

# Modeling the Interaction of Binary and Ternary Mixtures of Estradiol with Bisphenol A and Bisphenol AF in an *In Vitro* Estrogen-Mediated Transcriptional Activation Assay (T47D-KBluc)

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Exposure to xenoestrogens occurs against a backdrop to physiological levels of endogenous estrogens. Endogenous estrogen levels vary from low levels in early childhood to high levels during pregnancy and in young women. However, few studies have addressed how xenoestrogens interact with endogenous estrogens. The current study was designed to characterize the individual dose-response curves of estradiol-17 $\beta$  (E<sub>2</sub>), bisphenol A (BPA), tetrabromo-bisphenol A (TBBPA), and bisphenol AF (BPAF, 4,4'-hexafluoroisopropylidene diphenol) on estrogen-dependent luciferase expression in T47D-KBluc cells and to determine how binary (8  $\times$  8 factorial) and ternary (4  $\times$  4  $\times$  4 factorial) mixtures of an endogenous estrogen (E<sub>2</sub>) interact with BPA and/or BPAF. Log EC<sub>50</sub> and hillslope values with SEs, respectively, for individual compounds were as follows: E<sub>2</sub>,  $-12.10\text{M} \pm 0.06071$ ,  $0.7702 \pm 0.1739$ ; BPA,  $-6.679\text{M} \pm 0.08505$ ,  $1.194 \pm 0.2137$ ; and BPAF,  $-7.648\text{M} \pm 0.05527$ ,  $1.273 \pm 0.1739$ . TBBPA was not evaluated in mixture studies because of its minimally estrogenic response at  $3 \times 10^{-5}\text{M}$  and elicited cytotoxicity at higher concentrations. Both the binary mixtures of E<sub>2</sub> with BPA and BPAF and the ternary mixture of E<sub>2</sub>, BPA, and BPAF behaved in an additive manner. For binary mixtures, as E<sub>2</sub> concentration increased, higher concentrations of BPA and BPAF were necessary to induce a significant increase in the estrogenic response. Understanding the behavior of mixture interactions of xenoestrogens, like BPA and BPAF, with endogenous estrogens will allow a better assessment of the potential risk associated with exposure to these chemicals, individually or as mixtures.

**Key Words:** *in vitro*; mixtures; estradiol; bisphenol A; mixtures; bisphenol AF.

Bisphenol A (BPA) is a high production volume chemical used to manufacture polycarbonate plastics, as well as epoxy resins used as protective liners in metallic cans, and as an additive in various plastics. Tetrabromo-bisphenol A (TBBPA) is used primarily as a flame retardant in epoxy resin circuit boards, electronic enclosures, paper, plastic, and textiles (NTP, 2002; Olsen *et al.*, 2003) and bisphenol AF (BPAF) is used in fluoroelastomers, polyamides, polyesters, polycarbonate copolymers, and other specialty polymers (Akahori *et al.*, 2008; NTP, 2008; Perez *et al.*, 1998). BPA and BPAF have been shown to induce estrogen-dependent responses *in vivo* and *in vitro* via binding to estrogen receptor (ER) ER $\alpha$  and ER $\beta$  (Akahori *et al.*, 2008; Bay *et al.*, 2004; Wetherill *et al.*, 2007; Yamasaki *et al.*, 2003), whereas studies on the estrogenicity of TBBPA *in vitro* are inconsistent. Both BPAF and TBBPA have been nominated for toxicological characterization by the NTP, National Institute of Environmental Health Sciences (NTP, 2002, 2008). Exposure to BPA and BPAF may co-occur as there is concern of potential exposure of the general population to BPAF from its use as a monomer of polycarbonate and other polymers and resins and the use of fluoroelastomer gaskets and hoses in food processing equipment; however, information on specific use and potential exposure were not available (NTP, 2008). Exposure of BPA and its analogs to human and wildlife populations is widespread and well documented (Bay *et al.*, 2004; CERHR, 2008; NTP, 2008); but association of exposure to these compounds with adverse health effects remains controversial.

In different life stages, environmental estrogen exposures would occur concurrently with endogenous hormones. Furthermore, humans display widely varying concentrations of circulating estradiol-17 $\beta$  (E<sub>2</sub>) depending on their gender and life stage. For example, children have circulating E<sub>2</sub> concentrations ranging from 0.3 to 50pM (0.08–13 ng/l) with boys falling on the lower end of the range (Aks glaede *et al.*, 2006; Bay *et al.*, 2004; Ikegami *et al.*, 2001; Klein *et al.*, 1994).

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During pregnancy, especially in late pregnancy, circulating concentrations of  $E_2$  are as high as 60–88nM (16.35–24  $\mu\text{g/l}$ ) (Smith *et al.*, 2009; Troisi *et al.*, 2003a, 2003b). Therefore, data examining the interaction of environmental estrogens like BPA or its analogues with the ER when combined with endogenous estrogens such as  $E_2$  are needed to provide a more accurate representation of the potential effects of these anthropogenic chemicals *in vivo*. The objective of the current study was to assess *in vitro* mixtures of BPA and BPAF with a wide range of concentrations of the natural estrogen  $E_2$  to elucidate whether these mixtures behave, in an additive, synergistic, or antagonistic manner. The study was also designed to determine concentrations of BPA and BPAF that enhanced the estrogenic effect of increasing concentrations of  $E_2$  in order to simulate how these xenoestrogens might interact with physiological levels of estrogens during different life stages and for different genders.

Using a transcriptional activation assay, individual compounds and binary and ternary mixtures of an endogenous estrogen ( $E_2$ ) with BPA or its analogues (TBBPA and BPAF) were tested. Dose-response parameters ( $EC_{50}$  and hillslope) obtained from individual chemical experiments were incorporated into a dose-addition model to predict the responses elicited by mixtures of  $E_2$  and BPA,  $E_2$  and BPAF, and  $E_2$ , BPA, and BPAF in order to develop a predicted response surface plot. Additionally,  $EC_{50}$  values and mixture concentrations were incorporated into the toxic equivalence (TEQ) model of additivity to develop an estrogen equivalence (EEQ) model, in order to compare the EEQ predictions with those obtained with dose-addition modeling. The TEQ model is a specific type of dose-addition model that assumes same slopes and requires less data to calculate (Safe, 1998a, 1998b; Van den Berg *et al.*, 1998).

Modeled predictions were tested experimentally to determine if the dose-addition model provided accurate predictions of mixture effects and whether predictions based upon the EEQ model differed significantly from dose-addition model. Although several studies have investigated the estrogenic effects of BPA *in vitro*, few address whether the estrogenic activity of BPA functions in an additive manner with endogenous estrogens. Understanding the behavior of mixture interactions of BPA with endogenous estrogens will allow better assessment of the potential risk of individual xenoestrogen exposures and exposure to mixtures of xenoestrogens.

