Electronic Supplementary Information

Introducing BPA-equivalents: Mixture Toxicity and Substitution of BPA in Environmental Exposure Scenarios*

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S1 Bisphenol A alternative occurrence data

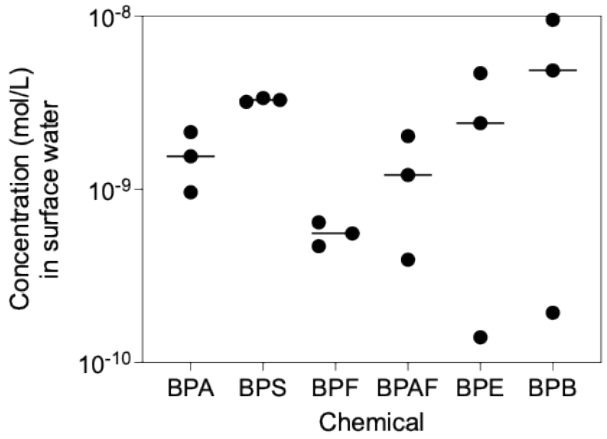

Figure S1: Occurrence data of bisphenol A and and BPA alternatives compiled by Adamovsky et al.{Adamovsky, 2024 #1}. Lines are medians that were used to design the mixture I and II.

S2 Bioassays

S2.1 Cell Culturing Conditions. All suppliers and sources of cells are given in Table S2. The bioassay media for the different assays were prepared as follows: GeneBlazer-bioassay medium for ERα was prepared with 98% Opti-MEM, supplemented with 2% charcoal-stripped FBS, and 100 U/mL penicillin-streptomycin. AREc32 and AhR bioassay medium was comprised of 90% DMEM with GlutaMAX, supplemented with 10% FBS, and 100 U/mL penicillin-streptomycin. The neurobasal medium was supplemented with 2% B-27 and 2% GlutaMAX.

S2.2 Cell Seeding and Incubation. For all bioassays, cell suspensions in the respective assay medium were dispensed into each well of 384-well plates using a MultiFlo Dispenser (Biotek, (now Agilent), Winooski, VT, U.S.). After seeding, the plates were incubated at 37°°C and 5% CO_2 for 24°h. The final cell densities and plate types were as follows: Black poly-D-lysine coated 384-well plates (Product°No.°354663, Corning Inc., NYC, U.S.). ERα-bla: 5000°cells/well. White 384-well plates with a clear bottom (Product No.°781098, Greiner, Kremsmünster, Austria) were utilized for AREc32 (MitoOxTox assay): 2650°cells/well and white poly-D-lysine coated 384-well plates (Product No.°354660, Corning Inc., NYC, U.S.).

S2.3 Chemical Dosing. The dosing procedure and high-throughput assay workflow have been described in detail before.[9] The highest dosing concentrations for each chemical were estimated as 3× $IC_{10,baseline}$ (Table S1). Specifically for mixture, the dosing concentrations were 3× EC_{10} of the mixture (Table S4). Stock solutions were then either added directly to the respective cell culture medium or evaporated under nitrogen, followed by replenishment with fresh medium. The final solvent content in each well was kept below 1% for methanol.

Dosing plates were prepared as 11-step serial dilutions using a Hamilton Microlab Star robotic system (Hamilton, Bonaduz, Switzerland), and chemicals were dosed in duplicates by transferring 10 µL twice

from the dosing plates to the cell plates. The cell plates were incubated at 37°C and 5% CO_2 for an additional 24h. All chemicals were tested in all assays with at least three independent replicates.

S2.4 Error Propagation Equations.

$$\mathrm{SE\ IC_{10}}\left(\mathrm{slope_i}\right) \approx \frac{10}{\mathrm{IC_{10}^2}} \times \mathrm{SE\ IC_{10}} \tag{S1}$$

$$\mathrm{SE\ (slope_{CA})} \approx \sum_{i=1}^{n} p_i \times \mathrm{SE\ (slope_i)} \tag{S2}$$

$$\mathrm{SE\ IC_{10}(CA)} \approx \frac{10}{\mathrm{slope_{CA}}^2} \times \mathrm{SE\ (slope_{CA})} \tag{S3}$$

Additional figures

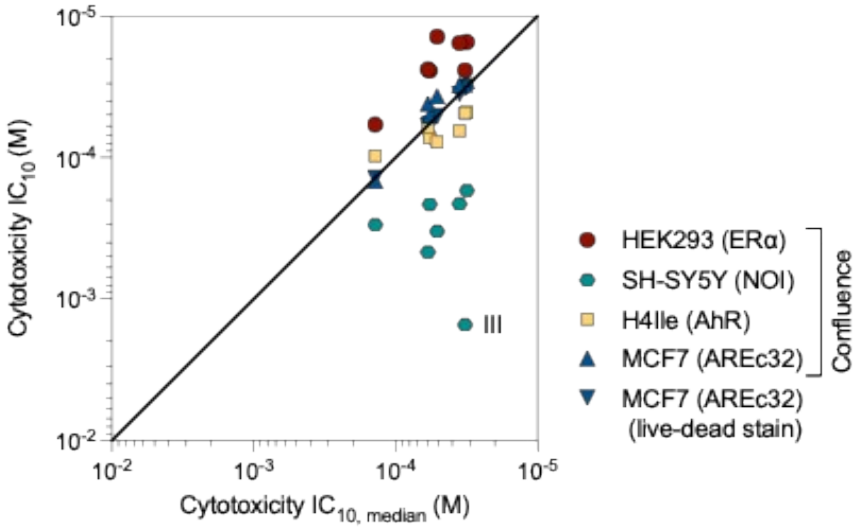

Figure S2: Cytotoxicity IC_{10} of the mixtures of the various cell lines compared to the median $IC_{10,median}$. Data in Table S5. As the live-dead stain gave very similar results as the confluence for MCF7 (AREc32), only the confluency was included in the calculation of the median.

