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Combined effects of estrogenic chemicals with the same mode of action using an estrogen receptor binding bioassay

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ABSTRACT

The increasing amounts of various estrogenic chemicals coexisting in the aquatic environment may pose environmental risks. While the concept of estradiol equivalent (EEQ) has been frequently applied in studying estrogenic mixtures, few experiments have been done to prove its reliability. In this study, the reliability of EEQ and the related model concentration addition (CA) was verified based on the two-hybrid recombinant yeast bioassay when all mixture components had the same mode of action and target of action. Our results showed that the measured estrogenic effects could be well predicted by CA and EEQ for all laboratory-made mixtures using two designs, despite the varying estrogenic activity, concentration levels and ratios of the test chemicals. This suggests that when an appropriate endpoint and its relevant bioassay are chosen, CA should be valid and the application of EEQ in predicting the effect of non-equi-effect mixtures is feasible.

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1. Introduction

The development of analytical measurement and the constant pollutant discharges lead to the increasing detection rates and concentrations of endocrine disrupting chemicals (EDCs) (Petrovic et al., 2004). The reproductive and developmental toxicities of EDCs to the aquatic organisms evoke worries about the impacts on human health, and their adverse effects

after the long-term exposure to environmental observed concentrations (ng/L) aggravate these worries (Hotchkiss et al., 2008; Vandenberg et al., 2012; Zha et al., 2007). Particularly, steroid estrogens (both natural and synthetic) and phenolic xenoestrogens receive more attention because of their non-ignorable estrogenicities and widespread applications (Peng et al., 2006). These substances have high or moderate affinity to the estrogen receptor (ER), mimicking the normal function of natural estrogen and disrupting the endocrine

Abbreviations: 4-NP, 4-nonylphenol; 4-t-OP, 4-tert-octylphenol; BPA, bisphenol A; CA, concentration addition; DES, diethylstilbestrol; E1, estrone; E2, 17 β -estradiol; E3, estriol; EC50, half maximal effective concentration; EDCs, endocrine disrupting chemicals; EE2, 17 α -ethinylestradiol; EED, equivalent effect design; EEQ, estradiol equivalent; ER, estrogen receptor; EV, estradiol valerate; MoA, mode of action; NOEC, no observable effect concentration; OD, optical density; RA, response addition; REP, relative potency; RMD, randomly mixed design; ToA, target of action; YES, yeast estrogen screen.

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Table 1 – Information, properties, EC₅₀s and REPs of the nine test chemicals.

Abbr.	Name	CAS no.	MW	EC ₅₀	REP
E2	17-β-estradiol	50-28-2	272.39	18.3	1.00
E1	Estrone	53-16-7	270.37	2.52E+2	7.28E–2
EE2	17α-Ethinylestradiol	57-63-6	296.44	13.1	1.40
E3	Estriol	50-27-1	288.39	6.06E+3	3.03E–3
DES	Diethylstilbestrol	56-53-1	268.38	5.69E+2	3.22E–2
EV	Estradiol valerate	979-32-8	356.50	4.53E+4	4.04E–4
4-t-OP	4-Tert-octylphenol	140-66-9	206.36	1.33E+5	1.38E–4
4-NP	4-Nonylphenol	104-40-5	220.39	1.14E+5	1.60E–4
BPA	Bisphenol A	80-05-7	228.31	1.87E+6	9.82E–6

Abbr. = abbreviation; CAS = chemical abstract services registry number; MW = molecular weight (g/mol); EC₅₀ = half maximal effective concentration (ng/L); REP = relative potency, REP(i) = EC₅₀(E2)/EC₅₀(i).

and reproductive systems. They are emitted from a variety of sources and seldom exist individually in any environmental compartment, leading to the combined adverse effects on organisms. There have been a number of surveys measuring either the estrogenic activity by use of bioassay, or concentration levels of the estrogenic substances by chemical analysis in water samples (Beck et al., 2006; Lavado et al., 2009; Mahomed et al., 2008; Matthiessen et al., 2006). To bridge the two measurements, concentrations could be transformed into biological activity using the estrogenic relative potency (REP).