## MATERIALS AND METHODS

**Test chemicals.** The following chemicals were tested individually, stated by common name, abbreviation, CAS number, purchase source, purity (as reported by the manufacturer), and IUPAC name.  $E_2$  (CAS# 50-28-2, Sigma-Aldrich, 98%, (17 $\beta$ )-estra-1,3,5(10)-triene-3,17-diol); BPA (CAS# 80-05-7, Sigma-Aldrich, 99+%, 4,4'-dihydroxy-2,2-diphenylpropane); TBBPA (CAS# 79-94-7, Sigma-Aldrich, 97%, 2,2',6,6'-tetrabromo-4,4'-isopropylidenediphenol); BPAF (CAS# 1478-61-1, AccuStandard, 100%, 4,4'-(hexafluoroisopro-

pylidene)diphenol); ICI 182,780 (ICI; CAS# 129453-61-8, ICI Pharmaceuticals (7R,8R,9S,13S,14S,17S)-13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfanyl)-nonyl]-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthrene-3,17-diol).

**T47D-KBluc transcriptional activation assay.** T47D-KBluc cells naturally express both ER $\alpha$  and ER $\beta$  and are stably transfected with a triplet estrogen-responsive element promoter-luciferase reporter gene construct. The T47D-KBluc cells are sensitive to potent estrogens like  $E_2$ , ethynyl estradiol, and diethylstilbestrol and also to weaker environmental estrogens like genistein and 4-nonylphenol (Wilson *et al.*, 2004). A brief summary of the T47D-KBluc assay is presented below; please refer to Wilson *et al.* (2004) for greater detail.

The stock cells (T47D-KBluc, ATCC # CRL-2865) were maintained in 75-cm<sup>2</sup> culture flasks with vented caps (Corning 430641) at 37°C, 5% CO<sub>2</sub> in RPMI (Gibco 13200-076) growth media supplemented with 10% fetal bovine serum (FBS) (Hyclone #SH30071.03), 2mM glutamine, and 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 0.25  $\mu\text{g/ml}$  amphotericin. Cell passage numbers 40 through 50 were used during the performance of this study. Stock cells were subcultured once per week onto a new culture flask with fresh growth media and fed at midweek. One week prior to the assay, cells were placed in withdrawal media consisting of RPMI supplemented with 10% dextran-coated charcoal-stripped FBS (Hyclone #SH30068.03) (DCC-FBS RPMI) to remove all estrogens from the culture. Cells were incubated 1 week in withdrawal media with media replacement at midweek before being used in an assay. After incubation in low estrogen media, cells were seeded onto 96-well luminometer plate (Costar 3610) at 10<sup>4</sup> cells/100  $\mu\text{l}$ /well in 5% DCC-FBS RPMI and allowed to attach overnight. Cells were dosed with test chemical the following day.

Chemical stock solutions were prepared in 100% ethanol in glass amber vials with Teflon-lined caps and stored at room temperature. Dosing solutions were prepared by diluting chemical stocks in fresh 5% DCC-FBS RPMI to desired concentrations. Ethanol concentration did not exceed 0.3%; a vehicle concentration which has been shown to result in no adverse impact on the assay results (Wilson *et al.*, 2004). Chemically treated cells were incubated overnight and harvested the following day. Luciferase activity was determined using a BMG LUMIstar 96-well plate luminometer (BMG Labteck, Durham, NC) and quantified in relative light units (RLUs) (Wilson *et al.*, 2004).

**Binary mixtures.** Binary mixtures of  $E_2$  with either BPA or BPAF were run in an 8  $\times$  8 factorial design. Each plate had four replicates of an ethanol vehicle control, an ICI (1 $\mu\text{M}$ ) antagonist control with 100pM  $E_2$  or 1 $\mu\text{M}$  BPA/BPAF and a positive control of 100pM  $E_2$  and 1  $\mu\text{M}$  BPA or BPAF depending on the mixture being tested. The ICI (1 $\mu\text{M}$ ) antagonist control was used to demonstrate that the observed effect with the test chemical is solely ER mediated. Each plate also had one binary mixture for each point in the 8  $\times$  8 factorial design. Concentrations used encompassed the complete dose-response range for each compound. For  $E_2$ , the concentrations were as follows: vehicle control, 30fM, 100fM, 300fM, 1pM, 3pM, 10pM, and 30pM. For BPA, the concentrations were vehicle control, 3nM, 10nM, 30nM, 100nM, 300nM, 1 $\mu\text{M}$ , and 3 $\mu\text{M}$ . For BPAF, the concentrations were vehicle control, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, and 1 $\mu\text{M}$ . The binary mixtures of  $E_2$  and BPA had a total of 12 runs (each run is equivalent to one 96-well assay plate), whereas the binary mixtures of  $E_2$  and BPAF had a total of 15 runs.

**Ternary mixtures.** Ternary mixtures of  $E_2$  with BPA and BPAF were tested using a 4  $\times$  4  $\times$  4 factorial design. The ternary mixtures were repeated for a total of six runs. In addition, each run had an ethanol vehicle control and an  $E_2$  dose-response curve each with four replicate wells at each concentration. The  $E_2$  dose-response curve had the following concentrations: 30fM, 100fM, 300fM, 1pM, 3pM, 10pM, and 100pM. The concentrations used in the ternary mixture were as follows: for  $E_2$ , 1fM, 30fM, 300fM, and 10pM; for BPA, 1nM, 10nM, 30nM, and 300nM; for BPAF, 300pM, 3nM, 10nM, and 100nM. The concentrations of  $E_2$ , BPA, and BPAF chosen for the ternary mixtures were 0, 10, 35, and 85% response for each compound. The percent response concentrations used for the ternary mixture ensured that if antagonism or

synergism occurred among the chemicals, the mixture spectrum was broad enough to detect such phenomena.

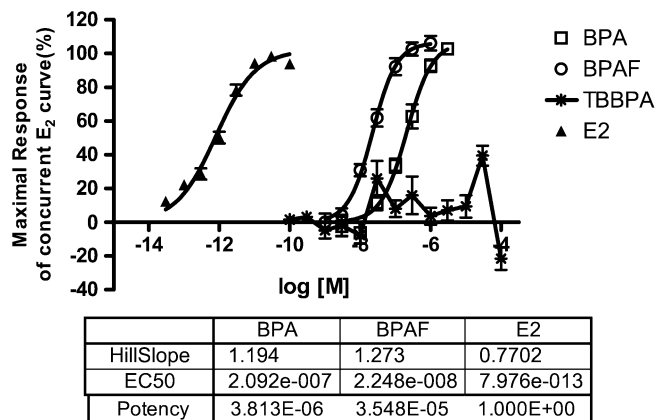
**Data and statistical analysis.** RLU for each run were normalized by converting values to fold induction by dividing the RLU for each well by the average RLU for the ethanol vehicle control. Fold induction values were then log transformed to control for heterogeneity of variance.  $\log_{10}$ -transformed fold induction (log-fold) values for each plate (run) were further converted to percent of maximal  $E_2$  response using the following procedure. The concurrent  $E_2$  curve for each run in log-fold (y-axis) was plotted in GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) versus chemical concentration. Concentration values (x-axis) were log transformed and then a nonlinear regression curve fit analysis (four-parameter logistic regression) were performed on the  $E_2$  data using a constrained bottom parameter held constant equal to 0.0. The calculated top parameter (maximal response) of the nonlinear regression analysis for  $E_2$  was used to convert the log-fold data into percent response for each test well. Specifically, the log-fold response was divided by the top parameter (in log fold) and then multiplied by 100. Once each run was converted to percent of the maximal  $E_2$  response, data from all runs for each chemical were combined.