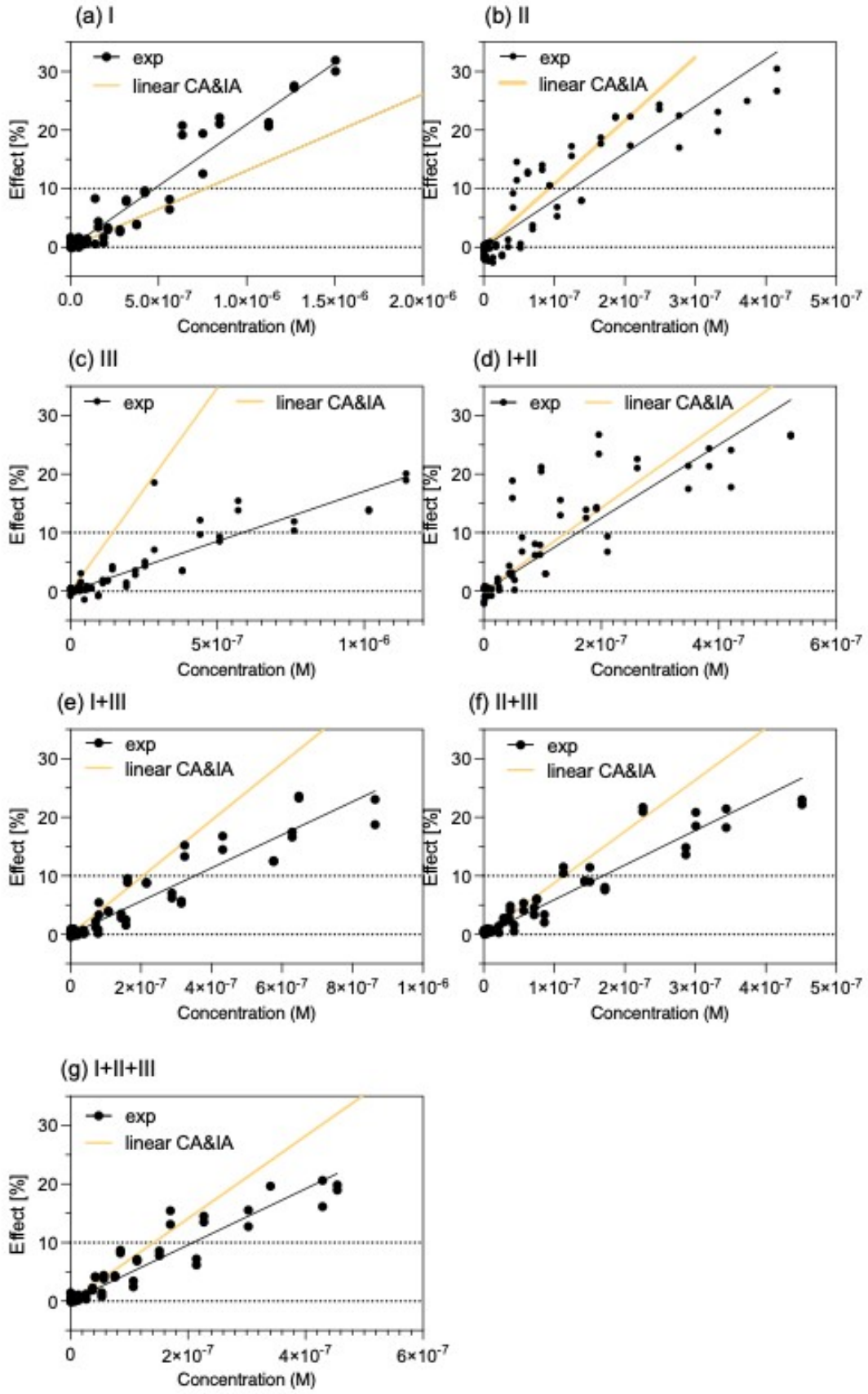

Figure S3: Comparison of the linear prediction model for the estrogen receptor ERα activation in ERα-UAS-bla (a) Mixtures I, (b) II, (c) III, (d) I+II, (e) I+III, (f) II+III, (g) I+II+III. Black dots are the experimental data points and black thin line best fit with the linear CRC model (eqn (3)). Linear CA/IA refers to the linear low-effect model for prediction of concentration addition (eqn (10. All data from Table S7.

