A Variety of Environmentally Persistent Chemicals, Including Some Phthalate Plasticizers, Are Weakly Estrogenic

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Sewage, a complex mixture of organic and inorganic chemicals, is considered to be a major source of environmental pollution. A random screen of 20 organic man-made chemicals present in liquid effluents revealed that half appeared able to interact with the estradiol receptor. This was demonstrated by their ability to inhibit binding of 17β-estradiol to the fish estrogen receptor. Further studies, using mammalian estrogen screens in vitro, revealed that the two phthalate esters butylbenzyl phthalate (BBP) and di-n-butyl phthalate (DBP) and a food antioxidant, butylated hydroxyanisole (BHA) were estrogenic; however, they were all less estrogenic than the environmental estrogen octylphenol. Phthalate esters, used in the production of various plastics (including PVC), are among the most common industrial chemicals. Their ubiquity in the environment and tendency to bioconcentrate in animal fat are well known. Neither BBP nor DBP were able to act as antagonists, indicating that, in the presence of endogenous estrogens, their overall effect would be cumulative. Recently, it has been suggested that environmental estrogens may be etiological agents in several human diseases, including disorders of the male reproductive tract and breast and testicular cancers. The current finding that some phthalate compounds and some food additives are weakly estrogenic in vitro, needs to be supported by further studies on their effects in vivo before any conclusions can be made regarding their possible role in the development of these conditions. Key words: butylbenzyl phthalate, butylated hydroxyanisole, di-n-butyl phthalate, phthalates, estrogenicity, sewage. Environ Health Perspect 103: 582–587 (1995)

Over the last 50 years, large amounts of some estrogenic man-made chemicals have been released into the environment (1). These chemicals include classical environmental estrogens, such as o,p'-DDT and its metabolites, methoxychlor, and many of the polychlorinated biphenyls (PCBs). More recently, chemicals originating from the plastics and detergent industries, such as alkylphenols (2,3) and bisphenol-A (4), have been discovered to be estrogenic. Evidence suggests that in many instances the presence of these chemicals has had deleterious effects on exposed wildlife populations (5,6). Estrogens influence many developmental and physiological responses in target cells by regulating the activity of specific genes. Their action is mediated by a soluble intracellular receptor that functions as a transcription factor (7). Estrogens have been shown to have multiple sites of activity and exert biological actions on the reproductive tract and the mammary gland. They also influence the neuroendocrine system (8) and have skeletal effects (9,10). Untimely exposure to natural or synthetic estrogens can adversely affect human health, particularly with regard to the reproductive cycle and reproductive function. In addition to decreased sperm counts in men and increased incidence of disorders of the male reproductive tract (11,12), recent epidemiological studies suggest that cumulative exposure to estrogenic chemicals is related to the incidence of reproductive cancers (13).

As many of the estrogen xenobiotics discovered to date have an anthropogenic source, the highest concentrations would be expected to occur near urbanized or industrial areas. Sewage is considered to be a major input source of organic contaminants into the environment. The release of liquid effluents into the rivers and oceans, the disposal of dry sludge onto the land, and the release of volatile organics into the atmosphere all contribute to this source of pollution. This fact, coupled with the report that sewage effluents are estrogenic (14), increases the possibility that there may be other estrogenic chemicals in the environment not yet discovered.

Extensive information exists on the occurrence and concentrations of organic micropollutants in raw, potable, and waste waters (15,16), yet only about 3,000 man-made organic compounds have been identified out of a probable 60,000 (17). The sources of these compounds range from domestic and industrial effluents and leachates from solid waste disposal sites to agricultural or urban run-off and atmospheric fall-out. The range of compounds found includes aliphatic and aromatic hydrocarbons, polycylic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, organochlorine pesticides, PCBs, and phthalate esters (18-22), all of which are present in various environments at highly variable concentrations. For example, phthalates are present in waters at concentrations ranging from nanograms to milligrams per liter. The reasons for the reported variability in concentrations in the aquatic environment include the use of different methodologies for analysis, geographical variation, and variations in the source of the water sample (e.g., influent, effluent, river).

The estrogenic activity of environmental chemicals has nearly always been discovered because an estrogenic effect, either in vivo or in vitro, has occurred upon exposure to the chemical. With the exception of studies conducted by Soto et al. (22), no systematic screening of chemicals has been reported. Because the estrogenic activities of various widely used industrial chemicals continue to be discovered, it seems likely that additional chemicals also exhibit activity. Our interest in the aquatic environment led us to test some of the major chemicals present in sewage effluent to determine whether any of these chemicals are estrogenic.