Percent response data were combined, and observed means were compared with predicted values. Observed and predicted data were plotted in GraphPad Prism using a nonlinear regression curve fit. Dose-addition predicted values were calculated using the concentration addition model (power function written in Microsoft excel format):  $R = (1/(1 + (1/(\text{POWER}((\Sigma C_i/EC_{50}^i)^{\text{HillSlope}})))))) \times 100$ , where  $C$  = concentration of each chemical in mixture/ $EC_{50}$  of said chemical and “ $i$ ” refers to individual compounds (Rider and LeBlanc, 2005). Data were visualized with three-dimensional response surface plots and scatter plots created with SigmaPlot 11.0 (Systat Software, San Jose, CA). The EEQ model (the EEQ model equation used:  $EEQ = \Sigma[E_{2i}] \times EQF_i + \Sigma[BPA_i] \times EQF_i + \Sigma[BPAF_i] \times EQF_i$ ) was modified from the TEQ model used for dioxins and dioxin-like chemicals (Bhavsar *et al.*, 2008; Safe, 1998a, 1998b; Van den Berg *et al.*, 2006). Individual compound estrogen equivalent factors were calculated by dividing the  $E_2$   $EC_{50}$  by each individual compound  $EC_{50}$ .

The combined dose-response data for each chemical for all binary and ternary mixture assays were plotted in GraphPad Prism and a nonlinear regression curve fit analysis performed to obtain  $EC_{50}$  and hillslope values.  $EC_{50}$  and hillslope values for each chemical in the binary and ternary mixtures were used to calculate the predicted responses. Statistical analysis to determine if the dose-response curves were different from one another for BPA and BPAF were computed using a sum of squares  $F$ -test run ( $\alpha = 0.05$ ) on bottom, top, log  $EC_{50}$ , and hillslope parameters in GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). Statistical calculations of 95% confidence limits (CLs) for binary and ternary mixtures were calculated using Proc Means SAS 9.1 (SAS Institute, Cary, NC). In order to determine at which concentrations BPA or BPAF would induce an observable response in our mixtures with  $E_2$ , a one-way ANOVA was run on log-transformed fold data at each concentration of  $E_2$  within the binary mixtures to determine the “no-observed-effect concentration” (NOEC) for BPA or BPAF through Proc GLM LS Means using SAS 9.1.

## RESULTS

Dose-response curves for individual compounds,  $E_2$ , BPA, BPAF, and TBBPA, were plotted in Figure 1. The log  $EC_{50}$  and hillslope mean data with SE, respectively, were as follows:  $E_2$ ,  $-12.10M \pm 0.06071$ ,  $0.7702 \pm 0.1739$ ; BPA,  $-6.679M \pm 0.08505$ ,  $1.194 \pm 0.2137$ ; and BPAF,  $-7.648M \pm 0.05527$ ,  $1.273 \pm 0.1739$ . The log  $EC_{50}$  values were statically different between BPA and BPAF ( $p < 0.0001$ ) with BPAF being the slightly more potent environmental estrogen. No statistical difference was found in top, bottom, and hillslope parameters



**FIG. 1.** T47D-KBluc assay ER transcriptional activation dose-response of individual chemicals,  $E_2$ , BPA, BPAF, and TBBPA, fitted with a nonlinear regression (variable slope) curve. The  $EC_{50}$  and hillslope values were calculated through GraphPad Prism 5.01. Relative potency calculated by dividing  $EC_{50}$  values of individual compounds by  $E_2$   $EC_{50}$  value. Data are plotted as mean  $\pm$  SE.  $E_2$   $n = 12$ ; BPA  $n = 12$ ; BPAF  $n = 15$ ; TBBPA  $n = 15$ .

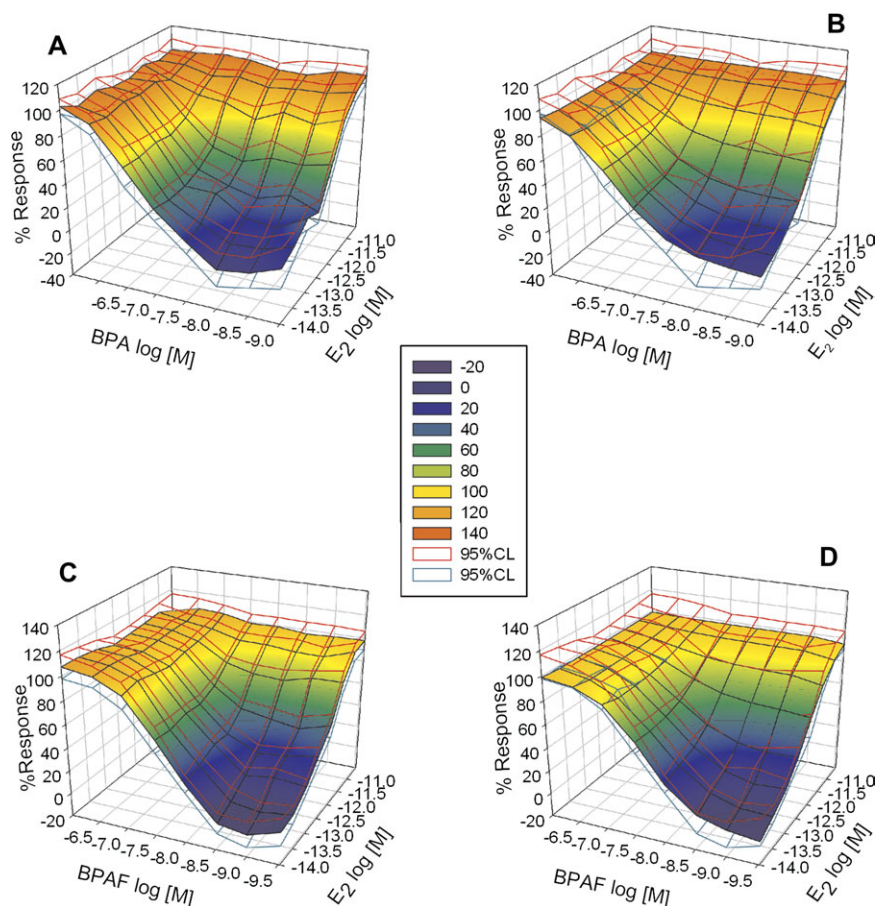
for BPAF and BPA response curves. TBBPA induced a weak, equivocal estrogenic response in the T47D-KBluc assay only at a concentration of  $30\mu M$ . Activity in the assay then declined at  $100\mu M$  because of cytotoxicity. Therefore, because of its ambiguous estrogenicity, TBBPA was excluded from further evaluation.

