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An evaluation of the combined effects of phenolic endocrine disruptors on vitellogenin induction in goldfish *Carassius auratus*

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Abstract Phenolic compounds are widely distributed in the natural environment, typically existing as a mixture at the nanogram or microgram per liter level. Among the phenolic compounds, 4-nonylphenol, 4-t-octylphenol, bisphenol A and 2,4-dichlorophenol attract the most concern due to their abundance and risks within the natural environment. The former three chemicals are known as endocrine disruptors causing feminization in various organisms, whereas the latter one requires further clarification concerning its feminization effect. This study aims to evaluate the combined effects of these chemicals using vitellogenin protein induction in male juvenile goldfish *Carassius auratus* as an endpoint after 15 days of exposure. The results showed that all these chemicals can induce vitellogenin with a relative potency of 4-t-octylphenol > bisphenol A > 4-nonylphenol ≫ 2,4-dichlorophenol. 2,4-dichlorophenol showed a very weak estrogenic effect with an induction of vitellogenin concentration of <1 % of positive control, and it is therefore omitted in further tests to evaluate their combined effect. The other three chemicals were mixed in two ways, at an equipotent ratio and at an equal environmental level ratio, and their combined effects were evaluated with both the toxicity units method and concentration addition model. The resulting effect of exposure to both mixtures showed that these chemicals generally exhibited an additive effect. The ecological risk of phenolic chemicals may therefore be underestimated if based on the presence of single chemicals whereas their combined effects warrant further consideration.

Keywords Combined effect · Endocrine disruptor · Vitellogenin · Concentration addition model

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Introduction

Endocrine disruptors are exogenous agents that interfere with the synthesis, secretion, transport, binding, action and elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and behavior (Gültekin and Ince 2007). The most studied endocrine disruptors are environmental estrogens that mimic the function of a sex steroid hormone 17β-estradiol (E2). Various natural and synthetic chemicals have been identified to induce estrogen-like responses including pharmaceuticals, pesticides, industrial chemicals and phytoestrogens (Giesy et al. 2002). Among the environmental estrogens, several phenolic compounds including 4-nonylphenol (4-NP), 4-tert-octylphenol (4-t-OP), bisphenol A (BPA), and 2,4-dichlorophenol (2,4-DCP) have received special concerns due to their ubiquitous distribution in the environment (Staples et al. 2011). Both 4-NP and 4-t-OP are mainly used as raw materials to produce alkylphenol ethoxylates, a group of non-ionic surfactants with a variety of applications such as detergents, paints, cosmetics, textiles and herbicides (Tubau et al. 2010). BPA is used as the monomer to manufacture polycarbonate plastics, the resin that lines most food and beverage cans, dental sealants, and as an additive in other plastics with a global production capacity of 4.7 million metric tonnes in 2007 (Huang et al. 2012). 2,4-DCP is mainly used as a raw

material to synthesize 2,4-D, an effectual component of various types of herbicides. There have been numerous reports on the occurrence of these phenolic chemicals in rivers, lakes and coastal seas at the nanogram or microgram per liter level (Fu et al. 2007; Rubin 2011; Writer et al. 2010). Blackburn and Waldock (1995), for example, reported high concentrations of nonylphenol of 330 and 130 μg/l, respectively, in the effluent of a sewage treatment plant (STP) and in the water of Eire River in England.

Both in vitro and in vivo assays have been developed to screen for estrogenic activity of chemicals (Li et al. 2010; Richter et al. 2007). In vitro assays, such as estrogen receptor binding assay, mammalian cell reporter gene (MCRG) assay and the MCF-7 breast cancer cell proliferation assay (E-screen), have been widely used for the rapid screening of the estrogen-mimicking potential of fairly unknown compounds or samples (Connolly et al. 2011; Pomatto et al. 2011; Vanparys et al. 2010). Although in vitro estrogenicity screening systems are unable to fully predict the risk of adverse effects in humans and wildlife, they are well suited to give a first rapid ranking of the estrogenic potency of compounds or to indicate the overall estrogenic load in environmental matrices. Concurrently, in vivo assays, such as vitellogenin induction assay, uterotropic assay, pubertal onset assay, have also been designed for estrogenicity confirmation and the study of chemical metabolism and bioavailability (Borgert et al. 2011; Knudsen et al. 2011; Pillon et al. 2005).

Vitellogenin (Vtg) is a precursor of the egg-yolk protein, vitellin, which provides energy reserves for embryonic development in oviparous organisms (Matozzo et al. 2008). Vtg is generally synthesized in mature females, in response to endogenous estrogens, such as 17β-estradiol (E2). Under exposure to environmental estrogens, however, Vtg can also be induced in male juveniles. Vtg induction is therefore widely used as a biomarker of exposure to estrogenic compounds (Isidori et al. 2010; Matozzo et al. 2008).

