Prediction and Assessment of the Effects of Mixtures of Four Xenoestrogens

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The assessment of mixture effects of estrogenic agents is regarded as an issue of high priority by many governmental agencies and expert decision-making bodies all over the world. However, the few mixture studies published so far have suffered from conceptual and experimental problems and are considered to be inconclusive. Here, we report the results of assessments of two-, three- and four-component mixtures of *o,p´*-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol, all compounds with well-documented estrogenic activity. Extensive concentration–response analyses with the single agents were carried out using a recombinant yeast screen (yeast estrogen screen, YES). Based on the activity of the single agents in the YES assay we calculated predictions of entire concentration–response curves for mixtures of our chosen test agents assuming additive combination effects. For this purpose we employed the models of concentration addition and independent action, both well-established models for the calculation of mixture effects. Experimental concentration–response analyses revealed good agreement between predicted and observed mixture effects in all cases. Our results show that the combined effect of *o,p´*-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol in the YES assay does not deviate from expected additivity. We consider both reference models as useful tools for the assessment of combination effects of multiple mixtures of xenoestrogens. *Key words*: additivity, combination effects, genistein, mixtures, 4-*n*-octylphenol, 4-nonylphenol, *o,p´*-DDT, xenoestrogens, yeast estrogen screen. *Environ Health Perspect* 108:983–987 (2000). [Online 8 September 2000]
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There is considerable concern about the increasing incidence of endocrine-related cancers and deteriorating reproductive health in man and wildlife (*1–3*). It is apparent that a large number of natural and man-made chemicals have the ability to mimic the action of the endogenous steroid hormone 17β-estradiol by binding to and activating the estrogen receptor (*4,5*).

At any given time, people are exposed to a multitude of such xenoestrogens, yet research has tended to focus predominantly on the activity of single compounds. The need to assess the effects of mixtures of estrogenic agents is widely acknowledged and considered to be an issue of high priority (*6*). However, the few mixture studies conducted to date with xenoestrogens have suffered from inadequate theoretical and conceptual foundations or could not be reproduced experimentally in other laboratories [for reviews and comments, see (*7–9*)].

Assessments of mixture effects in terms of synergisms, antagonisms, or additivity rely crucially on definitions of what the expected effect of a mixture should be. If the observed effects are stronger than expected, there is said to be synergism; likewise, if they are weaker there is antagonism. When expectations are met, the combination effect can be called additive (*10*).

A popular misconception not only in the estrogen field is the idea that the combined effect of mixtures of multiple compounds should always be equal to the arithmetic sum of the effects of its constituents. Deviations from this expectation are then diagnosed as synergisms or antagonisms. It is frequently overlooked that this method, termed effect summation, is only applicable to agents that exhibit linear dose–response curves (*10*). It produces unreliable results when dealing with mixtures of agents showing sigmoidal curves with differing maximal effects and slopes, such as xenoestrogens (*8*).

Much of the literature about combination effects (*10–14*) is concerned with providing a theoretical foundation for computing expected (additive) mixture effects for agents with nonlinear dose–response curves. The task is to predict combination effects on the basis of the dose–response relationships of individual mixture constituents. Two reference models have evolved that allow such computations—the models of concentration addition and independent action.

The model of concentration addition assumes that chemicals act in a similar manner. In its original form the model was conceived by Loewe and Muischnek (*11*). The model states that effects can be produced by replacing one compound totally or in part with other constituents. Each individual component is thought to contribute to the overall mixture effect by acting in proportion to its concentration, even below threshold concentrations.

Independent action was developed by Bliss (*12*) and later evolved to assume that compounds act on different subsystems in organisms. When present at subthreshold doses, mixture components will not contribute to mixture effects.

In extensive studies with mixtures of 20 and more aquatic toxicants, Faust and colleagues (*15*) recently demonstrated that concentration addition yielded more accurate predictions with agents that interact with the same molecular site (inhibitors of photosystem II in algae). Conversely, independent action performed better with mixtures of chemicals with diverse modes of action. Similar results were obtained in studies with the luminescent bacterium *Vibrio fischeri* (*16,17*). Thus, each of the models has its merits.