Materials and Methods

Chemicals tested. We searched the scientific literature (using the Institute of Scientific Information database and also government reports, both published and unpublished) in order to discover what chemicals had been reported to be present in sewage effluents and at what concentrations. None of these reports quantified all of the chemicals present in effluent; many of them tended to focus on one group of chemicals rather than the whole range likely to be present. It is not known how many chemicals are present in effluent, although the number is probably high.

Based on this literature search, we made a list (Table 1) of selected man-made chemicals present in sewage effluent. We do not claim that this table is representative of all sewage effluents, but the chemicals listed are likely to be present at significant concentrations in most effluents (see Discussion for fuller explanation of this point).

Fish studies. Because of their document- ed presence in the aquatic environment, the initial examination for estrogenicity was carried out by measuring direct binding of the chemicals to the fish estrogen receptor. This initial screening process was both rapid and economical and was carried out using a cysotolic extract from the liver of rainbow trout; it is well documented that estradiol receptor-binding sites are present here in both male and female fish (23). Livers were removed from rainbow trout,

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A portion of this work was financially supported by the Department of the Environment, UK. Received 23 March 1991; accepted 5 December 1994.
frozen immediately in liquid nitrogen, and subsequently stored at -80°C until required. They were then thawed and homogenized on ice in 2.5 volumes of buffer (50 mM TrisHCl, 0.1 mM EDTA, 10 mM sodium molybdate, and 1 mM monothioglycerol, pH 7.4). The homogenate was centrifuged at 10,000 g for 30 min at 2°C to yield a crude nuclear pellet and a crude cytosolic supernatant. The cytosol was then incubated on ice for 30 min in the presence of dextran-coated charcoal to remove any endogenous steroids and then spun at 50,000 g for 1 hr at 2°C. The final supernatant was carefully aspirated, decanted, and a saturation analysis was carried out on this cytosolic extract to establish the concentration of [2,3,7,3H]17β-estradiol (86 Ci/mmol) that saturated the receptor preparation (generally between 2 and 10 nM). Thereafter, cytosol samples with a protein content of 2–5 mg/ml were incubated in triplicate with a saturating concentration of 5 nM tritiated 17β-estradiol, both alone and in the presence of competing ligands at a wide range of concentrations (up to 1 mM). We removed the unbound fraction by addition of charcoal and specific binding was quantitated [as described by Pottinger (23)]. These experiments were repeated at least three times.

Mammalian studies. Apart from their presence in waters, many of the compounds identified as putative environmental estrogens originate either from the diet or from the human usage of plastics and cosmetics, and therefore humans could be exposed to them via many other routes. In view of this potential for human exposure, we tested several of the compounds further using mammalian-based assays employing two human breast cancer cell lines in vitro, ZR-75 and MCF7.

Human breast cancer ZR-75 cells were grown initially in phenol red-free Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% (v/v) charcoal-stripped fetal calf serum containing no hormone for 7 days. They were then transferred into medium containing no hormone (NH), 10 nM 17β-estradiol (E2), 10–9 M octyphenol (OP), or 10–8 M of each of the environmental pollutants n-butylbenzene, di-n-butyl phthalate (DBP), butylbenzyl phthalate (BBP), 4-nitrotoluene, bis(2-ethylhexyl)adipate (DEHA), 2,4-dichlorophenol, benzophenone, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or bis(2-ethylhexyl)phthalate (DEHP). Cells were cultured for a further 10 days and counted on days 0, 3, 6, 8, and 10. All experiments were carried out in duplicate and repeated twice.

To determine whether the estrogenic compounds stimulated transcriptional activity of the estrogen receptor directly, we examined their effects on transiently transfected MCF7 cells using the reporter plasmids pTKLUC and pERE-TKLUC. MCF7 cells were plated to 80% confluence in phenol red-free DMEM plus 10% charcoal-stripped fetal calf serum and transfected using the calcium phosphate coprecipitation method, as previously described (24). The reporter plasmid pTKLUC contains the herpes simplex virus thymidine kinase (TK) promoter from -105 to +55 inserted in the Bgl II site of the luciferase reporter plasmid pGL2-Basic (Promega). pERE-TKLUC contains a single copy of the vitellogenin A2 estrogen response element (ERE) inserted upstream of the TK promoter in pTKLUC. The transfected DNA included the reporter (0.8 μg) and an internal control plasmid (pJ7LacZ; 0.2 μg). After transfection, cells were maintained with no hormone, E2, OP, BBP, DBP, DEHP, BHA, or BHT at the concentrations indicated. After 24 hr, the cells were harvested, and extracts were assayed for luciferase (25) and β-galactosidase (Galactolight, Tropix Inc, Bedford, Massachusetts) activities. We used β-galactosidase to correct for differences in transfection efficiency. All experiments were carried out in duplicate and repeated at least twice.