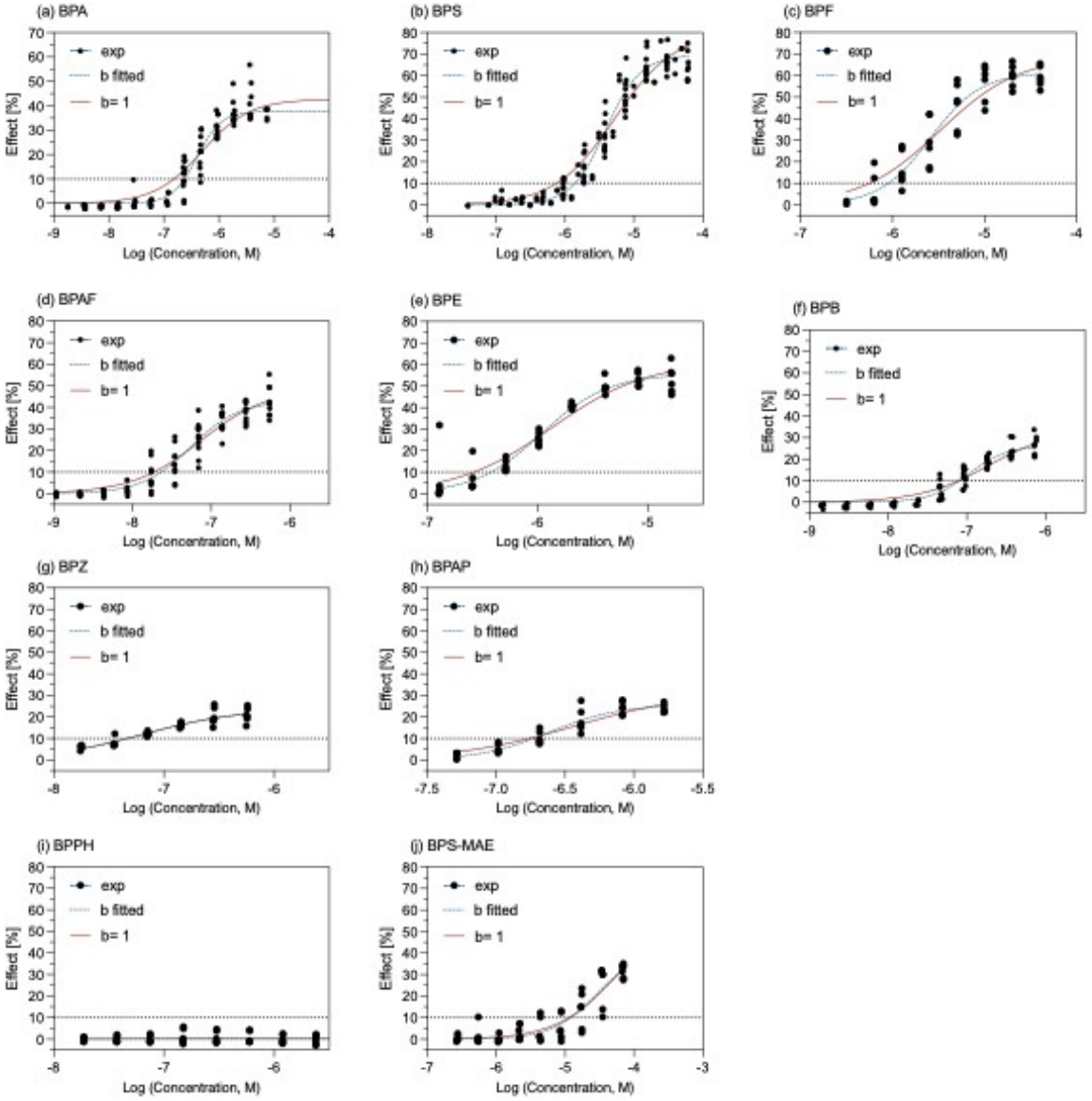

Figure S4: Full concentration-effect curves for estrogen receptor ERα activation in ERα-UAS-bla GripTite for the individual mixture components using the log-logistic CRC (eqn (5)) with adjustable slope b or b=1.

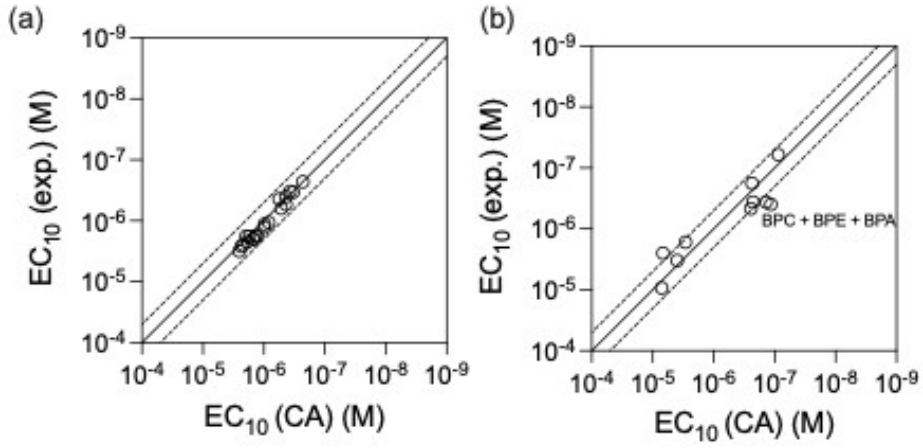

Figure S5: Comparison of experiment vs. prediction for estrogen receptor ERα activation in the literature (a) Skledar et al.[21], (b) Lee et al.[22]

