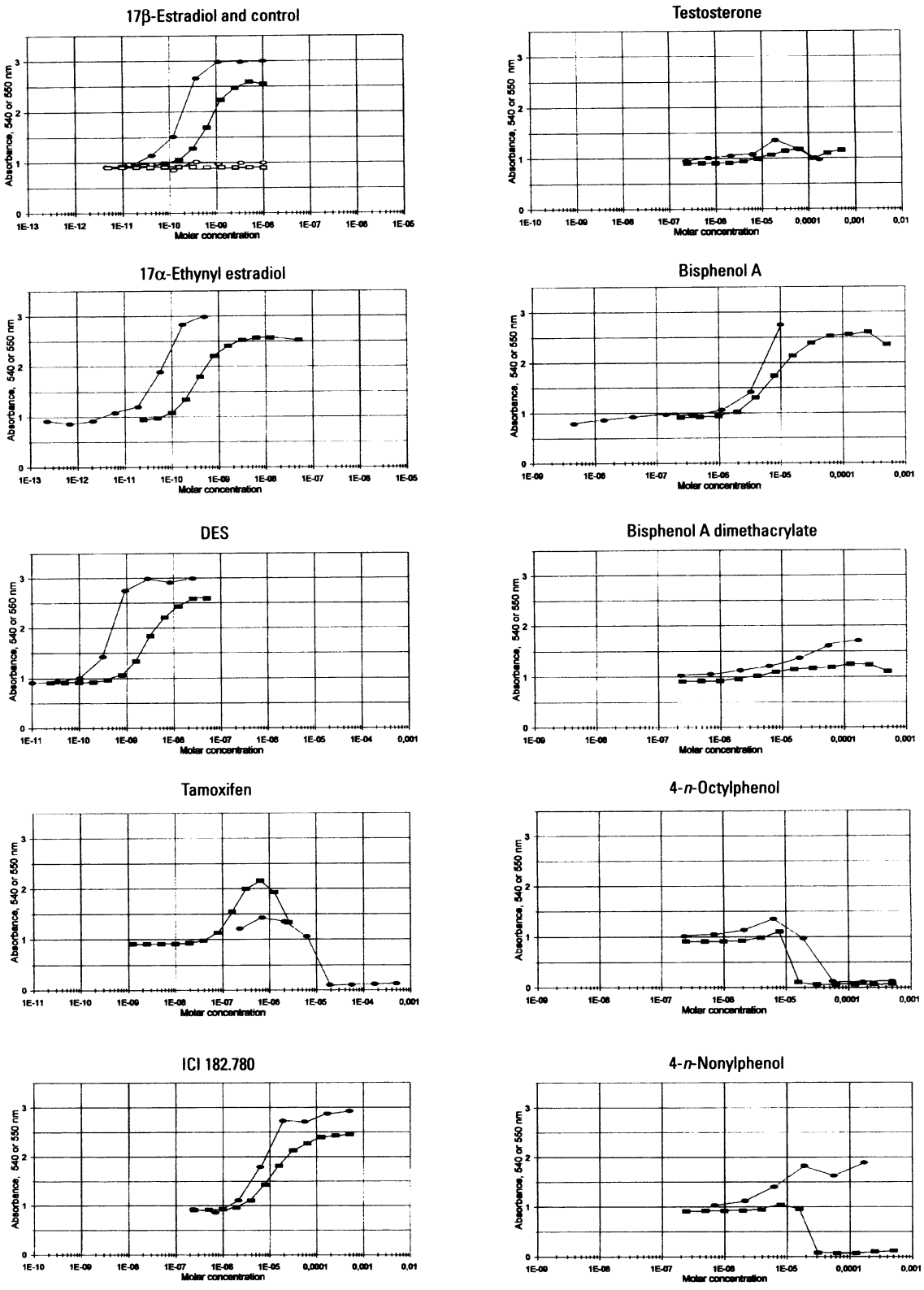

Figure 4. Effects of the test chemicals on β-galactosidase activity in yeast (Saccharomyces) expressing the hER plotted against the log concentration of the test compounds. Incubation time was 72 hr. Laboratory 4 (■) and laboratory 6 (●); control, □ and ○. (*Continued on next page*)

