The E-SCREEN Assay as a Tool to Identify Estrogens: An Update on Estrogenic Environmental Pollutants

Ana M. Soto,1 Carlos Sonnenschein,1 Kerrie L. Chung,1 Mariana F. Fernandez,1 Nicolas Olea,2 and Fatima Olea Serrano3

1Tufts University School of Medicine, Department of Anatomy and Cellular Biology, Boston, Massachusetts; 2School of Medicine, University of Granada, Granada, Spain; 3School of Pharmacy, University of Granada, Granada, Spain

Estrogens are defined by their ability to induce the proliferation of cells of the female genital tract. The wide chemical diversity of estrogenic compounds precludes an accurate prediction of estrogenic activity on the basis of chemical structure. Rodent bioassays are not suited for the large-scale screening of chemicals before their release into the environment because of their cost, complexity, and ethical concerns. The E-SCREEN assay was developed to assess the estrogenicity of environmental chemicals using the proliferative effect of estrogens on their target cells as an end point. This quantitative assay compares the cell number achieved by similar inocula of MCF-7 cells in the absence of estrogens (negative control) and in the presence of 17β-estradiol (positive control) and a range of concentrations of chemicals suspected to be estrogenic. Among the compounds tested, those that mimicked estradiol are considered "new" estrogens were found; alkylphenols, phthalates, some PCB congeners and hydroxylated PCBs, and the insecticides dieldrin, endosulfan, and toxaphene were estrogenic by the E-SCREEN assay. In addition, these compounds competed with estradiol for binding to the estrogen receptor and increased the levels of progesterone receptor and pS2 in MCF-7 cells, as expected from estrogen mimics. Recombinant human growth factors (bFGF, EGF, IGF-1) and insulin did not increase cell yields. The aims of the work summarized in this paper were a) to validate the E-SCREEN assay; b) to screen a variety of chemicals present in the environment to identify those that may be causing reproductive effects in wildlife and humans; c) to assess whether environmental estrogens may act cumulatively; and finally d) to discuss the reliability of this and other assays to screen chemicals for their estrogenicity before they are released into the environment. — Environ Health Perspect 103(Suppl 7):113–122 (1995)

Key words: xenobiotics, cell proliferation, endosulfan, phthalates, antioxidants, alkylphenols

Introduction

For the last 40 years, substantial evidence has surfaced on the hormonelike effects of many xenobiotics in fish, wildlife, and humans (1). The endocrine and reproductive effects of xenobiotics are believed to be due to their a) mimicking effects of endogenous hormones such as estrogens and androgens; b) antagonizing the effects of normal, endogenous hormones; c) altering the pattern of synthesis and metabolism of natural hormones; and d) modifying hormone receptor levels.

Among environmental chemicals found to cause reproductive impairment in wildlife and humans there are estrogen mimics (xenoestrogens). Natural estrogens promote cell proliferation and hypertrophy of female secondary sex organs and induce the synthesis of cell type-specific proteins (2). Xenobiotics of widely diverse chemical structure have estrogenic properties (3,4).

This diversity makes it difficult to predict the estrogenicity of xenobiotics solely on structural bases. To overcome this shortcoming, their identification as estrogens has relied on rodent bioassays. These assays measure either vaginal cornification or the increase in uterine wet weight; however, the latter is not a specific estrogen response (5). To obviate problems inherent to animal testing, quantitative bioassays using cells in culture have been developed. For example, the induction of prolactin in primary sheep pituitary cell culture has been proposed as a measure of estrogen action (6); in this model, estrogens induce protein synthesis but are ineffective at inducing cell proliferation. The limitations of this assay are that some estrogen-inducible genes could also be induced by nonestrogenic substances. For example, prolactin synthesis may be induced by EGF, thyrotropin releasing factor, and phorbol esters (7). Another estrogen-inducible marker, ovalbumin synthesis, is stimulated by other steroids such as progesterone and glucocorticoids (8). Also, induction of reporter genes under control by estrogen-responsive elements has been
proposed to assess estrogenicity; however, elevated basal expression in the absence of estrogen often occurs, and this may raise concern about the reliability of these assays. Therefore, the proliferative effect of natural estrogens on the female genital tract remains the hallmark of estrogen action.

Hertz argued convincingly that this proliferative property should be adopted as the one method to determine whether or not a chemical is an estrogen (2). This requires measuring the increase of mitotic activity in tissues of the female genital tract after estrogen administration. However, this method is not suitable for large-scale screening of suspected chemicals and an equally reliable, easy, and rapid-to-perform method would be preferable. The novel E-SCREEN assay fulfills these requirements (9). This assay measures estrogen-induced increase of the number of human breast MCF-7 cells and is recognized as biologically equivalent to the increase of mitotic activity in the rodent endometrium (9,10). The objectives of this study were to validate the E-SCREEN assay and to test the estrogenicity of chemicals released into the environment in large volumes.

Materials and Methods

Cell Line and Cell Culture Conditions

Human breast cancer estrogen-sensitive MCF-7 cells were obtained from the Michigan Cancer Foundation (Detroit, MI) (11). For routine maintenance, cells were grown in Dulbecco’s modification of Eagle’s medium (DME) (GIBCo, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) at 37°C in an atmosphere of 5% CO₂/95% air under saturating humidity.

Steroids, Xenobiotics, and Growth Factors Tested

17β-Estradiol (E₂) was obtained from Calbiochem (Richmond, CA). Other steroids were purchased from Steraloids (Keene, NH). R26008 (allenolic acid) was supplied by Roussel-UCLA, Romainville, France. Toxaphene (technical grade) and endosulfan (technical grade) were obtained from Chem Services (West Chester, PA). Endosulfan α and β isomers, p, p’-DDT, p,p’-DDT, p,p’-DDD, p,p’-DDE, PCB congener, methoxychlor, dieldrin, phtha-
late esters, and antioxidants were from Ultra Scientific (North Kingstown, RI). Hydroxylated biphenyls were a gift from J. A. McLachlan (National Institute of Environmental Health Sciences, Research Triangle Park, NC [NIEHS]). DES metabolites were a gift of K.S. Korach (NIEHS). Estradiol was stored as a 1-mM stock solution in ethanol at −20°C. Pesticides were dissolved in ethanol to a final concentration of 10 mM, except endosulfan mixed isomers, dieldrin, and toxaphene, which were dissolved in dimethyl sulfoxide (DMSO); they were all diluted to desired concentrations in phenol red-free DME immediately before use. The final solvent concentration in culture medium did not exceed 0.1%; this concentration did not affect cell yields. Human recombinant EGF, basic FGF, and IGF-1 were purchased from Collaborative Research (Lexington, MA).