With various in vitro and in vivo bioassays, 4-NP, 4-t-OP and BPA have been confirmed as environmental estrogens with a relative estrogenicity of 10^{-4}–10^{-5} compared to natural estrogen 17β-estradiol (E2) (Gutendorf and Westendorf 2001; Rubin 2011). The estrogenicity of 2,4-DCP, however, remains unconfirmed with various controversial reports. For example, it cannot bind with estrogen receptor (Li et al. 2010). It can however induce Vtg production in female individuals of the minnow Gobiocypris rarus, but cannot induce Vtg in males (Zhang et al. 2008). Considering the ubiquitous occurrence of these phenolic compounds, their ecological risks have already raised widespread concern (Fu et al. 2007). Since these compounds usually coexist in the natural environment, their combined effect warrants further attention. There have already been some reports on the combined effects of these phenolic compounds. Tan et al. (2003), for example, studied the combined effects of 4-NP and BPA with a less than additive effect through an in vivo rat pubertal onset assay. Duan et al. (2008) reported that bisphenol A and pentachlorophenol acted in either synergistic or antagonistic mode depending on different endpoints. These studies usually employed binary combination with two chemicals in the mixture. There are also some reports on the combined effects of more than three estrogenic chemicals. Fent et al. (2006), for example, examined the combined effects of mixtures of up to five active estrogenic pharmaceuticals with either additive or synergistic effects through an in vitro yeast reporter gene assay. There are however few studies on the combined effects of multiple phenolic compounds with an in vivo assay such as vitellogenin induction assay. This paper therefore aims to study the combined effects of the four most abundant phenolic endocrine disruptors with vitellogenin induction in male fish as an endpoint. The type of combination effects was evaluated through the toxicity units (TUs) method and the concentration addition (CA) model.

Materials and methods

Chemicals and reagents

The test compounds, 4-nonylphenol (CAS number: 84852-15-3), 4-tert-octylphenol (CAS number: 140-66-9), bisphenol A (CAS number: 80-05-7) and 2,4-dichlorophenol (CAS number: 120-83-2), were purchased from Sigma Aldrich. 17β-estradiol (E2, CAS number: 50-28-2) was also from Sigma Aldrich. The vitellogenin ELISA (enzyme-linked immunosorbent assay) kit was from Trans Genic (Japan). The pancreatic trypsin inhibitor (aprotinin) was from Amresco (USA). Solvents including ethanol, methanol, hexane, acetone and dichloromethane at HPLC grade were from Tedia.

Test species

The experimental individuals of juvenile goldfish *Carassius auratus* were purchased from a local fish farm in Qingdao, China. The individuals with a body weight of 10.7 ± 2.46 g and a body length of 7.0 ± 0.60 cm, were transported in aerated water and acclimated to laboratory conditions for 1 week prior to exposure experiments. During the acclimatization and exposure periods, the fish were kept in 30 cm × 25 cm × 25 cm aquaria, at 25 ± 1 °C in aerated (dissolved oxygen: 7.5 ± 0.4 mg/l) and sterilized water, with a pH of 7.6 ± 0.2. The individuals were fed with dry feed and surplus food was removed each day.

Chemical exposure

For single chemical treatment, the juvenile fish individuals were exposed to one phenolic compound at sub-lethal

nominal concentrations of 5–500 μg/l for 4-NP and 4-t-OP, 10–1,000 μg/l for BPA, and 20–2,000 μg/l for 2,4-DCP, respectively. 17β-estradiol (E2) was used as a positive control and the individuals were exposed to E2 at nominal concentrations of 5–500 ng/l, respectively. The test concentrations were chosen on the basis of previous studies carried out on several fish species (Cionna et al. 2006; Pomatto et al. 2011; Staples et al. 2011). For each treatment group, there were two replicate tanks with eight individual fish in each tank. For all the phenolic chemicals and E2, the ethanol was used as a solvent and the carrier never exceeded 0.1 ml/l. Two control groups were reared, one in an ethanol solution of 0.1 ml/l and the other in water without any other chemicals. The exposure was static renewal and a half of the exposure solution was replaced each day.

For multiple chemicals exposure, the juvenile fish individuals were exposed to a mixture of 4-NP, 4-t-OP and BPA at two ratios, since combination effects may vary with different mixture ratios (Fent et al. 2006). For the equipotent ratio, the chemicals were mixed according to their relative estrogenic potency (REP, see "Statistical analysis" section for details) of 1:1.38:3.19 (4-t-OP:BPA:4-NP). The individuals were exposed to mixtures with a sum nominal concentration of 22–667 μg/l. For equal environmental level ratio, the chemicals were mixed according to their realistic concentrations of 1:8:15 (4-t-OP:BPA:4-NP) in Jiaozhou Bay of Qingdao and its adjacent rivers (Fu et al. 2007). The individuals were exposed to mixtures with a sum nominal concentration of 24–720 μg/l. The chemicals in each group were quantified at the beginning and upon the end of exposure. At the beginning of the exposure, water samples were taken immediately after the exposure solutions were made, whereas upon the end of exposure, the samples were taken 24 h after the exposure solutions were renewed. The actual concentrations in the water samples were quantified by GC–MS with methods defined in Li et al. (2004). Briefly, the water samples were extracted with dichloromethane (DCM). The extracts were concentrated with a rotary evaporator to about 1 ml and further concentrated under a gentle flow of dry nitrogen to <0.5 ml. The acetone was then added to change solvent, followed by derivatization with BSTFA. Internal standards were added and the final volume adjusted to 1.0 ml before GC/MS analysis. The measured concentrations generally deviated from the nominal concentrations by less than 20 % (Table 1).