The use of concentration addition and independent action for the prediction of combination effects requires comprehensive descriptions of dose–response curves of all mixture components in terms of shape and maximal effect. Although it is clear that the characterization of agents as "estrogenic" necessitates the integrated use of *in vivo* and *in vitro* assays, such thorough concentration–response analyses are currently only feasible at reasonable cost with *in vitro* assay systems that allow high through-put testing with minimum biologic variability and maximum reproducibility. For this reason we have chosen the yeast estrogen screen (YES) to conduct studies of the combination effects of mixtures of up to four xenoestrogens. The assay is rapid, sensitive, and yields reproducible results (*5,18*). It utilizes yeast cells genetically modified to harbor DNA coding for the α human estrogen receptor protein. Estrogen receptor activation becomes discernible in the presence of expression plasmids that carry estrogen response elements (ERE) in tandem with the reporter gene *lac-Z*. Upon binding of the receptor protein to ERE, β-galactosidase is expressed and secreted into the culture medium where it reacts with its substrate

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chlorophenol red β-D-galactopyranoside (CPRG) to cause a color change from yellow to red (*5*).

The aim of our studies was to evaluate whether additive combination effects of mixtures of xenoestrogens can be reliably predicted on the basis of the concentration–response relationships of their individual components. To this end we have selected test compounds that are well known to produce estrogenic effects in the YES assay: the ubiquitous organochlorine pesticide *o,p´*-DDT, the phytoestrogen genistein, and the alkylphenols 4-*n*-octylphenol and 4-nonylphenol. There is no particular environmental relevance to this mixture. The choice of compounds was motivated by our interest to explore the predictability of combination effects, rather than to emulate "real world" mixtures. Because all the chosen test agents interact with the same molecular site, the binding domain of the estrogen receptor, our expectation was that the model of concentration addition would produce more accurate mixture effect predictions than the model of independent action. We became interested in putting this hypothesis to the test by using both models for a comparative assessment of additive combination effects.

Materials and Methods

Test agents. 17β-Estradiol and genistein were purchased from Sigma (Poole, Dorset, UK), 4-*n*-octylphenol and 4-nonylphenol (technical grade) from Aldrich (Dorset, UK), and *o,p´*-DDT from Lancaster (Morecambe, UK). All agents were used as supplied and prepared in ethanol as 1-mM stock solutions. Equimolar mixtures of test agents were made by combining equal volumes of ethanolic stock solutions (1 mM). All stock solutions were kept in critically cleaned glass containers and stored at –20°C. Chlorophenol red β-D-galactopyranoside (CPRG) was obtained from Boehringer (Mannheim, Germany).

Yeast estrogen screen. The YES assay was carried out exactly as described previously by Routledge and Sumpter (*5*). Briefly, 50 mL of growth medium was inoculated with 125 µL of a concentrated yeast suspension and incubated at 28°C in an orbital shaker (150 rpm) until an absorbance of 1.0 at 640 nm was obtained. Assay medium was then prepared by adding 0.5 mL of the chromogenic substrate CPRG and 2 mL of the yeast suspension to 50 mL of fresh growth medium.

Test agents and mixture solutions were serially diluted in ethanol. Aliquots of 10 µL of these solutions were transferred to 96-well optically flat-bottom microtiter plates and allowed to evaporate to dryness. A volume of 200 µL of the assay medium containing yeast was then added to the wells. Each individual plate also incorporated ethanol controls (i.e., no test agents), positive controls with 17β-estradiol (0.1 and 10 nM), and blanks without yeast cells. To keep variations in background readings (ethanol controls with yeast, but no test compounds) to a minimum, we took great care to administer similar numbers of yeast cells to individual wells in different experiments. This was achieved by monitoring the absorbance of the growth medium immediately before addition to growth medium and adjusting to readings close to 1.0 by diluting with medium, if necessary.

4-*n*-Octylphenol and 4-nonylphenol are able to permeate the plastic walls of 96-well plates (*19*). To avoid cross-contamination of neighboring wells, empty wells were left between differing concentrations of these agents.