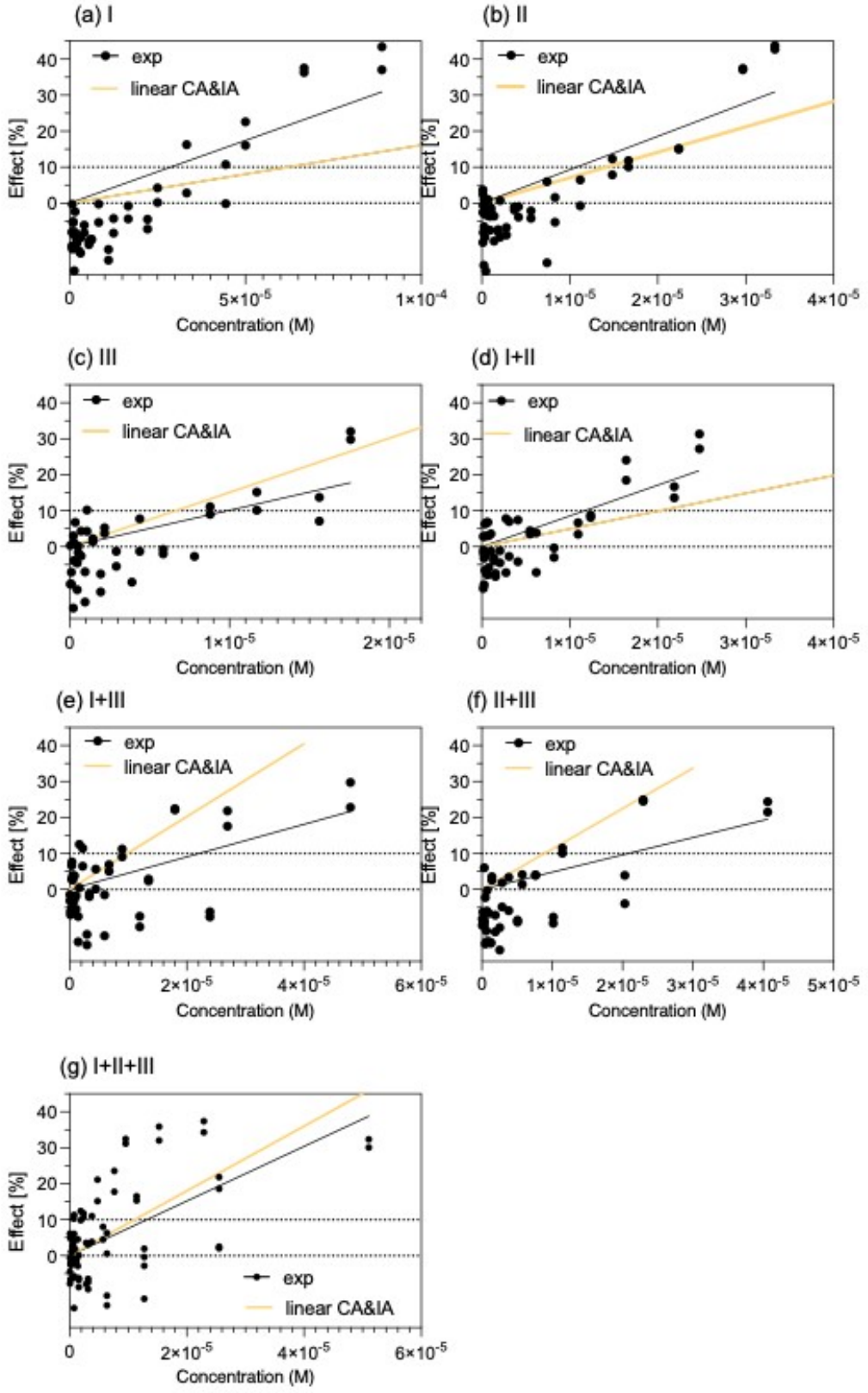

Figure S6: Comparison of the linear prediction model for the mitochondrial membrane potential (MMP) inhibition assay (a) Mixtures I, (b) II, (c) III, (d) I+II, (e) I+III, (f) II+III, (g) I+II+III. Black dots are the experimental data points and black thin line best fit with the linear CRC model (eqn (3)). Linear CA/IA refers to the linear low-effect model for prediction of concentration addition (eqn (10. All data from Table S9.

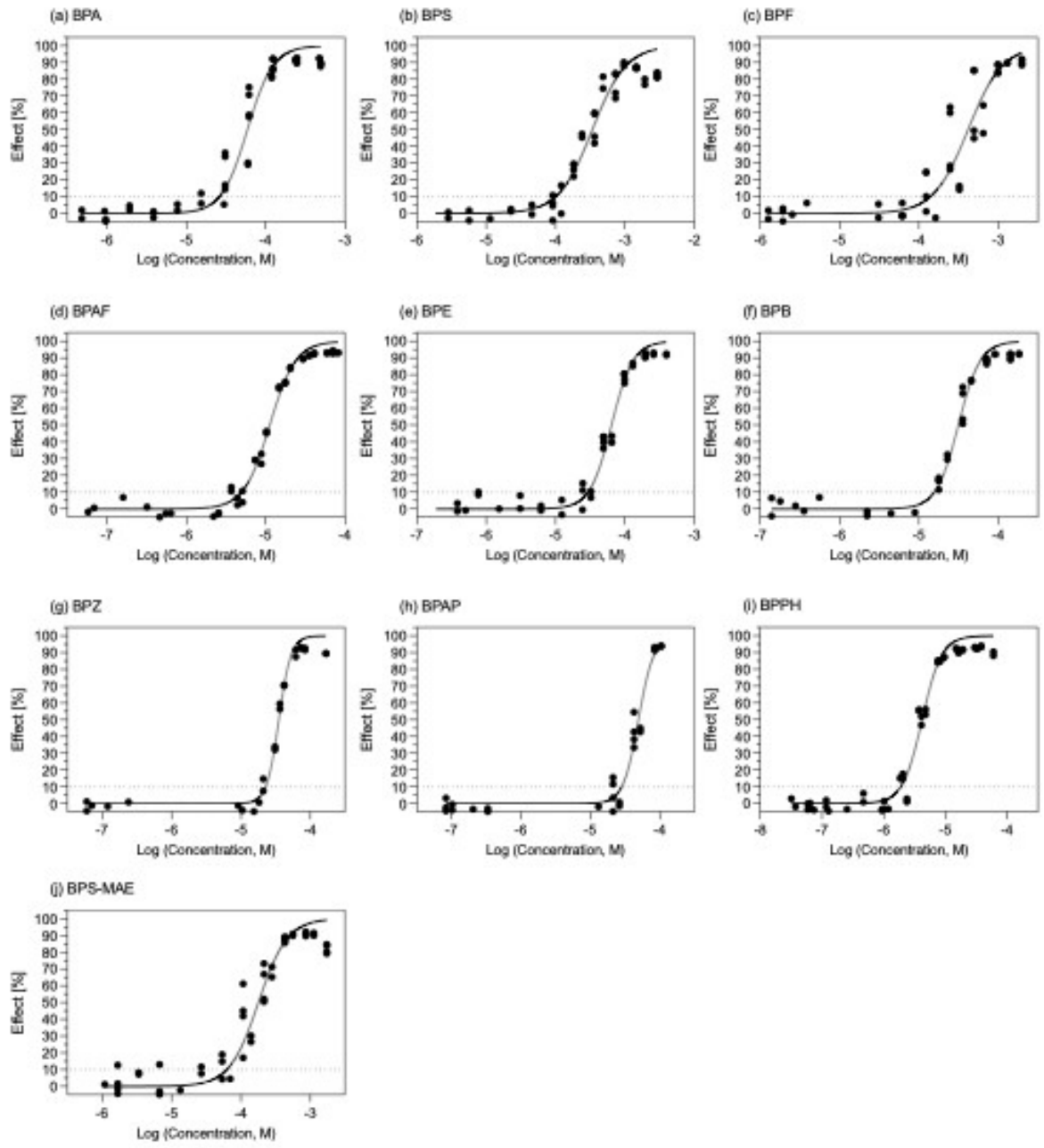

Figure S7: Full concentration-effect curves prediction models for the mitochondrial membrane potential (MMP) inhibition assay for the individual mixture components using the log-logistic CRC model (eqn (5)) with E_{max} = 100%. Fitted descriptor in Table S6.

