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Something from "Nothing" — Eight Weak Estrogenic Chemicals Combined at Concentrations below NOECs Produce Significant Mixture Effects

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We tested whether multicomponent mixtures of xenoestrogens would produce significant effects when each component was combined at concentrations below its individual NOEC or EC_{01} level. The estrogenic effects of eight chemicals of environmental relevance, including hydroxylated PCBs, benzophenones, parabenes, bisphenol A, and genistein, were recorded using a recombinant yeast estrogen screen (YES). To ensure that no chemical contributed disproportionately to the overall combination effect, a mixture was prepared at a mixture ratio proportional to the potency of each individual component. The performance of four approaches for the calculation of additive combination effects (*concentration addition, toxicity equivalency factors, effect summation,* and *independent action*) was compared. Experimental testing of the predictions revealed that *concentration addition* and its application, the *toxicity equivalency factor* approach, were valid methods for the calculation of additive mixture effects. There was excellent agreement between prediction and observation. In contrast, *independent action* and *effect summation* led to clear underestimations of the experimentally observed responses. Crucially, there were substantial mixture effects even though each chemical was present at levels well below its NOEC and EC_{01}. We conclude that estrogenic agents are able to act together to produce significant effects when combined at concentrations below their NOECs. Our results highlight the limitations of the traditional focus on the effects of single agents. Hazard assessments that ignore the possibility of joint action of estrogenic chemicals will almost certainly lead to significant underestimations of risk.

Introduction

The discrepancies between the high concentrations of estrogenic chemicals that are needed to elicit effects in laboratory assays and their low levels in the environment have lent credence to the belief that risks to human health or wildlife are negligible (*1*). An alternative view seeks to take into account that humans and wildlife are exposed not to single agents but to mixtures of multiple estrogenic agents, so-called xenoestrogens. The low concentrations of the incriminated chemicals in environmental media and human tissues have fueled the expectation that synergisms between estrogenic chemicals need to be invoked to explain possible health risks. This has motivated a systematic search for synergistic combination effects (reviewed in ref *2*). In 1996, a report claiming spectacular synergisms between binary mixtures of estrogenic pesticides was published (*3*). However, the paper had to be withdrawn (*4*) because the experimental results could not be reproduced by other laboratories (*5, 6*). This episode has led many to question the overall importance of combined effects of endocrine disrupters, since synergisms were absent.

Although the successful demonstration of synergisms will justifiably always heighten concerns about health risks, the possible implications of apparently less spectacular additive combination effects have not received adequate attention. Furthermore, it is crucial to explore whether xenoestrogens can act together to yield measurable responses when combined at concentrations which individually produce undetectable effects. Here, we present work that addresses these problems experimentally. We studied the activation of the human estrogen receptor (alpha) in a recombinant yeast system, the Yeast Estrogen Screen (YES). The assay utilizes yeast cells genetically modified to harbor DNA coding for the alpha human estrogen receptor. 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−ligand complex to ERE, beta-galactosidase is expressed and secreted into the culture medium where it reacts with its substrate chlorophenol red beta-D-galactopyranoside (CPRG) to cause a color change from yellow to red (*7*).

The assessment of joint exposures to xenobiotics presents considerable theoretical challenges and is fraught with conceptual controversies. Synergisms or antagonisms are generally identified as outcomes that exceed, or fall short of, responses expected from additive interactions of the individual mixture components. There is much debate as to how additive combination effects should be calculated (*8*−*10*). All too often, mixture effects have been assessed without explicit reference to additivity expectations (*3, 11*−*14*).

A widely used and intuitively appealing way of predicting additive effects is based on the expectation that the effect of a mixture should be the arithmetic sum of the effects of its individual components. It is not always appreciated that the generalized use of this method, termed *effect summation*, leads to logical inconsistencies with agents that exhibit sigmoidal dose−effect curves (discussed in ref *2*). For this reason, there are serious doubts as to whether *effect summation* can be regarded as a reliable method (*8, 9*).

