Environmental Pollution 240 (2018) 396–402

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

The evaluation of endocrine disrupting effects of *tert*-butylphenols towards estrogenic receptor α, androgen receptor and thyroid hormone receptor β and aquatic toxicities towards freshwater organisms☆

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ARTICLE INFO

Article history:
Received 4 January 2018
Received in revised form
24 April 2018
Accepted 24 April 2018
Available online 9 May 2018

Keywords:
Phenolic compound
Drinking water source
Ecotoxicity
Chlorophyll content
Luminescent bacteria
Green algae

ABSTRACT

The phenolic compounds have posed public concern for potential threats to human health and ecosystem. *Tert*-butylphenols (TBPs), as one group of emerging contaminants, showed potential endocrine disrupting effects and aquatic toxicities. In the present study, we detected concentrations of 2,4-DTBP ranging from <0.001 to 0.057 μg/L (detection limit: 0.001 μg/L) in drinking water source from the Qiantang River in East China in April 2016. The endocrine disrupting effects of 2-TBP, 2,4-DTBP and 2,6-DTBP toward human estrogen receptor α (ERα), androgen receptor (AR) and thyroid hormone receptor β (TRβ) were evaluated using human recombinant two-hybrid yeast bioassay. Their aquatic toxicities were investigated with indicator organisms including *Photobacterium phosphoreum, Vibrio fischeri* and freshwater green alga *Chlamydomonas reinhardtii.* 2-TBP and 2,4-DTBP exhibited moderate antagonistic effects toward human ERα and AR in a concentration-dependent manner. 2-TBP significantly inhibited the light emission of *P. phosphoreum.* 2-TBP, 2,4-DTBP and 2,6-DTBP significantly inhibited the growth of *C. reinhardtii* and reduced the chlorophyll content. Our results suggest the potential adverse effects of TBPs on human health and aquatic organisms. The data will facilitate further risk assessment of TBPs and related contaminants.

1. Introduction

Phenolic compounds have been widely used in industries and some of them have been listed as priority pollutants by the U.S. Environmental Protection Agency and the European Community (EC, 2001; EPA, 1977). *Tert*-butylphenols (TBPs) are one group of alkylated phenolic compounds with one or more *tert*-butyls at the benzene ring. Their residues have been detected from consumer products (Dekiff et al., 2014; Jonker et al., 2016), dust (Liu et al., 2017a), surface water (Liu et al., 2016, 2017b) and effluents from waste water treatment plants (Xu et al., 2016). TBPs caused increasing public concern due to their unique environmental behavior and toxicities (Haavisto et al., 2003; Rudel et al., 2003; Ying et al., 2002).

Manufactures and suppliers of TBPs in the vicinity of drinking water source are the frequent contributors to the residues of TBPs in surface water, causing potential aquatic ecotoxicities. As the largest source of drinking water in Zhejiang Province in East China, Qiantang River has been an important commercial artery and many kind of industries are located along it (Chen et al., 2017; Lu et al.,

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.

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https://doi.org/10.1016/j.envpol.2018.04.117
0269-7491/

2017). Till now it lacks the data on residues of TBPs in drinking water source from the Qiantang River in East China. It also remains unclear on how TBPs exhibit their aquatic toxicities toward various indicator organisms such as bioluminescent bacteria and freshwater green algae. Thus, work to monitor the residues of TBPs in Qiantang River and determine the potential aquatic toxicities after exposure to TBPs is necessary.

2-*tert*-butylphenol (2-TBP) and 2,6-di-*tert*-butylphenol (2,6-DTBP) caused cytotoxicity toward human submandibular gland carcinoma cells (Kadoma et al., 2009). 2-Alkyl- and 2,6-dialkyl-4-X-phenols induced cytotoxicity of tumor cell L1210 (Selassie et al., 2002). 2-TBP and 2,4-DTBP caused adverse effects on the liver of newborn and young rats (Hiratakoizumi et al., 2005). TBPs were also reported to exhibit endocrine disrupting effects, for example, 2-TBP was revealed as a weak inhibitor towards progesterone receptor (Li et al., 2010a), and 2-TBP, 4-TBP and 2,4-DTBP showed estrogenic disrupting effects (Akahori et al., 2008; Tollefsen and Nilsen, 2008). It is thus essential to investigate the adverse effects of TBPs toward these different nuclear receptors.

In the present study, samples were collected at 14 sites along the main stream and tributaries of Qiantang River (Fig. 1) and three TBPs were analyzed (Table S1). The endocrine disrupting effects toward human estrogenic receptor α (ERα), androgenic receptor (AR) and thyroid hormone receptor β (TRβ) were evaluated by the human recombinant yeast two-hybrid bioassay. The comprehensive toxicity of TBPs were fully investigated using two bioluminescent bacterium including *Photobacterium phosphoreum* and *Vibrio fischeri,* and freshwater green alga *Chlamydomonas reinhardtii*. The results are essential for a comprehensive evaluation of toxicity of TBPs, further facilitating the risk assessment of TBPs towards human health and aquatic organisms.