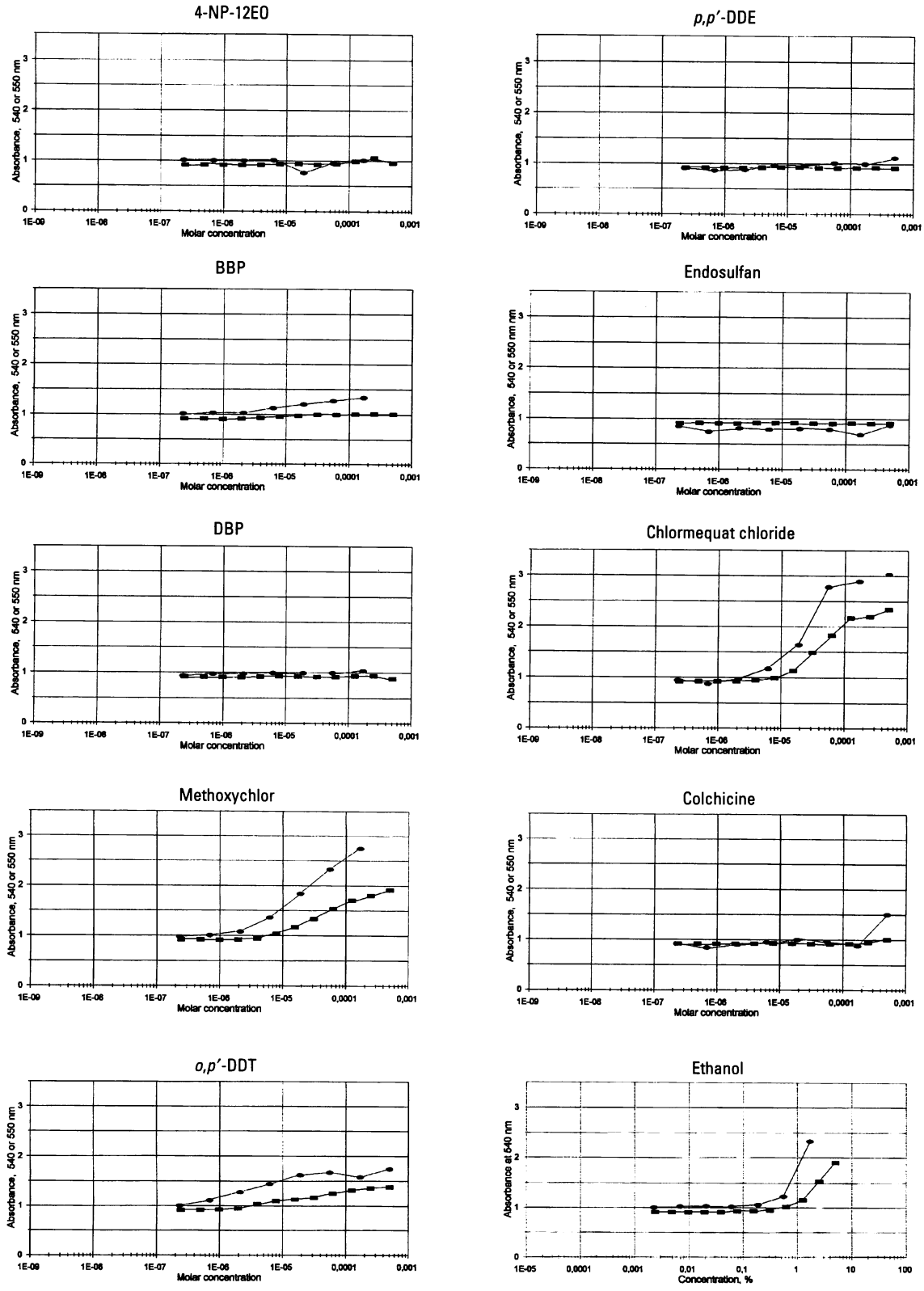

Figure 4. *Continued.*

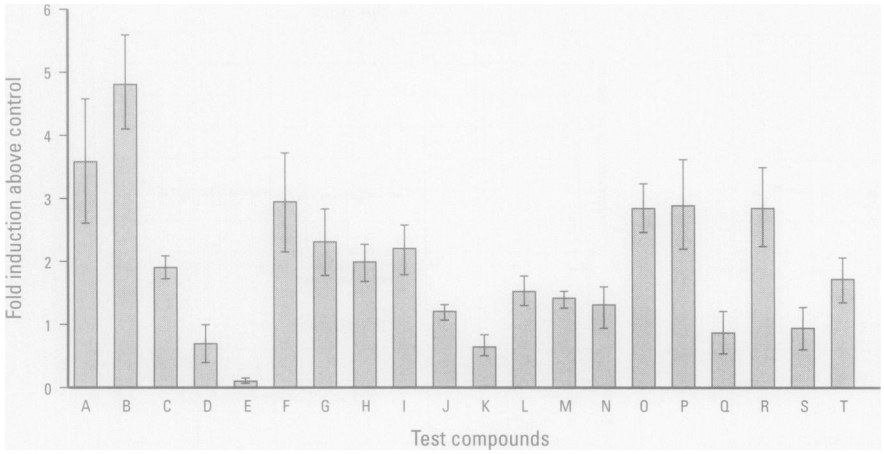

Figure 5. Transient reporter gene expression in MCF-7 cells. The results are expressed as the ratio between the activity of CAT in cells exposed to test compounds (concentrations as stated below) and cells exposed to hormone-free solvent after 48-hr incubation. *A*, 17β-Estradiol (0.01 μM); *B*, 17α-Ethynyl estradiol (1.11 μM); *C*, DES (0.63 μM); *D*, tamoxifen (2.5 μM); *E*, ICI 182.780 (2.5 μM); *F*, testosterone (10 μM); *G*, bisphenol A (10 μM); *H*, bisphenol A dimethacrylate (10 μM); *I*, 4-*n*-octylphenol (2.5 μM); *J*, 4-*n*-nonylphenol (2.5 μM); *K*, nonylphenol dodecylethoxylate (0.63 μM); *L*, benzylbutylphthalate (5 μM); *M*, dibutylphthalate (10 μM); *N*, methoxychlor (2.5 μM); *O*, *o,p′*-DDT (2.5 μM); *P*, *p,p′*-DDE (10 μM); *Q*, endosulfan (2.5 μM); *R*, chlormequat chloride (2.5 μM); *T*, ethanol (0.1%).

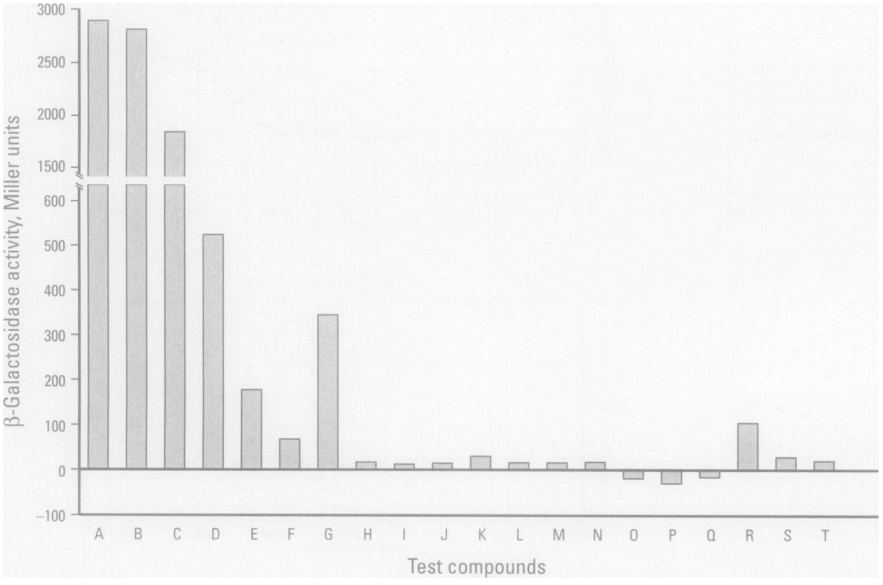

Figure 6. β-Galactosidase activity in yeast (DY150) expressing the hER after 18-hr incubation with the test compounds. The final concentration tested was 10 nM for 17β-estradiol and 10 μM for all other test compounds. *A*, 17β-estradiol; *B*, 17α-ethynylestradiol; *C*, DES; *D*, tamoxifen; *E*, ICI 182.780; *F*, testosterone; *G*, bisphenol A; *H*, bisphenol A dimethacrylate; *I*, 4-*n*-octylphenol; *J*, 4-*n*-nonylphenol; *K*, nonylphenol dodecylethoxylate; *L*, benzylbutylphthalate; *M*, dibutylphthalate; *N*, methoxychlor; *O*, *o,p′*-DDT; *P*, *p,p′*-DDE; *Q*, endosulfan; *R*, chlormequat chloride; *T*, ethanol (0.1%).

chemicals had IC_{50} values below 1000 μM, i.e., the limit for nonspecific binding in this assay (Table 3). Of the environmental chemicals, bisphenol A and 4-*n*-NP had the highest binding affinities of 1:8000 and 1:90,000, respectively, compared to the binding affinity of 17β-estradiol, whereas *o,p′*-DDT showed an affinity ratio of 1:170,000. Bisphenol A dimethacrylate and methoxychlor also bind to the ER, although with low affinity. No binding of these two compounds was detected in the other binding assay based on recombinant hER.