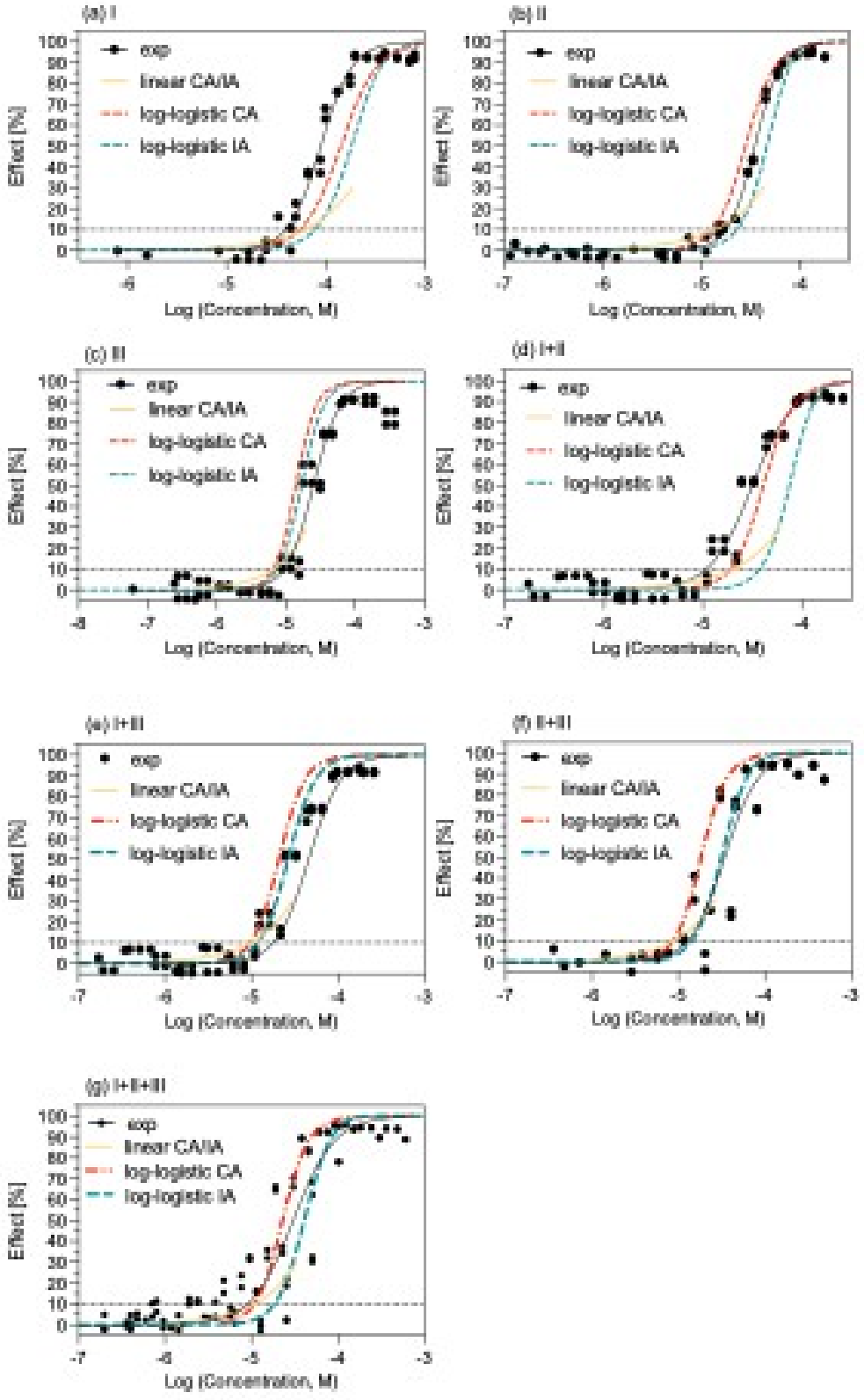

Figure S8: Comparison of the different prediction models for the mitochondrial membrane potential (MMP) inhibition assay (a) Mixtures I, (b) II, (c) III, (d) I+II, (e) I+III, (f) II+III, (g) I+II+III. Black dots are the experimental data points and black thin line best fit with the log-logistic CRC model (eqn (5)). Linear CA/IA refers to the linear low-effect model for prediction of concentration addition (eqn (10)).Log-logistic CA refers to log-logistic CRC model (eqn (5)) and prediction with eqn (8), IA refers

to log-logistic CRC for experiments (eqn (5)) and prediction with eqn (9). All data from Table S6 and Table S9.

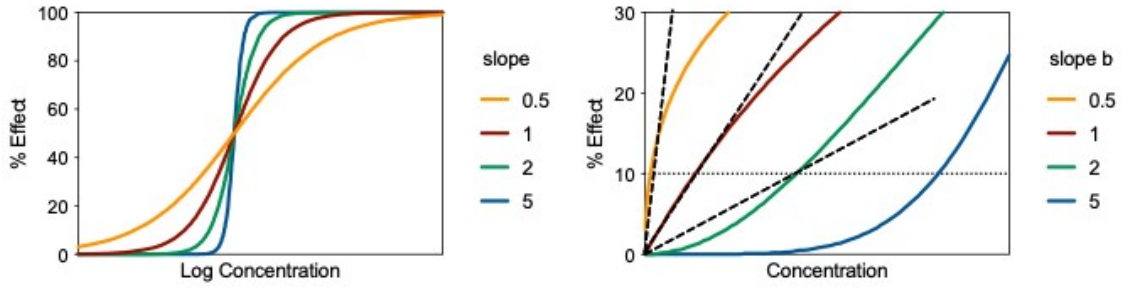

Figure S9: Simulation of the effect of changes of the slope b of the CRC.