Two competing pharmacological concepts for the calculation of expected additive mixture effects have gained broad acceptance, *concentration addition*, and *independent action*. The origins of *concentration addition* can be traced to Fraser (*15*) and Loewe (*16*). The concept rests on the assumption that the components of a mixture act in a similar way, such that one can be replaced by an equal fraction of an equieffective concentration of another, without diminishing the overall mixture effect. By implication, this means that every mixture component contributes to the overall combination effect in proportion to its concentration, even below zero effect levels. There is a consensus that *concentration addition* is a suitable and valid concept for the prediction of mixture effects of similarly acting agents (*17, 18*). Whether

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TABLE 1. Summary of Parameters for Test Agents in the YES

compound[b]	fraction in mix[c]	NOEC (μM)	EC_{01} (μM)[d]	concn (μM) in 1.43 μM mixture[e]	asymmetric Hill function parameters[a]		
					X_{50} (μM)	p	max
1. 2′,3′,4′,5′-tetrachloro-4-biphenylol	0.0027	0.005	0.008	0.004	0.061	2.20	1.71
2. 2′,5′-dichloro-4-biphenylol	0.004	0.011	0.011	0.0057	0.060	2.70	1.71
3. 4′-chloro-4-biphenylol	0.027	0.054	0.074	0.039	0.600	2.16	1.67
4. genistein	0.025	0.038	0.086	0.036	0.600	2.34	1.67
5. 2,4-dihydroxybenzophenone	0.045	0.073	0.124	0.064	0.700	2.62	1.71
6. benzyl-4-hydroxyparabene	0.040	0.116	0.13	0.057	0.790	2.52	1.70
7. bisphenol A	0.128	0.422	0.632	0.183	2.88	2.98	1.68
8. resorcinol monobenzoate	0.728	0.99	2.24	1.04	13.5	2.55	1.77

[a] Defined in Materials and Methods. Absorbance units for max. [b] Numbered as in Figure 2. [c] Ratio of the concentration of each component to total mixture concentration. [d] Concentration producing effect 0.017 absorbance units, i.e., EC_{01} of maximal response in YES. [e] Calculated from the total mixture concentration (1.43 μM) by the multiplication with the fraction of each component in the mixture, see Figure 4.

it is applicable to mixtures of agents that show differing modes of action is the topic of a long-standing controversy (*8*, *10*). *Concentration addition* has found application in the method of isoboles (*16*), toxic unit summation (*19*), and in the *toxic equivalency factor* approach (*20*).

Originally under the name "independent joint action", the concept of *independent action* was developed by Bliss (*21*) on the basis of stochastic considerations. It assumes that mixture effects are the result of interactions of individual mixture constituents with different subsystems of an organism. It is often applied to mixtures composed of agents with dissimilar modes of action. Chemicals that are present below zero effect levels are not expected to contribute to the total mixture effect. There are doubts concerning the general utility of the concept of *independent action* (*9*). Applications of *independent action* include *effect multiplication* (*22*) and *joint independent action* (*23*).

The above approaches can be utilized to predict the combined effects of mixtures from knowledge of the potency of individual mixture components, but it is unclear which of the methods should be applied to xenoestrogens in experiments with the YES. In previous work with xenoestrogen mixtures of up to four components we have found that *concentration addition* and *independent action* produced almost identical additivity expectations, both with mixtures of estrogen receptor agonists in the YES (similarly acting agents) (*24*) and with combinations of mitogenic agents showing diverse modes of action in the E-SCREEN assay (*25*). In both cases, observed and expected combination effects agreed well. Since the YES responds to agents that activate the estrogen receptor (alpha) and is blind to any other effects, it may be expected that *concentration addition* should produce valid calculations of additive combination effects with estrogen receptor agonists. The *toxicity equivalency factor* approach should also produce accurate predictions, provided the equivalency factors are accurately estimated and concentration−response curves of all mixture components are parallel (*26*). Conversely, the concepts of *effect summation* and *independent action* should prove to be unreliable for the prediction of joint effects of xenoestrogens. However, decisive evidence to support these notions is not yet available.