Overall for both test compounds, the dose-addition and EEQ models accurately predicted the observed response of the binary mixtures. Figures 2A and 2C, 3, and 4 depict the observed data (means with 95% CLs) for the binary mixtures of BPA with  $E_2$  and BPAF with  $E_2$ , respectively. There were some areas of the response surface curves where the model slightly over- or underpredicted the experimental response. Overlaying the predicted values with the 95% CLs of the observed data indicate that the deviations from the predicted response were infrequent for both BPA and BPAF (Figs. 2B and D, 3, and 4). The scatter plots of the observed means for the binary mixtures of BPA and BPAF both compared well with the predicted regression lines of the EEQ model (BPA  $r^2 = 0.971$ , BPAF  $r^2 = 0.969$ ) with only minor deviations from predicted values (Figs. 5 and 6).

With an increase in  $E_2$  concentrations, both the BPA and BPAF dose-response curves shifted to the right resulting in increasing  $EC_{50}$  values with each  $E_2$  increment. To further evaluate the impact of BPA or BPAF on the  $E_2$  dose-response, the NOECs for binary mixtures of BPA or BPAF with  $E_2$  were determined as increasing concentrations of each test compound were added (Fig. 7).

The ternary mixture also behaved in a dose-additive manner (Fig. 8). At an  $E_2$  concentration of  $10pM$ , the model system reached saturation, and addition of BPA and BPAF at any higher concentrations did not produce any additional increase in activity. Similar to the predicted results from the binary





**FIG. 2.** Predictions of dose-addition data generally fall within 95% CLs of the observed data. Observed (A and C) and dose-addition modeled data (B and D) compared with 95% CLs from observed data (red = upper CL, dark cyan = lower CL) for binary mixtures of BPA plus E<sub>2</sub> (A and B) and BPAF plus E<sub>2</sub> (C and D).

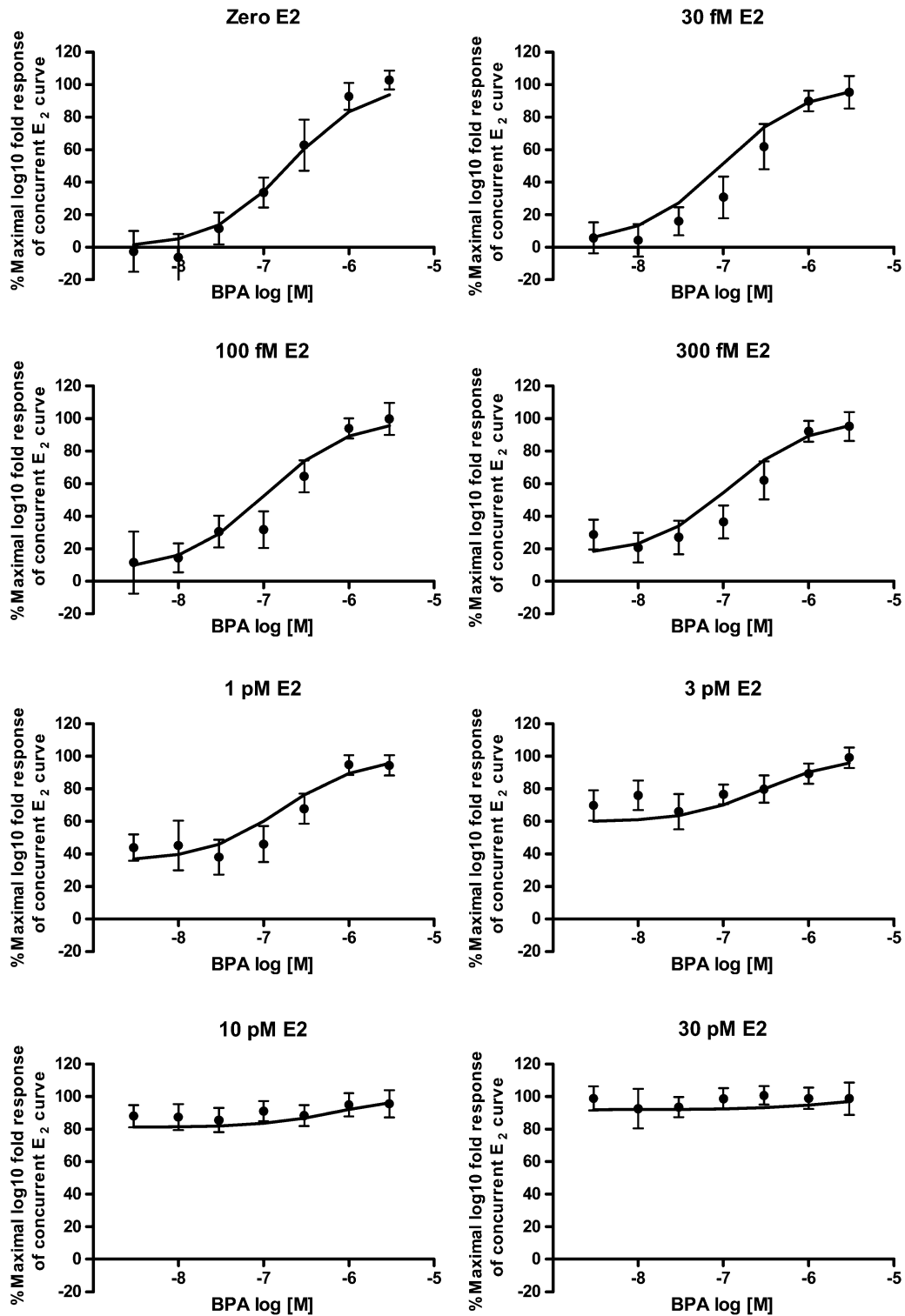
mixtures, some areas of the ternary response surface curve were slightly different with minor deviations from the observed responses (Fig. 9). The majority of those differences occurred at an E<sub>2</sub> concentration of 300fM. The EEQ model predicted the observed responses as well as the dose-addition model (Fig. 10) and observed means compared well with the predicted regression line ( $r^2 = 0.949$ ).