Prepared plates were sealed with autoclave tape and shaken vigorously for 2 min on a titer plate shaker. They were then incubated at 32°C in a humidified box for 72 hr. Plates were again shaken at 24 hr and at the end of the experiment. After the final shake, plates were left to stand for 60 min before spectrophotometric analysis at 540 nm (colour change) and 620 nm (turbidity) using a Labsystems Multiskan Multisoft plate reader (Basingstoke, UK). Readings were corrected for untreated controls and turbidity as follows:

$$Corrected\ readings = test_{540\ nm} - [test_{620\ nm} - control_{620\ nm}] - control_{540\ nm}$$

Samples were run in duplicate and experiments repeated at least twice so that each dose–response curve was based on a minimum of 30 single observations. Nominal concentrations were used.

Dosimetry. Scatter plots of corrected absorbance readings ("effect") versus log concentration were constructed and the data fitted to the asymmetric Hill function

$$Effect = Min + (Max - Min)/[1 + (c/EC_{50})\exp(-p)],$$

where *Min* and *Max* are the minimal and maximal observed effects, respectively, *c* the concentration of test agent, EC_{50} the concentration of test agent yielding half-maximal effects and *p* a slope parameter. The 95% confidence intervals of mean effects were also estimated. Nonlinear curve-fitting was carried out by using Fig P for Windows software (Biosoft, Cambridge, UK).

Mixture testing. In designing our experiments we have employed the so-called fixed ratio design: additive mixture effects were computed for equimolar mixtures over the entire effect range and the predictions tested experimentally. This experimental design is particularly well suited for analyzing multiple mixtures by employing the models of concentration addition and independent action. The frequently used alternative approach of varying the concentration of one agent while keeping the others fixed leads to complications in the computation of expected responses, because mixture ratios change continuously.

Calculation of predicted mixture effects. The model of concentration addition predicts a concentration of a mixture of agents that produces a predetermined effect. Such calculations are possible if *a*) the relative abundance of an agent in the mixture (mixture ratio) is known and *b*) data are available on the concentrations of each mixture component that individually produce the same effect as the mixture. Thus, assuming that the combined effect of the mixture with *n* components is concentration additive, the following Equation 1 will hold for any effect level *E*:

$$\Sigma c_i/EC_i = 1, \qquad [1]$$

where c_i denotes the concentration of agent *i* in a mixture yielding an effect *E* and EC_i the concentration of *i* needed to produce effect *E* on its own. The concentration c_i of agent *i* in the mixture is related to the total mixture concentration:

$$c_i = p_i\, EC_{mix} \qquad [2]$$

where p_i is the concentration of the *i*th compound relative to the total mixture concentration EC_{mix} that is required to produce effect *E*. Substitution of c_i in Equation [1] gives

$$\Sigma p_i\, EC_{mix}/EC_i = 1, \qquad [3]$$

and rearranging yields

$$EC_{mix} = [\Sigma p_i/EC_i]^{-1}. \qquad [4]$$

The effect concentrations EC_i were calculated from the parameters describing the best fits of the concentration–response models of single agents (Table 1) by using the inverse expression of the Hill function.

The model of independent action allows it to calculate the predicted effects e_{mix} of a mixture of known composition by using the expression

$$e_{mix} = 1 - \Pi[1 - E(c_i)], \qquad [5]$$

where $E(c_i)$ is the effect E produced by compound *i* at concentration *c*, when applied singly. Independent action is a probabilistic model, i.e., $E(c_i)$ is a fraction of a maximal possible effect that cannot exceed 1.

Thus, when applying this model to measures of estrogen receptor activation (in our case absorbance readings), here termed activating effects $AE(c_i)$, a maximal effect, E_{max}, has to be defined. For this purpose, the maximal activating effect of saturating concentrations of 17β-estradiol (i.e., > 1 nM) was chosen as a reference point and the effects of test agents expressed relative to the maximal effect of 17β-estradiol (i.e., 1.4 on the corrected absorbance scale):

$$E(c_i) = AE(c_i)/E_{max}. \quad [6]$$

Since the concentration–response relationships of all mixture constituents i are described by an appropriate regression model F_i (Hill function), $AE(c_i)$ can be estimated from the mean effect F_i (c_i) predicted by the regression model. Thus,

$$AE(c_i) = F_i(c_i), \text{ and } E(c_i) = F_i\,(c_i)/E_{max}. \quad [7]$$

Substitution of $E(c_i)$ in Equation 5 yields

$$e_{mix} = 1 - \Pi[1 - F_i\,(c_i)/E_{max}]. \quad [8]$$

To ensure comparability of the independent action predictions with those of concentration addition, the fractional effects in Equation 8 were rescaled by multiplication with E_{max}, thus:

$$E_{mix} = E_{max}\, e_{mix} \quad [9]$$

and

$$E_{mix} = E_{max}(1 - \Pi[1 - F_i\,(c_i)/E_{max}]). \quad [10]$$

Results

Concentration–response analysis for single agents. Each of our four chosen test agents induced activation of the estrogen receptor in a concentration-dependent fashion. Absorbance readings were normalized relative to untreated control cultures and corrected for cell number, as described in "Materials and Methods." Data could be reproduced on several different occasions and were fitted to the asymmetric Hill function. Figure 1 depicts scattergrams and nonlinear fits to the regression model, including 95% confidence intervals for mean effects. The resulting concentration–response plots show considerable differences in shape and position. Table 1 gives parameters that characterise these concentration–response curves in terms of slope, maximal effect, and median effect concentrations (EC_{50}).

17β-Estradiol (data not shown) was employed as a positive control and yielded a maximal absorbance (corrected for readings in untreated control cultures) of 1.4 with an EC_{50} of 0.16 nM, in good agreement with data reported in the literature (*5*,*20*). Two of the test agents, genistein and 4-nonylphenol, produced maximal responses similar to those seen with 17β-estradiol, again in good agreement with previous communications (*5*). In terms of potency, *o*,*p*´-DDT was similar to 4-nonylphenol, but elicited considerably lower maximal responses, in line with earlier observations (*5*). However, in our hands 4-*n*-octylphenol was less potent (i.e., showed a higher median effect concentration) than reported by Coldham and coworkers (*21*), although their maximal effects agreed well with our data.

On the basis of the single agent concentration–response relationships in Figure 1 we constructed predicted concentration-effect curves for equimolar mixtures of *o*,*p*´-DDT and genistein (Figure 2); *o*,*p*'-DDT, genistein, and 4-nonylphenol (Figure 3); and *o*,*p*´-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol (Figure 4). The predictions were made assuming additive combination effects.

In all cases the two models produced almost identical concentration–response curves, although concentration addition signaled marginally stronger effects in the range between 0.1 and 1 µM with the three- and four-component mixtures than independent action. Independent action was able to model responses over the entire range of effect levels. In contrast, the largest effects predictable with concentration addition were determined by the mixture component with the lowest maximal effect, in our case *o*,*p*´-DDT. This was due to the fact that the model computes concentrations of mixtures that yield the same effects as its components, when applied individually. Thus, effects exceeding those elicited by the weakest agonist in the mixture could not be calculated.

The agreement between predicted and experimentally observed mixture effects was

Table 1. Parameters derived from nonlinear fits of single agent concentration–response data (Figure 1) to the asymmetric Hill function. These parameters were used to compute the predicted mixture effect curves shown in Figures 2–4.

Parameter	*o*,*p*'-DDT	Genistein	4-Nonylphenol	4-*n*-Octylphenol
EC_{50} (µM)[a]	1.67	0.31	1.94	9.29
p[b]	1.57	1.67	1.80	1.71
Max[c]	0.59	1.56	1.43	0.61
Min[d]	0	0	0	0

[a]Median effect concentration, i.e., concentration yielding 50% of the maximal effect produced by the agent in question. [b]Slope parameter of the Hill function. [c]Maximal effect, expressed as corrected absorbance readings. [d]Minimal effect, i.e., responses seen with control cultures treated with ethanol.