Quantitative ELISA analysis of Vtg

After 15 days of exposure, six fish individuals were randomly selected from each tank and anesthetized with 200 mg/l MS222 (Sigma Aldrich). Blood samples were collected by caudal puncture with a heparinized syringe. For each exposure group, at least three blood samples were collected from different individuals. To avoid proteolysis, 10 μl aprotinin (Amresco, USA) at 1 mM in 0.65 % NaCl was added to the blood at sampling. The blood samples were then centrifuged at 3,000×g and 4 °C for 15 min and the plasma samples were frozen at −80 °C until analysis. The gender of juvenile fishes was difficult to discriminate by physical appearance and it was, therefore, determined through dissection.

Plasma Vtg was quantified by a competitive ELISA using an ELISA kit for carp Vtg (Transgenic, Japan) since goldfish Vtg cross reacts well with monoclonal antibody made against carp Vtg (Toyoizumi et al. 2008). The linear range of the ELISA assay was 7.8–500 ng/ml. The plasma samples were diluted when Vtg concentration exceeded the linear range. The antigen coating and antibody incubation were performed according to the instruction of the ELISA kit manufacturer. Briefly, standards and the plasma samples were added to microplate wells pre-coated with the primary Vtg antibody and the plates were incubated at 37 °C for 2 h. Then the antibody against Vtg labeled with horseradish peroxidase was added and incubated at 37 °C for 1 h. After that, the substrate (*o*-phenylenediamine) was added and the visualization of the reaction was performed with horseradish peroxidase. The reaction was stopped after 30 min by adding 50 μl/well of 4 M sulphuric acid. The absorbance of each well was measured at 490 nm using a Microplate Reader (Benchmark, BIO-RAD). The detection limit with the ELISA kit was 7.8 ng/ml of Vtg.

Statistical analysis

One-way ANOVA (analysis of variance) was employed to evaluate differences of Vtg induction (log transformed) among exposure groups. If ANOVA rejected a multi-sample hypothesis of equal means, Tukey multiple comparison tests were undertaken to evaluate the significance of difference between the control and exposure groups. To simulate the dose–response relationship, the Vtg induction in each group was expressed as a percentage of the logarithm of the maximum induction in E2 exposure groups and the exposure concentrations were also on a log axis. The dose–response curves showed an "S" shape and were fitted with Weibull distribution. The half effective concentration (EC_{50}) was defined as the dose required to induce 50 % of the logarithm of the maximum Vtg induction in E2 positive control. The relative estrogenic potency (REP) of each phenolic compound was defined as: $REP_{(i)} = EC_{50(E2)}/EC_{50(i)}$, where $EC_{50(E2)}$ and $EC_{50(i)}$ represent EC_{50} values of E2 and the phenolic compounds respectively. The statistical analysis and plotting were done with SPSS 13.0 and Origin 7.5.

Table 1 Nominal and measured phenolic concentrations in the exposure solutions (μg/l)

Phenolics	Nominal	Measured at the beginning[a]	Measured in the end[b]	Measured average[c]	Percentage[d]
Single chemical exposure					
4-*tert*-octylphenol	5	5.2	3.8	4.5	90.0
	15	14.0	11.2	12.6	84.0
	50	42.3	38.4	40.4	80.7
	150	128	132	130	86.7
	500	487	416	452	90.3
Bisphenol A	10	8.3	7.6	8.0	79.5
	30	31.2	21.9	26.6	88.5
	100	87.6	91.2	89.4	89.4
	300	272	228	250	83.3
	1,000	965	783	874	87.4
4-Nonylphenol	5	4.4	3.2	3.8	76.0
	15	13.5	11.7	12.6	84.0
	50	40.8	42.2	41.5	83.0
	150	146	118	132	88.0
	500	478	398	438	87.6
2,4-Dichlorophenol	20	18.5	14.2	16.4	81.8
	60	52.8	40.8	46.8	78.0
	200	158	163	161	80.3
	2,000	1,820	1,680	1,750	87.5
Multiple chemicals exposure					
Equipotent ratio[e]	22	22.6	14.7	18.7	84.8
	67	58.7	52.1	55.4	82.7
	223	212	164	188	84.3
	445	379	305	342	76.9
	667	633	486	560	83.9
Equal environmental ratio[e]	24	22.2	14.7	18.5	76.9
	72	62.8	47.8	55.3	76.8
	240	205	178	192	79.8
	480	486	355	421	87.6
	720	696	588	642	89.2