Among the environmental chemicals, bisphenol A, bisphenol A dimethacrylate, BBP, *o,p′*-DDT, and chlormequat chloride reacted as full agonists in the MCF-7 cell proliferation assays by inducing cell proliferation to an extent (75 to 100%) similar to that caused by 0.1 nM 17β-estradiol in at least two of the three laboratories using this assay (Figure 4). Bisphenol A and bisphenol A dimethacrylate were the most potent chemicals because they induced a full estrogenic response at 1 μM or 1/10,000th of the concentration needed of 17β-estradiol to induce maximal response. Although 4-*n*-OP and 4-*n*-NP had higher binding affinities than bisphenol A and bisphenol A dimethacrylate in the recombinant hER binding assay, these two compounds reacted only as partial agonists in the E-SCREEN assay, inducing maximal proliferation of 70 and 50% 17β-estradiol, respectively, at 10 μM. NP12EO did not induce proliferation of MCF-7 cells in any of the laboratories. *o,p′*-DDT induced a full agonistic response at concentrations of 2 and 10 μM in two of the laboratories using this assay and a maximal response of 46% of 17β-estradiol at 1 μM in the third laboratory. *p,p′*-DDE reacted as a partial agonist in two laboratories and as a full agonist in one laboratory, inducing maximal responses of 30, 60, and 90% of 17β-estradiol at 10 or 100 μM in the three laboratories, respectively. Endosulfan reacted as a partial agonist in two laboratories and as a weak agonist in one laboratory, inducing maximal responses of 60, 33, or 21% of 17β-estradiol at 5 to 10 μM. At higher concentrations the cell number was lower than in control cells, indicating a toxic response. Chlormequat chloride reacted as a full agonist in two laboratories, inducing proliferations of approximately 80% of 17β-estradiol at concentrations of 5 or 10 μM, and as a partial agonist in the third laboratory, inducing maximal proliferative response of 50% of 17β-estradiol at 1 μM and a lower response at higher concentrations. Methoxychlor reacted as a partial agonist, inducing maximal proliferative responses of approximately 40 to 60% of 17β-estradiol at 5, 10, or 100 μM in the three laboratories. DBP induced a response of 30 to 40% of 17β-estradiol in all three laboratories at relatively high concentrations (50 to 100 μM), whereas BBP induced a considerably higher response of 45% at 100 μM, 83% at 10 μM, or 100% at 10 μM of 17β-estradiol in the three laboratories, respectively.

When the environmental chemicals were tested for their ability to stimulate the transcriptional activity of the ER in MCF-7 cells, the strongest response was observed for

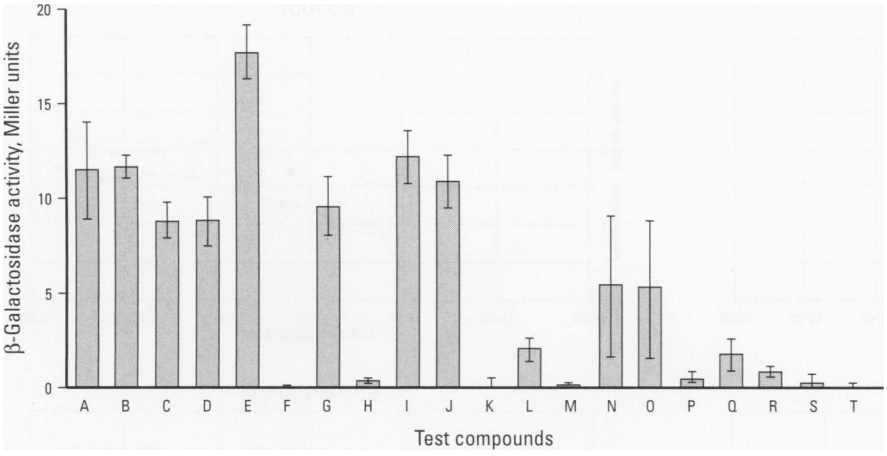

Figure 7. β-Galactosidase activity in yeast (Saccharomyces) expressing the rtER after 4-hr incubation with the test compounds at 28°C. The final concentration tested was 10 nM for 17β-estradiol and 10 μM for all other test compounds. *A*, 17β-estradiol; *B*, 17α-ethynyl estradiol; *C*, DES; *D*, tamoxifen; *E*, ICI 182.780; *F*, testosterone; *G*, bisphenol A; *H*, bisphenol A dimethacrylate; *I*, 4-*n*-octylphenol; *J*, 4-*n*-nonylphenol; *K*, nonylphenol dodecylethoxylate; *L*, benzylbutylphthalate; M, dibutylphthalate; *N*, methoxychlor; *O*, *o,p'*-DDT; *P*, *p,p'*-DDE; *Q*, endosulfan; *R*, chlormequat chloride; *T*, ethanol (0.1%).

o,p'-DDT, *p,p'*-DDE, and chlormequat chloride, which at concentrations of 2.5, 10, and 2.5 μM, respectively, induced increases in CAT activity 2.5 to 3 times higher than the hormone-free control level (Figure 5) or 70 to 75% of the response induced by 10 nM 17β-estradiol. Bisphenol A (10 μM), bisphenol A dimethacrylate (10 μM), and 4-*n*-OP (2.5 μM) reacted as partial estrogen agonists by increasing CAT activity 2.3-, 2.0-, or 2.2-fold above control (or 50, 38, or 46% of 10 nM 17β-estradiol), respectively. The response to 4-*n*-NP (2.5 μM) was considerably weaker; it was only 1.2-fold above control or 7% of 10 nM 17β-estradiol. Also methoxychlor (2.5 μM), BBP (5 μM), and DBP (10 μM) induced only weak responses of 1.3- to 1.5-fold above controls (11 to 19 % of 10 nM 17β-estradiol). NP12EO, endosulfan, and colchicine did not induce CAT activity above the control level. As expected, colchicine was toxic to the cells at low concentrations of 0.01 μM and above.

The ability of the test compounds to induce reporter gene activity in yeast (Saccharomyces) expressing the hER was investigated by two laboratories. The response to all test chemicals, including 17β-estradiol, in one of these laboratories was in general slightly higher than the corresponding response in the other laboratory. The concentration of 17β-estradiol that induced maximal activity was 0.001 or 0.005 μM in the two laboratories, respectively (Figure 4). If 17β-estradiol was added directly to the medium, a maximum response was reached at a lower concentration than when 17β-estradiol was evaporated to dryness on the assay plate (Figure 8). A similar effect was observed for DES and 17α-ethynyl estradiol (data not shown).

At 10 or 60 μM, bisphenol A increased reporter gene activity in yeast to a level similar to that of 17β-estradiol (Figure 4). Bisphenol A dimethacrylate induced a weak or partial estrogenic response (20 or 33% of 17β-estradiol) at 100 to 200 μM. Besides bisphenol A, only chlormequat chloride induced a full estrogenic response in both laboratories. Methoxychlor reacted as a full agonist, inducing β-galactosidase activity to 84% of 17β-estradiol at 170 μM in one laboratory and as a partial agonist, inducing a response of 60% of 17β-estradiol at 500 μM in the other laboratory. *o,p'*-DDT induced a partial agonistic response of approximately 30% of 17β-estradiol at 500 μM and a flat dose–response curve in both laboratories, whereas *p,p'*-DDE was inactive, in both laboratories. Of the two phthalates, BBP induced a weak response of 7 to 14% that of 17β-estradiol at 170 to 250 μM in both laboratories and DBP was inactive, at the concentrations tested. Both 4-*n*-OP and 4-*n*-NP were toxic to the yeast. The toxic effect was pronounced in one of the laboratories in which the yeast was killed at concentrations above 10 μM, thus possibly obscuring an estrogenic effect. In the other laboratory a partial estrogenic response was induced by 4-*n*-NP showing a maximum of 40% of the 17β-estradiol response at 20 μM 4-*n*-NP. Endosulfan and NP12EO did not induce β-galactosidase activity above control levels in either of the two laboratories when the chemicals were evaporated on the assay plate. In laboratory 6 all test chemicals were retested by adding the chemicals directly to the medium instead of evaporating the test compound before adding the medium and yeast. This procedure led to a higher response for some of the test chemicals: bisphenol A dimethacrylate, *o,p'*-DDT, and BBP (Figure 8). NP12EO, which did not induce activity when the chemicals were evaporated to dryness on the assay plate, now induced a full agonistic response (85% of 17β-estradiol) at 380 μM when the chemical was added directly to the medium. In addition, 4-*n*-OP and 4-*n*-NP induced a more pronounced toxic response when the chemicals were added directly to the medium. These results indicate that at least some chemicals may adhere to the surface of the assay plates when the test compounds are evaporated to dryness on the plate before yeast and medium are added, thus leading to lower availability of the test compound.