## DISCUSSION

Understanding the behavior of mixture interactions of xenoestrogens like BPA and BPAF with endogenous estrogens is important in assessing their potential risk to humans. The current study characterized the dose-response effects of E<sub>2</sub>, BPA, BPAF, and TBBPA in the T47D-KBluc transcriptional activation assay. Based on the individual chemical dose-response analyses, binary and ternary mixtures of an endogenous estrogen (E<sub>2</sub>) with BPA or BPAF were tested and their interactions evaluated. The current study demonstrates that the binary and ternary mixtures of selected estrogens behaved in a dose-additive manner. Both the dose-

addition model and the EEQ model predicted the mixtures results well. Additionally, NOECs of the binary mixtures of BPA and BPAF with E<sub>2</sub> demonstrates that increasing levels of BPA or BPAF are required to enhance the estrogenicity of the *in vitro* mixture as the E<sub>2</sub> levels increase. There was no evidence that BPA or BPAF acted synergistically or in an antagonistic manner when combined with E<sub>2</sub>.

Estrogens have numerous functions in vertebrates. In males, estrogens are essential for normal testicular (Hess *et al.*, 2001), prostate (Chen *et al.*, 2009), brain (McCarthy *et al.*, 2008), and behavioral development (McCarthy, 2008). Estrogens play a role in adipocyte development and function as well as in bone growth and epiphyseal plate closure in both sexes (Cooke and Naaz, 2004; Syed and Khosla, 2005; Vandenput and Ohlsson, 2009). In females, the role of estrogens is fundamental during puberty and reproductive cycling (Johnson and Everitt, 2004; Knobil and Neill, 1993). An important factor when investigating environmental estrogens is how they interact with the varying concentrations of endogenous estrogens in different genders and at different human life stages. The circulating estrogen concentration varies at different stages of development and maturation. For reference, at birth, E<sub>2</sub> plasma



**FIG. 3.** Predictions of dose-addition data generally fall within 95% CLs of the observed data. Binary mixture response of E<sub>2</sub> and BPA, fitted with a nonlinear regression (variable slope) curve fit and 95% CLs. Line represents the predicted dose-addition model.

concentrations are extremely high in both male and female neonates (5–6 µg/l, approximately 18.4–22nM) (Johnson and Everitt, 2004; Rohrmann *et al.*, 2009; Shibata *et al.*, 2002)

and adult females can fluctuate from approximately 140pM (38 ng/l) (Finstad *et al.*, 2009; Hatta and Nagaya, 2009; Knobil and Neill, 1993; Lemieux *et al.*, 2009) to 455pM (124 ng/l)

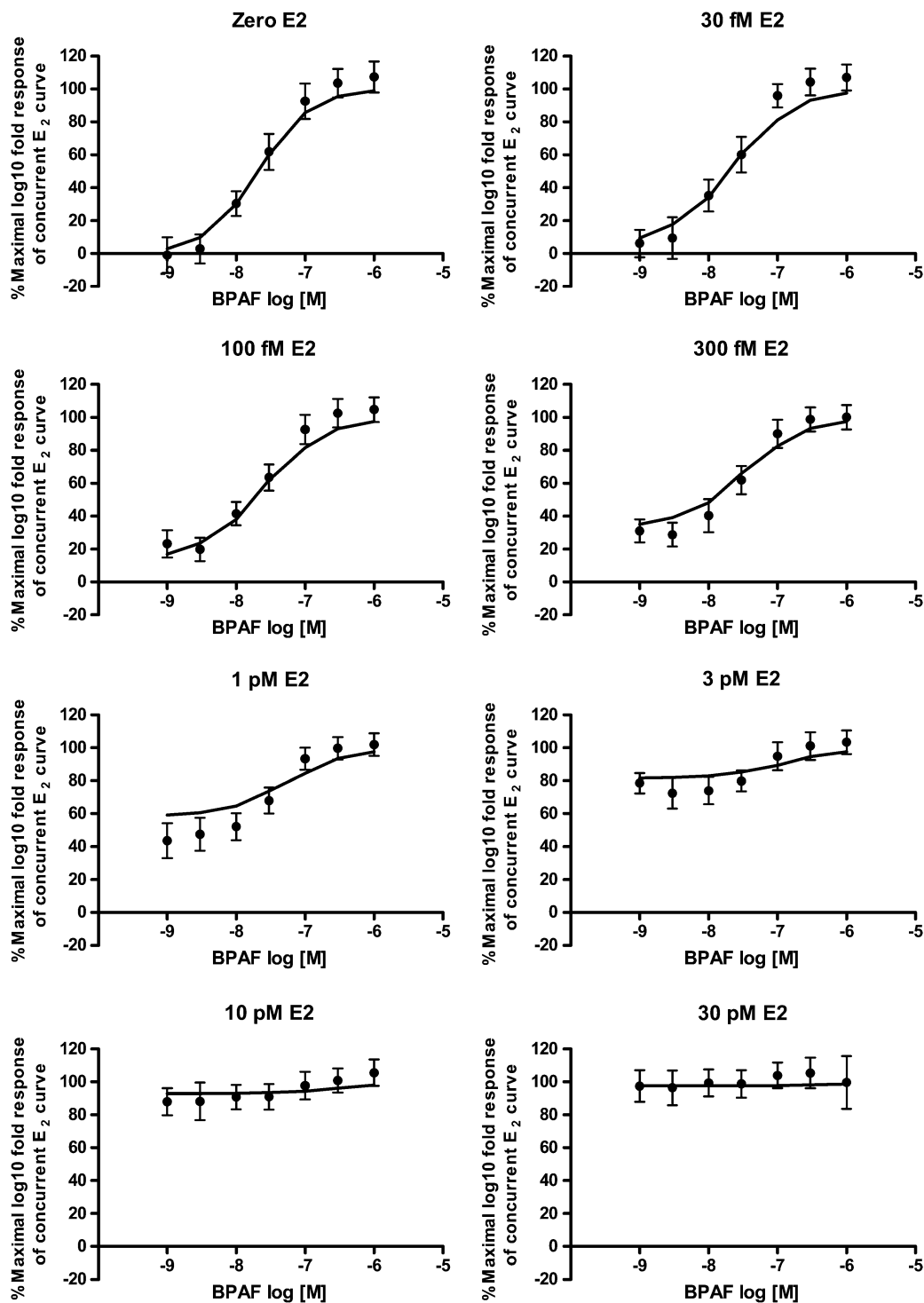
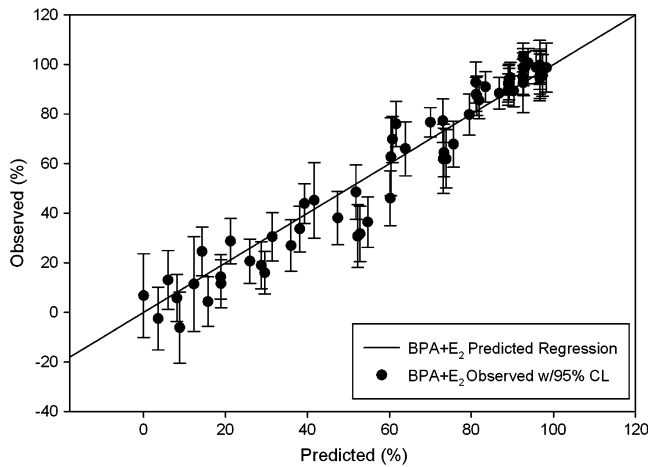


FIG. 4. Predictions of dose-addition data generally fall within 95% CLs of the observed data. Binary mixture response of E<sub>2</sub> and BPAF, fitted with a nonlinear regression (variable slope) curve fit and 95% CLs. Line represents the predicted dose-addition model.

