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Evaluation of ecotoxicological effects of benzophenone UV filters: Luminescent bacteria toxicity, genotoxicity and hormonal activity

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ABSTRACT

The widespread use of organic ultraviolet (UV) filters in personal care products raises concerns about their potentially hazardous effects on human and ecosystem health. In this study, the toxicities of four commonly used benzophenones (BPs) UV filters including benzophenone (BP), 2-Hydroxybenzophenone (2HB), 2-Hydroxy-4-methoxybenzophenone (BP3), and 2-Hydroxy-4-methoxybenzophenone-5-sulfonicacid (BP4) in water were assayed *in vitro* using *Vibrio fischeri*, SOS/umu assay, and yeast estrogen screen (YES) assay, as well as *in vivo* using *zebrafish* larvae. The results showed that the luminescent bacteria toxicity, expressed as $\log EC_{50}$, increased with the lipophilicity ($\log Kow$) of BPs UV filters. Especially, since 2HB, BP3 and BP4 had different substituent groups, namely -OH, $-OCH_3$ and $-SO_3H$, respectively, these substituent functional groups had a major contribution to the lipophilicity and acute toxicity of these BPs. Similar tendency was observed for the genotoxicity, expressed as the value of induction ratio = 1.5. Moreover, all the target BPs UV filters showed estrogenic activity, but no significant influences of lipophilicity on the estrogenicity were observed, with BP3 having the weakest estrogenic efficiency *in vitro*. Although BP3 displayed no noticeable adverse effects in any *in vitro* assays, multiple hormonal activities were observed in *zebrafish* larvae including estrogenicity, anti-estrogenicity and anti-androgenicity by regulating the expression of target genes. The results indicated potential hazardous effects of BPs UV filters and the importance of the combination of toxicological evaluation methods including *in vitro* and *in vivo* assays.

1. Introduction

Rapid economic development and increasing demand for health protection have promoted the widespread use of personal care products worldwide. UV filters are chemical compounds extensively used in sunscreen and a variety of cosmetics, such as creams, lipsticks, and even agricultural chemicals and pharmaceuticals (Balmer et al., 2005; Roelandts et al., 1983) to protect humans and materials from the harmful effects of UV irradiation. Currently, 28 organic UV filters are registered in the European Union (Schlumpf et al., 2008) and 14 are authorized for use in the USA (Rodil et al., 2009). It is estimated that about 10,000 t of UV filters are produced annually for the global markets (Danovaro et al., 2008). Inorganic UV filters, such as titanium dioxide and/or zinc oxide, are used to scatter and/or reflect UV light, whereas organic UV filters absorb UV light. Benzophenones (BPs) are the most important members of the organic UV filters family (Suzuki et al., 2005). These aromatic UV filters are added to sunscreen products, as dominant components, in different proportions. For example, the US Food and Drugs Administration (US FDA, 2013) regulated BP3 and BP4 in sunscreen products at maximal levels of 6% and 10%, respectively.

The BPs UV filters are released into aquatic ecosystems either directly through recreational activities, such as bathing and swimming (Buser et al., 2006), or indirectly through discharges from wastewater treatment plants (WWTPs) (Balmer et al., 2005). Due to the various applications of BPs UV filters, their occurrence and contamination of the environment have been recorded in concentrations ranging from ng L^{-1} to μg L^{-1} in raw sewage, surface water, tap water and even indoor dust (Tsui et al., 2015; Díaz-Cruz et al., 2012; Wang et al., 2013). In fact, some studies have discovered the occurrence of BP3 and 4-Methylbenzylidene-camphor in samples of human urine, breast milk

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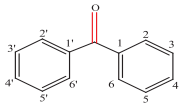
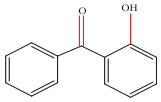
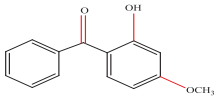
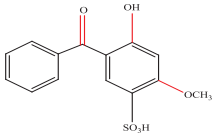
Table 1
Chemical structures, CAS numbers, purity, molecular weight and log*K*ow of target UV filters.

Chemicals	Structure	CAS number	Purity	M (g mol^{-1})	log *K*ow[a]	pH[d]
BP		119-61-9	≥99%	182.22	3.15[b]	6.12
2HB		117-99-7	≥99%	198.22	3.44[b]	6.50
BP3		131-57-7	≥99%	228.22	3.79[c]	7.20
BP4		4065-45-6	≥97%	308.31	0.37[c]	4.14

[a] octanol-water partition coefficient
[b] Liu et al. (2015);
[c] Molins-Delgado et al. (2016)
[d] the actual pH values of medium used for all experiments

and placental tissue (Frederiksen et al., 2013; Schlumpf et al., 2010). Although the acute and sub-chronic systemic toxicity of these UV filters after dermal application is rather low, problems caused by photoallergic reactions in patients have been reported (Schauder and Ippen, 1997). Therefore, the widespread occurrence of BPs UV filters warns us of potential harmful impacts on human and ecosystem health.

In fact, increasing evidence to support growing concerns regarding the eco-toxicity of BPs UV filters has been reported. The most impressive was that of multiple hormonal activities reported by Kunz and Fent (2006). In addition, the estrogenic activity of BPs UV filters was demonstrated with an assay using MCF-7 breast cancer cells and an immature rat uterotrophic assay (Schlumpf et al., 2001; Yamasaki et al., 2003). Recently, an acute toxicity level (50% effective concentration, EC_{50}) of BP3 on the larvae of *Mytilus galloprovincialis* of 3.42 mg L^{-1} was reported (Paredes et al., 2014). The 48 h-EC_{50} of BP4 on *Daphnia magna* was estimated to be 30.40 mg L^{-1} (Molins-Delgado et al., 2016), and the 15-min EC_{50} of BP on *Photobacterium phosphoreum* was estimated to be 34.26 mg L^{-1} (Liu et al., 2015). Nevertheless, the toxicological profile and modes of action of BPs UV filters are poorly understood. Most especially, the information on the non-specific toxicity to luminescent bacteria and the genotoxicity of UV filters remains scarce, except for a few fragmentary studies (Abramsson-Zetterberg and Svensson, 2011; Jeon et al., 2007).