We also examined the possibility that some of these chemicals might act as antagonists in the presence of 17β-estradiol. In these experiments, MCF7 cells were transfected with pERE-TKLUC and pJ7LacZ, and then incubated with 10–7 M 17β-estradiol alone, simultaneously with DBP or BBP, or simultaneously with the antiestrogens 4-hydroxytamoxifen (4-OHT) or ICI 182780. Both the phthalates and the antiestrogens were added at the concentrations indicated. The experiment was carried out in duplicate and repeated three times.

Results

Many of the compounds tested in this initial screen reduced the binding of the tritiated natural estrogen, 17β-estradiol, to the receptor. BBP, DBP, DEHP, DEHA, benzophenone, n-butylbenzene, 4-nitrotoluene, BHA, and 2,4-dichlorophenol reduced the binding of tritiated 17β-estradiol to the receptor, although whether this inhibitory effect was due to direct competition was not determined. Concentrations as high as 1 mM may have approached the limits of solubility of some chemicals in the solvent system used, as suggested by the observation that some of the curves appeared to flatten. In these cases, higher concentrations were not tested and hence full displacement curves were not obtained. No accurate estimations of the affinities of these chemicals for the receptor could be obtained because

<table>
<thead>
<tr>
<th>Compound</th>
<th>Primary uses/sources</th>
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<tbody>
<tr>
<td>Bis(2-ethylhexyl)phthalate (DEHP)</td>
<td>Plasticizer</td>
</tr>
<tr>
<td>Benzophenone</td>
<td>Manufacture of insecticides and antihistamines; fixative in strong perfumes (soaps, shampoo)</td>
</tr>
<tr>
<td>Butylated hydroxyanisole (BHA)</td>
<td>Antioxidant, especially in foods</td>
</tr>
<tr>
<td>Butyl benzyl phthalate (BBP)</td>
<td>Plasticizer, especially in the production of vinyl floor tiles, adhesives and synthetic leather</td>
</tr>
<tr>
<td>n-Butylbenzene</td>
<td>Petrochemical origin</td>
</tr>
<tr>
<td>p-tolyl Butyl benzoic acid</td>
<td>Plastics industry; corrosion inhibitor; in polyester manufacture in dyeing</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Drinks and pharmaceuticals</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Excreted steroid, emulsifying agent</td>
</tr>
<tr>
<td>p-Cresol (4-methylphenol)</td>
<td>To produce antioxidants; UV stabilizer</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT)</td>
<td>Antioxidant in food, petrol products, rubbers, plastics, and soaps</td>
</tr>
<tr>
<td>Di-n-butyl phthalate (DBP)</td>
<td>Plasticizer in food packaging, PVC, cellulosics and certain elastomers; insect repellent</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>Fungicide and germicide products</td>
</tr>
<tr>
<td>3,4-Dimethylphenol</td>
<td>Disinfectant/microbicidal</td>
</tr>
<tr>
<td>Bis(2-ethylhexyl)adipate (DEHA)</td>
<td>Manufacture of plastics (PVC)</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>Cosmetic, food, and pharmaceutical preservative</td>
</tr>
<tr>
<td>2-Methylphenol</td>
<td>Herbicide products, phenolic resins</td>
</tr>
<tr>
<td>Musk xylene</td>
<td>Scent</td>
</tr>
<tr>
<td>Musk ketone</td>
<td>Scent</td>
</tr>
<tr>
<td>4-Nitrotoluene</td>
<td>Manufacture of dyes</td>
</tr>
<tr>
<td>p-Toluene</td>
<td>Industrial solvent</td>
</tr>
</tbody>
</table>
in most cases the displacement curves were not parallel to that of 17β-estradiol (Fig. 1).