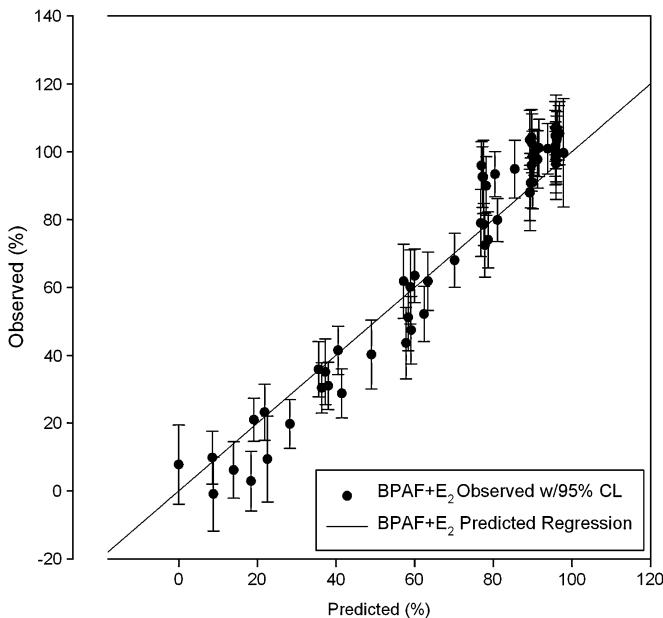
(Hatta and Nagaya, 2009; Knobil and Neill, 1993; Lemieux *et al.*, 2009) depending on the stage in their menstrual cycle. Children have “low” circulating estradiol concentrations, from 0.3 to 50pM (0.08–13 ng/l) (Aksglaede *et al.*, 2006; Bay *et al.*,

2004; Ikegami *et al.*, 2001; Klein *et al.*, 1994). Additionally, because the reported E<sub>2</sub> concentrations in children fall in the lower picomolar range, this suggest that high circulating levels of BPA (>1μM BPA) would be necessary to induce an effect.

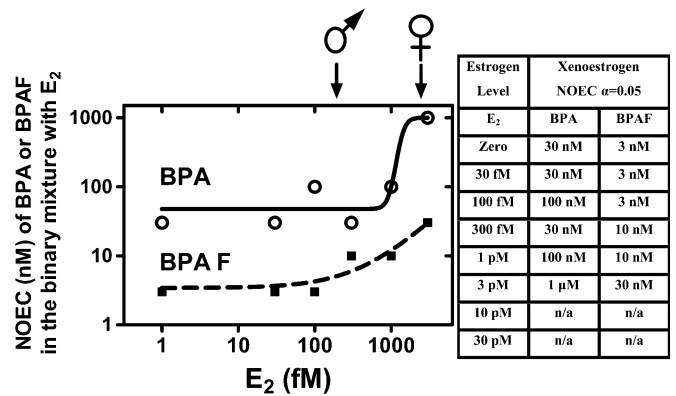


**FIG. 5.** Binary mixture of BPA plus E<sub>2</sub> shows good correlation between predicted and observed data. Regression line plots the predicted EEQ of the binary mixtures for both axes x and y. Individual points plot the means with 95% CLs of observed results on the y-axis and predicted EEQ on the x-axis. The scatter allows for the evaluation of the efficacy of the EEQ model.

However, the above suggestion does not account for several important factors, including (1) a considerable percentage of the E<sub>2</sub> in serum is bound to SHBG, (2) a small proportion of BPA exists in serum as “free” BPA whereas most BPA exists in the inactive glucuronidated form, and (3) some of the “free” BPA also is bound to plasma proteins. For these reasons, the stated circulating serum E<sub>2</sub> and BPA levels may not necessarily reflect the E<sub>2</sub> and BPA concentration levels found at the cellular



**FIG. 6.** Binary mixture of BPAF plus E<sub>2</sub> shows good correlation between predicted and observed data. Regression line plots the predicted EEQ of the binary mixtures for both axis x and y. Individual points plot the means with 95% CL of observed results on the y-axis and predicted EEQ on the x-axis. The scatter allows for the evaluation of the efficacy of the EEQ model.