[a] Concentrations measured at the beginning of exposure

[b] Concentrations measured in the end of exposure

[c] The average concentrations of the two measurements

[d] The average measured concentration as a percentage of the nominal concentration

[e] Sum concentrations of the mixture

Combination effect analysis

The type of combination effects of the phenolic compounds was evaluated with the toxicity units (TUs) method and the concentration addition (CA) model, respectively. For the TUs method, the toxicity unit of a single compound is defined as: $TU_i = C_i/EC_{50(i)}$, where C_i is the concentration of the phenolic compound in the mixture and $EC_{50(i)}$ is the half effective concentration of the compound. The total TU of the mixture (TU_{mix}) is defined as the sum of the TU_i of each compound in the mixture. If the TU_{mix} is equal to 1.0 or its 95 % confidence interval covers 1.0, the mixture is categorized as an additive type. For CA model, the expected EC_{50} of the mixture is calculated based on the $EC_{50(i)}$ of each component. If the expected EC_{50} lies within the 95 % confidence interval of the observed EC_{50}, the mixture was identified as an additive type.

Results

During the exposure period, no fish mortality occurred and no difference in animal behavior between phenolic exposure and control groups existed, which implies that the phenolic chemicals at the exposure concentrations did not exert acute toxicity on the fish. The Vtg induction in both water and carrier control groups was below the detection limit of the ELISA kit (7.8 ng/ml).

Vtg induction by single phenolic chemicals

All of the four target phenolic chemicals can induce Vtg in male fish (Fig. 1). The lowest concentration for significant Vtg induction for 4-t-OP, 4-NP, BPA and 2,4-DCP was 15, 50, 100 and 200 μg/l, respectively. A maximum Vtg induction of 9.40×10^6 ng/ml was measured in the E2

positive control and all of the phenolic chemicals induced Vtg comparable to the maximum induction at the highest exposure concentration except for 2,4-DCP with a maximum Vtg induction of only 2.86×10^3 ng/ml.

The dose–response relationship for E2, 4-NP, 4-t-OP and BPA generally shows an "S" shape and can be fitted with a Weibull distribution (Fig. 2). The half effective concentration (EC_{50}) can be calculated based on the Weibull function as follows:

$$Y = 1 - \exp(-\exp(\alpha + \beta \times \lg X),$$

where Y is the percentage of Vtg induction relative to the maximum of positive control, X is the exposure concentration, and α and β are regression parameters.

The results of Weibull regression are shown in Table 2. Among the test compounds, 4-t-OP exhibited the strongest estrogenicity with a half effective concentration (EC_{50}) of 79.6 μg/l and a relative estrogenic potency (REP) of 9.80×10^{-4}. BPA and 4-NP showed a relative estrogenic potency of 6.88×10^{-4} and 3.05×10^{-4}, respectively. 2,4-DCP showed a very weak estrogenicity and therefore no dose–response regression could be made.

Vtg induction by mixture of phenolic chemicals

The three chemicals were mixed to an equipotent ratio and an equal environmental level ratio, respectively. For the equipotent ratio, the chemicals were mixed at 1:1.38:3.19 (4-t-OP:BPA:4-NP) according to their relative estrogenic potency (REP). For the equal environmental level ratio, the chemicals were mixed at 1:8:15 (4-t-OP:BPA:4-NP) (see "Chemical exposure" section for details). The results showed that the mixture also exhibited an "S" shaped dose–response curve. The Weibull regression was then made and the results presented in Table 3. For the mixture at the equipotent ratio, the half effective concentration (EC_{50}) was 144.9 μg/l and the relative estrogenic potency (REP) was 5.38×10^{-4}. For the mixture at the equal environmental level ratio, the EC_{50} was 174.2 μg/l and the relative estrogenic potency was 4.48×10^{-4}. The former mixture was therefore slightly more estrogenic than the latter.

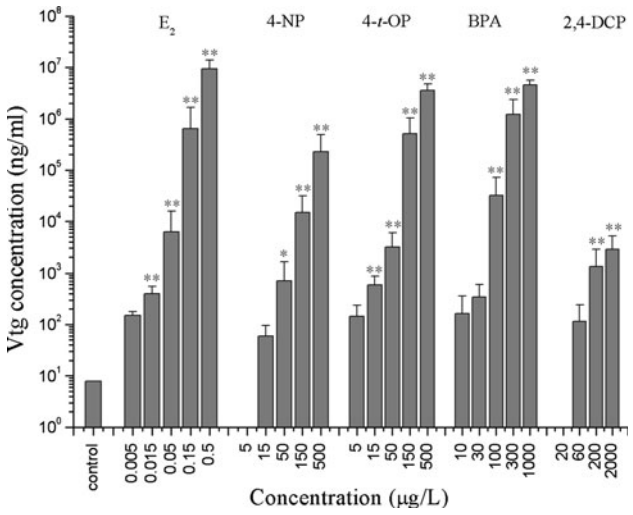

Fig. 1 Vitellogenin concentrations in male goldfish induced by single phenolic compounds and 17β-estradiol (E2) (*$0.01 < P \leq 0.05$; **$P \leq 0.01$). Data are given as means of at least three replicate samples and *error bars* represent standard deviation. Vtg in solvent control was not detected and was expressed as the detection limit of the ELISA kit)