Thus, before addressing the main topic of this paper—combination effects of xenoestrogens at very low effect concentrations—it was necessary to resolve the issue of valid mixture assessment concepts. The challenge was to construct a mixture of xenoestrogens where the differences in combination effect predictions derived from *concentration addition* and *independent action* were large enough to be discriminated experimentally. Drescher and Bödeker (*27*) have shown that *concentration addition* may give higher or lower (and sometimes identical) mixture effect predictions than those derived from *independent action*. This is dependent on the number of mixture components, their concentration ratio, the steepness of their concentration−response functions, and the biometrical model used to describe the relationship between concentration and effects of single agents. To avoid the disproportionate contribution of a single xenoestrogen to the overall mixture effect, it was imperative to choose concentration ratios that reflected the individual potency of mixture components. Assuming that the steepness of concentration−response functions of xenoestrogens as well as a suitable biometric model are not open to experimental manipulation, large discriminations between mixture effect predictions could only be achieved by increasing the number of mixture components. We therefore selected eight xenoestrogens for in-depth mixture experiments.

The main motivation of our work, i.e., to assess the occurrence of joint action when each xenoestrogen is present at levels that elicit effects indistinguishable from those seen with untreated controls, made it necessary to estimate as accurately as possible low effect concentrations of all tested agents. To achieve this, concentration−response relationships of all test agents had to be recorded. These data formed the basis for predictions of entire concentration−response curves for mixtures of defined composition, assuming additive combination effects. The predictions were made using *concentration addition*, the *toxic equivalency factor* approach, and *independent action*. Because of its widespread use and appeal, *effect summation* was also included here, although the approach is theoretically ill-founded. The predicted (additive) combination effects were then tested experimentally.

The eight estrogenic chemicals (*28*, *29*) listed in Table 1 were selected for in-depth studies. They include plastisizers, such as bisphenol A, a number of 4-hydroxylated polychlorinated biphenyls, parabene, benzophenone, and related compounds.

Experimental Section

17beta-Estradiol (98% purity) and genistein (98% purity) were purchased from Sigma (Poole, Dorset, U.K.), resorcinol monobenzoate (99% purity), benzyl-4-hydroxyparabene (99% purity), and 2,4-dihydroxybenzophenone (99% purity) from Aldrich Chemicals (Poole, Dorset, U.K.), bisphenol A (97% purity) from Acros Organics (Geel, Belgium), 4′-chlorobiphenyl-4-ol (>95% purity), 2′,5′-dichlorobiphenyl-4-ol (>95% purity) and 2′,3′,4′,5′-tetrachlorobiphenyl-4-ol (>95% purity) from UltraScientific (North Kingston, RI). All agents were used as supplied and prepared in HPLC-grade ethanol as 1 mM stock solutions. A mixture of test agents was made by combining appropriate volumes of stock solutions. All stock solutions were kept in critically cleaned glass containers and

stored at −20 °C. Chlorophenol red-beta-D-galactopyranoside (CPRG) was obtained from Boehringer (Mannheim, Germany).

The yeast estrogen screen was carried out exactly as described previously (*30*), following the protocol developed by Routledge and Sumpter (*7*). Single agent samples were run in duplicate and experiments repeated twice, often three times. Nominal concentrations were used.

Dosimetry. The colorimetric readings in the YES assay were corrected for absorbances seen with untreated control cultures (for details see ref *30*). Scatterplots of corrected absorbance readings ("effect") vs log concentration were constructed. Nonlinear regression analysis was carried out using the asymmetric Hill function

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

where Min and Max are the minimal and maximal observed effects, respectively, c is the concentration of test agent, EC_{50} is the concentration of test agent yielding half-maximal effects, and p is a slope parameter. Because of the correction for readings in untreated controls, Min equaled zero. The 95% confidence intervals of the best estimate of mean effects were also calculated. Nonlinear regression analysis was carried out by using SigmaPlot software (version 5.0, SPSS Inc., Chicago, U.S.A.).