Musk ketone, musk xylene, p-toluene, BHT, caffeine, cholesterol, p-hydroxybenzoic acid, p-tert butylbenzoic acid, 3,4-dimethylphenol, and 2-methylphenol did not impair binding of tritiated estradiol to the estradiol receptor (results not shown).

When the compounds were tested for their mitogenic effects on cell growth at 10⁻⁵ M, the three most potent were BBP, DBP, and BHA (Fig. 2). Many of the other compounds were either inactive or only weakly active at concentrations in excess of 10⁻⁴ M. The growth responses to these chemicals were all less than the maximal responses shown by the natural estrogen 17β-estradiol and the environmental estrogen OP, which we have tested in this system previously (26).

When tested for their ability to stimulate the transcriptional activity of the estrogen receptor directly (Fig. 3), BBP stimulated transcription at concentrations in the range 10⁻⁶ to 10⁻⁴ M DBP, and to a lesser extent BHA, also stimulated transcription at concentrations between 10⁻⁵ and 10⁻³ M (Fig. 3). Two closely related compounds, DEHP (a phthalate) and BHT (an antioxidant), did not stimulate transcription to any appreciable degree until concentrations in excess of 10⁻³ M were reached. At these high concentrations, the response to these latter two chemicals was less than 15% of the maximum response obtained with estradiol (results not shown).

OP stimulated transcription of the reporter gene (LUC) to a similar extent as 17β-estradiol (albeit at a concentration 1000-fold greater) and was used for comparison because it is a recognized environmental estrogen (26). No ligand-dependent transactivation was detected with any of the compounds in transfections using the reporter plasmid pTKLUC, which lacks the consensus ERE (results not shown).

Of the 20 compounds initially tested (Table 1), the action of the two most potent compounds (the phthalates) was compared with the action of two antiestrogens (4-OHT and ICI 182780). The compounds were tested for their ability to inhibit transcription of the reporter caused by the presence of 17β-estradiol at concentrations of 10⁻¹¹ M (Fig. 4) and 10⁻⁸ M (data not shown). In view of the relative binding affinities of the phthalates for the receptor (Fig. 1), the lower concentration of 17β-estradiol used would allow competition by the compounds in binding to the receptor. In contrast to the two antiestrogens, which inhibited the response in a dose-dependent manner, DBP and BBP increased the transcriptional activity of the receptor in the presence of 10⁻¹¹ M 17β-estradiol (Fig. 4).

**Figure 1.** Inhibitory effects of organic chemicals present in sewage effluent on the binding of tritiated 17β-estradiol to the rainbow trout estrogen receptor. Butylbenzyl phthalate (BBP), di-n-butyl phthalate (DBP), bis(2-ethylhexyl)phthalate (DEHP), bis(2-ethylhexyl)adipate (DEHA), benzophenone (BP), n-butylbenzene (BB), 4-nitrotoluene (NT), butylated hydroxyanisole (BHA), and 2,4 dichlorophenol (DCP) reduced the binding of tritiated 17β-estradiol to the receptor. Musk ketone, musk xylene, p-toluene, butylated hydroxytoluene (BHT), caffeine, cholesterol (CHO), p-hydroxybenzoic acid, p-tert butylbenzoic acid (BHA), or tert-butylbenzene (BHA), 3,4-dimethylphenol, and 2-methylphenol did not impair binding of tritiated estradiol to the estradiol receptor (most results not shown). All experiments were repeated three times. The error bars are too small and are therefore not shown on the figure.

**Figure 2.** Mitogenic effects of various environmental chemicals on breast cancer cells. Cells were exposed to no hormone (NH), 10 nM 17β-estradiol (E), 10⁻⁶ M octylphenol (OP), or 10⁻⁶ M of each of the environmental pollutants n-butylbenzene, di-n-butyl phthalate (DBP), butylbenzyl phthalate (BBP), 4-nitrotoluene, bis(2-ethylhexyl) adipate, 2,4 dichlorophenol, benzophenone, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or bis(2-ethylhexyl)phthalate. Cells were cultured for 10 days and counted on days 0, 3, 6, 8, and 10. Only those compounds that enhanced cell growth are shown. All other compounds did not enhance breast cancer cell growth at this concentration to any significant degree. All experiments were carried out in duplicate and repeated twice. Similar results were observed in a replicate experiment, although the number of cells used per well at the beginning of the experiment differed. Mean values are presented from a single experiment.
Phthalate plasticizers are weakly estrogenic

**Discussion**

Microbial degradation of chemicals present in sewage results in a wide range of products, many of which are unidentified. Some of these products will be transient intermediates in the degradation process, while others will be more persistent. Thus, we do not know exactly what is in effluent, and we are left with the task of testing only the compounds that have been positively identified. This group of identified chemicals may represent only 20% of the total chemicals present. Of the 20 chemicals tested, 9 reduced the binding of tritiated 17β-estradiol to the fish estrogen receptor.