The incubation time used in both laboratories was 72 hr for all test substances. However, incubation time has a pronounced effect on the level of β-galactosidase activity induced by several chemicals. In laboratory 6 the activity induced for all test chemicals was determined after both 72 and 93 hr. After 93 hr, β-galactosidase activities induced by 4-*n*-OP, 4-*n*-NP, bisphenol A dimethacrylate, and *o,p'*-DDT were considerably higher than after 72 hr (data not shown).

In the yeast strain DY150, a single concentration of 10 μM of each compound (10 nM for 17β-estradiol) was tested. Bisphenol A induced a weak estrogenic response after 18 hr incubation; the other environmental chemicals were inactive. The β-galactosidase activity after addition of *o,p'*-DDT, *p,p'*-DDE, and endosulfan was even below control, thus possibly indicating a toxic response to the high concentrations used in this assay (Figure 6).

Also in the yeast system expressing the rtER, only a single concentration of 10 μM of each compound (10 nM for 17β-estradiol) was tested. The yeast was incubated with the test chemicals for 4 hr before the activity of β-galactosidase activity was measured. Among the environmental chemicals, 4-*n*-NP, 4-*n*-OP, and bisphenol A reacted as full agonists in this assay; *o,p'*-DDT and methoxychlor were partial agonists, inducing responses approximately half that of 10 nM 17β-estradiol. BBP and endosulfan were weak agonists, inducing responses of 18 and 16% of 17β-estradiol;

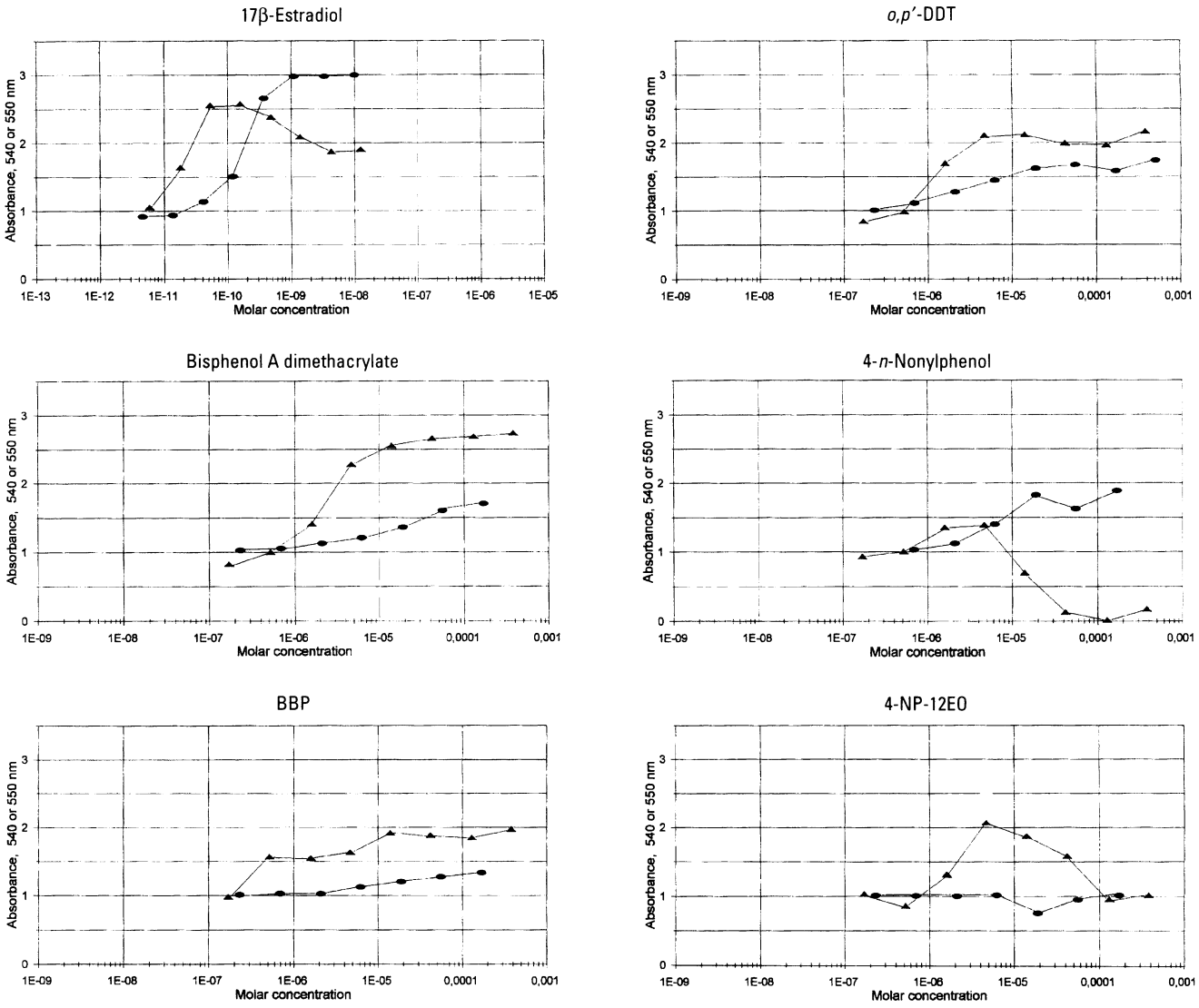

Figure 8. Activity of β-galactosidase activity in yeast (Saccharomyces) expressing the hER in laboratory 6 when the test compounds were allowed to evaporate on the assay plate (●) and when the test compounds were added directly to the medium (▲). The results are plotted against the log concentration of the test compounds.

the remaining compounds induced a response similar to the hormone-free control (Figure 7). Cell density was not affected by any of the test compounds, indicating the absence of cytotoxic effects in this assay.

Vitellogenin is a classic estrogen-inducible protein synthesized in the liver of oviparous vertebrates under the control of estrogen. It is normally synthesized in females and the production increases markedly in serum during oocyte development. The vitellogenin gene is present but usually not expressed in males or juveniles. However, exposure to estrogens, estrogen mimics, or aromatase inducers can elicit vitellogenin synthesis in males (*4*). Only three of the environmental chemicals tested induced increased vitellogenin production in juvenile rainbow trout using the present test conditions and concentrations of test substances (Figure 2). The doses were selected to be approximately one-tenth of the median lethal dose values reported in mammals or fish if the data were available. The strongest response was induced by bisphenol A administered in a dose of 50 mg/kg. BBP (500 mg/kg) and bisphenol A dimethacrylate (50 mg/kg) induced a weak increase in vitellogenin production. 4-*n*-NP (50 mg/kg) induced a low increase in vitellogenin production of only 0.003% of the response induced by 17β-estradiol (0.5 mg/kg); the vitellogenin production was enhanced only in one of six fish and, considering the standard deviation, the response could not be regarded as different from those of untreated fish. Chlormequat chloride at the dose used (250 mg/kg) killed all six fish in the group within a few minutes; therefore, the ability of this chemical to induce vitellogenin synthesis could not be evaluated within the limits of this study.