In mixture toxicity research, concentration addition (CA) and response addition (RA) are two best-known and widely used models (USEPA, 2000). When taking no interaction into account, their differences mainly depend on whether all components of the mixture are toxicologically similar (Bliss, 1939; Loewe, 1928). Estradiol equivalent (EEQ) is a derived approach of CA, which just simplifies the calculation process by summing the component concentrations after adjusting them for each component's potency. In risk assessment, by comparing EEQs obtained from both analytical determination and bioassay, the major and minor contributors of a complex sample could be identified. When this was applied to environmental waters, some work reported that the predicted overall EEQs were similar to observed ones (Beck et al., 2006; Jiang et al., 2012; Liscio et al., 2009), but some found that the predictions were higher or lower (Cargouët et al., 2004; Furuichi et al., 2004). These contradictory conclusions may result from the contribution of non-targets chemicals such as unknown estrogen agonist, antagonist and humics, or the multiple involved modes of action (MoAs) and the resulting interaction of mixture components (Villeneuve et al., 2000). However, few studies have focused on the adaptations of EEQ and REP which are essential in the causality analysis. Thus, there is a need to confirm the validity of CA and EEQ for the most common ER-binding effects and EDCs with high detection rates by conducting a certain bioassay and eliminating the disturbance of other chemicals.

In the present study, we aimed to test the validity of CA and EEQ in calculating the binding capability to ER when the MoA and target of action (ToA) of all components are the same. To achieve the goal, we adopted a two-hybrid recombinant yeast bioassay to study the combined effects: the dimerization of ERs with estrogens leads to the conformational change of the ligand–receptor complex; then, the recruitment of co-activator and the transcription and translation of downstream

reporter gene occur. Like the one-hybrid yeast estrogen screen (YES), this assay also offers single MoA (binding to ER) and ToA (ER). However, it is consistent with the in vivo tests better through introducing the co-activator (Li et al., 2008b; Sheeler et al., 1999). During each experiment, the high purity of yeast strain and setting of control groups should be guaranteed for the validity of test results.

2. Materials and methods

2.1. Chemicals

Estrone (E1, 99%), 17β-estradiol (E2, 97%), estriol (E3, 97%), bisphenol A (BPA, 99%), and dimethyl sulfoxide (DMSO, 99.5%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 17α-ethinylestradiol (EE2, 99%), diethylstilbestrol (DES, 98%), and estradiol valerate (EV, 99.5%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 4-Tert-octylphenol (4-t-OP, 97%) was obtained from Sigma–Aldrich and 4-nonylphenol (4-NP, a mixture of branched chain isomers) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Information on the test chemicals is listed in Table 1. All the test chemicals were dissolved in DMSO and stored below –20 °C. They solved well and existed stably in the standard solution and diluted gradients, and the short exposure time would not change their concentrations in culture medium (Knox et al., 2011; Tetko et al., 2005). Therefore, the actual and nominal concentrations were considered in good agreement with each other.

2.2. Experimental design for the mixtures

Nine representative estrogenic chemicals were selected for mixture preparation, which have been frequently detected in aquatic environment and demonstrated to play a role in estrogenic pollution. They included three natural estrogens (E1, E2, and E3), three synthetic estrogens (EE2, DES, and EV) and three phenolic compounds (4-t-OP, 4-NP, and BPA). Two experimental designs were applied to create the laboratory produced mixtures: some were made to cause equal estrogenic effects, and some were arranged according to the related environmental concentrations.

Initially, twelve mixtures (M1–M12), including two mixtures with three components, six with four components, three with five components, and one with nine components, were

designed by an L12(9²) orthogonal array. M13–M16 were gained in a similar way, containing the three phenols only to test the performance of models for weaker estrogenic effectors. Each mixture was prepared using a fixed ratio in which all components were combined at equipotent concentrations (according to their REP values), to ensure that every one of them lead to even effects, and their total agonistic activities equaled to the maximum one of E2 (an equivalent effect design, EED, Table 2). Seven concentration gradients of each of the 16 mixtures were obtained by double dilution to cover the main part of the concentration–response sigmoid curve.