Successful completion of our studies required the preparation of mixtures with each component present at levels that did not produce statistically significant effects. One approach to realizing such "ineffective" concentrations is to choose the no-observed-effect-concentrations (NOECs) of the tested agents. However, the limitations of the hypothesis testing methods used in estimating NOECs are increasingly recognized. It has been shown (*31*) that such procedures all too easily overlook low-dose effects. It has been argued that more reliable estimations of low dose effects can be made by interpolation on the basis of complete dose−response curves ("benchmark concentrations") (*31*). In light of these considerations we have not only estimated NOECs by using Dunnett's test (*32*) but also based our assessments on effect concentrations yielding 1% of the maximal observed effect in the YES, i.e., that of saturating concentrations of resorcinol monobenzoate (EC_{01}). EC_{01} values were estimated by interpolation using the Hill regression model.

Experimental Approach to Mixture Testing. The estrogenic activity of the eight-component mixture was determined and assessed using the fixed mixture ratio design described by Altenburger et al. (*33*) and Backhaus et al. (*34*). A master solution (1 mM) of the eight selected xenoestrogens was made, with the concentration ratios given in Table 1. These reflect equieffective concentrations of each chemical which were established in prescreening runs. The total concentration of the mixture was varied, and complete concentration−response relationships were recorded.

Calculation of Predicted Mixture Effects. On the basis of the Hill regression models for each mixture component, the joint effects of a mixture with known composition were calculated by using *concentration addition*, the *toxicity equivalency factor* approach, *effect summation*, and *independent action*. Detailed descriptions of the approaches and their mathematical underpinnings can be found in refs *25* and *30*.

Results

Estrogenicity of Individual Mixture Components. All tested compounds induced concentration-dependent increases in the activity of beta-galactosidase, indicative of activation of the estrogen receptor (alpha) in recombinant yeast cells. The data showed little experimental variation, as exemplified by the scatter plot and the regression model for 2′,3′,4′,5′-tetrachlorobiphenyl-4-ol depicted in Figure 1. The remaining chemicals gave plots of similar quality, and the data proved to be reproducible. Figure 2 shows regression models of all tested chemicals, including the steroid hormone 17 beta-estradiol. Important parameters of the regression models of the single chemicals employed in our studies are summarized in Table 1. The regression models were used to estimate EC_{01} values of these chemicals (Table 1); NOEC were determined using Dunnett's test. In many cases, the NOEC of the individual chemicals were lower than their EC_{01} values. The potencies of the eight estrogenic chemicals varied considerably. By far the most potent of the tested chemicals were 2′,3′,4′,5′-tetrachlorobiphenyl-4-ol and 2′,5′-dichlorobiphenyl-4-ol, and the least potent was resorcinol monobenzoate. The median effect concentrations and the EC_{01} values of these compounds spanned almost 3 orders of magnitude. Slope parameters and maximal effects of all chemicals were very similar. Our data agree well with the values reported by Miller et al. (*28*) for benzyl-4-hydroxyparabene, bisphenol A, and resorcinol monobenzoate. In our hands, however, dihydroxybenzophenone was more potent than communicated

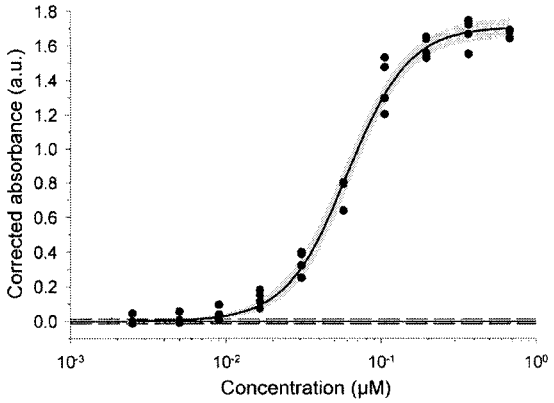

FIGURE 1. Concentration−response relationship for 2′,3′,4′,5′-tetrachlorobiphenyl-4-ol in the YES. Experimental effect data (solid circles) from three independent experiments, with the regression model (black solid sigmoidal line) and the upper and lower confidence limits of the best estimate of mean responses (shading). The horizontal solid and dashed lines are the mean ± 95% confidence interval of the population mean of untreated controls ($n = 20$).