Plasma-derived and Blood-derived Human Serum

Plasma-derived human serum was prepared from outdated plasma supplied by the New England Medical Center Blood Bank, (Boston, MA). Calcium chloride was added to a final concentration of 30 mM to facilitate clot formation. Blood-derived serum was obtained using blood from healthy adult volunteers; blood was allowed to clot in glass centrifuge tubes for 2 to 4 hr to obtain serum. Plasma- and blood-derived serum were clarified by centrifugation (2000×g for 10 min), heat-inactivated (56°C for 30 min), centrifuged, charcoaled, dextran stripped, and stored in glass tubes at −20°C until use.

Removal of Sex Steroids by Charcoal–Dextran Treatment of Serum

Charcoal (Norit A, acid washed; Sigma Chemical Co, St. Louis, MO) was washed twice with cold sterile water immediately before use. A 5% charcoal–0.5% dextran T70 (Pharmacia-LKB, Uppsala, Sweden) suspension was prepared. Charcoal–dextran (CD) suspension aliquots of a volume similar to the serum aliquots to be processed were centrifuged at 2500 rpm for 10 min. Supernatants were aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal–serum mixture was maintained in suspension by rolling at 4 cycles/min at 37°C for 1 hr. This suspension was centrifuged at 2000×g for 20 min. The supernatant was then filtered through a 0.45-μm Nalgene filter. Over 99% of serum sex steroids were removed by this treatment when determined by removal of 3H-E₂ (12); E₂ levels after CD treatment were less than 0.01 pg/ml when measured by radioimmunoassay. CD sera were stored at −20°C until needed. Samples kept for 1 year in the freezer maintained their inhibitory properties on the proliferation of human estrogen-sensitive breast tumor MCF-7 cells; plasma- and blood-derived sera were equally effective.

The E-SCREEN Test

The E-SCREEN assay was developed based on the following premises: (a) a human serum-borne molecule specifically inhibits the proliferation of human estrogen-sensitive cells (12–16); and (b) estrogens induce cell proliferation by canceling this inhibitory effect (12,13,16). Nonestrogenic steroids and growth factors did not abolish the proliferative inhibition by mammalian serum (12,13).

Cloned MCF-7 cells were trypsinized and plated into 12-well plates (Costar, Cambridge, MA) at initial concentrations of 20,000 cells per well (9,10). Cells were allowed to attach for 24 hr; then, the seeding medium (5% FBS in DME) was removed and replaced by the experimental medium [5% CD human serum supplemented to phenol red-free DME (CDHuS)]. A range of concentrations of the test compounds was added to this medium. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells, adding a cell lysing solution (10% ethylhexadecyl–dimethylammonium bromide [Eastman Kodak Co, Rochester, NY] in 0.5% Triton X-100, 2 mM MgCl₂, 15 mM NaCl, 5 mM phosphate buffer, pH 7.4) and counting the nuclei in a Coulter Counter Apparatus, Model ZM (Coulter Electronics, Hialeah, FL).

The best estimate of the proliferative behavior of a cell population is t₅₀ or doubling time. t₅₀ is the time interval in which an exponentially growing culture doubles its cell number. Determining t₅₀ requires measuring cell yields at several time intervals during the exponential proliferation phase. A less cumbersome alternative to measuring proliferation rates is comparing the cell yield achieved by similar cell inocula harvested simultaneously during the late exponential phase of proliferation. The proliferative effect (PE) is measured as the ratio between the highest cell yield obtained with the test chemical and with the hormone-free control. Under these experimental conditions, cell yield represents a reliable estimate of the relative proliferation rate achieved by similar inocula exposed to different proliferation regulators. In our experimental design, MCF-7 cell yields were measured 6 days after t₅₀; however, significant differences between
control and estrogen-treated cultures are apparent after 4 days (16).

The estrogenic activity of xenoestrogens was assessed a) by determining their relative proliferative potency (RPP), which measures the ratio between the minimal concentration of estradiol needed for maximal cell yield and the minimal dose of the test compound needed to achieve a similar effect; and b) by measuring their relative proliferative effect (RPE), which is 100 times the ratio between the highest cell yield obtained with the chemical and with E2. RPE is calculated as 100×(PE-1) of the test compound/PE-1 of E2. Thus, the RPE indicates whether the compound being tested induces a proliferative response quantitatively similar to the one obtained with E2, that is, a full agonist (RPE = 100), or a proliferative yield significantly lower than the one obtained with E2, that is, a partial agonist (9). Figure 1 displays a schematic representation of these concepts. For screening purposes, the range of xenobiotic concentrations was from 1 nM to 10 μM, and for E2 from 0.1 pM to 1 nM, measured at intervals of one order of magnitude.

**Progesterone Receptor Assay and Estrogen Receptor Processing**

MCF-7 cells were seeded in 25-cm² flasks in 5% PBS-supplemented DME. Twenty-four hours later, the medium was changed to 5% CDHuS, and the chemicals to be tested were added. Control flasks were treated with vehicle. After 72 hr of exposure to the test xenoestrogens, medium was aspirated, the cell layer was rinsed with PBS, and the cells were frozen in liquid N₂. To extract receptor molecules, cells were incubated with 1 ml of extraction buffer (0.5 M KCl, 10 mM potassium phosphate, 1.5 mM EDTA, and 1 mM monothioglycerol, pH 7.4) at 4°C for 30 min (17). After centrifugation to pellet the cell debris, receptor levels were measured in 100-μl extract aliquots by enzyme immunoassay using the Abbott estrogen and progesterone receptors kits (Abbott Diagnostics, Chicago, IL) according to the manufacturer’s instructions.

**pS2 Assay**

Culture media were harvested after 72 hr of exposure to the test chemicals and centrifuged to eliminate floating and detached cells; samples were kept frozen at −80°C until the immunoradiometric assay was performed following the manufacturer’s protocol (ELSA-PS2, CIS Bio International, Gif-sur-Yvette, France).

**Determination of Relative Binding Affinities**

MCF-7 cells were grown in 150-cm² flasks in 5% FBS; they were harvested during the late exponential phase after 24 hr of exposure to 5% CDHuS. Cells were rinsed with PBS, and a suspension of 20×10⁶ cells/ml of buffer (500 mM KCl, 1.5 mM EDTA, 10 mM Tris-HCl, pH 7.4, at 4°C) was sonified at 4°C (5-sec pulses with 30-sec intervals). The cell homogenate was centrifuged at 100,000×g for 40 min, and supernatant aliquots were incubated with 2 nM [3H]-E2 alone and in combination with unlabeled competitors at concentrations ranging from 1 pM to 1 μM E2 or 1 nM to 1 μM xenoestrogens for 16 hr at 4°C. The reaction mixture contained 15% DMSO to solubilize hydrophobic xenoestrogens. This treatment did not alter the shape of the competition curve for E2 and nonylphenol, the only two compounds from which a competition curve could be obtained in the absence of DMSO. Separation of bound and free hormone was done by CD adsorption (18).