To further confirm the results of EED test, another 15 mixtures (S1–S15) were created by mimicking some actual environmental levels of the test chemicals. Their compositions and concentrations were references to several surveys of estrogenic chemicals in surface water and wastewater samples from different cities and river valleys of China (see details in Table 3). Thus, the 15 chemical profiles of these samples were used for a randomly mixed design (RMD) test. For each synthetic mimic mixture, we tested five concentration gradients which were 0.1, 1, 10, 100 and 1000 folds of actual concentrations in water samples to get its concentration–response relationship and the observed EEQ value. During dilution, DMSO was also served as the solvent, with a 0.5% final concentration in exposure cultures which will cause no cytotoxicity to the yeast cell.

2.3. Bioassay

The assays were carried out as previously described (Li et al., 2008a), and all tests were conducted in quadruplicate. Each group consisted of a positive control (E2 for the ER inductive effect), a negative control (DMSO), and test samples. After overnight culture of the yeast strain at 30 °C and mixing at 130 rpm, 5 µL of the control or test sample was added to 995 µL of medium containing 5×10^3 yeast cells/mL. Two hundred microliters of the control/test culture was transferred to a well in a 96-well plate and incubated at 30 °C with vigorous orbital shaking (800 rpm) on a titer plate shaker (Heidolph TITRAMAX 1000, Hamburg, Germany) for 2 h. The optical density (OD) at a wavelength of 600 nm, which indicated cell density of the culture, was measured (TECAN GENios A-5002, Salzburg, Austria), and then a 50 µL aliquot of the culture was transferred to a new well plate. After adding 120 µL of Z-buffer (16.1 g/L Na₂HPO₄·7H₂O; 5.5 g/L NaH₂PO₄·H₂O; 0.75 g/L KCl; 0.246 g/L MgSO₄·7H₂O) and 20 µL of chloroform, the assay solution was carefully mixed at 30 °C for 10 min. The enzymatic reactions were started by adding 40 µL o-nitrophenyl-β-D-galactopyranoside (13.3 mmol/L in Z-buffer), and the sample was incubated at 30 °C for 60 min. The reactions were terminated by adding 100 µL Na₂CO_{3(aq)} (1 mol/L). 200 µL of the supernatant was then transferred into a new well plate and the absorbance at 420 nm was determined. The β-galactosidase activity was calculated using Eq. (1):

$$U = \frac{(OD_{420s} - OD_{420b})D}{t \times V \times OD_{600}} \quad (1)$$

where U is the β-galactosidase activity, OD_{420s} and OD_{420b} are OD values of the enzymatic reaction supernatant of the

Table 2 – Concentrations (ng/L) of test chemicals in EED mixtures and the predictive results of CA and RA.

N _{test}	Test no.	Composition and concentration										EC ₅₀	
		E2	E1	EE2	E3	DES	EV	4-t-OP	4-NP	BPA	Obs.	CA	RA
3	M1	22.7	–	–	–	7.06E+2	–	–	1.42E+5	–	23.5	18.3	45.0
	M2	–	3.11E+2	16.2	–	–	–	1.65E+5	–	–	26.0	18.3	50.6
4	M3	17.0	–	12.2	–	–	4.20E+4	–	–	1.74E+6	25.3	18.3	43.2
	M4	17.0	–	–	5.62E+3	–	–	1.24E+5	–	1.74E+6	21.2	18.3	36.8
	M5	–	2.34E+2	–	–	5.29E+2	4.20E+4	–	–	1.74E+6	25.2	18.3	48.9
	M6	–	2.34E+2	–	5.62E+3	–	–	–	1.06E+5	1.74E+6	18.2	18.3	42.8
	M7	–	–	–	5.62E+3	5.29E+2	4.20E+4	1.24E+5	–	–	22.7	18.3	50.1
	M8	–	–	12.2	5.62E+3	–	4.20E+4	–	1.06E+5	–	26.0	18.3	54.0
5	M9	13.6	1.87E+2	9.72	4.50E+3	4.24E+2	–	–	–	–	16.9	18.3	46.6
	M10	13.6	1.87E+2	–	–	–	3.37E+4	9.91E+4	8.51E+4	–	21.0	18.3	67.4
	M11	–	–	9.72	–	4.24E+2	–	9.91E+4	8.51E+4	1.39E+6	22.1	18.3	67.0
9	M12	7.57	1.04E+2	5.40	2.50E+3	2.35E+2	1.87E+4	5.51E+4	4.72E+4	7.72E+5	22.9	18.3	84.7
3	M13	–	–	–	–	–	–	2.47E+5	2.13E+5	–	18.7	18.3	26.0
	M14	–	–	–	–	–	–	2.47E+5	–	3.47E+6	20.3	18.3	24.2
	M15	–	–	–	–	–	–	–	2.13E+5	3.47E+6	20.8	18.3	27.0
4	M16	–	–	–	–	–	–	1.64E+5	1.42E+5	2.31E+6	18.0	18.3	32.4