Evaluation on phenolic combined effects

The type of combination effects was evaluated with the toxicity units (TUs) method and the concentration addition (CA) model, respectively. With the toxicity units method, the TU_{mix} for the mixture in equipotent ratio was calculated as 0.968 with a 95 % confidence interval of between 0.927 and 1.102 (Table 4). The confidence interval covers 1.0 and the combined effect was therefore categorized as an additive type. Similarly, the combined effect for the mixture in equal environmental levels was also categorized as an additive type (Table 4).

Based on the concentration addition model, the effects of the mixture at different concentrations can be predicted based on the specific concentration and relative estrogenic potency (REP) of each chemical. The predicted effect of the mixtures was shown in Fig. 3. The dose–responsive relationship of the mixtures was then regressed with a Weibull distribution and the predicted EC_{50} of the mixtures was calculated as 149.2 and 168.7 μg/l for the mixture at the equipotent ratio and at the equal environmental level ratio, respectively (Table 4). The predicted EC_{50} values in both mixture ratios fall within the 95 % confidence interval of the observed EC_{50} and the combined effect was therefore categorized as an additive type. For the mixture at equipotent ratio, the prediction effect line is however slightly less than the lower confidence interval at the high concentration end (400–667 μg/l), indicating a slight synergistic effect (Fig. 3). For the equal environmental level ratio, the predicted effect line is also slightly less than the lower confidence interval at high concentrations (500–720 μg/l).

Discussion

The relative estrogenic potency of 4-t-OP and BPA in this study generally matches those reported (Staples et al. 2011; Pomatto et al. 2011). The estrogenicity of 4-NP in this study was however weaker than those reported previously (Jayne et al. 2005). In addition, the dose–response curve of 4-NP is not satisfactory and higher exposure doses are expected to exhibit a full "S" shape (Fig. 2). This is

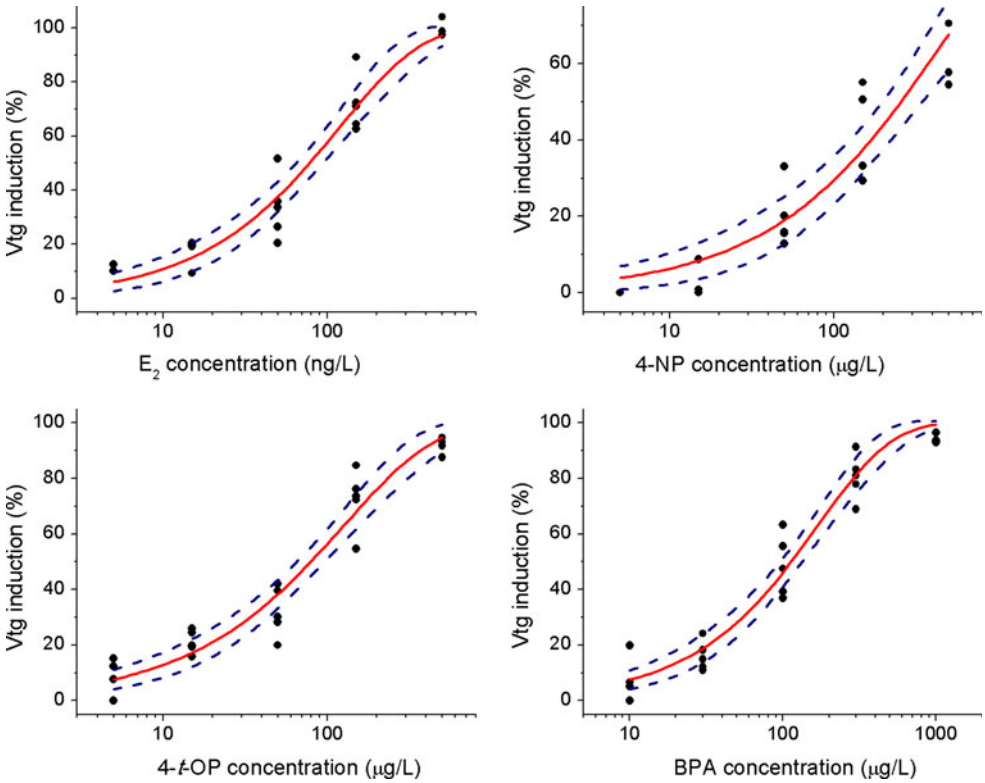

Fig. 2 Dose–responsive curves (*solid line*) with 95 % confidence intervals (*dash line*) for the effects of single phenolic compounds and 17β-estradiol (E2) on Vtg induction in male goldfish (Vtg concentrations expressed as percentages of the maximum induction in E2 control)