In some of the assays (E-SCREEN, transient reporter gene expression in MCF-7 cells, and the recombinant yeast assay expressing the hER) an estrogenic response was induced by the ethanol used as solvent control in the test panel (Figures 3–5). In the E-SCREEN the highest dilution of ethanol that induced a response above hormone-free controls was 10,000- to 100,000-fold (or 0.01 to 0.001% ethanol), corresponding to a concentration of 0.1 to

1 μM of the other test chemicals. A maximal response was induced at 500- to 1000-fold dilutions, corresponding to 10 to 20 μM of the test compounds. In yeast a response was induced at dilutions lower than 100-fold, corresponding to concentrations higher than 100 μM of the test compounds or 1% ethanol. In the transient reporter gene assay in MCF-7 cells, 0.1% ethanol induced a response of 27% of the response seen with 10 nM 17β-estradiol. The ethanol used was analyzed by mass spectrometry, and no contaminants were detected even after the ethanol was concentrated 100-fold. However, the detection limit of mass spectrometry might not be low enough to detect trace amounts of strong estrogens. When samples of ethanol of the same brand but from a previously unopened bottle were retested, no response was induced in the two MCF-7 cell assays. In addition, testing for memory effect or contamination from test vials, pipette tips, and gloves using this ethanol showed no sign of estrogenicity.

Discussion

This comparison study in 10 different laboratories compares results obtained after blind testing 20 compounds by a range of short-term assays for estrogenicity. The testing was successfully performed, and the results allow comparison of the sensitivity of the assays for different types of chemicals and the reproducibility between laboratories of the E-SCREEN and the yeast estrogen assay. We will review the results in relation to previous findings for the test chemicals. On this basis we will then discuss the validity and usefulness of the test methods used.

As expected, 17β-estradiol, 17α-ethynyl estradiol, and DES induced an estrogenic responses in all test systems. The binding affinity of DES for ER was higher than the binding affinity of 17β-estradiol, and the potency of DES in the MCF-7 proliferation assay was approximately one-tenth that for 17β-estradiol. These results are in good agreement with those in earlier reports (*48*).

The binding affinities of the test chemicals varied considerably between the two binding assays (Table 3). The binding affinity of tamoxifen to recombinant hER from MCF-7 cells was similar to that of 17β-estradiol, whereas the binding affinity of tamoxifen to ER from rabbit uterus was only 1/6000th of the binding affinity of 17β-estradiol. Only three of the environmental chemicals had binding affinities above the level of nonspecific binding in both binding assays. These chemicals were bisphenol A, 4-*n*-NP, and *o,p'*-DDT. Kuiper et al. (*55*) show that the ER exists as two different subtypes: ER-α and ER-β. Differences in relative ligand binding affinity and tissue distribution of the two ER subtypes could possibly explain some of the discrepancies in binding affinities observed in the two binding assays. Another possible explanation is that the recombinant hER binding assay is based on isolated ER without any other cellular constituents, whereas the LBA based on rabbit uterus includes cytosol-containing cellular proteins with possible metabolizing capacity. The existence of metabolizing enzymes is supported by the ability of methoxychlor to inhibit the binding of ^{3}H-17β-estradiol to the ER in this assay because only the demethylated metabolite, not methoxychlor itself, binds to the ER (*40,56*).

The E-SCREEN assay was performed in three different laboratories. In one laboratory the MCF-7 cell proliferation was determined by direct cell counting; a staining technique was used in the other two laboratories. For several of the test compounds, good agreement was observed among the results obtained in the three laboratories. However, tamoxifen, testosterone, BBP, *o,p'*-DDT, *p,p'*-DDE, and chlormequat chloride showed discrepant responses in the three laboratories (Figure 3). ER expression in MCF-7 cells is modified by factors such as growth rate and cell density (*57*), and different MCF-7 stocks may exhibit different responses to estradiol and chemicals with estrogenic activity (*49*). However, cells from the same MCF-7 stock (BUS) were used in all three laboratories. Binding proteins (SHBG and albumin) seem to be present in charcoal-stripped serum and approximately 85% of estradiol is bound to plasma proteins in the E-SCREEN assay when 10% CD-treated serum was added to the medium (*58*). In this study CD-treated serum was prepared in the individual laboratories, and minor differences in protein content cannot be excluded. In two of the laboratories, 10% CD-treated serum—and in one laboratory 5% CD-treated serum—was added to the test medium. Because the proliferation was induced to similar degrees by 17β-estradiol in the three laboratories, this difference in procedure apparently does not affect the results.

It has been discussed whether cell proliferation or activation of reporter genes is the most reliable end point to estimate estrogenic potency (*5*). In this study, the proliferation assay (E-SCREEN) and the reporter gene expression assay in MCF-7 cells showed good agreement regarding chemicals identified as estrogenic or nonestrogenic, although the classification as full, partial, or weak estrogens varied somewhat between the assays (Figures 3 and 5). In the reporter gene expression assay, the test compounds were examined at only one concentration after ensuring that it was the highest nontoxic concentration to the cells. Hence, the response obtained will probably be close to the maximal estrogenic response of the test chemical in this assay. The responses calculated as percentages of the response induced by 17β-estradiol were in general lower in the reporter gene expression assay than in the proliferation assay; therefore most chemicals identified as full or partial estrogenic agonists in the proliferation assay were judged as partial or weak agonists, respectively, in the reporter gene assay. Only one of the environmental chemicals (bisphenol A) induced a full estrogenic response in both assays. Endosulfan induced no reporter gene activation above the hormone-free control at 25 μM but a weak proliferation response at 5 to 10 μM. Differences in sensitivity to estrogens between different MCF-7 cell stocks (*49*) may contribute to deviations in the response pattern between the two assay systems because different stocks of MCF-7 cells were used.

The assay based on hER expression in Saccharomyces was used in two different laboratories. Their results were similar, although one laboratory consistently tended to obtain slightly higher results than the other laboratory (Figure 4). However, for most of the chemicals the two laboratories agreed on classification as strong, partial, or weak estrogens, or as nonestrogenic. The yeast assay is not always capable of discriminating between antagonists and agonists, as the pure antiestrogen ICI 182.780 and the partial antiestrogen tamoxifen both induced increased β-galactosidase activity. This finding is in agreement with earlier observations (*59*) and thus indicates that both tamoxifen and ICI 182.780 bound to ER are able to induce expression of an ERE-linked reporter gene in yeast. The nature of agonist-receptor versus antagonist-receptor interaction and the resulting altered transcriptional activity are poorly understood. Metzger et al. (*60*) reported that the regions of hER that are important for activation of the transcription of estrogen-sensitive reporter genes in yeast and mammalian cells may be different. These

regions are not believed to be involved in the binding of estrogens to the receptor. By comparing results obtained in yeast with results obtained in, e.g., MCF-7 cells, more information may be revealed regarding the mechanisms involved in antagonistic versus agonistic responses.

The stimulation of proliferation and transcriptional activity in MCF-7 cells by testosterone is in agreement with earlier findings and is thought to be due to conversion of testosterone to estradiol catalyzed by a high activity of aromatase present in these cells (*61,62*). In the different yeast strains, testosterone induced no response or a very weak response—a finding that is in accordance with earlier observations (*15,27*) and the notion that yeast has no aromatase activity.