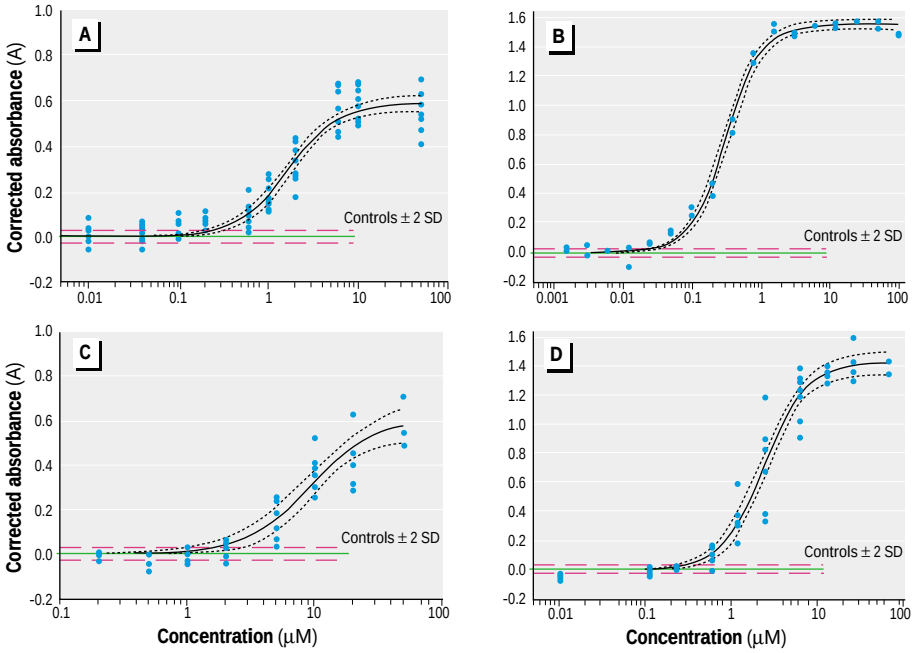

Figure 1. Concentration–response curves for (*A*) *o*,*p*'-DDT, (*B*) genistein, (*C*) 4-*n*-octylphenol, and (*D*) 4-nonylphenol in the yeast estrogen screen. Data are from at least two independent experiments and were fitted to the asymmetric Hill function (best fit: solid lines). Dotted lines show 95% confidence intervals of the fit (mean absorbance readings). The solid horizontal line shows corrected readings from untreated cultures ± 2 SD (dashed lines).

very good. In the case of the binary mixture (Figure 2) both model predictions were almost congruent with the best fit of the experimental data to the regression model. With the other two mixtures, there was overlap between the prediction curves and the 95% confidence intervals of the best-fit regression models. Both concentration addition and independent action slightly underestimated the effects of the three-component mixture in the low-effect range (Figure 3), whereas the opposite was true for the four-component mixture (Figure 4). Our data demonstrate that the combined effect of *o,p´*-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol does not deviate from the additivity assumption.

At total mixture concentrations exceeding 20 µM, marked reductions in absorbance readings were observed. These effects were reproducible and were most pronounced with the four-component mixture (Figure 4). They are very likely the consequence of toxic effects on the yeast cells and were not considered for the regression analyses in Figures 2–4.

Discussion

The results of our studies show that additive mixture effects could be predicted on the basis of the concentration–response curves of individual mixture components. When we applied the criterion of overlap between the calculated effects and the 95% confidence interval of the best fit of the regression model, no marked deviations between predictions and observations could be identified. The degree of agreement between calculated and measured mixture effects was almost complete with the binary mixture but decreased somewhat as the number of mixture components increased. It is conceivable that this is due to multiplication of errors during the preparation, dilution, and administration of the mixtures. Nevertheless, given the multitude of sources of experimental errors, the agreement between predicted and observed responses is very good.

The success of our assessments was not least dependent on the robustness of the YES assay in providing reproducible data with relatively small variations. The data shown were produced on several occasions by different operators using independently prepared serial dilutions of single agents and mixtures. There was always good agreement from experiment to experiment. Our results for single-agent responses are also in line with previously reported literature values.

We found that the fixed mixture ratio design worked well with multiple mixtures. Alternative experimental approaches, such as the one proposed by Pöch (*13*) in which the influence of varying the concentration of one mixture component is studied while all others are held constant, were not pursued because the study of combination effects at low concentrations of all mixture components is not possible using this design.

Our data show that *o,p´*-DDT, genistein, 4-nonylphenol, and 4-octylphenol act additively in stimulating the estrogen receptor. This observation may to a certain degree be due to the intrinsic features of the YES assay. Interactions at receptor domains are often additive and the assay is blind to other effects. The system largely precludes the detection of synergistic or antagonistic effects that may be the result of toxicokinetic interactions between agents, where deviations from additivity are seen because, for example, one compound induces or inhibits metabolic activation of another mixture constituent. Therefore, it remains to be seen whether additive combination effects between our chosen test agents will also occur in more complex experimental systems. We are currently addressing this point by studying mixture effects in the MCF-7 cell proliferation assay.