On the one hand, as knowledge of the physicochemical properties, fate, and eco-toxicological effects is fundamental to the preliminary assessments, it is necessary to obtain the “basic set” of data, such as EC_{50}, from experimental tests using standardized test protocols. *Vibrio fischeri* is a luminescent marine bacterium and one of the aquatic organisms most used in non-specific toxicity assessment (ISO 11348, 2008). In addition, the SOS/umu test using *Salmonella typhimurium* (*S. typhimurium*) TA1535/pSK1002 has been used world-wide as a standard method to analyze the genotoxicity of individual compound and pollutants in water samples (ISO 13829, 2000). Moreover, the yeast estrogen screen (YES) assay is recommended for detecting estrogenic activity of chemicals, and mainly to create a useful reference for *in vivo* assay. Yeast cells have been transfected with expression plasmids carrying a reporter gene (lac-z) situated downstream from a promoter sequence, which incorporates an estrogen response element (Alvarez et al., 2013). In addition, the *in vivo assay* has the capability to metabolize chemicals resulting in the general outcome that chemicals are less active *in vitro*, but nevertheless are still more active *in vivo* than *in vitro* (Miller et al., 2001). Especially, the inactivity can translate into *in vivo* activity. *Zebrafish* has been proposed as an excellent vertebrate model for assessing the toxic effects of chemicals *in vivo*, which is especially useful for elucidating their mode of action (Hutchinson et al., 2003).

On the other hand, the toxicity of chemicals is related to their physicochemical characteristics, such as lipophilicity and substituent groups (Ranke et al., 2007; Liu et al., 2015). Zhao et al. (2013) studied the contribution of substituent group of UV filters to genetic effect by conducting the SOS/umu assay. Li et al. (2012) reported the acute toxicities of 14 BPs to a freshwater organism-*Dugesia japonica*. However, to date, little information is available on assessment of the overall biological effect of BPs from different aspects of toxicities and there is no study concerning the systematic research regarding the impacts of chemical structures on the eco-toxicity of BPs UV filters. Moreover, investigating the relationship between the substituent group of UV filters and their toxicity will provide a good chance to fill gaps of toxicity prediction for UV filters, and is in favor of the quantitative structure-activity relationship model building.

Accordingly, the aim of this study is to comprehensively analyze the biological effects of four commonly used BPs UV filters, namely BP, 2HB, BP3 and BP4 including acute toxicity, genotoxicity and endocrine disrupting effects using *in vitro* and *in vivo* bioassays. To the best of our knowledge, this is the first report to study the contribution of related substituent groups of UV filters to different aspects of toxicities. Moreover, the mechanisms of estrogenicity *in vitro* and multiple hormone activities *in vivo* of these BPs UV filters are preliminary

discussed. It will deepen the understandings of the mechanisms of toxicities of BPs UV filters to organisms, and the awareness of safe usage of UV filters can also be enhanced.

2. Materials and methods

2.1. Chemicals and test organisms

All BPs UV filters (≥99.0% purity) purchased from TCI (Tokyo, Japan) are listed in Table 1. Phenol, 17β-estradiol (E2), 4-nitroquinoline-N-oxide (4-NQO) and o-nitrophenyl-β-Dgalactopyranoside (ONPG) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All target UV filters were dissolved in ≤1% (v/v) dimethylsulfoxide.

2.2. Bioassays methodology

2.2.1. V. fischeri test

The marine bacterium *V. fischeri* used in the luminescent bacteria toxicity tests, was purchased in a freeze-dried form from the China Center for Industrial Culture Collection and activated by rehydration with a reconstitution solution. The test was performed in 96-well microplates, and the luminescence was measured using the CentrolIApc LB962 Microplate Luminometer (Berthold Technologies Company, Germany) according to the standard method (ISO 11348, 2008). Briefly, luminescent bacteria were incubated at a constant temperature in glass vials including 1 mL of 2% NaCl solution and target UV filters or positive control (phenol). Ultimately, the target UV filters were present at various concentrations achieved by a series of 2:3 dilutions in 96-well microplates. The luminescence of each well was measured at 15 min time intervals. The relative intensity of the luminescence in samples (R) and control (R_0) was measured to calculate the potential luminescence inhibition (I) of the chemicals according to Eq. (1). Then, effective concentration of the tested solution resulting in a 50% decrease in bioluminescence (EC_{50}) was obtained. For each UV filter, the concentrations analyzed were within the scope of the water solubility at 25 °C.

$$I = \frac{R_0 - R}{R_0} \quad (1)$$

2.2.2. SOS/umu assay

The SOS/umu assay was performed to evaluate the genotoxic effect of the positive control (4-NQO) and the target UV filters with *S. typhimurium* TA1535/pSK1002 without S9 activation according to ISO 13829, (2000). Briefly, the bacteria were cultured in a LB medium containing 50 mg L^{-1} ampicillin for 12 h. Then, the bacterial suspension was diluted at 1:10 with fresh TGA medium and incubated for 1.5 h at 37 °C on a shaker. When the bacteria reached the exponential growth phase, the test was performed on a 96-well microplate as follows: a mixture of 180 μL of each target UV filter and 70 μL of bacterial suspension was incubated in a microplate A for 2 h at 37 °C by shaking. Then, 30 μL of the above bacterial suspension was transferred to a new microplate B containing 270 μL of fresh TGA, which was incubated for 2 h. The bacterial growth rate was determined at 600 nm (A_{600}). A volume of 30 μL of the above incubated bacterial suspension was transferred to another microplate C containing 120 μL of B-buffer, then 30 μL of ONPG was added to microplate C, which was then incubated for 0.5 h at 28 ± 1 °C. Afterward, 120 μL of Na_2CO_3 was added to terminate the reaction. Finally, the activity of β-galactosidase was measured at 420 nm (A_{420}) using a microplate reader (Sun RISE, TECAN, Switzerland). The genotoxicity was calculation according to Eqs. (2) and (3).

$$G = \frac{A_{600,T} - A_{600,B}}{A_{600,N} - A_{600,B}} \quad (2)$$

$$IR = \frac{1}{G} \times \frac{A_{420,T} - A_{420,B}}{A_{420,N} - A_{420,B}} \quad (3)$$

Note: G: growth factor; *IR*: induction ratio; *S*: samples; *B*: blank control; *N*: negative control. When $G \geq 0.5$, the concentration with $IR = 1.5$ was considered as the lowest positive effect concentration (LPEC) at which the compound was regarded as genotoxic (Zhao et al., 2013).

2.2.3. Yeast estrogen screen (YES)

The β-galactosidase activity induced by UV filters was evaluated according to the method used in a previous study (Gaido et al., 1997). Briefly, the yeast strain in an Erlenmeyer flask was pre-incubated at 30 °C with on a shaker. After 36 h the yeast suspension reached the exponential phase at 600 nm, the bacteria suspension was diluted to 0.6–0.7. An aliquot of 5 μL of the toxicant solution was added into 1.5 mL eppendorf tubes containing 995 μL of diluted yeast suspension and mixed. The 200 μL of the test culture was transferred into 96-well microplate and incubated at 30 °C. Cell density was determined at 600 nm (A_{600}) after 4 h. Then 150 μL of the culture was removed from each well. After addition of 120 μL buffer and 20 μL chloroform, the samples were mixed for 10 min by shaking. The coloring reaction proceeded for 60 min after adding 40 μL of ONPG, and then was terminated by the addition of 100 μL Na_2CO_3. Finally, the absorption of the supernatants at 420 nm (A_{420}) was recorded using a microplate reader (SUNRISE-Basic, TECAN) and β-galactosidase activity was calculated by Eq. (4).

$$U = \frac{(OD_{420,S} - OD_{420,B}) \times 6.6}{T \times 0.2 \times OD_{600,S}} \quad (4)$$

Note: U: β-galactosidase activity; *S*: samples; *B*: blank control; 6.6: dilution factor; *T*: time for coloration.