This initial screening process isolated a subset of chemicals that were likely to be able to bind to the estrogen receptor, but it was not possible to determine whether these chemicals were agonists or antagonists. Using more specific tests, designed to assess whether any of these chemicals were estrogenic, we showed that three of these compounds had significant effects on transactivation of the estrogen receptor and breast cancer cell growth.

BHA is commonly used as an antioxidant, particularly in foods. Therefore, its route of exposure to humans is likely to be mainly via ingestion. It has a low oral toxicity, and it has been estimated that the mean human intake of BHA averages 0.13 mg/kg body weight/day (27). Our studies indicate that BHA is six or more orders of magnitude less potent than 17β-estradiol, and hence causes stimulatory effects on both the transcriptional activity of the human estrogen receptor and the growth of breast cancer cells in vitro only at concentrations of $10^{-5}$ M (2–3 ppm) and above. However, it is impossible to make predictions on its activity in vivo because no such studies have been carried out. BHA may bioconcentrate to a low degree in humans, although it is not certain whether the lack of full recovery of BHA from urine after ingestion is due to bioaccumulation of intact BHA or its metabolites or to unknown routes of biotransformation (28). Although it is reported to be present in some sewage effluents, BHA is not as ubiquitous as its chemical cousin BHT, which was found to be even less estrogenic than BHA.

In contrast, phthalates are the most abundant man-made chemicals in the environment (29). They are produced industrially in large quantities, mainly to impart flexibility into plastics, and can leach out of these materials into water, soil, or food over time. BBP is also used in the production of vinyl floor tiles, adhesives, and synthetic leather; DEP is more common as a plasticizer in food-packaging materials, PVC, the cellulose, and certain types of elastomers (30–32). Thousands of tons of plastics are disposed of annually in landfill sites, thus enabling phthalate esters to migrate into groundwater via the soil. The ubiquity of these compounds in the aquatic environment is well known, and their presence is reported in river, waste, and drinking waters as well as in fish sediments (33–39). Commonly detected species include DBP, dimethyl phthalate, diethyl phthalate, DEHP, di-n-octyl phthalate, BBP, and DEHA (18).

We have not tested many of these phthalates to determine whether any of them are estrogenic (we have tested only those phthalates listed in Table 1). However, our results indicate that a comprehensive survey of the estrogenic activities (if any) of all commonly used phthalates would be justified. The general population may be exposed to these compounds via their diet, either from food contamination, or from food or drinks directly contaminated by plastic wraps containing phthalates, or from polluted drinking water (31,34,40). In most cases, the greatest exposure is from food. Levels of DBP in foods range from 50 to 500 µg/kg in the United States (41). A 1987 study in the UK estimated that the average intake of DBP of food packaged in cellulose film
was 230 μg/day (42). Indeed, up to 14 mg DBP/kg was found in chocolate bars and potato snacks wrapped in printed polypropylene films (43).

In our studies, the phthalates DBP and BBP were estrogenic in vitro at concentrations between 10^{-6} and 10^{-3} M. However, these figures cannot be used to predict estrogenic activity in vivo. Because they are lipophilic, all phthalates have a tendency to accumulate in fatty tissues and can be absorbed through human skin very efficiently. However, once they are absorbed and ingested, they may be metabolically cleared from the body; little is known about the absorption and metabolism of phthalates. The oral toxicities of phthalate compounds in humans are generally low (30), although at high concentrations, they are testicular toxicants. It has been suggested that the concentration of these compounds (particularly DBP) in the cellular fraction of sperm from adult men is negatively correlated with either sperm density or the total numbers of sperm (29). Indeed, when administered to rats in high doses phthalates are embroyotoxic toads as well as testicular toxicants (44-48). In the female rat, the primary effect on reproduction is spontaneous abortion and decreased litter size. Recent studies on the embryolethality of BBP have shown that this effect is correlated with a lowering of plasma progesterone levels (49), and it is possible that this is a consequence of an estrogenic effect.