**Statistical Analysis**

Results were expressed as the mean ± SE. Proliferation yield experiments conducted in duplicate wells were repeated at least a minimum of 5 times. Mean cell numbers from each experiment were normalized to the steroid-free control (100%) to correct for differences in the initial plating density. Differences between the diverse steroid treatment groups were assessed by analysis of variance and the a posteriori Shaffe’s test (19). A p value of ≤0.05 was regarded as significant.

**Results**

**Proliferative Effect of Compounds Known To Be Estrogenic in Animal Models**

E₂ induced maximal cell yields at 10 to 100 μM using the E-SCREEN assay. Twenty-two compounds reported to have estrogenic activity were also tested. Their RPP is listed in Table 1. Their relative potency measured by the E-SCREEN assay correlated with their relative binding affinity to the estrogen receptor and with their biological effect in uterotropic assays. Exceptions to these correlations have been reported in the literature; they reflect rates of clearance and metabolism of estrogens (20). The E-SCREEN assay mimics exposure to a constant level of hormone, much like that achieved in animals by using estrogen-filled silastic implants. Estriol behaved as a full agonist in the E-SCREEN assay as it did when administered to animals in multiple doses (21). Similarly, the proliferative potency of DES metabolites measured by the E-SCREEN assay paralleled that in the uterotropic assay; however, pseudo-DES and indaneestrol had poor uterotropic activity but were full agonists when assayed by the E-SCREEN test (RPP = 10). The lowered

| Compound                  | RPP, % |
|---------------------------|-------|
| 17β-Estradiol             | 100   |
| 17α-Estradiol             | 10    |
| Estrone                   | 1     |
| Estriol                   | 10    |
| Moxestrol                 | 1000  |
| 16-Hydroxyestrone         | 0.1   |
| Diethylstilbestrol (DES)  | 1000  |
| cis-Tamoxifen             | 0.001 |
| Metabolite E (from tamoxifen) | 0.001 |
| R28008                    | 0.1   |
| 17β-Chloromethylsteradiol | 1000  |
| Indanestril               | 10    |
| Indanestril-A             | 100   |
| Indanestril-B             | 10    |
| Pseudo-DES                | 0.1   |
| Pseudo-DES-e              | 10    |
| Pseudo-DES-z              | 10    |
| Zearalenol                | 1     |
| Zearalenone               | 1     |
| d-Equilenil               | 1     |
| Coumestrol                | 0.001 |
| Ethynyl-estradiol         | 100   |

Relative proliferative potency (RPP) is the ratio between 17β-estradiol and xenoestrogen doses needed to produce maximal cell yields × 100.
The estrogenic potency of these two compounds was attributed to slow processing or clearance of the ligand-bound receptor (20). This notion is at variance with the data discussed below regarding alkylphenols, which are active both in uterotropic and E-SCREEN assays, while displaying diminished processing of estrogen receptors.

Non estrogenic compounds (natural and synthetic progestagens, glucocorticoids, and pesticide derivatives such as mirex, chlordane-α isomer, chlordane, and heptachlor) did not affect the proliferation of MCF-7 cells. No false positives were observed. Moreover, insulin, transferrin, and EGF did not reverse the inhibitory effect of serum (12, 13, 16). This conclusion was validated further by using human recombinant bFGF, EGF, and IGF-1 (Table 2). These results strengthen the reliability and specificity of this "in culture" assay.

Identification of New Xenoestrogens among Antioxidants and Plasticizers

An estrogenic contaminant was isolated from modified polystyrene centrifuge tubes (Corning Glass Co., Corning, NY, Cat. No. 25310-15). After purification by flash chromatography and reverse-phase HPLC, the estrogenic compound was identified by gas chromatography-mass spectrometry as a p-nonylphenol isomer (10). This nonylphenol was a full estrogen for MCF-7 cells (RPP=100; RPP=0.0003 %, Table 3). Nonylphenol also increased the mitotic index of the endometrial epithelium in adult ovariectomized rats. As expected from a genuine estrogen, it also induced progesterone receptors in MCF-7 cells. p-Nonylphenol is 10 to 50 times more potent an estrogen than ethynylestradiol and p,p'-DDT, and it mimics both the proliferative and inductive properties of natural estrogens. Alkylphenols with at least a three-carbon alkyl chain were also found to be estrogenic; p-octylphenol was the more potent one (RPP = 0.03). Other phenolic antioxidants were tested; among them polyclalylated, hindered phenols such as butylated hydroxytoluene (BHT) and Irganox 1640 were not estrogenic, whereas t-butylhydroxyanisole (BHA) was estrogenic. Among phthalate esters used as plasticizers, those derived from alkylalcohols such as dibutylphthalate and dimethylphthalate were not estrogenic, whereas butylbenzylphthalate was estrogenic.

| Table 3. Estrogenic effect of industrial chemicals measured by the E-SCREEN assay. |
|-----------------|---------|--------|-------|--------|
| Compound | Concentration | RPE, % | RPP, % |
| Estradiol | 30 μM | 100 | 100 |
| Phenol | 10 μM | 0 | - |
| 4-Ethylphenol | 10 μM | 5 | - |
| 4-Propylphenol | 10 μM | 17 | - |
| 4-sec-Butylphenol | 10 μM | 76 | 0.0003 |
| 4-tet-Butylphenol | 10 μM | 71 | 0.0003 |
| 4-tet-Pentylphenol | 10 μM | 105 | 0.0003 |
| 4-isopentylphenol | 10 μM | 93 | 0.0003 |
| 4-butoxphenol | 10 μM | 0 | - |
| 4-hexyloxphenol | 10 μM | 0 | - |
| 4-Hydroxybiphenyl | 10 μM | 87 | 0.0003 |
| 4,4'-Dihydroxybiphenyl | 10 μM | 84 | 0.0003 |
| 1-Naphthol | 10 μM | 0 | - |
| 2-Naphthol | 10 μM | 0 | - |
| 5,6,7,8-Tetrahydronaphthol-2 | 10 μM | 0 | - |
| 6-Bromonaphthol-2 | 10 μM | 38 | - |
| 5-Octyphenol | 100 nM | 100 | 0.03 |
| 4-Nonylphenol | 1 μM | 100 | 0.003 |
| Nonylphenol, technical grade | 10 μM | 102 | 0.0003 |
| t-Butylhydroxyanisole | 50 μM | 30 | 0.00006 |
| Benzylbutylphthalate | 10 μM | 90 | 0.0003 |