2.2.4. Zebrafish larvae assay

Zebrafish larvae were obtained by in-house breeding using unexposed adult male and female fishes from a circulatory culture system. About 1 h after spawning, fertilized embryos were examined under an inverted microscope (Olympus, CKX 41). After embryos had hatched out, the normally developed larvae were transferred to glass beakers that contained 300 mL of reconstituted water with appropriate concentrations of BP3 (0, 1, 10, 100 and 500 μg L^{-1}) and positive control (E2, 100 ng L^{-1}). Water was entirely replaced every 24 h by transferring larvae to a new beaker containing the appropriate nominal BP3 concentrations, so that constant experimental conditions would be established to evaluate the final toxicological effects of target chemicals. The larvae were continuously exposed for 144 h at 26 ± 1 °C under a photoperiod of 14:10 h light/dark, and were not fed during exposure. Appearance, mortality, development and abnormal behavior were recorded daily during the entire exposure period. At the end of the exposure, 30–50 larvae were selected randomly to extract RNA.

2.2.5. Analytical confirmation

To verify the actual BP3 concentrations during exposure, aliquots of 100 or 200 mL exposure water from each treatment group were sampled at the beginning (0 h) and after 24 h (prior to water renewal). Water samples were also collected after 1, 3, and 5 d of exposure and stored at −20 °C in brown glass bottles until analysis. Watersample extraction and chemical analysis were performed following the procedure reported by Zhang et al. (2016). A linear regression model was applied to the concentration range of 0.10–10 μg mL^{-1} according to some successful applications (Valipour et al., 2013, 2017; Valipour, 2016a, 2016b; Rezaei et al., 2016). All analytes were quantified individually using an external calibration. To determine the influence of illumination on the stability of BP3, a control experiment including a solution of 10 μg L^{-1} BP3 without fish was performed for 24 h.

Table 2
Primer sequences for RT-qPCR analysis: *vtg1*, *esr1*, *ar*, *cyp19b*, *rbp2a* and *18 S rRNA*.

Target gene	GenBank number	Sense primer (5′−3′)	Antisenseprimer (5′−3′)	Product size (bp)	Reference
vtg1	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94	Hoffmann et al. (2006)
esr1	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163	Martyniuk et al. (2007)
ar	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237	Hossain et al. (2008)
cyp19b	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94	Arukwe et al. (2008)
rbp2a	AF363957	GGAGATGCTCAGCAATGACA	TCTGCACAATGACCTTCGTC	110	Zucchi et al. (2010)
18s rRNA	Y855349.1	AAACGGCTACCACATCCAAG	TTACAGGGCCTCGAAAGAGA	116	Wintz et al. (2006)

2.2.6. Quantitative real-time PCR experiments (RT-qPCR)

Total RNA was extracted from embryos using the Takara MiniBest Universal RNA Extraction kit (Takara, Japan) following the manufacturer's instructions. mRNA templates were reverse transcribed to cDNA and stored at −20 °C until RT-qPCR analysis. The three-step real-time PCR profile was performed according to a previous study (Bluthgen et al., 2014). In this study, several target genes involved in hormonal activity were selected (Table 2) including estrogen responsive gene (*vtg1*), nuclear receptor (*esr1, ar*) and steroid metabolism (*cyp19b, rbp2a*). The 18S ribosomal RNA (*18S rRNA*) gene was selected as a housekeeping gene. The relative linear amount of target molecules relative to the calibrator was calculated by the $2^{-\triangle\triangle ct}$ method (Livak and Schmittgen, 2001). Transcriptional alterations of different genes are expressed as fold change (log 2).

2.3. Data analysis and statistics

Data are graphically illustrated below using GraphPad®Prism5 (GraphPad Software, La Jolla, CA, USA). Data distributions were assessed for normality using the Kolmogorov-Smirnov test. Differences among the control and exposure groups were analyzed by one-way analysis of variance (ANOVA). Newman-Keulstests were used for post-ANOVA pair-wise comparisons to identify significant differences among the means. Linear regression analysis was also performed to determine concentration-dependent trends in the data. *p*-Values less than 0.05 were considered statistically significant. Graphical results are shown as mean ± standard deviation (SD) of the mean.

3. Result and discussion

3.1. Toxicity of UV filters to V. fischeri

The linear toxicity responses of the test bacteria to the positive control (phenol) and target UV filters are depicted in Fig. 1. The values of R^2 ($R^2 \geq 0.98$) indicated that the fitting curve had excellent statistical significance for the dose-effect relationship. Evidently, the inhibitory effect increased with the increase of the concentration within the scope of the extent of water solubility. The effective concentration (EC_{50}) was obtained from the dose-response curve, which used to quantify the acute toxicity of UV filters. The inhibition of ≥0.5 was regarded as the threshold at which the compound was considered as acutely toxic. Thus, the acute toxicity was obtained from the inhibition-concentration only in the case of BP, 2HB and BP4. Specifically, BP and 2HB showed 15 min-EC_{50} values of 18.85 and 14.55 mg L^{-1}, respectively. BP4 induced a toxic effect at the 15 min-EC_{50} values of 858.95 mg L^{-1}, whereas BP3 exhibited no toxic effect. This can be explained by the insufficient membrane concentration caused by the decrease in bioavailability *via* sorption and uptake by organisms and/or by reduced chemical activity of the introduced chemical species because of its higher log*K*ow values and higher melting points (Mayer and Reichenberg, 2006). However, when the dose-response curve of BP3 was extended, a lower EC_{50} value of 13.39 mg L^{-1} was obtained. Based on these EC_{50} values, BP3 was the most toxic UV filter to *V. fischeri*, followed by 2HB, BP, and finally BP4. This toxicity ranking is consistent with that reported previously on *photobacterium phosphoreum* (Liu et al., 2015). According to the EC_{50} toxicity categories established by the US EPA (Callow, 1998) that considers compounds with EC_{50} of 1–100 mg L^{-1} as "harmful", these aromatic compounds have intermediate-low toxicity. Molins-Delgado et al. (2016) also showed that BP3 and BP4 were "toxic" to *Daphnia magna* (EC_{50} = 1.9–30.4 mg L^{-1}). On this basis, the acute toxic effect of target UV filters to aquatic organisms is undeniable.