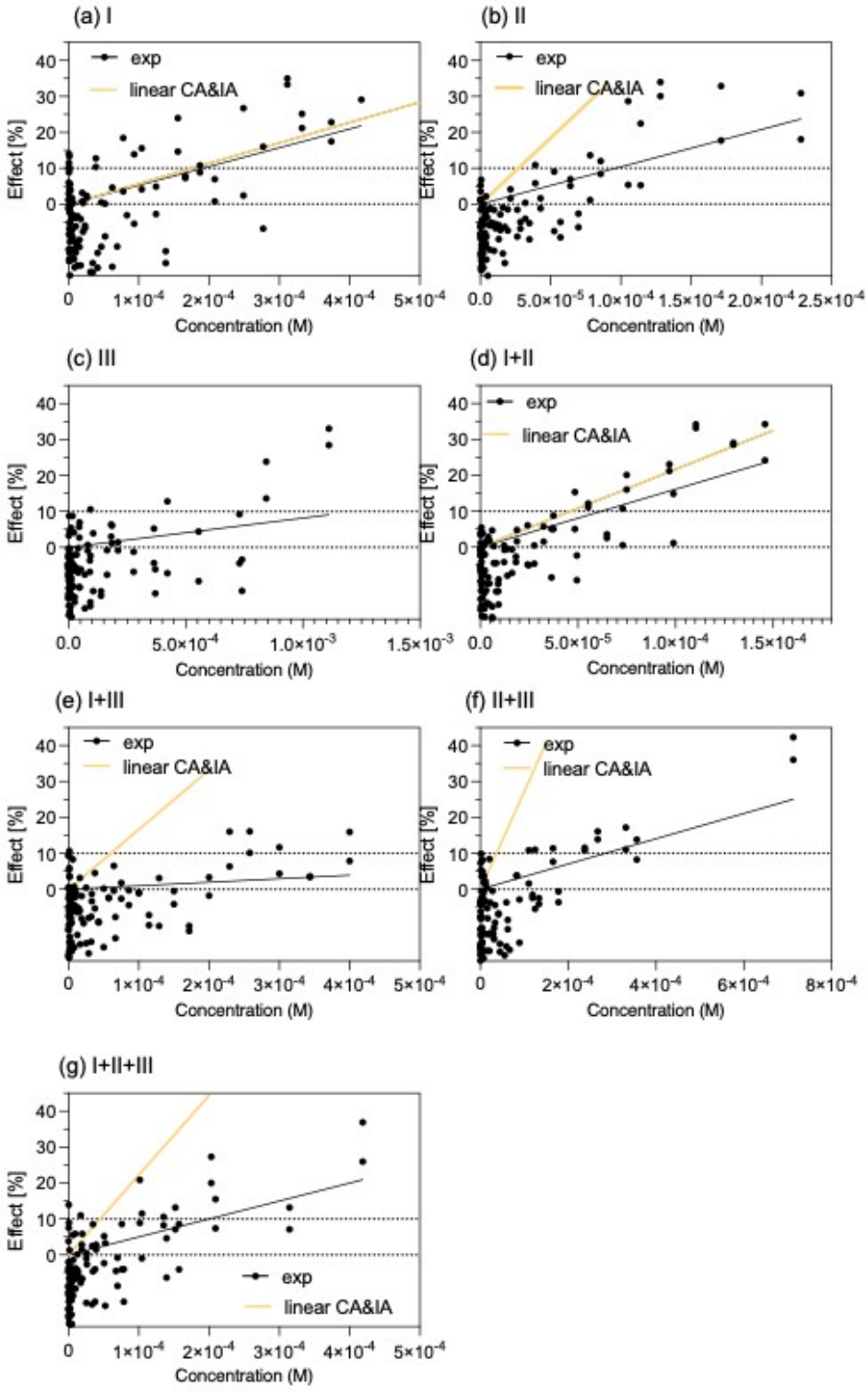

Figure S10: Comparison of the different prediction models for the neurite outgrowth inhibition (NOI) assay. (a) Mixtures I, (b) II, (c) III, (d) I+II, (e) I+III, (f) II+III, (g) I+II+III. Black dots are the experimental data points and black thin line best fit with the linear CRC model (eqn (3)). Linear CA/IA refers to the linear low-effect model for prediction of concentration addition (eqn (10)). All data from Table S10.

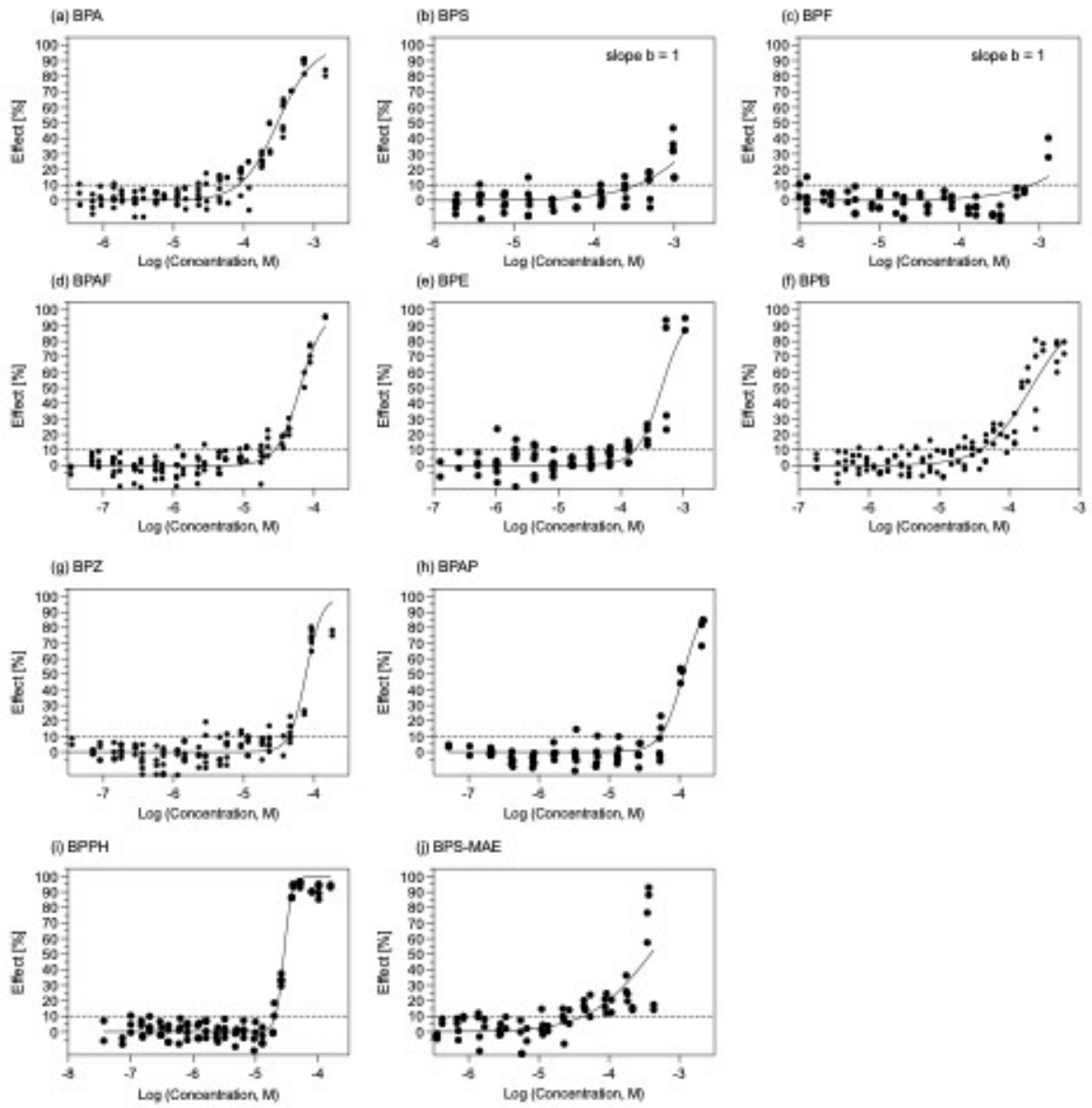

Figure S11: Full concentration-effect curves prediction models for the neurite outgrowth inhibition (NOI) assay for the individual mixture components using the log-logistic CRC (eqn (5)) with E_{max} = 100% and slope b fitted.Fitted descriptor in Table S6.