Because all our test agents interact with the same domain of the estrogen receptor, we expected the model of concentration addition to perform better in predicting mixture effects. However, the prediction of the effects of mixtures of agents with differing maximal effects proved to be a challenge that the model of concentration addition could only meet to a limited degree. Effects exceeding those of the least potent partial agonist in the mixture could not be computed and this may be a problem in the future with mixtures containing very weak xenoestrogens. Fortunately, the mixtures assessed here did not present this complication, largely because both models yielded almost identical predictions. Unlike concentration addition, the model of independent action is able to adequately compute higher effect levels. However, it is necessary to emphasise that both models implicitly assume that concentration–response curves enter a plateau at high effect concentrations. Reductions in responses, which may be the result of toxic effects to yeast cells, cannot be modeled.

To resolve the question concerning which of the two models is valid in predicting mixture effects in the yeast estrogen screen, it will be necessary to explore mixtures where concentration addition and independent action yield predictions with large differences which can be discriminated experimentally. Recently, Faust (*22*) was able to demonstrate that the maximal possible separation depends on the steepness of the concentration–response curves of individual mixture components, their number and the mixture ratio.

In conclusion, we have shown that the effects of multiple mixtures of xenoestrogens can be accurately predicted from concentration–response curves of individual mixture components. When used with the fixed mixture ratio design, the models of concentration addition and independent action provide useful tools for the assessment of multiple mixtures of xenoestrogens.

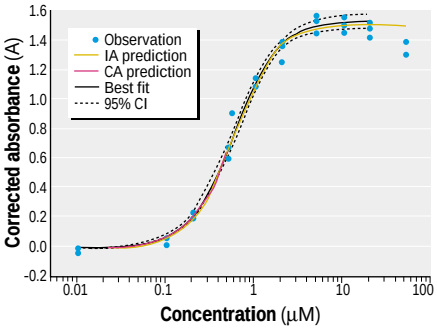

Figure 2. Comparison of predicted and observed mixture effects of an equimolar mixture of *o,p´*-DDT and genistein. On the basis of the single agent concentration–response relationships shown in Figure 1, additive combination effects were predicted using the models of concentration addition (CA) and independent action (IA). Closed circles are the observed mixture responses, with the best fit to the Hill function and 95% confidence belt (mean absorbance readings) of the fit.

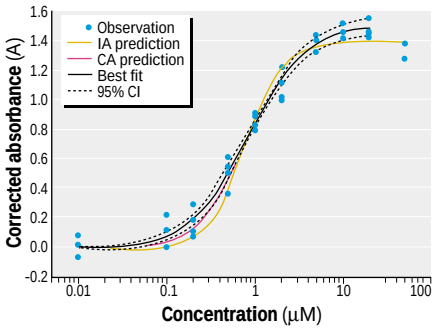

Figure 3. Predicted and observed mixture effects of an equimolar mixture of *o,p´*-DDT, genistein, and 4-nonylphenol.

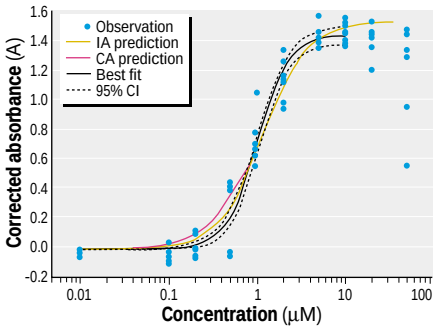

Figure 4. Predicted and observed mixture effects of an equimolar mixture of *o,p´*-DDT, genistein, 4-nonylphenol, and *n*-4-octylphenol.

Our data indicate that one estrogenic agent may be replaced by equi-effective concentrations of a second one to produce the same overall response. Should this prove to be true, estrogenic agents, when present in mixtures, may act together additively even when each component is present at concentrations that individually produce no detectable effects. In this case, it may not be necessary to invoke synergistic combination effects to explain how low, seemingly insignificant, levels of xenoestrogens may produce significant effects as mixtures.

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