Thomulka et al. (1993) pointed out that the enzymes involved in the light reaction activity would influence the toxic effect of compounds on *V. fischeri*. The active centers of pollutants react with luciferase and/or coenzyme in bacteria, and thus display luminescent inhibition. Notably, the luminescence of bacteria will be inhibited, due to the formation of hydrogen generated by the reactions between a hydrogen bonding donor and the original form of flavin mononucleotide (FMWH2) (Su, 2008). Consequently, BPs UV filters, with electron-donating groups, such as -OH and -OCH_3, are bound to induce acute toxicity to *V. fischeri*. Besides, log*K*ow affects the absorption, accumulation and interface transmission of compounds by the cell membrane, as well as the combination of compounds and enzyme protein. Lee et al. (2013) demonstrated that logK_{OW} is one of the most important molecular descriptors in predictive toxicity. Therefore, the acute toxicity data of BPs UV filters from both the literature and our experimental results were used to try to find the correlation between logEC_{50} and logK_{OW} (Fig. 2). In light of this result, the lipophilicity of BPs UV filters has a positive contribution to the acute toxicity effect on luminescent bacteria, whereby the higher the log*K*ow is, the stronger the acute toxicity.

On the other hand, results of the comparative molecular field analysis performed by Liu et al. (2015) indicated that the electrostatic effect was the principal factor influencing the toxicity of UV filters. The selected UV filters in this study contained the same benzophenone skeleton but had different types of chemical substituents on the benzene rings (Table 1). Therefore, the electronegative substituents would cause higher toxicity. Consequently, 2HB, which has one hydroxyl group, showed higher toxicity than BP, due to the high electronegativity of this group. By contrast, BP3 with one hydroxyl group at the 2-position and one methoxy group at the 5-position, shows strong negative charges, resulting in BP3 being the most acutely toxic compound among the selected BPs UV filters. However, Liu et al. (2015) found that the increase in the negative charge at the 5-position (Table 1) would decrease the toxicity. In addition, BP4 introduced a strong electron-withdrawing group (-SO_3H), which resulted in significantly low log*K*ow value of 0.37 (Table 1). Thus, according to the aforementioned correlation between log*K*ow and logEC_{50} (Fig. 2), the finding that BP4 exhibits the lowest acute toxicity to *V. fischeri* is reasonable.

3.2. Genotoxicity of UV filters

A concentration-dependent increase in β-galactosidase activity was observed when the strain *S. typhimurium* TA1535/pSK1002 was exposed to target UV filters in the absence of S9, as shown in Fig. 3. The increase of the genotoxicity of BPs was also within the scope of water solubility. The basis of the *in vitro* SOS/umu test is that the strain *S. typhimurium* TA1535/pSK1002 has a deficient lipopolysaccharide and contains the umuDC-lacZ genes, which allow the *in vitro* bioassay to

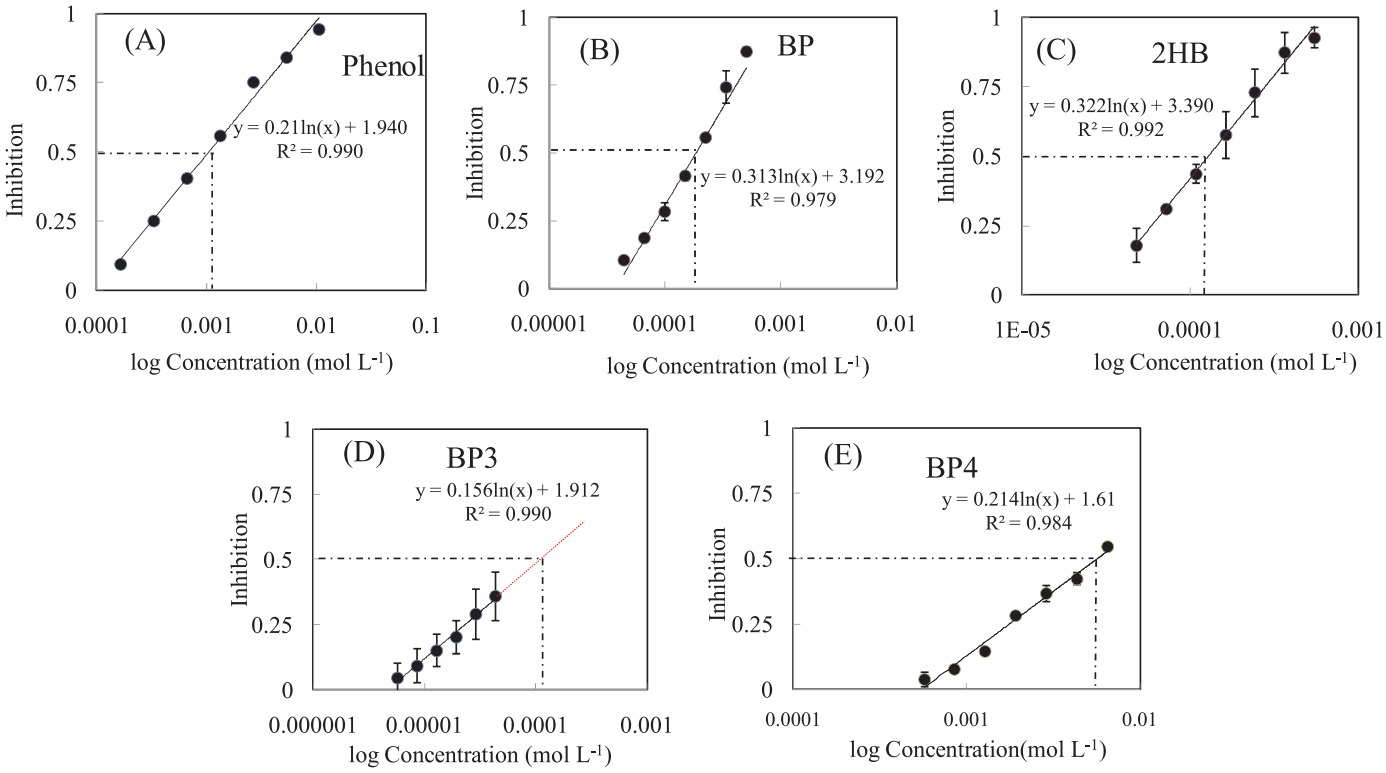


Fig. 1. Inhibition-concentration relationships of UV filters in the luminescent bacteria toxicity test.