The lowest concentration needed for maximal cell yield. Indicates the highest concentration tested in culture. The relative proliferative effect is calculated as 100 x (PE-1) of the test compound/PE-1 of E2. A value of 100 indicates that the compound tested is a full agonist, a value of 0 indicates that the compound lacks estrogenicity at the doses tested, and intermediate values suggest that the xenobiotic is a partial agonist. Relative proliferative potency is the ratio between E2 and xenobiotic doses needed to produce maximal cell yields x 100. All compounds designated as full or partial agonists increased cell yields significantly over the hormoneless control (p<0.05).
Table 4. Estrogenic effect of polychlorinated biphenyl (PCB) congeners and hydroxylated PCBs measured by the E-SCREEN assay.

| Compound | Name | Concentration | RPE, % | RPP, % |
|----------|------|---------------|--------|--------|
| 1        | 2,4,6-Trichlorobiphenyl | 10 μM | 77.0 | 0.0001 |
| 2        | 2,4,5-Trichlorobiphenyl | 10 μM | 75.7 | 0.0001 |
| 3        | 2,3,4-Trichlorobiphenyl | 10 μM | 71.2 | 0.0001 |
| 4        | 2,3,5-Trichlorobiphenyl | 10 μM | 61.6 | 0.0001 |
| 5        | 2,3,6-Trichlorobiphenyl | 10 μM | 4.4 | - |
| 6        | 2,4,5,6-Tetrachlorobiphenyl | 10 μM | 3.7 | - |
| 7        | 2,3,4,5,6-Pentachlorobiphenyl | 10 μM | 3.4 | - |
| 8        | 2,3,4,6,5-Hexachlorobiphenyl | 10 μM | 2.7 | - |
| 9        | 2,2',3,5,4'-Hexachlorobiphenyl | 10 μM | 2.2 | - |
| 10       | 2,2',3,3',4'-Hexachlorobiphenyl | 10 μM | 2.2 | - |
| 11       | 2,2',3,4,5'-Pentafluorobiphenyl | 10 μM | 2.0 | - |
| 12       | 2,2',3,4,5'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 13       | 2,2',3,4,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 14       | 2,2',3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 15       | 2,2',3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 16       | 2,2',3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 17       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 18       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 19       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 20       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 21       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 22       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 23       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 24       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 25       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 26       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 27       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |
| 28       | 2,3,4,5,6'-Pentafluorobiphenyl | 10 μM | 1.0 | - |

*Indicates the lowest concentration needed for maximal cell yield. * Indicates the highest concentration tested in culture. Relative proliferative effect is calculated as 100 x (RPE-1) of the test compound/(RPE-1) of E2; a value of 100 indicates that the compound tested is a full agonist, a value of 0 indicates that the compound lacks estrogenicity at the doses tested, and intermediate values suggest that the xenobiotic is a partial agonist. Relative proliferative potency is the ratio between E2 and xenobiotic doses needed to produce maximal cell yields × 100. All compounds designated as full or partial agonists increased cell yields significantly over the hormoneless control (p<0.05). *This compound exhibited submaximal proliferative activity at 100 nM (RPE:62%).

Figure 2. Proliferative activity of HC, hormoneless control; A, estradiol; B, 2,3,4,5,6-pentafluorobiphenyl; C, 2,3,4,5,6-pentafluorobiphenyl; D, 2,3,4,5,6-pentafluorobiphenyl; E, 2,3,4,5,6-pentafluorobiphenyl; F, 2,3,4,5,6-pentafluorobiphenyl. Asterisks (*) indicate significant differences with hormoneless control (p<0.05).

The E-SCREEN test and novel xenoestrogens

Induction of Progesterone Receptor and pS2 by the Newly Identified Xenoestrogens

Pesticides found to be estrogenic by the E-SCREEN assay were also effective inducers of markers of estrogen action such as progesterone receptors (PR) and pS2 in MCF-7 cells (Table 7). Dielndrin and toxaphene were found to be estrogenic at 10 μM. The RPEs of these compounds were lower than those of endosulfan (dieldrin, RPE = 55%; toxaphene, RPE = 52%) (23). The fact that these compounds have toxic effects at concentrations one order of magnitude higher than those needed to evoke a proliferative response precluded assessing whether higher concentrations would attain full estrogenic activity in this bioassay.

Discussion

The deleterious impact of xenoestrogens on the reproductive success, development, and health of animals is well documented; the realization that humans are also exposed and at risk has become increasingly obvious (26). Data showing a lowering of sperm quality and quantity, increased infertility, and spontaneous abortion rates in humans suggest that environmental estrogens play a role in the toxicology of human reproduction and development (27,28). An objective causal relationship between detrimental
Table 5. Xenobiotics tested by the E-SCREEN assay.

| Herbicides | Estrogenic xenobiotics | Nonestrogenic xenobiotics |
|------------|------------------------|--------------------------|
| None       | 2,4-D                  | 2,4-DB                   |
|            | Atachlor               | Atrazine                 |
|            | Butylate               | Cyanazine                |
|            | Dacthal                | Dinoseb                  |
|            | Hexazinone             | Metolachlor             |
|            | Propazine              | Picloram                 |
|            | Trifuraline            | Simazine                 |

| Insecticides |                      |
|--------------|----------------------|
| p,p'-DDT     | Bendiocarb           |
| o,p'-DDT     | Chlorfenvin           |
| o,p'-DDE     | Dieldrin             |
| p,p'-DDE     | Diethane             |
| DDT*         | Lindane              |
| Dieldrin     | Malathion            |
| Chlorodene   | Methoprene           |
| (kepone)     | Parathion            |
| Endosulfan   | Rotenone             |
| α-Endosulfan |                      |
| β-Endosulfan |                      |
| Methoxychlor |                      |
| Toxaphene    |                      |

| Fungicides |                      |
|------------|----------------------|
| None       | Chlorothalonil       |
|            | Hexachlorobenzene    |
|            | Maneb                |
|            | Ziram                |
|            | Metiram              |

| Industrial chemicals | Estrogenic xenobiotics | Nonestrogenic xenobiotics |
|----------------------|------------------------|--------------------------|
| 2,3,4-TCB            | Butylated hydroxytoluene | 2-CB                     |
| 2,2',4,5-TCB         | 2,5-DCB                | 4-5-TCB                  |
| 2,3,4,5,6-TCB        | 2.6-DCB                | 2.3,5-TCB                |
| 2,2',3,3',6',6'-HCB  | 3.5-DCB                | 2.3,5'-TCB               |
| 2-OH-2',5'-DCB       | 2.3,4,5-PCB            | 2,3',4',5',-PCB           |
| 3-OH-2',5'-DCB       | 2,3,4,5-PCB            | DecaCB                   |
| 4-OH-2',5'-DCB       | 2-OH-3,5-DCB           | Diamyl phthalate         |
| 4-OH-2',4',6'-TCB    | 2-OH-3,5-DCB           | Dibutyl phthalate        |
| 3-OH-2',3',4',5'-TCB | 2-3,5'-TCB             | Dimethyl isophthalate    |
| 4-OH-2',3',4',5'-TCB | 4,6-TCDD               | Dimethyl terephthalate   |
| 4-OH-alkyl-phenols   | 2,3,7,8-TCCD           | Dinonyl phthalate        |
| Bisphenol-A          | 2,3,7,8,9-TCCD         | Octachlorostyrene        |
| 4-OH-biphenyl        | Tetracloroethylene     | Styrene                  |
| t-Butylhydroxyanisole|                      |
| Benzylbutyphthalate  |                      |

* Denotes a technical grade isomer mixture.