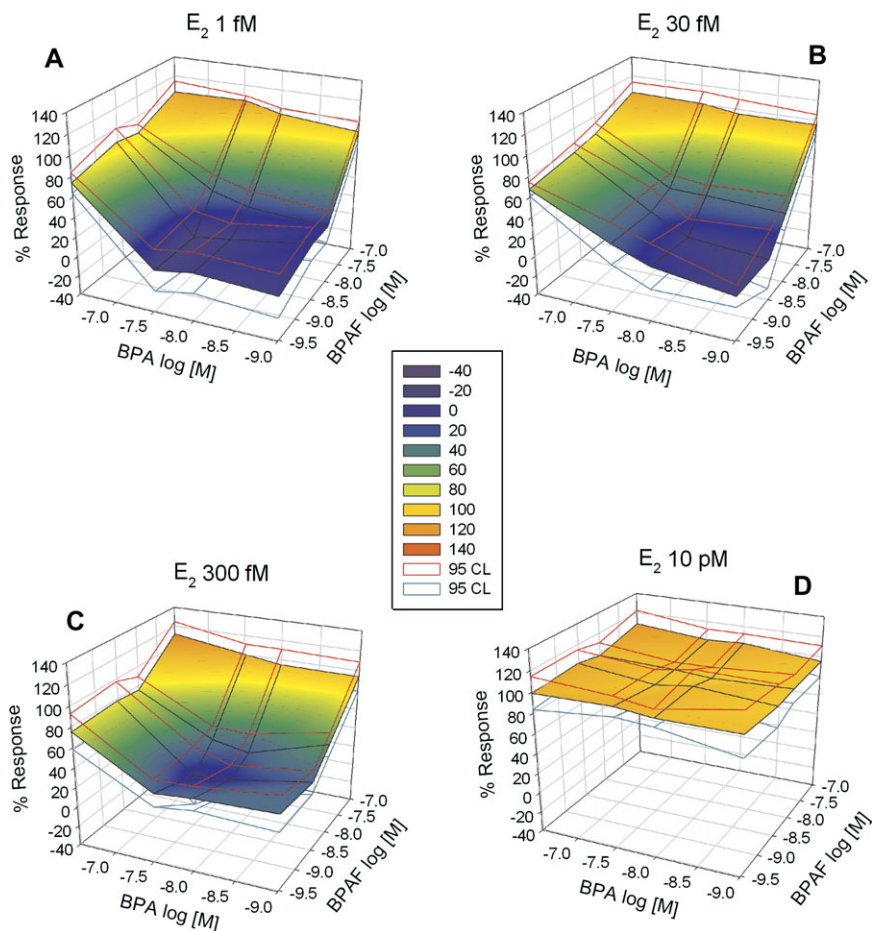


**FIG. 7.** The NOEC for E<sub>2</sub> plus BPA or BPAF. The NOECs are for BPA or BPAF when combined with the stated concentrations of E<sub>2</sub> and indicate which concentration of BPA or BPAF is necessary to induce a statistically significant response in the binary mixture. Increasing levels of BPA or BPAF are required to enhance the estrogenicity of the in vitro mixture as the E<sub>2</sub> levels increase. Arrows indicate lowest measured E<sub>2</sub> levels in humans and prepubertal boys and girls, respectively. T47D-KBluc assay reached saturation and maximal response was induced; hence, NOEC cannot be calculated; n/a, not applicable.

and subcellular level interacting with ERs. Several studies measuring BPA levels in human blood using High Performance Liquid Chromatography/Mass Spectrometry/Mass Spectrometry showed BPA blood levels in adults under 1  $\mu$ g/l (CERHR, 2008; Volkel *et al.*, 2005). It is also noted that there is a dearth of high-quality data for human serum and tissue levels of “free” BPA and glucuronidated BPA, NTP/NIH studies are currently investigating these gaps in the data.

In our dose-response analyses for E<sub>2</sub>, BPA, and BPAF, a full response curve was used to ensure that relevant concentrations of each compound were incorporated into the binary and ternary mixtures. When examining the dose-response curves, both BPA and BPAF have similar responses with BPAF being the slightly more potent environmental estrogen. Relative potency of BPA and BPAF to E<sub>2</sub> was evaluated (Fig. 1) showing BPA six orders of magnitude weaker than E<sub>2</sub> and BPAF was five orders of magnitude weaker than E<sub>2</sub>. These results were corroborated in another study where the EC<sub>50s</sub> of BPAF and BPA were  $0.05 \times 10^{-6}$ M and  $0.63 \times 10^{-6}$ M, respectively, and corresponded well with observations from other published studies (Kitamura *et al.*, 2005; Olsen *et al.*, 2003).

TBBPA induced an estrogenic response in our assay only at the concentration of  $3 \times 10^{-5}$ M (approximately 40% response) followed by a sharp drop in activity because of cytotoxicity at the next highest concentration. Therefore, no clear dose-response was observed. Others studies have shown it to behave like an estrogen albeit only at high concentrations (Kitamura *et al.*, 2005; Li *et al.*, 2010; NTP, 2002; Olsen *et al.*, 2003). The maximal concentration of  $1 \times 10^{-4}$ M used in the T47D-KBluc assay fell within the activity range reported by Kitamura *et al.* (2005) of  $1 \times 10^{-6}$  to  $1 \times 10^{-4}$ M but was cytotoxic in our assay. TBBPA has been shown to be a partial agonist with



**FIG. 8.** Observed ternary mixtures of BPA and BPAF plus  $E_2$  (A = 1fM, B = 30fM, C = 300fM, and D = 10pM) a with 95% CLs from observed data (red = upper CL, dark cyan = lower CL).

a low affinity to the ER, especially in the presence of binding proteins in serum, which corroborates TBBPA results seen in MCF-7 and T47D-KBluc cell lines which each requires 5% FBS (Kitamura *et al.*, 2005; Olsen *et al.*, 2003; Samuelsen *et al.*, 2001).

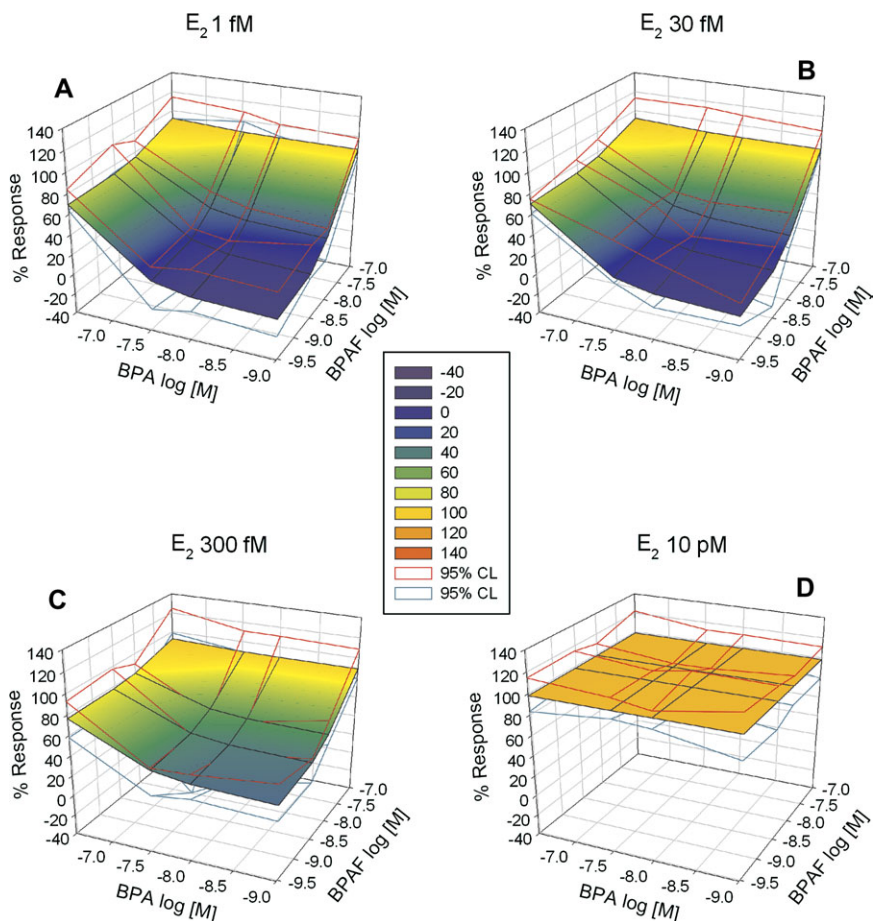
The overall trends reflected in the surface response plots for both binary mixtures with BPA or BPAF are consistent for both predicted and observed responses. Both the dose-addition model and the EEQ model accurately predicted the observed responses. The  $EC_{50}$  results for BPA and BPAF gradually increased as  $E_2$  concentration increased in the mixture, as expected. At  $E_2$  concentrations of 3pM and greater in the binary mixtures with BPA/BPAF,  $EC_{50}$  values could not be calculated because the curves approached maximal induction and flattened (Figs. 3 and 4).