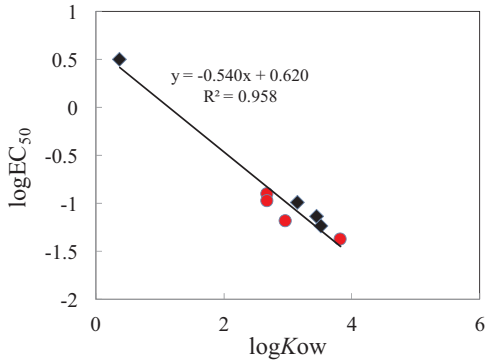


Fig. 2. Correlation between log*K*ow and logEC_{50}. Note: the data shown by red circles are from Liu et al. (2015) and Molins-Delgado et al. (2016); black square data are from this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

provide sensitive and simultaneous detection of single-stranded DNA, tri-nucleotides, oligo-nucleotides, DNA adducts, DNA dimmers and oxidative DNA damage (Rajagopalan et al., 1992; Kenyon, 1983). In the absence of S9, a direct DNA-damaging effect was detected in BP, 2HB and BP4. Specifically, the LPEC values for BP, 2HB and BP4 were 10.61, 9.52 and 113.75 mg L^{-1}, respectively. BP3 did not show a genotoxic effect at the tested concentrations. A similar finding was reported in an earlier study using the micronucleus assay (Abramsson-Zetterberg and Svensson, 2011). In addition, a study on BPs UV filters using an *in vitro* assay of genotoxicity in L5178Y(tk+/−) mouse lymphoma cells also demonstrated that BP3 did not significantly induce mutation frequencies either in the presence or absence of a metabolic activation system (Jeon et al., 2007). Zhao et al. (2013) reported that BP4 exhibited the least genotoxicity at a concentration higher than 1000 mg L^{-1} in the absence of a metabolic activation system. As with acute toxicity, a LPEC value of 4.96 mg L^{-1} for BP3 was obtained by extending the dose-response curve (Fig. 3d). Based on these LPEC values, the ranking of genotoxicity followed the same sequence as that of the acute toxicity results on *V. fischeri*.

According to these LPEC values, BP3 was the most genotoxic compound, followed by 2HB, BP, and finally BP4. As with the acute toxicity, a strong correlation (R^2 = 0.996) was obtained between the logLPEC and log*K*ow. Thus, the genotoxicity was also positively affected by the lipophilicity, whereby at higher log*K*ow values, a stronger genotoxicity is exhibited. In addition, the substituent groups on the benzene ring also influenced the genotoxic effect. For example, 2HB exhibited higher genotoxicity than BP due to the higher activity of the hydrogen bonding receptor. This phenomenon is consistent with previous reports that the hydroxy number influences the genotoxicity (Zhao et al., 2013; Jeon et al., 2007). Furthermore, the sulfonic acid group at the 5-position of BP4 increased its hydrophilicity, which decreased the concentration of compounds that enters into the cells, and consequently reduced its genotoxic effect.

3.3. Estrogenic activity of UV filters based on the yeast estrogen screen (YES) assay

The concentration-response curves for the BPs and positive control (E2) are shown in Fig. 4. All the target UV filters exhibited estrogenic activity by the YES assay. The EC_{50} values (causing a 50% increase in β-galactosidase activity) of the UV filters determined to compare estrogenic potency are listed in Table 3. For BP, 2HB, BP3 and BP4, the EC_{50} values were 0.48, 0.51, 5.14 and 8.85 mg L^{-1}, respectively. The most estrogenic potent UV filter was BP, which was 30,838- times less potent than the positive control (E2). While 2HB, BP3 and BP4 showed lower estrogenic activity, being 32,903-, 35,0322- and 55,3548-times less estrogenic potent than E2, respectively (Table 3). Accordingly, the ranking of estrogenic potency was in the following order: BP > 2HB > BP3 > BP4, with E2 being approximately five or six orders of magnitude more potent than the target UV filters. Previous studies widely demonstrated bisphenol A as an environmental estrogen which is also five to six orders of magnitude less estrogenic potency than E2 (Sun et al., 2013; Legler et al., 1999). BPs UV filters used in present study fall within the same order of magnitude potency of most estrogenic

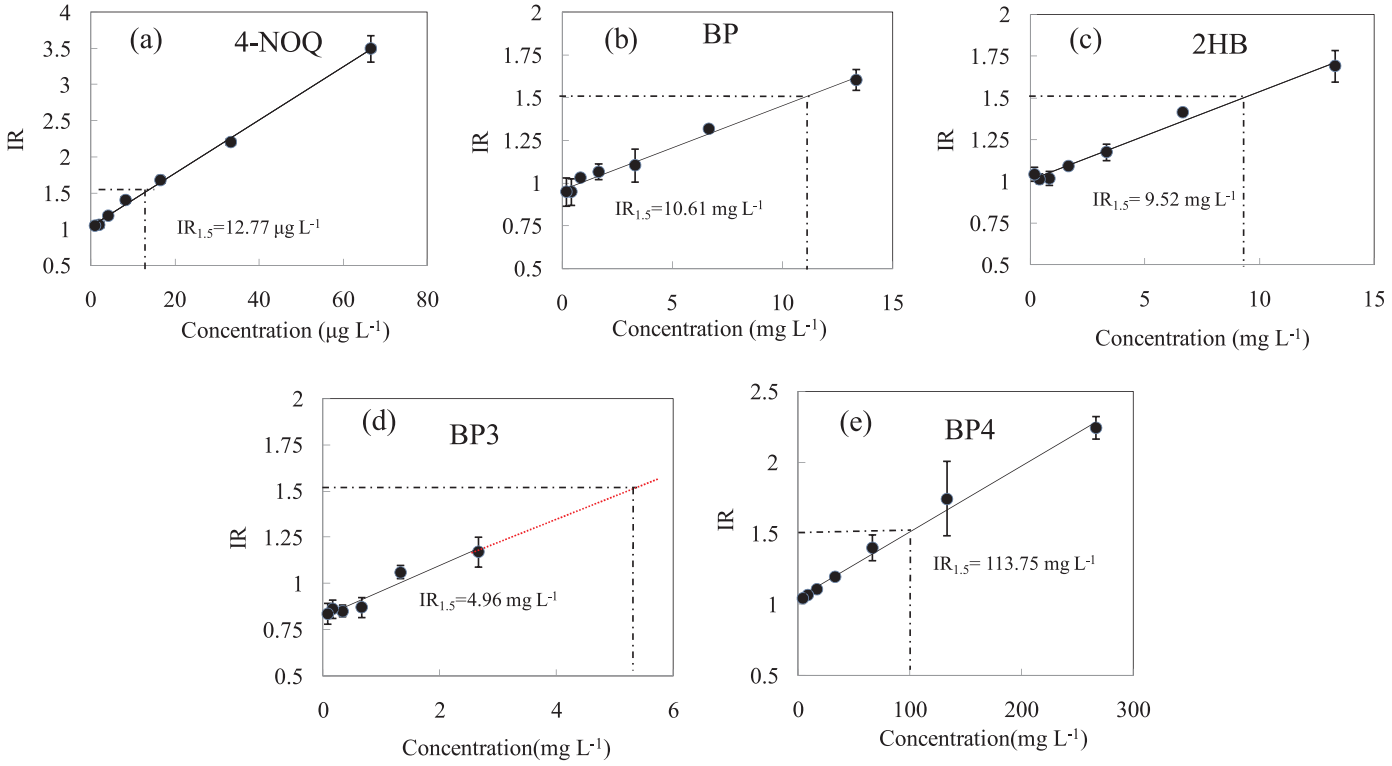


Fig. 3. Effects of UV filters on the induction of the SOS response in the SOS/umu genotoxic assay.

compounds, and thus they could also be considered as environmental estrogens. Nevertheless, this ranking of estrogenic activity results was different from those of acute toxicity and genotoxicity, as BP3 and BP4 showed lower estrogenic activity than BP and 2HB. High concentrations of BP3 was toxic to yeast cells (Miller et al., 2001), which could be regarded as one reason for its weak estrogenicity. More particularly, the EC_{50} values for the estrogenic activity showed threshold values of one or two orders of magnitude lower than the EC_{50} values for acute toxicity and the LPEC values for genotoxicity. Thus, the estrogenic effect was the most significant adverse effect of target BPs UV filters.