The table shows the maximum concentrations of chemical components. And for each mixture, seven concentration gradients were gained by double dilution. Unit: ng/L; N_{test} = number of test chemicals in mixture; Obs. = observation; CA = concentration addition; RA = response addition.

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Table 3 – Concentrations of estrogenic compounds and the predicted and observed EEQs from the RMD tests.

Area	Test no.	E2	E1	EE2	E3	DES	EV	4-t-OP	4-NP	BPA	EEQ _{pre}	EEQ _{obs}	Reference
Tianjin	S1 ^a	8.69	23.4	10.0	10.3	2.13	3.57	–	–	–	24.49	26.50	Lei et al., 2009
	S2 ^a	10.3	19.7	9.45	12.4	1.31	3.53	–	–	–	25.05	25.61	
	S3 ^a	7.26	10.5	3.54	5.76	1.88	1.29	–	–	–	13.06	13.29	
Yellow river	S4 ^a	ND	1.3	–	–	–	–	4.7	577.9	46.7	0.19	0.17	Wang et al., 2012
	S5 ^b	<LOQ	1.2	–	–	–	–	3.0	253.1	40.1	0.13	0.11	
	S6 ^b	2.3	15.6	–	–	–	–	4.3	709.4	171.5	3.55	3.60	
Chongqing	S7 ^b	32.7	88.6	29.1	338.7	ND	–	–	–	10566.7	81.02	77.87	Ye et al., 2012
	S8 ^b	1.9	30.4	ND	ND	ND	–	–	–	158.6	4.11	4.31	
Yangtze river (Nanjing)	S9 ^b	0.97	3.80	–	5.79	–	–	89.07	344.37	34.55	1.33	1.26	Lu et al., 2010
	S10 ^b	–	–	–	–	–	–	89.51	536.55	60.69	0.10	0.10	
	S11 ^b	0.81	2.33	–	–	–	–	95.77	503.72	37.44	1.07	1.11	
Tai lake	S12 ^b	5.18	1.01	ND	1.02	3.54	–	–	–	9.45	5.37	4.75	Yan et al., 2012
	S13 ^b	7.98	ND	6.03	1.39	3.30	–	–	–	3.95	16.53	15.08	
Pearl river	S14 ^a	<LOQ	3.2	–	–	<LOQ	–	31.8	1178	161	0.43	0.34	Gong et al., 2012
	S15 ^b	1.6	2.1	–	–	ND	–	20.1	898	245	1.90	1.74	

Units: ng/L; '–' = not available; ND = not detected; <LOQ = below the limit of quantification; EEQ_{pre} = predicted estradiol equivalent value; EEQ_{obs} = observed estradiol equivalent.^a Mean values of an area.^b Concentrations in a specific sampling spot.

sample and the blank at 420 nm, respectively, OD600 is the OD of the test culture at 600 nm, *t* is the duration of the enzymatic reaction incubation, *V* is the volume of the test culture, and *D* is the dilution factor.