Health effects and their presumed causation by xenoestrogens is tempered however by the lack of appropriate technology to explore this subject on a large scale. The first obstacle encountered is that the estrogenicity of chemicals cannot be predicted solely on structural bases; therefore, it is unknown how many xenoestrogens are present in the ecosystem. The E-SCREEN bioassay is a reliable tool to rapidly assess estrogenicity on a large number of compounds; in this paper we describe how its use helped to identify estrogens among environmental pollutants. The second obstacle to overcome is how to identify causal agents when signs of estrogen exposure have been verified. The finding of reproductive effects caused by xenobiotics has largely been accidental. For example, workers at a kepone-producing plant developed azoospermia and impotence (29); this was the first observation of reproductive toxicity by kepone. Because of the occupational nature of this case, the culprit kepone became readily apparent. In contrast, wildlife are exposed to a combination of xenobiotics. It became clear in the Great Lakes studies that it was difficult to sort out which one of the xenobiotics played a causal role or whether the signs of intoxication were due to cumulative interaction among the chemicals present in affected water bodies.

Table 6. Estrogenic effect of pesticides according to the E-SCREEN assay.

| Compound | Concentration | RPE, % | RPP, % |
|----------|---------------|--------|--------|
| Estradiol| 10 pM         | 100    | 100    |
| DDT*     | 10 pM         | 79.61  | 0.0001 |
| o,p'-DDT | 10 pM         | 86.14  | 0.0001 |
| p,p'-DDT | 10 pM         | 71.00  | 0.0001 |
| Endosulfan| 10 pM        | 54.89  | 0.0001 |
| p,p'-DDT | 10 pM         | 81.25  | 0.0001 |
| Dieldrin | 10 pM         | 78.28  | 0.0001 |
| β-Endosulfan| 10 pM      | 77.17  | 0.0001 |
| 1-Hydroxychlorodene| 10 pM | 40.00  | 0.0001 |
| Kepone   | 10 pM         | 84.00  | 0.0001 |
| Methoxychlor | 10 pM        | 51.90  | 0.0001 |

Concentration describes the dose at which an estrogenic effect is detected; maximal cell yield is obtained at concentrations between 10 and 100 pM estradiol. Most xenobiotics are active at 10 pM. The RPE measures the ratio between the maximal cell yield achieved by the xenobiotic and that of estradiol. The RPP measures the ratio between the dose of estradiol and that of xenobiotic needed to achieve a proliferative effect. Asterisks (*) denote that the compound referred to was of technical grade.

Table 7. Effect of xenoestrogens on progesterone receptor and pS2 levels.

| Compound | Concentration | PRa | pS2b |
|----------|---------------|-----|------|
| Control  | 7.9 ± 5.4     | 53.9 ± 16.7 |
| Estradiol| 1 nM          | 151.3 ± 49.5* | 179.6 ± 50.2 |
| Endosulfan| 1 μM         | 71.0 ± 13.1a  | 129.2 ± 6.2a  |
| Endosulfan| 10 μM        | 136.0 ± 27.2a | 172.1 ± 61.8a |
| Toxaphene| 1 μM          | 18.1 ± 9.2   | 59.5 ± 6.2   |
| Toxaphene| 10 μM         | 128.7 ± 19.1a| 108.0 ± 17.6a|
| Dieldrin | 1 μM          | 8.2 ± 1.7    | 210.3 ± 29.9a|
| Dieldrin | 10 μM         | 32.3 ± 11.7  | 232.7 ± 32.8a|

a Progesterone receptor (PR) levels are expressed as femtomoles per mg cellular protein. b pS2 levels in the culture medium are expressed as ng/106 cells. Each result represents the mean ± SD of three experiments. An asterisk (*) denotes a significant difference with the hormoneless control (p<0.05).

Table 8. Effect of xenoestrogens on estrogen receptor processing.

| Compound | Concentration | Estrogen receptora |
|----------|---------------|--------------------|
| Control  | 183.9 ± 59    |                    |
| Estradiol| 1 nM          | 92.3 ± 23.1        |
| Endosulfan| 1 μM         | 205.1 ± 21.7c     |
| Endosulfan| 10 μM        | 238.3 ± 17.6c     |
| Toxaphene| 1 μM          | 261.9 ± 15.2b,c   |
| Toxaphene| 10 μM         | 288.7 ± 31.7b,c   |
| Dieldrin | 1 μM          | 236.3 ± 21.2b,c   |
| Dieldrin | 10 μM         | 252.2 ± 17.1b,c   |

a Estrogen receptor levels are expressed as femtomoles/mg cellular protein. Each result represents the mean ± SD of at least three experiments. * Denotes a statistically significant difference with the estradiol-treated group (p<0.05).
animals (1). Exposure to environmental estrogens, singly or in combination, may be easily assessed in male fish, reptiles, or birds used as sentinels by measuring their vitellogenin plasma levels. Instead, exposure of females to xenoestrogens is more difficult to ascertain through a marker such as vitellogenin because the serum levels of this protein are high in animals laying eggs (30,31). In addition, there is no comparable marker to ascertain exposure in mammals. Again, the E-SCREEN assay represents the best alternative to resolve this second obstacle.

Animal Bioassays and the E-SCREEN Assay: Differences and Similarities

Diverse animal models and assays have been used to measure estrogenicity. Allen and Doisy (32) and other pioneers of estrogen research used mouse and rat activity units to follow their estrogen purification protocol; the end point of their assay was vaginal cornification. Dodds and Lawson (33) used both the Allen and Doisy assay (32) and the feminization of the feather pattern in brown leghorn capons (33). Others adopted the urogenital assay using single or multiple doses of estrogens over 24- to 72-hr periods in immature or ovariecestomized mice and rats. This diversity of end points indicates that there is no universal "gold standard" of estrogen action among animal bioassays. The E-SCREEN assay appears to be the best candidate for establishing a quantitative standard of estrogenic activity at the target organ level. As shown in Tables 1 through 6, no false positives or negatives were observed among the estrogens and nonestrogens tested.