The selected BPs UV filters can act as endocrine disruptors, partly because they carry at least one ring substituted hydroxyl group. However, they behaved as partial agonists to *hERα*, characterized by inducing submaximal dose-response curves, reducing estrogenic effi-

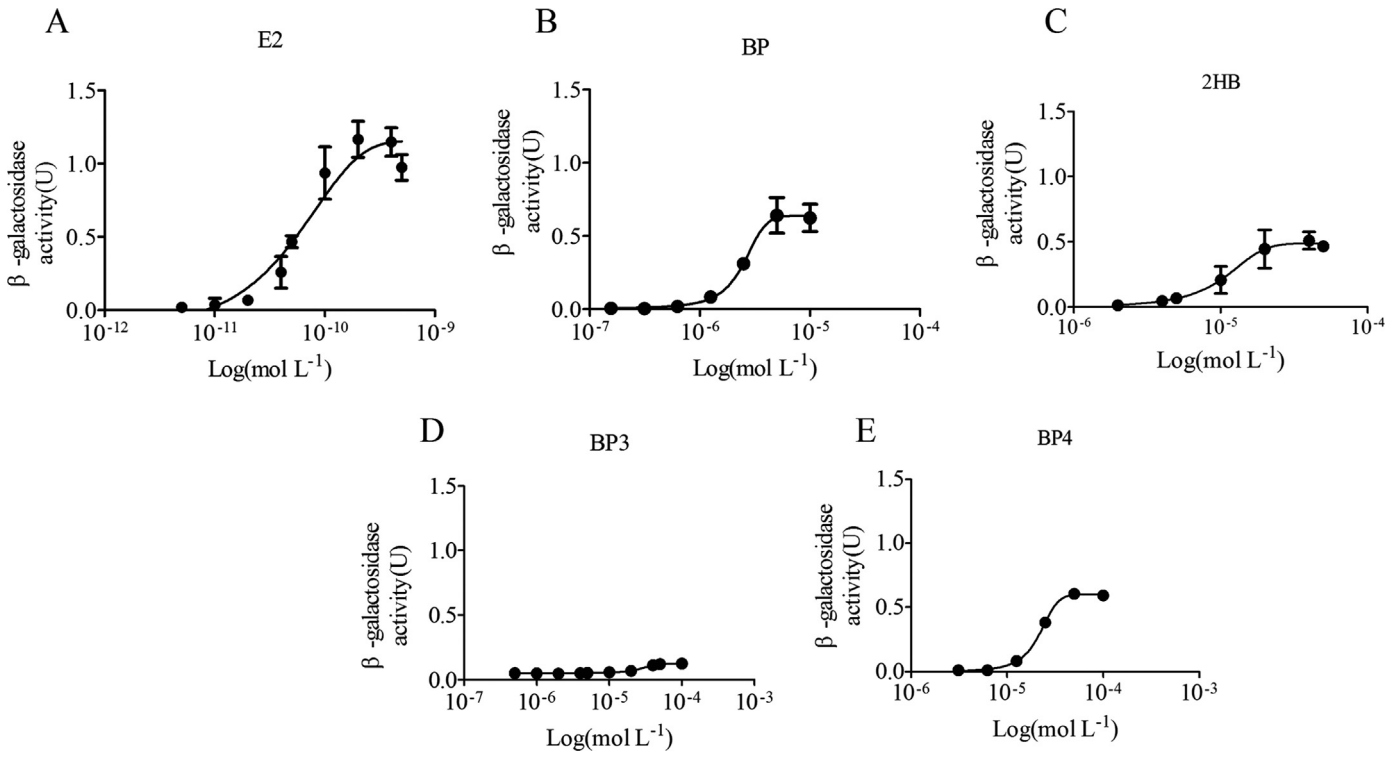


Fig. 4. Relationship between the concentrations of target UV filters and β-galactosidase activity.

Table 3
Effects of selected UV filters by YES assay.

Compounds	EC_{50}[a] (mg L^{-1})	Efficacy[b] (%)	Estrogenic activity[c]
E2	15.50E-6	100	1
BP	0.478	59.36	1/30838
2HB	0.51	44.65	1/32903
BP3	5.43	11.49	1/350322
BP4	8.85	55.66	1/553548

[a] concentration causing a 50% increase in β-galactosidase activity
[b] Effect (curve-height) of a compound given as the percentage of the effect of E2.
[c] Ratio of the EC_{50} of a compound divided by the corresponding positive control.

cacy or the ability to terminate the effect of a full agonist. In this study, the estrogenic efficiency of the target UV filters ranged from 11.49% to 59.36% as shown in Table 3. This partial agonist effect on hERα, such as those shown by BP3 and BP4, may be caused by additional substituents like hydroxyl-, methoxy- or sulfonic acid groups. This was also found in the study of the salicylates benzyl salicylate and phenyl salicylate (Kunz and Fent, 2006). An agonist can activate an optimal conformational change of the hERα, but these partial agonists (selected UV filters) can induce steric or ionic interferences, which result in reduced efficacy (Pike et al., 1999).

3.4. Zebrafish larvae assay

In the case of BP3, its luminescent bacteria inhibition was lower than 50%, and the *IR* value regarding genotoxicity was less than 1.5. Especially, BP3 showed the weakest estrogenic efficacy *in vitro*. Based on these *in vitro* assays results, environmental hazards produced by BP3 were not significant. However, the result of the *in vivo* assay may different from those of the *in vitro* assay, due to the metabolic capability *in vivo* (Blüthgen et al., 2014). To complete and realistically investigate the risk posed by BP3 to the environment, it is necessary to conduct *in vivo* assay in further research.