The pattern observed for the NOECs was that as  $E_2$  concentration increased so did the concentration of BPA or BPAF needed to significantly enhance the estrogenic response of the mixture. The NOECs are for BPA or BPAF when combined with the stated concentrations of  $E_2$  and indicate which concentration of BPA or BPAF is necessary to induce

a statistically significant response in the binary mixture. At the lowest  $E_2$  concentration tested of 30fM, the addition of 30nM BPA or 3nM BPAF or lower concentrations produced no change in the NOEC. At an  $E_2$  concentration of 3pM, the T47D-KBluc model system for the binary mixtures approached saturation and maximal response induction was reached. The NOEC at 3pM  $E_2$  for BPA was 1 $\mu$ M and 30nM for BPAF, meaning that a concentration higher than 1 $\mu$ M BPA and 30nM BPAF would be needed to induce statistically significant increases in binding response to the ER. At  $E_2$  concentration of 10pM and greater, the model was saturated, maximal induction was achieved, and further addition of BPA or BPAF induced no change in the assay's response (see Fig. 7).

Ternary mixtures were accurately predicted by the dose-addition model. The pattern observed in the binary mixtures was also evident in the ternary mixtures. The individual compounds in the ternary mixture behaved in a dose-additive manner; as individual concentrations increased, so did the ER-mediated activity in the assay. Similar to the binary mixtures, the majority of the variance in the predictions occurred at the higher concentrations of the ternary mixture. The increased variance



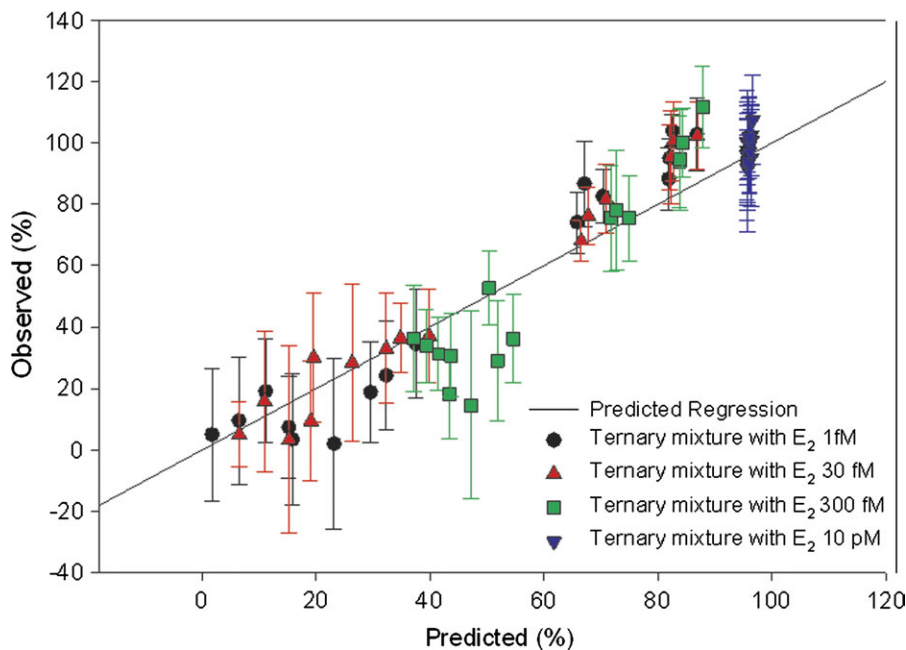


**FIG. 9.** Predictions of dose-addition data generally fall within 95% CLs of the observed data. Dose-addition predictions for ternary mixtures of BPA and BPAF plus  $E_2$  (A = 1fM, B = 30fM, C = 300fM, and D = 10pM) with 95% CLs from observed data (red = upper CL, dark cyan = lower CL).

found at the top portion of response curves may explain why the majority of missed predictions occurred at 300fM as this dose was approaching saturation in the model system. At the ternary mixture,  $E_2$  concentration of 10pM, the model system was saturated and maximal response had occurred.

The EEQ model of additivity is a modification of the TEQ model used for risk assessment of dioxin-like compounds (Safe, 1998b; Van den Berg *et al.*, 2006). The TEQ model has been used for the last two decades in risk assessment of closely related chemicals with identical mechanisms of action but different potencies (Bhavsar *et al.*, 2008; Finley *et al.*, 2003; Hong *et al.*, 2009). The TEQ/EEQ model has been shown to work well with *in vitro* systems and has been suggested to be most applicable for hazard/risk assessment of compounds that act through a common receptor (Safe, 1998a, 1998b; Van den Berg *et al.*, 2006). The EEQ model predictions for both the binary and ternary mixtures agreed with the observed mixture results. The differences in the EEQ model predictions compared with observed responses were similar to those observed with the dose-addition model. The EEQ model can be beneficial for risk assessment of environmental estrogens as it requires less data to calculate.

This study investigated interactions between compounds mediated through the ER in the T47D-KBluc *in vitro* assay system. Binary and ternary mixtures of an endogenous estrogen ( $E_2$ ) with exogenous environmental estrogens (BPA and BPAF) were tested. The results suggest that the binary and ternary mixtures of  $E_2$ , BPA, and BPAF can be modeled by the dose-addition and EEQ models of additivity. Results indicate that both additive models would be appropriate for predictions of environmental estrogen mixtures. The data suggest that at higher concentrations of  $E_2$ , higher BPA or BPAF concentrations would be needed to induce an effect. In terms of significant interactions in mixtures with BPA or BPAF and endogenous estrogens, the results of this study would suggest greater concern in scenarios when endogenous  $E_2$  concentrations were low as in prepubertal children and of lesser concern during pregnancy or in cycling women or women on hormone replacement therapies when endogenous  $E_2$  concentrations are high. These mixture studies are currently being extended to include several of the estrogens listed on Environmental Protection Agency's drinking water contaminant candidate list 3 (CCL3). The CCL3 are contaminants that are currently not subject to any proposed or promulgated



**FIG. 10.** Ternary mixture of BPA and BPAF plus  $E_2$  shows good correlation between predicted and observed data. Regression line plots the predicted EEQ of the ternary mixture for both axes  $x$  and  $y$ . Individual points plot the mean with 95% CLs of observed results on the  $y$ -axis and predicted EEQ on the  $x$ -axis. Black circles correspond to ternary mixtures with 1fM  $E_2$ ; red triangles correspond to ternary mixtures with 30fM  $E_2$ ; blue squares correspond to ternary mixtures with 300fM  $E_2$ ; and black cross with green lines correspond to ternary mixtures with 10pM  $E_2$ . The scatter allows for the evaluation of the efficacy of the EEQ model.

national primary drinking water regulations, but which are known or anticipated to occur in public water systems, and which may require future regulation under the Safe Drinking Water Act. These studies will identify how environmentally relevant concentrations of mixtures will interact and will determine if the mixtures display EEQ values that are likely to induce estrogen-like effects in aquatic vertebrates and whether these will potentially be of concern to human health and the environment.

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