No qualitative differences could be found when comparing animal assays and the E-SCREEN assay; that is, the estrogenic properties of compounds characterized using animal bioassays was also ascertained using the E-SCREEN test. From a pharmacokinetic perspective, the latter measures estrogenicity at the target cell level under conditions where estrogen levels are mostly constant, much like the ones achieved when animals are treated with estrogen-filled silastic implants. This approach is more relevant to chronic environmental exposure than that of measuring acute effects after a single dose. In both types of assays, metabolism of the suspected xenoestrogen into more or less active compounds is uncertain and should be defined individually for each compound. For example, nonylphenol diethoxy-lation is estrogenic for MCF-7 cells; since it does not compete for estradiol binding to the estrogen receptor, it is likely that estrogen activity results from nonylphenol diethoxy-late metabolism to the free phenol (22). Methoxychlor was also believed to be inactive until metabolized to free phenols, presumably in the liver; again, methoxychlor tested positive when assayed by the E-SCREEN test. Therefore, even though the putative proestrogens tested so far were estrogenic when assayed by the E-SCREEN test, an added step in the quest for identifying all xenoestrogens may include their metabolic activation by liver microsomal extracts prior to their testing by the E-SCREEN assay.

Regarding quantitative effects, while kepone is 100,000 to 1,000,000 times less potent than estradiol according to the E-SCREEN assay, an increase of the rat uterine wet weight comparable to that of estradiol occurred with a 1000- to 5000-fold higher dose of chlordecone than that of estradiol (3). This discrepancy may be due to rapid metabolism of estradiol and persistence and bioaccumulation of chlordecone in animals. Differences between results in culture and in live animals reflect the different parameters used as a measure of estrogenicity. On one hand, the rodent assay measures the increase of uterine wet weight [water imbibition, hypertrophy (which is also produced by estrogen antagonists), and hyperplasia] (5), while on the other hand, the human E-SCREEN bio-assay measures cell proliferation only. This is a necessary and sufficient parameter to define estrogen action (2).

Table 9. Relative binding affinities (RBAs) and IC50 values of xenoestrogens.

| **Compound** | **RBA** | **RPP** | **IC50** | **Concentration** |
|-------------|--------|--------|---------|-----------------|
| Estradiol   | 0.00024| 0.001  | 1.5±0.4 nM | 100 pM          |
| β-Endosulfan| 0.00032| 0.0001 | 63.1±0.88 μM| 10 μM           |
| Nonylphenol | 0.00001| 0.00001| 4.7±0.38 μM | 10 μM           |
| p-Nonylphenol| 0.0031 | 0.0001 | 485.0±42 μM | 10 μM           |
| p-Methoxytoluene | 0.021 | 0.001 | 7.2±3 μM | 1 μM           |

*Concentration needed for maximal proliferative effect. All xenoestrogen IC50 values were significantly different from that of estradiol.

New Estrogens Identified by the E-SCREEN Assay

Novel xenoestrogens were found among antioxidants, plasticizers, polychlorinated biphenyl (PCB) congeners, and pesticides.

- **Alkylphenols** are used as antioxidants and in the synthesis of detergents [alkylphenol polyethoxylates, (APEs)]. APEs are used as industrial detergents in the textile and paper industries, in toiletries, and as spermicides. Four hundred and fifty million pounds of APEs were sold in the United States in 1990. APEs are not estrogenic per se; however, they are degraded during sewage treatment. The polyethoxylate chain is shortened, and free alkylphenols as well as mono and diethoxylates are produced. The free phenols are estrogenic (9,10). Recently, White et al. (22) have shown that the diethoxylates are also estrogenic. These APE degradation products have been detected in drinking water (34). Nonylphenol has been reported to leach from PVC tubing for milk processing (35) and plastics used in food packaging (36). APEs such as those used as spermicides are degraded to free nonylphenol when administered to rodents (37). The contribution of APEs and alkylphenols to the xenoestrogen burden of humans is unknown; however, it has been reported that these chemicals are present in sewage outlets in concentrations sufficient to feminize sentinel fish (38). Alkylphenols accumulate in river sediment and in the fat of exposed fish (39). Some phenolic antioxidants such as butylated hydroxytoluene (BHT) and z-butylhydroxyanisole (BHA) are used to prolong the shelf life of foodstuffs and to reduce
nutritional losses by retarding oxidation. Interesting observations pertaining to structure–function relationships were made:  

- the alkyl chain must at least have 3 carbons;  
- the p-isomers are more potent estrogens than the m-isomers;  
- polyalkylated, hindered phenols like BHT and Irganox 1640 (Ciba-Geigy, Basel, Switzerland) are not estrogenic while being effective antioxidants; and  
- fused rings like naphthols are not estrogenic in spite of being an integral part of the A and B ring of natural steroids. Instead, substituted naphthols such as 6-Br naphthol and allenolic acid are estrogenic; more studies are needed to assess whether these substituted naphthols are active due to a bulk effect, electronegativity, or because flat molecules such as naphthols and coplanar PCBs are unable to bind tightly to the estrogen receptor.

In addition to the estrogenic alkyphenol antioxidants described above, we found that BHA was estrogenic. BHA is a widely used antioxidant; because it controls oxidation of short-chain fatty acids such as coconut oil (40). Maximal usage levels of BHA permitted by U.S. Food and Drug Administration (U.S. FDA) varies according to the food type, from 50 ppm in dry breakfast cereals to 1000 ppm in active yeast (41).

- Plasticizers are used to decrease the rigidity of certain polymers. For the most part, they are di- and triesters of organic acids. Phthalate esters are widely used plasticizers (42). These compounds leach from plastics, and they have been found to be ubiquitously distributed in the environment, including marine ecosystems (43,44). Among phthalate esters butyl benzyl phthalate was estrogenic whereas those derived from alkyl alcohols such as dibutyl phthalate and diethyl phthalate were not.

- Aroclor 1221 was not estrogenic by the E-SCREEN assay (data not shown). Many congeners are present in Aroclor mixtures; therefore, it is likely that the maximal concentration used, 10 µM, resulted in levels of individual congeners lower than those needed to induce cell proliferation. PCB mixtures such as Aroclor 1221 were reported to be estrogenic using uterotrophic assays as end points (increased uterine wet weight, increased uterine glycogen content) (45). However, the magnitude of the uterine wet weight increase was only marginally significant, analyzed by inappropriate statistical tests and lower than that achieved with E2 or DDT (45,46). Therefore, the estrogenicity of PCB mixtures would be best ascertained by first determining which congeners are estrogenic. Korach et al. (47) demonstrated the ability of certain hydroxy-PCBs to bind to estrogen receptors and to produce an uterotrophic effect that correlated with their relative binding affinity to the estrogen receptor. It is generally assumed that hydroxylated PCBs are estrogenic while nonhydroxylated PCBs are not. In this paper we show that 5 of the 18 congeners studied were estrogenic in the E-SCREEN assay (Table 4). It is unknown whether they were estrogenic per se or they were hydroxylated by MCF-7 cells; hydroxylated PCBs are more potent than their nonhydroxylated counterparts. The phenyl rings in the active compounds were not coplanar. Among the hydroxylated PCBs, those p-hydroxylated were more potent than m-hydroxylated; o-hydroxylated compounds were even less active. Hindered phenols, such as in 4-OH-3,5,-DCB were less estrogenic than unobstructed ones.