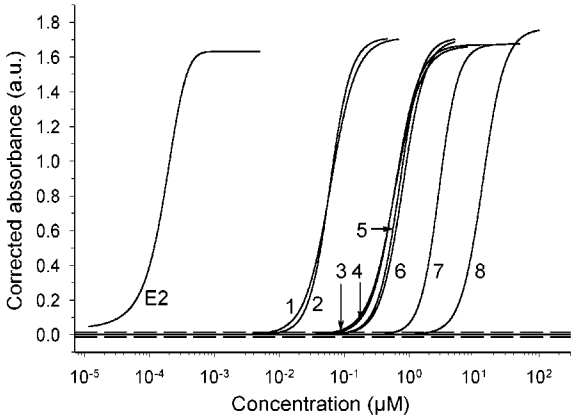

FIGURE 2. Regression models for the effects of all eight mixture components and of 17beta-estradiol (E2). The horizontal solid and dashed lines are the mean and 95% confidence interval of the mean of untreated controls ($n = 20$). The numbering of the chemicals is as follows: 1: 2′,3′,4′,5′-tetrachlorobiphenyl-4-ol, 2: 2′,5′-dichlorobiphenyl-4-ol, 3: 4-chlorobiphenyl-4-ol, 4: genistein, 5: dihydroxybenzophenone, 6: benzyl-4-hydroxyparabene, 7: bisphenol A, 8: resorcinol monobenzoate.

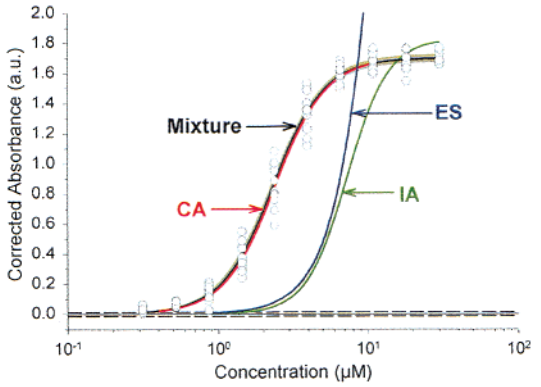

FIGURE 3. Predicted and observed effects of a mixture of all tested estrogenic chemicals 1−8 (see legend of Figure 2 for numbering). Experimental effect data (open circles) from three independent experiments, with the regression model (black solid sigmoidal line) and the upper and lower confidence limits of the best estimate of mean responses (shading). The red solid line shows the predicted combination effects derived from *concentration addition*. The curve produced using the *toxicity equivalency factor* approach is almost congruent with the *concentration addition* curve and is not shown here. The lines labeled IA and ES are the predictions derived from *independent action* and *effect summation*, respectively. The solid and dashed horizontal lines are the mean and 95% confidence interval of the mean of untreated controls ($n = 20$).

TABLE 2. Toxicity Equivalency Factors Derived for the Eight Tested Estrogenic Chemicals[a]

compound	toxicity equivalency factor
1. 2′,3′,4′,5′-tetrachlorobiphenyl-4-ol	2.86×10^{-3}
2. 2′,5′-dichlorobiphenyl-4-ol	3.00×10^{-3}
3. 4-chlorobiphenyl-4-ol	0.281×10^{-3}
4. genistein	0.288×10^{-3}
5. 2,4-dihydroxybenzophenone	0.253×10^{-3}
6. benzyl-4-hydroxyparabene	0.224×10^{-3}
7. bisphenol A	6.16×10^{-5}
8. resorcinol monobenzoate	1.35×10^{-5}

[a] Estradiol was used as the reference compound for these calculations.

by these authors (median effect concentrations of 0.7 μM vs 4 μM).