Table 2 Half effective concentrations (EC_{50}) and relative estrogenic potency (REP) of phenolic compounds for vitellogenin induction in male goldfish

Chemicals	EC_{50} (μg/l)	95 % CI[a] (μg/l)	REP[b]
E_2	0.078	0.065–0.094	1
4-*tert*-octylphenol	79.6	65.6–96.4	9.80×10^{-4}
Bisphenol A	113	95.9–134	6.88×10^{-4}
4-Nonylphenol	256	198–347	3.05×10^{-4}
2,4-Dichlorophenol	>2,000	NA[c]	$<3.90 \times 10^{-5}$

[a] *CI* Confidence interval

[b] *REP* Relative estrogenic potency

[c] *NA* Not applicable

Table 3 Half effective concentrations (EC_{50}) of phenolic mixtures for vitellogenin induction in male goldfish

Mixture type	Ratio (4-t-OP:BPA:4-NP)	EC_{50} (μg/l)	95 % CI[a] (μg/l)	REP[b]
Equipotent	1:1.38:3.19	144.9	119.0–172.8	5.38×10^{-4}
Equal environmental	1:8:15	174.2	145.3–204.6	4.48×10^{-4}

[a] *CI* Confidence interval

[b] *REP* Relative estrogenic potency

probably because the 4-NP used in this study is at a technical grade consisting of both linear and branched chain isomers. Uchiyama et al. (2008) have shown that the structural difference of the alkyl chain in 4-NP affects the estrogenic activity on the recombinant yeast screen assay. Pedersen et al. (1999) also reported that linear chain isomer of 4-NP could not induce Vtg production whereas branched ones could. The estrogenic potency of 4-NP with different ratios of branching therefore still warrants further study.

The estrogenic potency of 2,4-DCP is very weak in this study. The Vtg content in the highest exposure group (2,000 μg/l) is as low as 2.86×10^3 ng/ml, which is less than 1 % of the maximum induction in the E2 positive control. Concerning the estrogenicity of this chemical, Zhang et al. (2008) reported that it can induce Vtg induction in female individuals of minnow Gobiocypris rarus, but could not induce Vtg in male individuals. The chemical can also bind to estrogen receptor (Maekawa et al. 2004) and induce MCF-7 breast cancer cell proliferation (Jones et al. 1998). But Li et al. (2010) reported that 2,4-DCP cannot bind with estrogen receptor, although it can bind with androgen receptor. This chemical therefore warrants further clarification for its estrogenicity, but even positive, it should be much weaker in contrast to other phenolic compounds since it does not have a carbon chain on the phenol ring in its molecule structure.

The concentration addition model is based on the assumption that chemicals act in a similar way, such that effects can be produced by replacing one compound totally or in part with the other (Kortenkamp and Altenburger 1999). In this study, all of the phenolic compounds act

Table 4 The evaluation for the combined effects of phenolic compounds on Vtg induction in male goldfish

Mixture type	Toxic units method		Concentration addition model		
	TU_{mix}^{a}	Combined effect	Predicted EC_{50} (μg/l)	Within the 95 % CI[c]	Combined effect
Equipotent	0.968 (0.927–1.102)[b]	Additive	149.2	Yes	Additive
Equal environmental	1.028 (0.976–1.222)[b]	Additive	168.7	Yes	Additive

[a] Toxic unit of the mixture

[b] Values in brackets denote the 95 % confidence interval of TU_{mix}

[c] *CI* 95 % confidence interval of observed EC_{50}

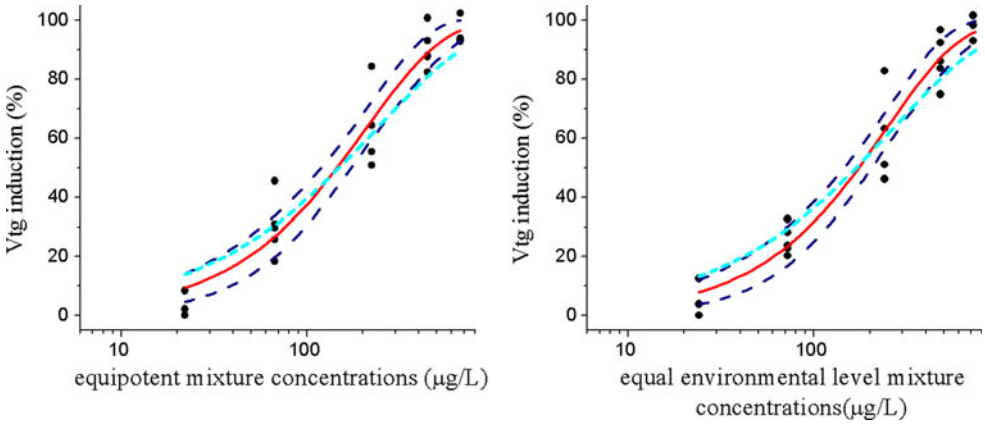

Fig. 3 Comparison between the observed (*solid line*) and CA (concentration addition) model predicted (*thick dash line*) mixture effects of phenolic compounds on Vtg induction in male goldfish (the 95 % confidence intervals of the observed effects shown in *dash lines*; Vtg concentrations expressed as percentages of the maximum induction in E2 control)