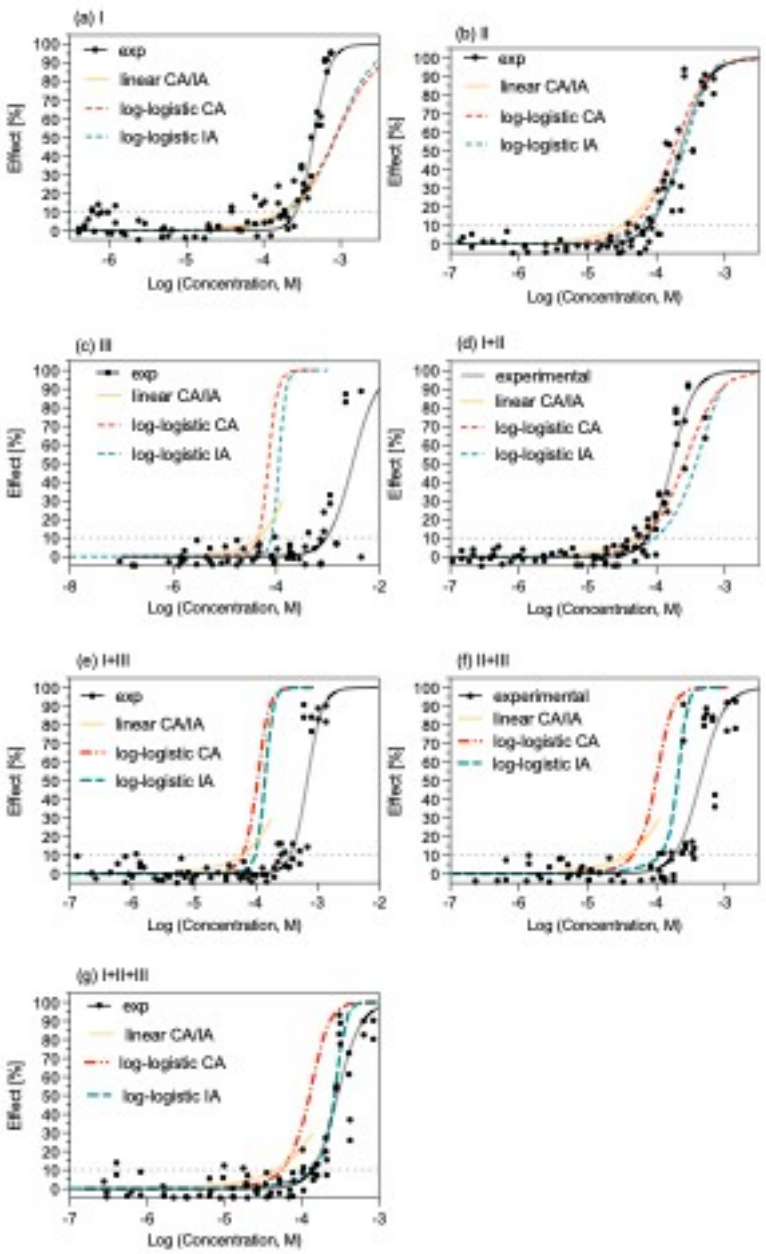

Figure S12: Comparison of the different prediction models for the neurite outgrowth inhibition (NOI) assay. (a) Mixtures I, (b) II, (c) III, (d) I+II, (e) I+III, (f) II+III, (g) I+II+III. Black dots are the experimental data points and black thin line best fit with the log-logistic CRC model (eqn (5)). Linear CA/IA refers to the linear low-effect model for prediction of concentration addition (eqn (10)).Log-logistic CA refers to log-logistic CRC model (eqn (5)) and prediction with eqn (8), IA refers to log-logistic CRC for experiments (eqn (5)) and CA prediction with eqn (9). All data from Table S10.