The Prognostic Value of Approaches for the Quantitative Estimation of Combination Effects. We used the regression models shown in Figure 2 to predict the mixture effects that are expected to occur from additive interactions of the eight estrogenic chemicals. The combined effects were calculated for a large range of responses by using *concentration addition*, the *toxicity equivalency factor* approach, *effect summation*, and *independent action*.

The predicted combination effects were tested experimentally. As shown in Figure 3, the eight-component mixture produced a complete concentration−response curve, with experimental variation highest in the linear portion of the curve. For the entire range of effect levels, the observed combination effects confirmed decisively the responses predicted by *concentration addition*. The regression model of the experimental mixture data was almost congruent with the *concentration addition* expectation.

The TEFs shown in Table 2 were used to estimate concentrations of 17beta-estradiol expected to be equieffective with the eight-component mixture. The regression model for the steroid hormone was employed to calculate the corresponding joint effects of the combination. To obtain a concentration−response curve that could be compared with the prediction curves of the remaining concepts, this

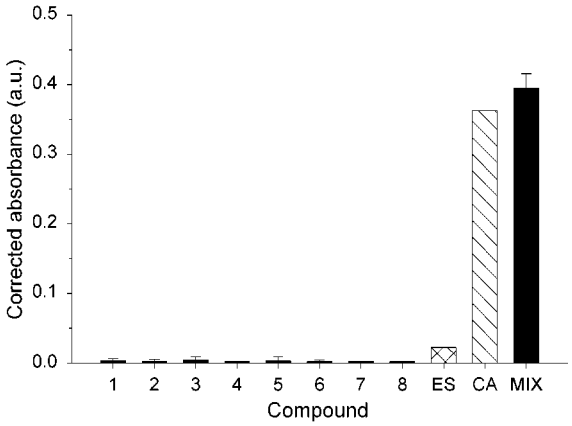

FIGURE 4. Effects of individual mixture components 1−8 at the concentrations present in 1.43 μM of the mixture. ES: *effect summation*, i.e., expected mixture effect obtained by calculating the arithmetic sum of individual effects of agents 1−8. CA: *concentration addition* prediction. MIX: observed mixture effect. Error bars are upper 95% confidence limits of the best estimate of mean responses. Concentrations of test agents in 1.43 μM of the mixture are depicted in Table 1.

procedure was repeated for a large number of concentrations. Confirming that the conditions of the TEF approach were met, the resulting "TEF-curve" was congruent with the one obtained by using *concentration addition* (Figure 3).

In contrast, the concepts of *effect summation* and *independent action* led to significant underestimations of the observed combination effects. The predicted median effect concentrations were approximately 3-fold higher than the experimentally observed ones. The steepness of the curves meant that mixture concentrations predicted to yield effects of 5% of the maximal effect, in reality produced significant responses (i.e. around the median effect level). The steepness of the *independent action* prediction curve was comparable to that of *concentration addition*. The maximal effect predicted on the basis of *independent action* was slightly higher than experimentally observed. As expected from its mathematical features, *effect summation* was unable to model the leveling off of responses normally seen at higher concentrations. Instead, this concept predicted a steep rise in response, even beyond the upper limits of maximally possible effects observable in the YES.

Combination Effects at Concentrations Producing Effects Indistinguishable from Those in Untreated Controls. Figure 4 displays the effects of individual mixture components that would be observed if they acted alone at levels corresponding to 50% their EC_{01} values. Although none of the eight xenoestrogens on their own would have produced measurable effects, they yielded a substantial response when combined at these concentrations. The observed mixture effect, corrected for untreated controls, was 0.39 ± 0.02 absorbance units (mean ± 95% CI of mean response), the effect predicted by *concentration addition* was 0.35 absorbance units. Simple summation of the individual effects of all the mixture components underestimated the observed response by a factor of 20 (0.02 predicted vs 0.39 ± 0.02 observed).