It is well established that, upon binding to 17β-estradiol, the estrogen receptor binds to DNA as a homodimer and activates transcription of estrogen-responsive gene products by means of two distinct activational regions on the estrogen receptor, AF1, in the N-terminal domain, which is estrogen independent, and AF2 in the estrogen-binding domain, which is active only in the presence of estrogen (50-53). The environmental estrogen OP mimics this action exactly; it binds to the estrogen receptor in the same region as 17β-estradiol and induces full activation (26). In contrast, the antiestrogenic/partial agonist tamoxifen promotes DNA binding but fails to induce the activity of AF2 and hence causes only a submaximal effect due to the constitutive activity of AF1 (54-56).

Because none of the active compounds listed in Table 1 could induce full activation, at least at the concentrations used, the possibility that they may also be antiestrogenic was considered. Indeed, the potential for harmful effects of these chemicals on humans or animals will depend not only on their agonistic activity, but also on their potential to act as antagonists in the presence of other environmental estrogens and/or endogenous estrogens.

Antiestrogens such as tamoxifen and ICI 182780 inhibit the action of estrogens by competing with 17β-estradiol for the estrogen receptor. In contrast, many of the halogenated aromatic compounds and dioxins such as TCDD have been shown to be antiestrogenic in human breast cancer cells (57), but their action is mediated by the Ah receptor rather than the estrogen receptor. Similarly, the antiestrogenic action of dietary estrogens, such as some phytoestrogens, is thought to be controlled by a nonestrogen receptor-mediated mechanism (58). Synthetic antiestrogens, which do act through the estrogen receptor, have been used in the treatment of estrogen-responsive breast cancers for several years (59). Antiestrogenic activity may be deleterious if it blocks the action of estrogen during sexual differentiation or puberty. Our results demonstrate that in vitro the phthalate compounds are acting as agonists only and do not act as antiestrogens at any concentration throughout their active range. Therefore, we suggest that rather than being contra-active, they would enhance the effects of endogenous estrogens if they were present.

Nothing is known about either the acute in vivo estrogenic effects or the possible chronic effects of phthalates on humans or wildlife if administered at low concentrations over long periods of time. Prior to this report, none of the chemicals we tested had ever been described as estrogenic. The fact that almost 50% of the compounds initially tested were found to inhibit the binding of tritiated estradiol to the fish estrogen receptor is provocative. More surprising is the fact that almost 30% of these "inhibitory" chemicals can have significant effects on transactivation of the receptor and breast cancer cell growth.

The possible implications of this scenario to man and wildlife will depend entirely on the estrogenic potencies of these chemicals in vivo; to a large extent this will depend on the processes of metabolic transformation and bioaccumulation. In addition, the effects of simultaneous exposure to a variety of estrogenic chemicals should be investigated. Since all of the estrogenic chemicals discovered to date are lipophilic, they probably co-exist in fat and body fluids of exposed individuals. Much of the current literature suggests that environmental estrogens may act cumulatively and that measuring the total estrogenic burden due to environmental contaminants may have more relevance than assessing exposure by measuring levels of individual estrogens alone (60,61). Estrogen-responsive sites such as the reproductive tract or neuroendocrine centers are highly sensitive and hence it is possible that exposure to many weakly active compounds either persistently at low concentrations, or acutely in high concentrations, may alter the natural hormonal balance.

In conclusion, we have discovered that a surprisingly large proportion of environmentally persistent chemicals are weakly estrogenic and thus have introduced the possibility that there may be hundreds, or even thousands, of chemicals in the environment which possess some estrogenic activity. Although the chemicals we tested possess some common structural features (such as a benzene ring), there is no obvious part of their molecular structure that might be expected to enable binding to the estrogen receptor, and hence one cannot easily deduce which chemicals are and which are not estrogenic. Aquatic organisms are probably exposed to these weakly estrogenic chemicals largely, if not exclusively, via water. However, terrestrial animals (including humans) are probably exposed via many routes. The concentrations required to induce effects in vivo are essentially unknown, particularly when an organism is exposed simultaneously to a cocktail of estrogenic chemicals. Even if the combined effect of exposure to a number of chemicals is additive, there is no evidence to suggest that the total concentration of estrogenic chemicals in humans or animals is high enough to cause any effects on estrogen-responsive tissues. However, no studies have been carried out to examine this possibility.

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