Among the environmental chemicals tested, bisphenol A induced the highest estrogenic response in all the assays, albeit the relative binding affinity for ER and the relative potency in the E-SCREEN and in the Saccharomyces hER assays were all a factor of 10,000 to 100,000 less than 17β-estradiol (Table 4). These results are in accordance with earlier reports on the binding affinity of bisphenol A for the ER (*16,26*) and the induced proliferation in MCF-7 cells (*26*). Low doses of this chemical may induce biologic responses *in vivo*. When bisphenol A was fed to pregnant mice, doses of only 2 and 20 μg/kg/day significantly increased the adult prostate weight of the males exposed *in utero* (*16*). In the present study a single dose of 50 mg/kg induced a marked increase in vitellogenin production in rainbow trout (Figure 2). Concentrations of bisphenol A capable of inducing proliferation in MCF-7 cells have been detected in the liquid phase of preserved vegetables from lacquer-coated cans and in water autoclaved in the cans (*24*). In addition bisphenol A and bisphenol A dimethacrylate have been detected in saliva collected during a 1-hr period after treatment with a dental sealant based on bisphenol A diglycidylether methacrylate (*26*). In Olea et al. (*26*) as well as in our study, bisphenol A dimethacrylate induced cell proliferation in MCF-7 cells with a potency similar to bisphenol A (Table 4). Also in Saccharomyces containing the hER, a weak positive response was induced by bisphenol A dimethacrylate (Figure 4). However, the binding of bisphenol A dimethacrylate to recombinant hER was below the limit for nonspecific binding, whereas a weak binding to the ER from rabbit uterus was observed (Table 3). Olea et al. (*26*) also reported a low binding affinity of bisphenol A dimethacrylate for cytosol ER. Despite the low binding affinity for the ER, bisphenol A dimethacrylate induced vitellogenin production in rainbow trout and a strong estrogenic response in MCF-7 cells. This indicates that bisphenol A dimethacrylate might be metabolized to bisphenol A in these test systems and probably also in the LBA based on cytosol from rabbit uterus.

Alkylphenol polyethoxylates (APEs) are nonionic surfactants produced worldwide at >300,000 tons annually (*4*).The alkyl group is typically a branched nonyl, octyl, or dodecyl chain. Nonylphenol ethoxylates (NPEs) are the most commonly used APEs, constituting about 80% of the production. The primary biodegradation of NPEs is the hydrolytic removal of ethoxylate groups. This step is relatively rapid and results in degradation intermediates NP, NP monoethoxylate, and NP diethoxylate, all of which are rather lipophilic and stable in the environment (*18*). For NP12EO, weak binding to the recombinant hER was detected, but the IC_{50} was near the range of nonspecific binding. No estrogenic response was induced in any of the other assays performed in this study except in the yeast assay when the chemical was added to the medium instead of evaporating it on the test plate (Figure 8). The reason for this lack of response needs further clarification. Although NP12EO seems rather toxic to all the cell types even at low concentrations, the compound itself does not seem to have any estrogenic potential and the cell types used in the assays included are probably not capable of metabolizing NP12EO to NP or NP diethoxylate (*31*).

Table 4. Relative potencies of the test compounds in the E-SCREEN and the yeast assay based on Saccharomyces expressing the hER.

	EC_{50}[a] E-SCREEN			RP[b] E-SCREEN			EC_{50}[a] yeast assay		RP[b] yeast assay	
Test compound	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6	Lab 4	Lab 6
17β-Estradiol	6×10^{-13}	7×10^{-12}	1×10^{-13}	1	1	1	8×10^{-10}	2×10^{-10}	1	1
17β-Ethynyl estradiol	NE	8×10^{-12}	3×10^{-12}	–	0.9	0.03	5×10^{-10}	6×10^{-11}	1.6	3.3
DES	NE	8×10^{-11}	NE	–	0.05	–	3×10^{-9}	5×10^{-10}	0.2	0.4
Tamoxifen	R<50	R<50	4×10^{-12}	–	–	0.03	R<50	3×10^{-7}	–	7×10^{-4}
ICI 182.780	R<50	R<50	R<50	–	–	–	2×10^{-5}	8×10^{-6}	4×10^{-5}	3×10^{-5}
Testosterone	2×10^{-7}	6×10^{-7}	R<50	3×10^{-6}	1×10^{-5}	–	R<50	R<50	–	–
Bisphenol A	2×10^{-7}	7×10^{-7}	2×10^{-7}	3×10^{-6}	1×10^{-5}	5×10^{-7}	1×10^{-5}	5×10^{-6}	8×10^{-5}	4×10^{-5}
Bisphenol A dimethacrylate	9×10^{-8}	3×10^{-7}	2×10^{-9}	7×10^{-6}	2×10^{-5}	5×10^{-5}	R<50	R<50	–	–
4-*n*-OP	4×10^{-6}	5×10^{-6}	5×10^{-6}	2×10^{-7}	1×10^{-6}	2×10^{-8}	R<50	R<50	–	–
4-*n*-NP	8×10^{-6}	1×10^{-5}	NE	8×10^{-8}	7×10^{-7}	–	R<50	R<50	–	–
NP12EO	R<50	R<50	R<50	–	–	–	R<50	R<50	–	–
BBP	R<50	5×10^{-6}	1×10^{-6}	–	1×10^{-6}	1×10^{-7}	R<50	R<50	–	–
DBP	R<50	R<50	R<50	–	–	–	R<50	R<50	–	–
Methoxychlor	4×10^{-6}	5×10^{-6}	R<50	2×10^{-7}	1×10^{-6}	–	4×10^{-4}	3×10^{-5}	2×10^{-6}	7×10^{-6}
o,p'-DDT	1×10^{-6}	5×10^{-7}	2×10^{-6}	6×10^{-7}	1×10^{-5}	5×10^{-8}	R<50	R<50	–	–
p,p'-DDE	2×10^{-6}	3×10^{-5}	7×10^{-5}	1×10^{-7}	2×10^{-7}	1×10^{-9}	R<50	R<50	–	–
Endosulfan	6×10^{-6}	R<50	R<50	9×10^{-7}	–	–	R<50	R<50	–	–
Chlormequat chloride	7×10^{-7}	2×10^{-6}	1×10^{-6}	–	4×10^{-6}	1×10^{-7}	7×10^{-5}	3×10^{-5}	1×10^{-5}	7×10^{-6}
Colchicine	R<50	R<50	R<50	–	–	–	R<50	R<50	–	–

Abbreviations: NE, value could not be estimated from the response curve; R<50, maximal response observed for the test chemical at the concentrations tested was below 50% of the maximal 17β-estradiol response; RP, relative potency. [a]EC_{50} expresses the concentration of test compound needed to produce 50% of the maximal response induced by 17β-estradiol. EC_{50} values were extrapolated from the response curves in Figures 2 and 3 for those test compounds inducing responses above 50% of the maximal 17β-estradiol response. [b]RP was calculated as the ratio between EC_{50} for 17β-estradiol and EC_{50} for the test compound.