Discussion

In view of the excellent agreement between prediction and experimental observation, there can be little doubt about the usefulness of *concentration addition* for the prediction and assessment of the joint action of xenoestrogens at the level of estrogen receptor activation. The predictive power of *concentration addition* was apparent for the entire range of effects. This is underlined by the complete overlap between

the prediction curve and the 95% confidence interval of the regression model of observed mixture effects—a criterion which we used to assess our results statistically.

Our data also show that the *toxicity equivalency factor* approach is a useful method for the assessment of joint effect of xenoestrogens. We attribute this to the fact that the assumptions (*26*) underlying the TEF approach were fulfilled in our case, namely that all mixture components acted through the same pathway (estrogen receptor activation) and that the concentration−response curves for all chemicals were essentially parallel. The TEF approach may, therefore, be regarded as a convenient and easy to use alternative to *concentration addition*. Against this however, we have to balance the resources and efforts needed to ascertain that all mixture components indeed produce parallel concentration−response curves. This is an essential requirement, because toxicity equivalency factors for individual mixture components will inevitably vary with the effect level chosen for analysis, if the curves show different slopes. A dilemma may arise with mixture components that elicit similar but not exactly parallel curves. How much deviation from the requirement of parallelism can be tolerated to justify application of the TEF approach? As far as we are aware, there are no clear-cut, rational criteria to decide this question. Considering these complications, we favor use of the *concentration addition* concept. Unlike the TEF approach, it copes well with agents showing response curves with differing slopes (*8, 33*).

The concepts of *effect summation* and *independent action* are clearly unsuitable for the assessment of the joint effects of xenoestrogens in this assay. This is highlighted by the fact that both concepts predicted effects of only 0.1 (corrected absorbance units) for the median effect concentration of the mixture (2.3 μM). This represents 11% of the experimentally observed value of 0.85.

The inappropriateness of *independent action* may be explained in terms of the features of the yeast estrogen screen. The assay only screens the events leading up to, and following, the activation of the estrogen receptor protein by binding to its ligand binding domain. It is blind to all other possible effects, except gross toxicity, which will induce a loss of signal through yeast cell killing. On theoretical grounds therefore, *independent action* could have been ruled out a priori as a concept suitable for dealing with xenoestrogens in receptor activation assays, because only similarly acting agents will be active in such systems. However, in previous studies (*24*) we have observed that *concentration addition* and *independent action* produced almost identical predictions for additive combination effects in the yeast estrogen screen, which agreed well with the experimentally observed mixture effects. Hence, evidence was required that *concentration addition* is indeed appropriate for use with estrogen receptor agonists in this assay. The present study provides proof of this hypothesis. This could only be achieved because the differences between the additivity expectations derived from *concentration addition* and *independent action* were sufficiently large as not to encounter problems with discriminating between prediction and observation.

Considering the widespread, and often naive, application of the *effect summation* concept, our demonstration of large underestimations of mixture effects with this approach is of considerable practical importance. We show that the combined effect of eight agents that individually produce estrogenic effects (corrected absorbance units) of e.g. 0.5, 0.4, 0.1, 0.15, 0.14, 0.17, 0.21, and 0.45 is **not** 2.12. The fatal flaw in simply adding up the individual effects of mixture components is underlined by the fact that this method produces combination effect predictions in wild excess of the biologically possible maximal effect of 1.765! Inevitably therefore, the *effect summation* curve in Figure 2 shoots up steeply to unattainably high responses, beyond all known biological reality. Crucially, the uncritical use of *effect summation* would have led us to the erroneous conclusion that the joint effect of the eight xenoestrogens studied here is synergistic. This is because the observed combination effects far exceeded those predicted by *effect summation*.

Concentration addition implies that every mixture component contributes to the total mixture effect in proportion to its concentration, even when present at concentrations below zero effect levels ("no effect concentrations", NEC). Therefore, does the excellent agreement between experimentally observed combination effects and the *concentration addition* prediction mean that not even NECs are safe when dealing with large numbers of xenoestrogens in combination?