3.4.1. Analytical confirmation

The actual measure concentration values of the freshly prepared BP3 approached those of the nominal concentration (Table 4). Moreover, the concentrations of BP3 showed no significant change following 24 h of exposure at the nominal concentrations of 10 (without fish), 1, 10, 100, and 500 μg L^{-1} (Table 4). Previous studies reported that O-demethylation is the main pathway for BP3 biodegradation, such as photolysis and hydrolysis (Blüthgen et al., 2012). However, the control concentration of BP3 at 10 μg L^{-1} without fish indicated that no photodegradation occurred during exposure. Moreover, *zebrafish* larvae have a low capability to metabolize BP3, probably because BP3 metabolizing enzymes in *zebrafish* are not yet fully active at this early life stage (Blüthgen et al., 2012). Furthermore, the more frequent exposure water renewal in this study kept BP3 concentrations rather stable for 24 h to adequately represent the environmental concentrations of ("E":1 μg L^{-1}), low dose ("L":11–10 μg L^{-1}), middle dose ("M": 105–96 μg L^{-1}), and highest dose ("H": 518–497 μg L^{-1}). As the concentration of BP3 was maintained within ± 20% of the initial concentration, test results were based on nominal values (OECD, 2012).

Table 4
Measured concentrations of BP3 in exposure waters during exposure.

Nominal concentration (μg L^{-1})	BP3		
	0 h	24 h (before renewal)	Average (μg L^{-1})
	Actual concentration[a] (μg L^{-1})	Measured concentration[a] (μg L^{-1})	
Water control	n.d.[b]	n.d.[b]	-[c]
10 (no fish)	11.88 ± 0.83	10.37 ± 0.29	11.01 ± 1.22
1	1.14 ± 0.30	1.08 ± 0.11	1.11 ± 0.04
10	11.42 ± 2.31	10.50 ± 0.98	10.96 ± 0.65
100	105.37 ± 6.23	96.26 ± 7.29	100.82 ± 6.44
500	518.70 ± 39.07	497.92 ± 31.40	508.31 ± 21.78

Each measurement at 0 and 24 h is based on three replicate samples during the experiment.
[a] mean ± SD
[b] not detected
[c] not available

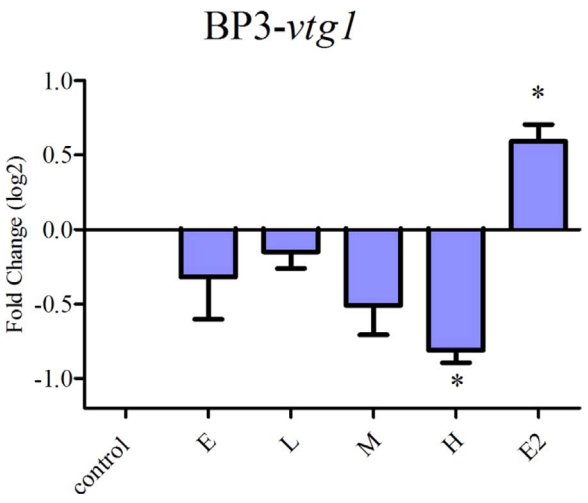


Fig. 5. Relative gene expression of *vtg1* in the larvae after exposure to BP3 at "E, 1 μg L^{-1}", "L, 11–10 μg L^{-1}", "M, 105–96 μg L^{-1}", "H, 518–497 μg L^{-1}", and "E2, 100 ng L^{-1}". Results are presented as mean ± SD; Asterisks (*) indicate significantly different expressions relative to the control ($^{*}p \leq 0.05$).

3.4.2. Targeted gene expression analysis by qRT-PCR

The hormonal activity and mode of action of BP3 at "E, 1 μg L^{-1}", "L, 11–10 μg L^{-1}", "M, 105–96 μg L^{-1}", and "H, 518–497 μg L^{-1}" were analyzed. During the exposure, BP3 showed no apparent toxicity to *zebrafish* larvae. Additionally, no statistically significant effects on the survival rate and behavior were observed.

After 144 h exposure, the effects of BP3 on the transcriptions of several genes involved in hormonal activity were observed (Fig. 5 and Fig. 6). Evidently, the E2 concentration of 100 ng L^{-1} as positive control significantly induced the up-regulation of *vtg1* in the larvae (Fig. 5). In addition, an overall inhibition of *vtg1* transcription was observed at all BP3 concentrations, significantly at the "H" level. Vitellogenin genes are already expressed in eleuthero-embryos, and estrogenic compounds can lead to induction of *vtg1* (Jin et al., 2009; Henry et al., 2009). The down-regulation of *vtg1* indicated the anti-estrogenic effect of BP3 *in vivo*.

Interference with the endocrine system can be induced through multiple mechanisms of action. The expression tendency of *ar* was identical to the transcriptional pattern of *vtg1* (Fig. 6b). This was in line with previous *in vitro* data showing that BP3 strongly inhibited the *ar*-related transactivation in recombinant yeast carrying the hAR, pointing to an anti-androgenic activity (Kunz and Fent, 2006).

The expression pattern in larvae showed an overall up-regulation tendency of the *esr1* transcript by all BP3 solutions (Fig. 6a). The interaction with estrogen receptors is a well established and key event in the initiation of adverse outcomes by xeno-estrogens (Sonavane et al., 2016). The induction of *esr1* in the larvae indicated an estrogenic activity of BP3. Moreover, the finding of the estrogenic activity of BP3 in larvae is consistent with our aforementioned *in vitro* data. On the other hand, the *in vitro* yeast based system lack the capability to metabolize chemicals, and complex biological effects of chemicals on organisms could not be accomplished (Blüthgen et al., 2014). Regarding the *in vivo* finding, metabolism might lead to loss of the parent