Although most of the alkylphenols used in different products are branched, this study included the well-defined unbranched alkylphenols 4-*n*-OP and 4-*n*-NP. Binding affinities to recombinant hER for 4-*n*-OP and 4-*n*-NP in this study were relatively high, with IC_{50} values of 4.0 and 4.3 µM, respectively, corresponding to binding affinities of approximately 1/3000th that of 17β-estradiol (Table 3). These affinities did not differ much from binding affinities to ER isolated from rainbow trout liver for mixed isomers of 4-*tert*-OP and 4-NP (*31*). 4-NP may be released from certain types of plastic centrifuge tubes and then induce cell proliferation and increases in progesterone receptors in MCF-7 cells, and it triggers mitotic activity in rat endometrium (*33*). In this study both 4-*n*-OP and 4-*n*-NP induced cell proliferation and transcriptional activation in MCF-7 cells, albeit to a different level than that of 17β-estradiol (Figures 3 and 5). 4-*n*-OP was the most potent of the two alkylphenols, which is in accordance with findings of most previous studies (*18,31*). However, a recent study reported that 4-NP had higher estrogenic potency than 4-OP (*23*). Maximal response in the proliferation assay was seen at concentrations of 10 µM, and toxic effects occurred at higher concentrations. Both chemicals were also toxic to the yeast strain expressing the hER (Figure 4). 4-*n*-OP killed the yeast in both laboratories using this assay and only a weak estrogenic response was induced at low doses not associated with cytotoxicity. 4-*n*-NP induced a partial agonistic response in one laboratory but killed the yeast in the other laboratory. In a previous study using the same assay conditions, both 4-*tert*-OP and 4-NP stimulated β-galactosidase activity to a similar extent as 17β-estradiol but with potencies of 1/1000th and 1/30,000th that of 17β-estradiol (*35*). In another yeast assay using the strain BJ2407 containing the hER, overnight incubation with 10 µM 4-OP significantly increased β-galactosidase activity to a similar extent as 17β-estradiol although the potency was 1000-fold less than 17β-estradiol (*15*).

In the assay using Saccharomyces containing the rtER, a strong response was induced by both alkylphenols at 10 µM (Figure 7). Differences in the primary structure of especially the *N*-terminal domain of rtER and hER and differences in binding affinity for 17β-estradiol between the two ER receptors have been reported (*53*). Thus, the discrepancy in the results between yeast containing the hER and yeast containing the rtER might be due to differences in transactivation of β-galactosidase activity caused by differences in binding affinities between the two receptors and the alkylphenols tested. Another possible explanation is that the shorter incubation time used in the rtER yeast assay (4 hr) than in the hER yeast assay (72 hr) may preclude a toxic effect.

Although both alkylphenols activated the rtER in the yeast assay, neither 4-*n*-OP nor 4-*n*-NP induced increased vitellogenin synthesis in juvenile rainbow trout at the doses investigated in this study (Figure 2). NP induces vitellogenin production in rainbow trout after exposure to only 10 ppb NP in a flow-through system for 72 hr (*32*). In addition, 4-NP and 4-*tert*-OP both induced synthesis of vitellogenin in rainbow trout hepatocytes *in vitro* (*63*). In rodents, low doses of NP (10 µg/day for 11 days) increased proliferation of the mammary epithelial cell of female rats by 200% compared to that of controls (*34*).

The apparent discrepancy between some of the results obtained for the two alkylphenols is probably due to the use of either unbranched or technical grade isomer mixtures, and interpretation is hampered by the lack of detail on the isomer characteristics of the alkylphenols used. The estrogenic potency of alkylphenols is likely to depend on both the position and branching of the alkyl group (*35*). 4-Tertiary branched alkyl groups of six to eight carbons located at the *para* position were most estrogenic and approximately 30-fold more potent than 4 normal and 4 secondary equivalents when evaluated in a yeast assay (*35*). In another yeast assay technical grade NP was approximately twice as potent as straight-chain *n*-NP in inducing β-galactosidase activity after 18 hr of incubation (*23*). In the rodent uterotropic assay technical NP, but not *n*-NP, induced a dose-related positive response (*12*).

With regard to the phthalates, both BBP and DBP bind to the ER from rainbow trout liver and induce proliferation and reporter gene activation in MCF-7 cells (*6*) and in yeast (*28*). BBP and DBP were the most potent of a range of phthalate esters evaluated in a recombinant yeast assay inducing maximal responses of 50 and 35% of the 17β-estradiol response, respectively. The most potent compound, BBP, was approximately 1,000,000-fold less potent than estradiol (*28*). In the present study BBP bound to the recombinant hER (but not rabbit uterus ER) (Table 3) and induced full or partial estrogenic responses in the E-SCREEN (Figure 3) and weak estrogenic responses in the reporter gene assays in MCF-7 cells (Figure 5), yeast (Figures 4,7), and in the *in vivo* vitellogenin assay. The potency of BBP in the E-SCREEN was estimated to be 1/10,000,000 to 1/1,000,000 that of 17β-estradiol. DBP was inactive in most of the assays, except the two MCF-7 cell assays in which a weak or partial estrogenic response was induced. Hence, the results obtained are in good agreement with previous findings that BBP and DBP are weakly estrogenic compounds, with BBP being the most potent.

Several organochlorine pesticides induce estrogenic responses *in vivo* (*64*) and *in vitro* (*25,65*). In agreement with other studies (*21*), a strong binding affinity of *o,p'*-DDT was found for recombinant hER, and a strong estrogenic response was demonstrated in MCF-7 cells either by increased proliferation or induction of transcriptional activity. The relatively low estrogenic response induced in yeast expressing the hER was similar to previously reported results when the same yeast strain and assay conditions were used (*27*) and may be caused by reduced availability of *o,p'*-DDT due to adherence to the surface of the assay plate. Thus, a higher and steeper response curve was induced if *o,p'*-DDT was added directly to the medium (Figure 8). In a study using another yeast strain (BJ2407) and direct addition of *o,p'*-DDT to the medium, a full estrogenic response to 1 and 10 µM *o,p'*-DDT was observed (*21*). The major DDT metabolite *p,p'*-DDE had a lower binding affinity for the ER and showed less estrogenicity in MCF-7 cells than *o,p'*-DDT—in agreement with earlier reports (*43*). In yeast, *p,p'*-DDE was unable to induce β-galactosidase activity at the concentrations tested. Besides the weak estrogenicity of *p,p'*-DDE, this compound is an androgen receptor antagonist (*43*).

In agreement with other studies, methoxychlor induced an estrogenic response in MCF-7 cells (*5*). A positive response was also seen in the yeast (Figures 4,7), thus indicating that both MCF-7 cells and yeast can provide the appropriate metabolic transformation of methoxychlor to the estrogenic demethylated metabolite (*40*).

Endosulfan induces proliferation of MCF-cells at 10 µM to a level similar to that of 10 pM 17β-estradiol and induces

progesterone receptors (*5*).The binding affinity of endosulfan for ER was approximately 1/10,000th that of 17β-estradiol (*5*), which was also found for recombinant hER in this study (Table 3). A weak increase in β-galactosidase activity induced by endosulfan has been reported in the yeast strain BJ2407 expressing the hER (*66*). In the present study endosulfan induced only a weak proliferative response in MCF-7 cells (Figure 3) and in yeast expressing the rtER (Figure 7) and no estrogenic response in the transactivation assay in MCF-7 cells (Figure 5) or in the yeast assays containing the hER (Figure 4).