similarly through estrogen receptor binding mechanisms to induce Vtg production (Blair et al. 2000; Hashimoto et al. 2000; Laws et al. 2000) and the model is therefore suitable for the evaluation of the combined effects of these chemicals. Based on the CA model, the predicted EC_{50} of the equipotent mixture was 149.2 μg/l (Table 4). The observed EC_{50} of 4-t-octylphenol, bisphenol A and 4-nonylphenol was 79.6, 113 and 256 μg/l, respectively. The observed EC_{50} of the equipotent mixture was 144.9 μg/l. The predicted EC_{50} was comparable to the observed one and the equipotent mixture therefore acted as an additive mode. Similarly, the equal environmental level mixture also acted as an additive mode. This is consistent with the results obtained in previous reports based on a binary mixture (Sun et al. 2009). This type of combination effect is understandable considering the similar action mechanism and similar molecular structure of the phenolic compounds. In the equipotent mixture, the predicted effect was however slightly lower than the observed effect at high concentrations, indicating a slight synergistic effect. For example, at the highest mixture concentration of 667 μg/l, the predicted effect was 89.9 % of the maximum induction, whereas the observed effect was 96.6 % with a confidence interval of between 93.0 and 100 % (Fig. 3). The predicted effect was therefore 3.1 % less than the lower confidence interval of the observed effect and a synergistic effect could be expected. The deviation was however small and more detailed study is warranted to confirm this hypothesis.

In natural environment, phenolic compounds can reach similar concentrations as those in this study (Blackburn and Waldock 1995; Fu et al. 2007). The chemicals typically exist in a mixture and consequently the risk based on a single chemical assessment may be under estimated. As pointed out by Fent et al. (2006) and Silva et al. (2002) with an in vitro Yeast E-Screen assay, a mixture of chemicals may cause considerable combined effects. The ecological risk of phenolic mixtures therefore warrants further attention.

Conclusions

With vitellogenin in male goldfish as an endpoint, all of the tested phenolic compounds showed positive induction with an estrogenic potency order of 4-t-OP > BPA > 4-NP ≫ 2,4-DCP. A nonlinear regression of Weibull function could be applied to the dose–response relationship for 4-t-OP, BPA and 4-NP with a median effective concentration (EC_{50}) of 79.6, 113 and 256 μg/l respectively. 2,4-DCP showed a very weak estrogenicity with an induction of Vtg concentration of <1 % of the positive control and was therefore omitted in the following mixture exposure study.

Vitellogenin induction is also detected for the exposure of mixtures of 4-t-OP, BPA and 4-NP, in both equipotent and equal environmental level ratios. The combined effects of the phenolic mixtures generally showed an additive mode both with the toxicity units method (TUs) and in the

CA model. The ecological risk of phenolics based on single chemicals may therefore be underestimated and their combined effects in natural environment can be predicted with the CA model.

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References

Blackburn MA, Waldock MJ (1995) Concentrations of alkylphenols in rivers and estuaries in England and Wales. Water Res 29:1623–1629

Blair RM, Fang H, Branham WS et al (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54:138–153

Borgert CJ, Mihaich EM, Quill TF et al (2011) Evaluation of EPA's tier 1 endocrine screening battery and recommendations for improving the interpretation of screening results. Regul Toxicol Pharmacol 59:397–411

Cionna C, Maradonna F, Olivotto I et al (2006) Effects of nonylphenol on juveniles and adults in the grey mullet, Liza aurata. Reprod Toxicol 22:449–454

Connolly L, Ropstad E, Verhaegen S (2011) In vitro bioassays for the study of endocrine-disrupting food additives and contaminants. Trend Anal Chem 30:227–238

Duan Z, Zhu L, Zhu L et al (2008) Individual and joint toxic effects of pentachlorophenol and bisphenol A on the development of zebrafish (Danio rerio) embryo. Ecotox Environ Safe 71:774–780

Fent K, Escher C, Caminada D (2006) Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reprod Toxicol 22:175–185

Fu M, Li Z, Gao H (2007) Distribution characteristics of nonylphenol in Jiaozhou Bay of Qingdao and its adjacent rivers. Chemosphere 69:1009–1016

Giesy JP, Hilscherova K, Jones PD et al (2002) Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Mar Pollut Bull 45:3–16

Gültekin I, Ince NH (2007) Synthetic endocrine disruptors in the environment and water remediation by advanced oxidation processes. J Environ Manage 85:816–832