2.4. Data analysis for single chemicals

Concentration–response curves (scatter plots of the normalized *U* value versus log concentration) for single chemicals were constructed and analyzed using regression analysis in Sigmaplot 11.0 (SPSS Inc., Chicago, IL, USA). Because of the good fit for the original data, the logistic function was chosen to run the regression, which can be expressed as the following:

$$\text{Logistic function: } E = b + \frac{a}{1 + (x/x_0)^{|p|}} \quad (2)$$

$$\text{Inverse logistic function: } x = x_0 \times \sqrt[|p|]{\left(\frac{a}{E-b}\right) - 1} \quad (3)$$

where *E* is the yeast assay inductive effect, *x* is the test chemical concentration, *a*, *b*, *x*₀, and *p* are parameters that represent the maximum effect, the minimum effect, the concentration for the half-maximum effect (i.e., EC₅₀), and the steepness of the concentration–response curve, respectively. REP was calculated using the EC₅₀ values of E2 and test chemicals.

2.5. Calculation of predicted mixture toxicities

For the EED mixtures, two classic non-interaction models, CA and RA, were used to predict the combined estrogenic effects. A common expression for CA is:

$$\text{ECx}_{\text{mix}} = \left(\sum_{i=1}^n \frac{p_i}{\text{ECx}_i} \right)^{-1} \quad (4)$$

where ECx_{mix} and ECx_{*i*} are the concentrations in the mixture and of the *i*th component which produces effect *x*, respectively, and *p_i* is the concentration proportion calculated as *p_i* = *c_i* / ∑ *c_i* (Backhaus et al., 2000).

The RA model is expressed using the following equation:

$$E(c_{\text{mix}}) = 1 - \prod_{i=1}^n (1 - E(c_i)) \quad (5)$$

where *c*_{mix} is the sum of the concentrations of all components, and *E*(*c*_{mix}) and *E*(*c_i*) are the effects of the whole mixture and the *i*th component, respectively (Faust et al., 2003).

For the RMD mixtures, the predicted EEQs of 15 samples were gained based on CA model, in a similar way to the toxic equivalency factor method (Van den Berg et al., 1998):

$$\text{EEQ}_{\text{pre}} = \sum_{i=1}^n (c_i \times \text{REP}_i) \quad (6)$$

where *c_i* and REP_{*i*} are the concentration and REP for each test chemical. The predictability of the CA was evaluated by comparing the predicted (calculated, or concentration-derived)

Fig. 1 – Concentration–response curves for individually tested mixture compounds.

EEQ with the observed (experimental, or bioassay-derived) EEQ.

3. Results

3.1. Individual estrogenic effects of the test chemicals

All chemicals showed concentration-dependent increases in β -galactosidase activity by inducing the enzyme production. Almost all of the concentration–response curves were parallel, and the logistic regression results were shown in Fig. 1. Based on the EC_{50} and REP for each chemical (Table 1), the estrogenic effect on the recombinant gene yeast was found to be in the order $EE2 > E2 > E1 > DES > E3 > EV > 4\text{-NP} > 4\text{-t-OP} > BPA$.

3.2. Combined effect of mixtures in EED tests

Sixteen mixtures containing different components and concentration ratios of the test chemicals were created by following the equivalent effect principle and using the test chemicals' EC_{50} s and REPs (Table 2), and the observed and predicted combined effects for these mixtures were shown in Fig. 2. From this graph, it was visualized that the CA line (black solid one) was always between the 95% confidence intervals (gray dashed lines) of the observed values at every concentration ratio and level. In addition, the EC_{50} s calculated using CA were closer to the observation than those obtained by RA (Table 2).

3.3. Combined effect of mixtures in RMD tests

Fig. 3a showed the relationship between concentration multiples and normalized Us for RMD mixtures. The EC_{50} was not a concentration of ng/L, but the folds of concentration levels at which 50% of the estrogenic activity was achieved. Observed EEQs were gained according to these EC_{50} s (Table 3). For all samples, the EEQ_{pres} were nearly equal to the corresponding EEQ_{obs} s. The plot with calculated EEQs on the x-axis and the experimental EEQs on the y-axis showed that the 15 data

points were very close to the theoretical line $x - y = 0$, with a coefficient of determination (R^2) of 0.9971 (Fig. 3b).