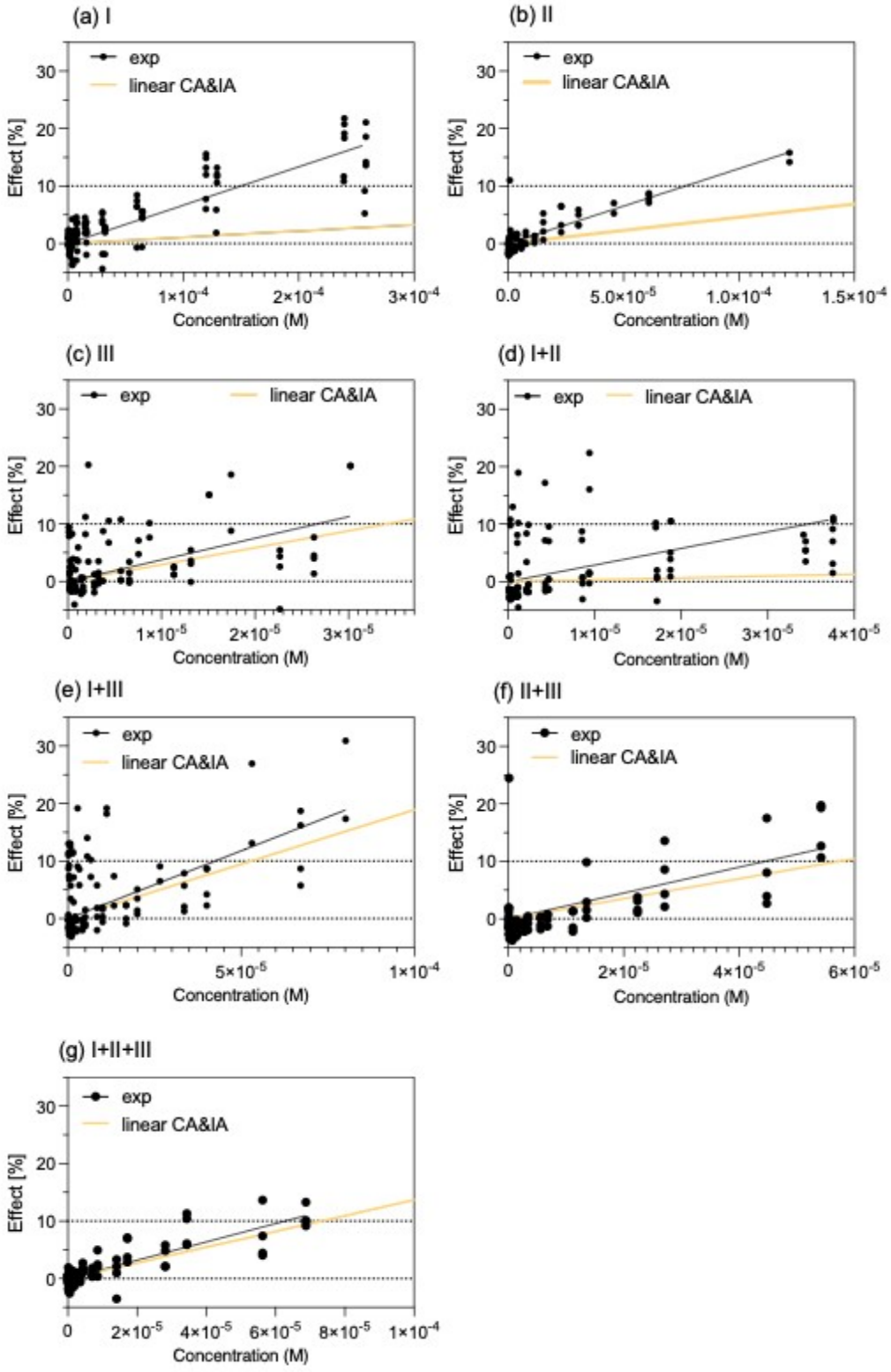

Figure S13: Full concentration-effect curves prediction models for the AhR activation assay. (a) Mixtures I, (b) II, (c) III, (d) I+II, (e) I+III, (f) II+III, (g) I+II+III. Black dots are the experimental data points and black thin line best fit with the linear CRC model (eqn (3)). Linear CA/IA refers to the linear low-effect model for prediction of concentration addition (eqn (10)). All data from Table S11.

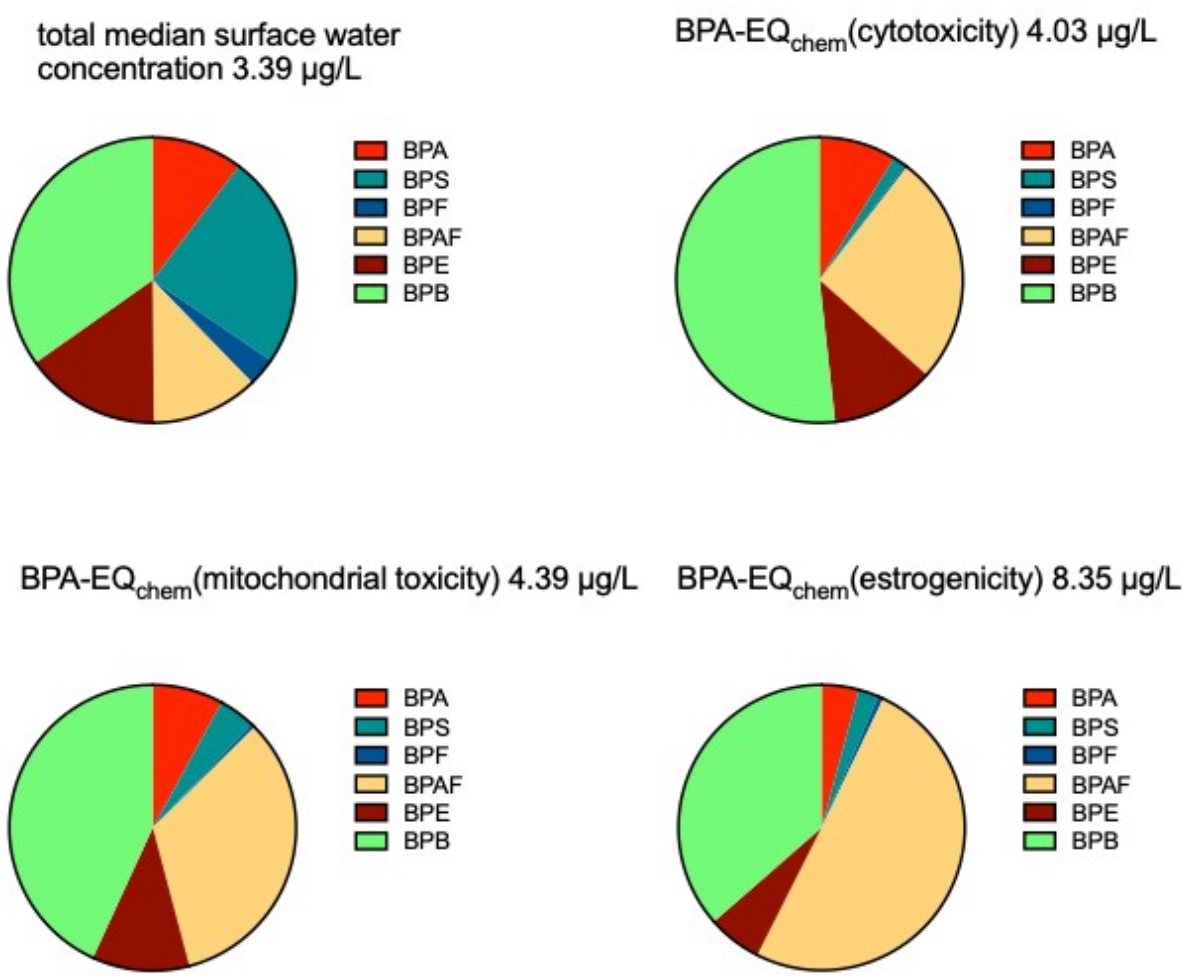

Figure S14: Comparison of total concentration in surface water and BPA-EQ$_{chem}$ in the three bioassays for mixture I+II. All data from Table S1 (REP), S3 (surface water concentrations) and S12 (BPA-EQ).