None of the pesticides—*o,p'*-DDT, *p,p'*-DDE, methoxychlor, or endosulfan—induced increased vitellogenin production in rainbow trout in this study (Figure 2). Repeated doses of *o,p'*-DDT, but not *p,p'*-DDE, induced vitellogenin in rainbow trout (*67*) at a total dose similar to the dose level used as a single dose in this study. In a study in catfish endosulfan caused a decrease in vitellogenesis that could be partially reversed by estradiol treatment (*42*).

Chlormequat chloride was included to represent a chlorinated chemical without a ring structure. To our knowledge this compound has not previously been tested for estrogenic effects *in vitro* or *in vivo*. In this study an estrogenic response was induced in several of the assay systems. After the termination of the blinded comparison study, another batch of chlormequat chloride from the same supplier (Ehrenstorfer) together with chlormequat chloride from another supplier (Riedel-de Häen) were retested in the E-SCREEN in laboratory 1 and in the reporter gene expression assay in MCF-7 cells in laboratory 7. Neither cell proliferation nor reporter gene expression was increased above control level for these two samples (data not shown). Hence, it was not possible to reproduce the estrogenic response to chlormequat chloride observed in the comparison study. The estrogenic response could potentially be due to contamination during preparation of the test sample or during synthesis or storage of the batch used.

The present comparison study provides evidence on several important aspects. Ethanol is often used as a solvent for test substances in different assay systems, including those involved in this study. In one study ethanol in concentrations between 0.0001 and 10% increased the growth, as estimated by ^{3}H-thymidine uptake in MCF-7 cells (*68*). To our knowledge, similar effects have not been observed in other studies on MCF-7 cells, and the laboratories participating in this study have no previous experience of ethanol-induced responses in the assays. Hence, the estrogenic response to the ethanol observed in the most sensitive assay systems in this study could theoretically be due to a sporadic ethanol-induced response, but it is most likely due to a cross-contamination by one of the three potent estrogens that were handled immediately before the vials with solvent controls were prepared. If the ethanol was contaminated with one of the strong estrogens included in the test panel, it must have occurred after the preparation of all the other test compounds. Therefore, the results obtained should not be disregarded because of the possibility of contamination. The overall findings are in good agreement with those of previous studies, and some chemicals induced responses lower than the ethanol. Further, for each test substance aliquots from the same stock solution were transferred to the test vials so that all participating laboratories (except for the vitellogenin assay in laboratory 9) tested exactly the same solutions. The inclusion of a solvent control prepared together with strongly estrogenic chemicals shows that laboratory contamination can occur despite rigorous hygiene procedures. Indeed, that possibility was considered when the study was designed. This experience therefore emphasizes that results identifying a new estrogenic compound should be reproducible in more than one laboratory, as there will always be a risk of contamination with potent estrogens present in the laboratory. In addition, it shows the importance of meticulous procedures for handling potent estrogens. Inclusion of solvent controls prepared before, concurrently, and after preparation of the test substances is recommended to document that no cross-contamination has occurred. Pretesting solvent controls in at least one sensitive assay system before distribution to the other participating laboratories could also be considered.

Finally, the data also allow a preliminary assessment of the validity of the tests used, based on the results obtained with similar methods used in different laboratories and with different methods based on related principles. The advantages and disadvantages must be carefully considered, in particular when choosing one or more tests for screening purposes.

The LBAs determine the degree of binding to ER in a simplified test situation and they cannot discriminate between antiestrogens and estrogens. The binding assay based on recombinant hER is sensitive, as determined from the number of substances that are positive in this assay, but estrogenic metabolites of the test chemicals will not be detected. In the binding assay based on ER from rabbit uterine tissue, some metabolism seems to occur, probably because of the presence of metabolizing enzymes in the cytosol during incubation. This assay has a lower sensitivity than the one using recombinant hER and it is more cumbersome.

Although cell cultures involve disadvantages associated with maintaining the cell line and avoiding contamination, their use in test systems offers definite advantages. The estrogenic response induced by methoxychlor and bisphenol A dimethacrylate in MCF-7 cells as well as in yeast cultures indicates that both cell types have the metabolic capacity to convert at least some proestrogens into estrogens. The E-SCREEN assay and the transient reporter gene expression assay in MCF-7 cells are capable of discriminating between antiestrogens and estrogens, and a high sensitivity is indicated by the response to most of the test chemicals at low concentrations. The E-SCREEN is easier to perform than the transient reporter gene activation assay, but transactivation may reveal more information regarding the ability of the test compounds to activate the ER. If the reporter gene construct could be stably transfected into the MCF-7 cells, this assay would be much easier to perform and more suitable for screening purposes. The proliferation assay carried out in three laboratories showed essentially identical results for some of the chemicals but somewhat deviating results for other chemicals. Some of that discrepancy may be due to slight methodological differences (5% CD-treated FBS versus 10% CD-treated serum human serum, cell counting versus SRB staining, plate size, etc). Further standardization of this assay may further improve the reproducibility of the results.

The yeast assays cannot necessarily discriminate between estrogenic and antiestrogenic compounds. Another disadvantage is that yeast seems to be rather sensitive to toxic effects of chemicals (e.g., 4-*n*-NP and 4-*n*-OP), which may complicate the interpretation of the results. Toxic effects probably account for at least part of the discrepancy between results obtained in the different yeast assays. In the assays using DY150 expressing the hER and the assay using Saccharomyces expressing the rtER,

only a single relatively high concentration (10 μM) of each test substance was used. In the former assay only a few chemicals showed a positive response after an 18-hr incubation. In several cases the response was less than the response in untreated controls, thus indicating a toxic response. This observation illustrates the importance of using several concentrations of the test substances or ensuring that the concentrations used are nontoxic to the assay system to avoid false negatives due to estrogenic responses hidden by toxicity. In the Saccharomyces assay with the rtER, toxicity may have been minimized by a shorter incubation time of 4 hr. Hence, the influence on the results of the specific assay conditions as well as the specific yeast strain, the origin of the ER, and the reporter gene construct must be further documented. Because the application method of the test compounds and the incubation time influenced the results, the assays must be optimized to obtain reliable results. When standardized assay conditions are used in different laboratories, the results obtained with the Saccharomyces expressing the hER appear reproducible.

The vitellogenin assay in rainbow trout is more cumbersome and expensive but has the advantage of being a short-term *in vivo* system. This method is less suitable for preliminary screening but may be useful for retesting chemicals that are positive in one of the preliminary assays. However, the exact procedure for the vitellogenin assay should be further evaluated regarding exposure time and dose level to avoid false negative results.

The specificity of the methods cannot be determined in this study because the true estrogenic response is unknown for several of the test compounds. However, as a first rapid screening assay for estrogenicity of chemicals, at least two assays should probably be carried out with a view to high sensitivity, standardized conditions, and ease in performance. Realistic candidates are the recombinant hER LBA and the E-SCREEN, as these assays have high sensitivity, are easy to perform, and in combination provide information on both receptor binding and a cellular response.

In the future development of short-term tests, additional comparisons will be necessary, standardization will be obligatory, and, quite likely, no single test will be found valid either for exclusion or for verification of estrogenicity. Better information on the metabolic capacity of the test systems is needed, and addition of metabolic enzymes to the *in vitro* assays may improve their reliability and comparability. Also, the subtype of ER in the individual assays must be taken into account. Although important differences between the tests have been documented in this study, the results suggest that it will be feasible to design a battery of tests for screening of estrogenicity of environmental chemicals.

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