4. Discussion

The combined effects of EDCs started to draw increased attention when the strong synergistic effects in estrogenic mixtures were reported (Arnold et al., 1996). Although the paper was retracted the following year because the result was lack of reproducibility in other laboratories (McLachlan, 1997), it led to academic concern and intense follow-up research. There have been lots of researches to study the effects of estrogenic mixtures on organisms ranging from recombinant yeasts and estrogen-sensitive cells (Arnold et al., 1997; Payne et al., 2001; Rajapakse et al., 2004; Silva et al., 2011, 2002; Suzuki et al., 2001) to vertebrates such as fish and mice (Brian et al., 2007; Ramamoorthy et al., 1997; Ribeiro et al., 2012; Sun et al., 2009; Thorpe et al., 2005; Tinwell and Ashby, 2004). Among these test organisms, the recombinant human ER gene yeast obtained wide application because it provided not only fast and sensitive detection but also single MoA and ToA. In our lab, a recombinant yeast strain – which was utilized in this study – was established previously (Li et al., 2008b) by using the two-hybrid yeast technique. When the yeast cells are exposed to an estrogenic substance such as E2, ER in the cytoplasm is activated by binding the ligand. Then, the ER–E2 complex interacts with the co-activator in nuclear to form the trimer. This brought the binding domain and activating domain of Gal4 protein into proximity, and permitted the transcription of downstream reporter gene lac-Z. However, if a variety of ligands exist simultaneously, the behavior between them and ERs may change due to their different concentrations and receptor binding abilities, both of which are critical influences on the final effects.

The nine selected substances have frequently been found, by chemical analysis, at relatively high concentrations in the aquatic system of China, and all of them have been associated with endocrine disrupting effects (Gong et al., 2012; Lei et al., 2009; Liu et al., 2012; Lu et al., 2010; Sun et al., 2008; Wang et al., 2012; Yan et al., 2012; Ye et al., 2012). Thus, we chose them as representative estrogen-like chemicals that are present in the water environment. Considering the estrogenic potencies of chemicals varying with species and bioassay endpoint, the potency values obtained from the two-hybrid yeast assay for individual substances were compared with those reported in the literature using other methods. We found that our estrogenic activity order for the test chemicals was close to, and the EC_{50} values were slightly lower than most of the published ones (Brian et al., 2005; Correia et al., 2007; Folmar et al., 2002; Liu et al., 2009; Thorpe et al., 2005), which illustrates the reliability and high sensitivity of our yeast strain.

The transcription and translation process in the recombinant gene two-hybrid yeast assay can only be driven when substances with the ability to bind to the assay-specific receptor are present. Therefore, every component in a mixture acts similarly, competitively interacting with the same molecular target sites, which meets the strict demands of CA model (Groten, 2000; Pösch, 1993). According to the results using different experimental designs, it is obvious that the observed

Fig. 2 – Observed results and predicted effects of each EED mixture. Concentrations are presented as estradiol equivalent (EEQ, ng/L). The black points are observed values, and each normalized U is expressed as mean value $\pm$ the standard deviation. The gray dashed lines, black solid lines, and black dashed lines are the 95% confidence intervals around the observations, the CA model predictions and the RA model predictions, respectively.

Fig. 3 – Observed and predicted results in RMD tests. (a) Regression models for the estrogenic effect of fifteen mixtures on the β -galactosidase activity in recombinant yeast cells. According to the concentrations of Table 3, 0.1, 1, 10, 100 and 1000 folds of levels which mimic the surface water or wastewater samples in China were tested. (b) Predicted and observed EEQs for each RMD mixture with the theoretical line $x - y = 0$.

effect of ER binding was always close to the prediction of CA. In EED study, no significant deviations were found from additivity, even for weak estrogenic chemicals. For the 16 mixtures, the results conformed to CA model better in the concentration range we tested. The excellent agreement between observations and predictions of the RMD study further proved that the EEQ method could provide a useful and convenient way for assessing the combined effects of xenoestrogens at concentrations below those used in EED (i.e., at environmental concentrations). Our results suggest that when all of the test chemicals acted through the same MoA, CA and EEQ are satisfactory predictors which are unaffected by the number, concentration ratio, or actual concentration of the mixture components.