As is well established, it is impossible to determine NECs with confidence, because true "zero effects" cannot be distinguished from very small, albeit statistically insignificant responses. In an attempt to deal with this uncertainty, NOECs were introduced as approximations of NECs. However, what is estimated as NOEC depends to a large degree on the biological variability of the test system and the number and spacing of tested concentrations (*31*). Often, effects below 10% of a maximal possible response cannot be distinguished reliably from effects seen in untreated controls (*31*) and NOECs tend to increase, the fewer concentrations were tested (*31, 35, 36*). NOECs define a range of concentrations where low effects can neither be quantified nor ruled out with certainty.

So-called benchmark concentrations (EC_x) are increasingly viewed as alternatives to NOEC determinations (*38, 39*), and we became interested in comparing NOECs with benchmark concentrations corresponding to low effects. These are estimated by interpolation using concentration−response relationships (*31*). Unlike NOEC determinations, which are derived from comparisons of only two concentrations ("treated" versus "controls"), the benchmark concentration procedure utilizes the information contained in entire concentration−response curves. EC_{05} or EC_{10} are considered to be appropriate alternatives to NOECs (*39*). For the purposes of our study, we chose EC_{01}. As shown in Table 1, the NOECs determined for each single agent were often lower than their corresponding EC_{01}. We attribute this to the low variability associated with YES and to the low tested concentrations of each chemical.

The results of our experiments show convincingly that xenoestrogens act together to produce effects when present at concentrations that individually yield responses indistinguishable from those of untreated controls. There were joint effects when the test agents were combined at concentrations equal to 50% of their individual EC_{01} values. These concentrations were below the NOECs in all cases (Table 1). These results put into sharp relief the limitations of the traditional focus on single agent effects during hazard and risk assessments of endocrine disrupting chemicals. The assertion that individual estrogenic chemicals pose no harm because they are present at low, ineffective levels in humans or in wildlife may be irrelevant when dealing with mixed exposures. If every estrogenic agent contributes to the overall mixture effect in an additive fashion, well below NOECs and EC_{01}, the task of identifying the potential risks associated with estrogenic exposures becomes first and foremost a question of establishing the sheer number of estrogenic chemicals in the environment.

These considerations force the conclusion that the recent focus on searching for synergistic mixture effects with estrogenic agents has been unnecessary. Clearly, additive combination effects are of importance and require urgent attention when assessing the potential risks estrogenic agents may pose for humans and wildlife.

Despite our success in predicting the joint effect of estrogenic chemicals we would like to highlight a number of factors that are likely to complicate the assessment of exposure to real existing mixtures of xenoestrogens:

1. Reproducibility of bioassays. To a large extent, the good agreement between prediction and observation was due to the high reproducibility of the YES. It remains to be seen whether the methodology employed here can be applied successfully to bioassays that yield less reproducible data. We have initiated such studies using the E-SCREEN, with encouraging results (*25*).

2. Nature of estrogenic mixture components. To produce prediction curves that covered a suitably large range of effects, we had to select xenoestrogens that yielded maximal effects very similar to those of steroidal estrogens. This is due to the fact that for mathematical reasons, the concept of *concentration addition* cannot predict mixture effects that exceed those of the mixture component with the lowest maximal effect. It is now necessary to explore the predictability of combination effects with mixtures composed of xenoestrogens that produce differing maximal effects (*30*).

3. Endpoints. In the present study we have explored combination effects at the molecular level, close to biological receptors. An important task will be to investigate whether our findings hold true for biological effects at higher levels of cellular organization, e.g. estrogen-mediated cell proliferation, or at the organism level. A study of in vivo effects of mixtures of estrogenic chemicals has been recently published (*40*).

4. The biological effects of endogenous steroidal estrogens. Endogenous steroidal estrogens exert quite strong effects at the levels normally found in body fluids and tissues. Therefore, it is crucial to explore whether relatively weak xenoestrogens are able to create an impact on the actions of endogenous estrogens, when combined at low levels that individually produce undetectable effects.

Studies addressing these points are underway in our laboratory.

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