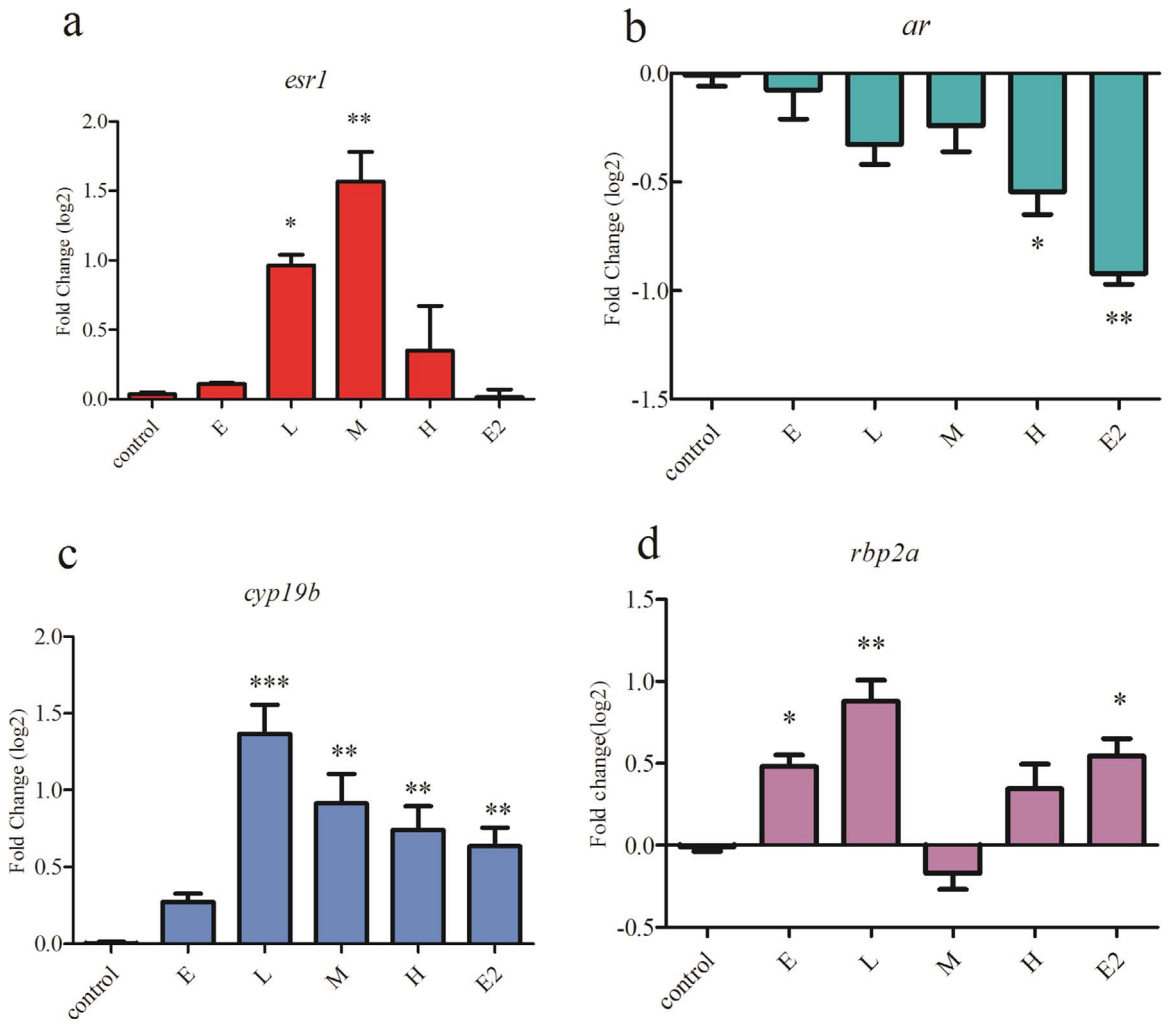


Fig. 6. Relative gene expression of *esr1, ar, cyp19b,* and *rbp2a* in the larvae after exposure to BP3 at "E, 1 μg L^{-1}", "L, 11–10 μg L^{-1}", "M, 105–96 μg L^{-1}", "H, 518–497 μg L^{-1}", and "E2,100 ng L^{-1}". Results are presented as mean ± SD; Asterisks (*) indicate significantly different expressions relative to the control ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

compounds, but their metabolites sometimes can be more harmful than the parent compounds. For example, the UV filter benzophenone-1 as a metabolite of BP3 had stronger estrogenic potency than BP3, which mainly possesses anti-estrogenic and anti-androgenic activities (Kunz and Fent, 2006). Thus, the display of estrogenic activity by BP3 was somewhat inevitable.

All BP3 concentrations and positive control (E2) led to a significant up-regulation of *cyp19b*, except at the "L" level of BP3 (Fig. 6c). The *cyp19b* gene encoding aromatase B is expressed during *zebrafish* embryogenesis (Mouriec et al., 2009). The time point of the up-regulation of *cyp19b* suggested an effect of BP3 on steroidogenesis. Mouriec et al. (2009) also demonstrated that *cyp19b* is fully functional in eleuthero-embryos, especially it can be considerably increased by E2 and relies on estrogen receptors. Thus, the up-regulation of *esr1* provided the conditions for the expression change of *cyp19b*. Moreover, these aromatases catalyze the final, rate-limiting step in the conversion of testosterone into estradiol in the gonads (Simpson et al., 1994). In addition, the organism has complex signaling pathway and neural networks, exogenous compounds can produce toxic effects through a variety of models. For example, *ar* belongs to the nucleoprotein receptors that can induce the transcription of aromatic hydrocarbons such as *cyp1a1*、*cyp1b1*、*cyp1a2* and some other enzyme genes (Kerkvliet, 2009). Thus, the aboved mentioned down-regulation of *ar* may have an influence on the transcription of *cyp19b*. Accordingly, there should be no doubt that the up-regulation of the *cyp19b* gene in larvae can be interpreted as an estrogenic response.

Moreover, the transcription of *rbp2a* was entirely induced by exposure to E2 and BP3 solutions, except for the "M" level of BP3 solution (Fig. 6d). *Rbp2a*, like retinoids, plays an important role in various physiological processes, such as cell growth, differentiation, angiogenesis, developmental processes, immunity and embryogenesis (Chen et al., 2012). Many studies have reported alterations of retinoids in various species by environmental contaminants. For instance, Levy et al. (2004) reported that retinoids were up-regulated by E2 in cultured *X.laevis hepatocytes* and proved that retinoids could be used as biomarkers for detecting specific actions of pure endocrine disrupting compounds. Thus, changes of *rbp2a* in this paper suggest an environmental endocrine disrupting effect of BP3 on *zebrafish* larvae.

Overall, BP3 showed significant estrogenic activity in *zebrafish* larvae, but simultaneously, BP3 acted as an anti-androgenic and anti-estrogenic agent *in vivo*. The endocrine disrupting activities and potencies *in vitro* will be manifested *in vivo* when using appropriate assays, end points and realistic routes of exposure, such as through the skin into the bloodstream or orally.

4. Conclusions

In the present study, the acute toxicity, genotoxicity, and estrogenic activity of four commonly used BPs UV filters were investigated using the luminescent bacteria assay, the SOS/umu assay, YES assay and *in vivo* assay in *zebrafish* larvae. The results of the *in vitro* bioassays revealed that the lipophilicity of BPs UV filters positively contributed to

the acute toxicity and genotoxicity, whereby the higher the log*K*ow was, the stronger the acute toxicity and genotoxicity. Especially, the relationship between lipophilicity and acute toxicity was elucidated, which provided the basic knowledge to fill gaps of toxicity prediction for BPs UV filters. Moreover, all the target BPs UV filters were identified as environmental estrogens with estrogenic potency being five or six orders of magnitude less potent than E2, but they all displayed sub-maximal *hERα* agonist activity, as the chemical structure was the dominant factor influencing the estrogenic effect in YES assay. However, although BP3 exhibited the weakest toxic effect among target BPs UV filters *in vitro*, multiple hormonal activities including estrogenicity, anti-estrogenicity and anti-androgenicity were discovered in *zebrafish* larvae by modulating related target genes expression. The present study provided direct toxicity data for the BPs UV filters, which is beneficial for the scientific evaluation of the environmental potential hazards of UV filters.

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