Gutendorf B, Westendorf J (2001) Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166:79–89

Hashimoto Y, Moriguchi Y, Oshima H et al (2000) Estrogenic activity of chemicals for dental and similar use in vitro. J Mater Sci Mater Med 11:465–468

Huang YQ, Wong CKC, Zheng JS et al (2012) Bisphenol A (BPA) in China: a review of sources, environmental levels, and potential human health impacts. Environ Int 42:91–99

Isidori M, Cangiano M, Palermo FA et al (2010) E-screen and vitellogenin assay for the detection of the estrogenic activity of alkylphenols and trace elements. Comp Biochem Physiol C: Toxicol Pharmacol 152:51–56

Jayne VB, Catherine AH, Martin S et al (2005) Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ Health Perspect 113:721–728

Jones PA, Baker VA, Irwin AKE et al (1998) Interpretation of the in vitro proliferation response of MCF-7 cells to potential oestrogens and non-estrogenic substances. Toxicol In Vitro 12:373–382

Knudsen JG, Holbech H, Madsen SS et al (2011) Uptake of 17β-estradiol and biomarker responses in brown trout (Salmo trutta) exposed to pulses. Environ Pollut 159:3374–3380

Kortenkamp A, Altenburger R (1999) Approaches to assessing combination effects of oestrogenic environmental pollutants. Sci Total Environ 233:131–140

Laws SC, Carey SA, Ferrell JM et al (2000) Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 54:154–167

Li Z, Li D, Oh J-R et al (2004) Seasonal and spatial distribution of nonylphenol in Shihwa Lake, Korea. Chemosphere 56:611–618

Li J, Ma M, Wang Z (2010) In vitro profiling of endocrine disrupting effects of phenols. Toxicol In Vitro 24:201–207

Maekawa S, Nishizuka M, Heitaku S et al (2004) Development of a competitive enzyme immunoassay for detection of capacity of chemicals to bind quail estrogen receptor α and β. J Health Sci 50:25–32

Matozzo V, Gagné F, Marin M et al (2008) Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ Int 34:531–545

Pedersen SN, Christiansen LB, Pedersen KL et al (1999) In vivo estrogenic activity of branched and linear alkylphenols in rainbow trout (Oncorhynchus mykiss). Sci Total Environ 223:89–96

Pillon A, Servant N, Vignon F et al (2005) In vivo bioluminescence imaging to evaluate estrogenic activities of endocrine disrupters. Anal Biochem 340:295–302

Pomatto V, Palermo F, Mosconi G et al (2011) Xenoestrogens elicit a modulation of endocannabinoid system and estrogen receptors in 4-NP treated goldfish, *Carassius auratus*. Gen Comp Endocrinol 174:30–35

Richter CA, Birnbaum LS, Farabollini F et al (2007) In vivo effects of bisphenol A in laboratory rodent studies. Reprod Toxicol 24:199–224

Rubin BS (2011) Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol 127:27–34

Silva E, Rajapakse N, Kortenkamp A (2002) Something from "nothing"—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Technol 36:1751–1756

Staples CA, Hall AT, Friederich U et al (2011) Early life-stage and multigeneration toxicity study with bisphenol A and fathead minnows (Pimephales promelas). Ecotoxicol Environ Saf 74: 1548–1557

Sun L, Zha J, Wang Z (2009) Interactions between estrogenic chemicals in binary mixtures investigated using vitellogenin induction and factorial analysis. Chemosphere 75:410–415

Tan BL, Kassim NM, Mohd MA (2003) Assessment of pubertal development in juvenile male rats after sub-acute exposure to bisphenol A and nonylphenol. Toxicol Lett 143:261–270

Toyoizumi T, Deguchi Y, Masuda S et al (2008) Genotoxicity and estrogenic activity of 3,3′-dinitrobisphenol A in goldfish. Biosci Biotechnol Biochem 72:2118–2123

Tubau I, Vázquez-Suñé E, Carrera J et al (2010) Occurrence and fate of alkylphenol polyethoxylate degradation products and linear alkylbenzene sulfonate surfactants in urban ground water: Barcelona case study. J Hydrol 383:102–110

Uchiyama T, Makino M, Saito H et al (2008) Syntheses and estrogenic activity of 4-nonylphenol isomers. Chemosphere 73: 60–65

Vanparys C, Depiereux S, Nadzialek S et al (2010) Performance of the flow cytometric E-screen assay in screening estrogenicity of

pure compounds and environmental samples. Sci Total Environ 408:4451–4460

Writer JH, Barber LB, Brown GK et al (2010) Anthropogenic tracers, endocrine disrupting chemicals, and endocrine disruption in Minnesota lakes. Sci Total Environ 409:100–111

Zhang X, Zha J, Li W et al (2008) Effects of 2,4-dichlorophenol on the expression of vitellogenin and estrogen receptor genes and physiology impairments in Chinese rare minnow (Gobiocypris rarus). Environ Toxicol 23:694–701