- The pesticides dieldrin and toxaphene are estrogenic. Their use has been restricted in the United States since 1974 and 1982, respectively (23). These compounds are highly lipophilic and bioaccumulate in ecosystems; they are still found in wildlife, coincidentally with signs of reproductive impairment. Toxaphene is a main airborne pollutant in North America, and its residues appeared in regions where it has never been used, like the Arctic and Scandinavia (48). It is present in Arctic and Baltic salmon muscle fat at concentrations of 700 to 7000 ppb (49); this concentration is well within those producing estrogenic effects in the E-SCREEN assay (10 µM = 4800 ppb). Endosulfan was introduced in 1954; it is presently used for agricultural purposes in the United States and other countries (50). Proliferative, estrogen-like effects in MCF-7 cells were found at doses of 10 µM (4060 ppb). Endosulfan was shown to produce testicular atrophy in male rats fed a diet containing 10 ppm (51,52); it also lowered gonadotrophin and testosterone plasma levels (53). These results are consistent with its estrogenicity revealed by the E-SCREEN test.

These newly identified estrogens not only induced cell proliferation but pS2 and PR as well; this confirms their estrogen-mimicking properties and the specificity of the E-SCREEN assay as a tool to identify estrogens. These xenoestrogens compete with estradiol for binding to its receptor; their RBA'S correlated well with their potency to induce cell proliferation, pS2, and PR. Recent data suggest that alkylphenols bind to the estrogen-binding domain of the estrogen receptor (22). Binding to the receptor is a necessary but insufficient property to define estrogenicity. Tetrahydroxynaphthol, which is not estrogenic, is effective in preventing the forward binding of estradiol to its receptor while alkylphenols, which are estrogenic, are effective in displacing prebound estradiol from its receptor (54). These data were interpreted as indicative of interactions with more than one site on the estradiol receptor.

Cumulative Effect of Xenoestrogens

Xenoestrogens may act cumulatively (Figure 3) and with endogenous estrogens thus disrupting the endocrine system of exposed wildlife and humans. Hence, measuring the total estrogen burden due to environmental contaminants present in a plasma/tissue sample may be more meaningful than to assess exposure by measuring the levels of each of the known xenoestrogens. Recently, an epidemiological study showed a positive correlation between breast cancer and serum levels of DDE, a DDT metabolite (55–57), leaving open the possibility that xenoestrogen exposure increases the incidence of breast cancer. Since xenoestrogens are postulated to be a risk factor for breast cancer, measuring a single xenoestrogen may not be a reliable indicator of exposure because different persons eating different diets may be exposed to different xenoestrogens. Therefore, measuring total xenoestrogen burden represents a more reliable approach to assess the link between xenoestrogens and breast cancer. The E-SCREEN test may be used to this end once a protocol is developed to separate environmental estrogens from endogenous ones. In addition, in a preventive approach, the E-SCREEN test may be used to screen chemicals for their estrogenicity before they are released into the environment.

REFERENCES

1. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).
2. Herr R. The estrogen problem — retrospective and prospect. In: Estrogens in the Environment II—Influences on Development (McLachlan JA, ed). New York:Elsevier, 1985:1-11.

3. Hammond B, Katzenellenbogen BS, Krahnhammer N, McConnell J. Estrogenic activity of the insecticide chlordane (kepone) and interaction with uterine estrogen receptors. Proc Natl Acad Sci USA 76:6641-6645 (1979).

4. Meyers CY, Mathews WS, Ho LL, Kolb VM, Parady TE. Carboxylic acid formation from kepone. In: Catalysis in Organic Synthesis (Smith GW, ed). New York:Academic Press, 1977:213-255.

5. Clark JH, Watson C, Upchurch S, Padykula H, Markavitch B, Hardin JW. Estrogen action in normal and abnormal cell growth. In: Estrogens in the Environment (McLachlan JA, ed). New York:Elsevier, 1980:53-67.

6. Lieberman ME, Maurer RA, Gorski J. Estrogen control of pro-lactin synthesis in vitro. Proc Natl Acad Sci USA 75:5946–5949 (1978).

7. Ramsdell JS, Tashjian AH. Thyro-tropin releasing hormone and epidermal growth factor stimulate prolactin synthesis by a pathway(s) that differs from that used by phorbol esters: dissociation of protein by calcium and cycloheximide additivity. Endocrinology 117:2050–2060 (1985).

8. Palmiter RD. Quantitation of parameters that determine the rate of ovalbumin synthesis. Cell 4:189–197 (1975).

9. Soto AM, Lin T-M, Justicia H, Silvia RM, Sonnenschein C. An "in culture" bioassay to assess the estrogenicity of xenobiotics. In: Chemically Induced Alterations in Sexual Development: The Wildlife/Human Connection (Colborn T, Clement C, eds). Princeton, NJ:Princeton Scientific Publishing, 1992:295–309.

10. Soto AM, Justicia H, Wray JW, Sonnenschein C. p-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyreny. Environ Health Perspect 92:167–173 (1991).

11. Soule HD, Vasquez J, Long A, Alberts S, Brennan MJ. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1413 (1973).

12. Soto AM, Sonnenschein C. The role of estrogens on the proliferation of human breast tumor cells (MCF-7). J Steroid Biochem 23:87–94 (1985).

13. Soto AM, Sonnenschein C. Mechanism of estrogen action on cellular proliferation: evidence for indirect and negative control on cloned breast tumor cells. Biochem Biophys Res Commun 122:1097–1103 (1984).

14. Lykkefeldt AE, Briand P. Indirect mechanism of oestradiol stimulation of cellular proliferation of human breast cancer cells. Br J Cancer 55:29–35 (1986).

15. Briand P, Lykkefeldt AE. Long-term cultivation of a human breast cancer cell line, MCF7, in chemically defined medium. Effect of oestradiol. Anticancer Res 6:85–90 (1986).

16. Soto AM, Silvia RM, Sonnenschein C. A plasma-borne specific inhibitor of the proliferation of human estrogen-sensitive breast tumor cells (estrocolyone-I). J Steroid Biochem Mol Biol 43:703–712 (1992).

17. Maddedu L, Legros N, Deveschouwer N, Bosman C, Piccart M, Leclercq G. Estrogen receptor status and estradiol sensitivity of MCF-7 cells in exponential growth phase. Eur J Cancer Clin Oncol 24:385–390 (1988).

18. Sonnenschein C, Olea N, Pasanen ME, Soto AM. Negative controls of cellular proliferation: human prostate cancer cells and androgens. Cancer Res 49:3474–3481 (1989).