We also investigated the validity of the RA model in the first part of our study. RA underestimated the effect of all 16 mixtures, demonstrating that it was unsuitable for assessing the effects of estrogenic chemicals in this assay. This may be because of the fundamentals of the yeast assay: RA model is not the optimal choice when only similarly acting chemicals can activate the yeast system (Backhaus et al., 2004; Drescher and Boedeker, 1995; Faust et al., 2003).

As shown above, if the number of involved MoAs can be limited to one, although the concentration of mixture components increases from environmental level (RMD) to tens of thousands of times (EED), CA model can offer preciser predictions on the two-hybrid yeast strain than RA. And for convenience, its derived EEQ method is recommended in environmental study. That also explains by using the chemical analysis data of mixtures, it is feasible to calculate the estrogenic effect (here refers to the ER-binding capacity) with considerable accuracy. The prediction deviations of EEQ in actual use may be the result of other impact factors, such as the disturbance of unknown chemicals in water sample or the interaction of different pathways in test organisms. Additionally, it is true that when the EEQ of chemical mixture is higher than the maximum-effect-concentration of E2 (68.1 ng/L in exposure culture or 13.6 µg/L in DMSO), the competitive binding of components to ER will result in the failure of CA prediction and create a false impression of antagonism. However, this can be avoided by diluting the test samples.

Here we take two cases as the example to explain how to get information about estrogenic effect and its main contributors by analyzing the concentrations of mixture chemicals and summing their EEQ values. Firstly, Gong et al. (2012) studied the distribution of EDCs in water from the Pearl River in China. They found that the mean concentrations (ng/L) of E1, 4-t-OP, 4-NP and BPA were 3.2, 31.8, 1178 and 161, respectively (S14 in RMD), while the EEQ values (ng/L) were 0.23, 4.4×10^{-3} , 0.19 and 1.6×10^{-3} after conversion. In spite of the higher concentration of three phenols, E1 is still the most potent estrogenic chemical due to its strong binding capacity with ER. Another study examined the content of EDCs in a sewage-treatment plant (Zhou et al., 2012), and the result showed that the average concentrations (ng/L) were 64.8 and 22.8 (E2), 171.5 and 49.9 (EE2), 115.3 and 50.9 (4-NP), 920.7 and 84.3 (BPA) in the influents and effluents, respectively. Thinking through the aspect of EEQ, the corresponding adjusted concentrations (ng/L) were 64.8 and 22.8 (E2), 240.5 and 70.0 (EE2), 0.019 and 0.008 (4-NP), 0.009 and 0.001 (BPA). The concentrations of these four

substances do not appear to be much different from each other, all of which are in the range of tens to hundreds of ng/L; whereas their biological effects vary by several orders of magnitude. Because of its high concentration and REP value, EE2 becomes the main contributor to estrogenic effect followed by E2, while 4-NP and BPA scarcely make a contribution.

The successful implementation of the CA and EEQ in single-MoA assays and the likelihood of synergism appearing in more complex organisms highlights the limitations of existing environmental standards and risk assessment approaches, which are always established upon the basis of a single compound. In our study, good agreement between predicted and observed effects showed that even if the concentration of every individual estrogenic substance in a mixture is less than its no observable effect concentration (NOEC), it is possible that several chemicals with similar MoAs may induce an observable and adverse effect when acting in combination, which is similar to the description of Hood (2005).

In summary, this study demonstrated that the logistic function could describe the concentration–response relationship of either single or multiple components well, while CA and EEQ could predict the recombinant yeast assay results effectively, even for mixtures containing a range of estrogenic chemical compositions and concentrations. At the molecular level without any disturbance of antiestrogens and other MoAs, the mixture toxicity was proved to be additive. Therefore, considering that the present control standards of pollutants and water quality guidelines are mainly based on single chemical toxicities, it is predictable that with the development of combined effect studies, the effect-based quality criteria for aquatic environment will receive more attention in the future.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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