19. Winer BJ. Design and analysis of single-factor experiments. In: Statistical Principles in Experimental Design. 2nd ed. New York:McGraw-Hill, 1962:149–260.

20. Korach KS, Metzler M, McLachlan JA. Diethylstilbestrol metabolites and analogues. J Biol Chem 254:8963–8966 (1979).

21. Martin L, Pollard JD, Page B, Oestridol; oestradiol-17β and the proliferation and death of uterine cells. J Endocrinol 69:103–115 (1976).

22. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds. Endocrinology 135:175–182 (1994).

23. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen sensitive cells. Environ Health Perspect 102:380–383 (1994).

24. Gyling M, Leclercq G. Estrogenic and antiestrogenic down-regulation of estrogen receptor levels: evidence for two different mechanisms. J Recept Res 10:217–234 (1990).

25. Reiner GCA, Katzenellenbogen BS. Characterization of estrogen and progesterone receptors and the dissociated regulation of growth and progesterone receptor stimulation by estrogen in MDA-MB-134 human breast cancer cells. Cancer Res 46:1124–1131 (1986).

26. Thomas KB, Colborn T. Organochlorine endocrine disruptors in human tissue. In: Chemically-induced Alterations in Sexual Development: The Wildlife/Human Connection (Colborn T, Clement C, eds). Princeton,NJ:Princeton Scientific Publishing, 1992:365–394.

27. Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm count and disorders of the male reproductive tract? Lancet 341:1392–1395 (1993).

28. Giwercman A, Carlsen E, Keiding N, Skakkebaek NE. Evidence for increasing incidence of abnormalities of the human testis: a review. Environ Health Perspect 101(Suppl 2):65–71 (1993).

29. Guzelian PS. Comparative toxicology of chloroform (kepone) in humans and experimental animals. Annu Rev Pharmacol Toxicol 22:89–113 (1982).

30. Bergink EW, Wallace RA, VandeBerg JA, Bos ES, Ab G. Estrogen-induced synthesis of yolk proteins in roosters. Am Zool 14:1177 (1974).

31. Redshaw MR, Follett BK. Physiology of egg yolk production by the fowl: the measurement of circulating levels of vitellogenin employing a specific radioimmunoassay. Comp Biochem Physiol A 55:395–405 (1976).

32. Allen E, Doisy EA. An ovarian hormone: preliminary report on its localization, extraction and partial purification, and action in test animals. JAMA 81:819–821 (1923).

33. Dodds EC, Lawson W. Molecular structure in relation to oestrogenic activity. Compounds without a phenanthrene nucleus. Proc Roy Soc Lond B Biol Sci 125:222–232 (1938).

34. Clark LB, Rosen RB, Hartman TG, Louis JB, Suffet IH, Lippincott RL, Rosen JD. Determination of alkylphenol ethoxylates and their acidic derivatives in drinking water by particle beam liquid chromatography/mass spectrometry. Int J Environ Anal Chem 47:167–180 (1992).

35. Junk GA, Svec HJ, Vick RD, Avery MJ. Contamination of water by synthetic polymer tubules. Environ Sci Technol 8:1100–1106 (1974).

36. Gilbert MA, Shepherd MK, Startin JR, Wallwork MA. Identification by gas chromatography-mass spectrometry of vinyl chloride oligomers and other low molecular weight components of poly(vinyl chloride) resins for food package applications. J Chromatogr 237:249–261 (1992).

37. Knaak JB, Elridge JM, Sullivan LJ. Excretion of certain polyethylene glycol ether adducts of nonylphenol by the rat. Toxicol Appl Pharmacol 9:331–340 (1966).

38. Purdom CE, Hardiman PA, Bye VJ, Enco NC, Tyler CR, Sumpter JP. Estrogenic effects from sewage treatment works. Chem Ecol 8:275–285 (1994).

39. Shiu H, Cantor D, Nielson RA. Identification and determination of tert-amylenolins in carp from the Trenton channel of the Detroit river, Michigan, U.S.A. Biomed Environ Mass Spectrom 18:478–483 (1989).

40. Shahidi F, Wanasundara PK. Phenolic antioxidants. Crit Rev Food Sci Nutr 32:67–103 (1992).

41. Singer PL. Occupational oligospermia. JAMA 140:1249–1250 (1949).

42. Tepper L. Phthalic acid esters - an overview. Environ Health Perspect 3:179–182 (1973).

43. Giam GS, Neff GS, Atlas EL, Chan HS. Phthalate ester plasticizers: a new class of marine pollutant. Science 199:419–421 (1978).
44. Randall R, Ozretich R, Boese B. Acute toxicity of butyl benzyl phthalate to the saltwater fish English sole, *Parophrys vetulus*. Environ Sci Technol 17:670-672 (1983).

45. Ecobichon DJ, MacKenzie DO. The uterotropic activity of commercial and isomerically-pure chlorobiphenyls in the rat. Res Commun Chem Path Pharmacol 9:85-94 (1974).

46. Gellert RJ. Uterotrophic activity of polychlorinated biphenyls (PCB) and induction of precocious reproductive aging in neonatally treated female rats. Environ Res 16:123-130 (1978).

47. Korach KS, Sarver P, Chae K, McLachlan JA, McKinney JD. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. Mol Pharmacol 33:120-126 (1987).

48. ATSDR. Toxicological Profile for Toxaphene. Rpt No TP-90-26. Atlanta:Agency for Toxic Substances and Disease Registry, 1990.

49. Paasivirta J, Rantio T. Chloroterpenes and other organochlorines in Baltic, Finnish and Arctic wildlife. Chemosphere 22(1):47-55 (1991).

50. ATSDR. Toxicological Profile for Endosulfan. Rpt No TP-91/16. Atlanta:Agency for Toxic Substances and Disease Registry, 1991.

51. NCI. Bioassay of endosulfan for possible carcinogenicity. Tech Rpt Ser NCI-CG-TR-62. National Cancer Institute 62:18-54 (1978).

52. Gupta PK, Gupta RC. Pharmacology, toxicology and degradation of endosulfan. A review. Toxicology 13:115-130 (1979).

53. Singh SK, Pandey RS. Effect of sub-chronic endosulfan exposures on plasma gonadotrophins, testosterone, testicular testosterone and enzymes of androgen biosynthesis in rat. Indian J Exp Biol 28:953-956 (1990).

54. Mueller GC, Kim U-H. Displacement of estradiol from estrogen receptors by simple alkylphenols. Endocrinology 102:1429-1435 (1978).

55. Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 85:648-652 (1993).

56. Krieger N, Wolff M. Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women. J Natl Cancer Inst 86:589-599 (1994).

57. Savitz D. Re: Breast cancer and serum organochlorines: a prospective study among white, black and Asian women. J Natl Cancer Inst 86:1255 (1994).