2. Materials and methods

2.1. Chemicals and reagents

2-TBP (99% purity), 2,4-DTBP (99% purity), 2,6-DTBP (99.5% purity) and 17β-Estradiol (E2, 99% purity) were purchased from J&K Chemical Ltd. (Shanghai, China). Dihydrotestosterone (DHT, 99% purity), 3,3′,5-triiodothyronine (T3, >98% purity) and dimethylsulfoxide (DMSO, 99.5% purity) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The synthetic defined (SD) broths and agar minimal media (lacking tryptophan and leucine, SD/-Leu/-Trp) was obtained from Mobitec Company (Catalogue: 4823-6). All other chemicals were of analytical grade. The test chemicals were dissolved in DMSO (v/v < 0.1%) and the corresponding stock solutions were prepared with Milli-Q water (18.2 MΩ cm, Millipore, Bedford, MA, USA).

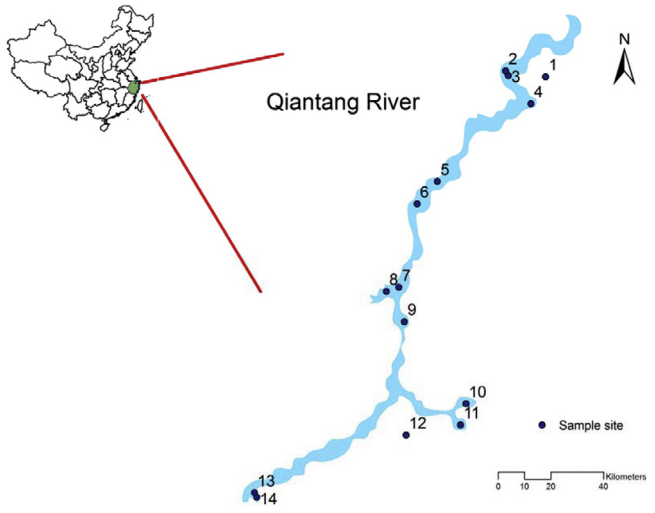

Fig. 1. Map of the sampling locations in Qiantang River. The green represented Zhejiang Province on the map of China. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Sampling and analysis

In total 42 water samples were collected from 14 sampling sites in April 2016 (Fig. 1, Table 1). The samples were transported to the laboratory under ice cold conditions and stored in the dark at 4 °C. All water samples were filtered firstly using glass fiber filters (Whatman GF/F, 0.45 μm pore size) to remove the particles. The water sample of 1 L was transferred into a separatory funnel for acidification to pH ≤ 2 by drop-wise addition of sulfuric acid solution, followed by the addition of 30 g sodium chloride and 70 mL dichloromethane. The organic phase was collected and dehydrated by anhydrous sodium sulfate. Each sample was extracted in triplicate. The organic phase was finally concentrated to 1 mL. Phenanthrene-d10 was used as the internal standard. The ultrapure water of 1 L was used as blank control. Extracts were stored at −20 °C prior to analysis.

TBPs were analyzed using gas chromatograph-mass spectrometry (Model GC-MS-QP2010 Plus, Shimadzu, Japan). TBPs were separated using an Agilent DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) in the splitless mode. A 1 μL extract was injected to the column with the column flow rate at 1.0 mL/min with injection temperature of 270 °C. The gas chromatograph was set at 50 °C for 2 min and ramped first at 20 °C/min to 160 °C for 2 min and ramped later at 5 °C/min to 260 °C for 2 min. The quadrupole mass spectrometer was set in selected ion monitoring mode with an electron ionization source at 70 eV. The ion source temperature and transfer line temperature were set at 230 °C and 280 °C, respectively. The used carrier gas helium has high purity of 99.999%.

2.3. Quality assurance/quality control

The GC-MS system was calibrated before sample analysis. A 1 μL standard solution containing trifluorotriphenyl phosphine (DFTPP) was injected into the GC-MS to ensure the quality control criteria (Table S2). The calibration curve was constructed with standard deviation of no more than 20% relative to the internal standard. The procedural blank and field blank were performed and the detection

Table 1
Information on 14 sampling sites in Qiantang River and detected concentrations of 2,4-DTBP (μg/L) in April 2016.

Sampling site	Coordinate	2,4-DTBP
1	119°29′8″E, 29°12′15″N	-[a]
2	120°9′48.17″E, 30°12′45.29″N	–
3	120°9′32.97″E, 30°12′37″N	–
4	120°10′57.28″E, 30°4′39.4″N	–
5	119°45′48.28″E, 29°52′30.29″N	0.040
6	119°45′48.28″E, 29°48′57.13″N	0.057
7	119°32′20.91″E, 29°32′35.76″N	–
8	119°28′8.8″E, 29°32′17.96″N	0.004
9	119°32′5.61″E, 29°25′37.80″N	–
10	119°42′36.28″E, 29°6′49.35″N	–
11	119°40′40″E, 29°3′37″N	0.018
12	119°27′36.87″E, 29°11′49.99″N	–
13	118°50′23.67″E, 28°56′41.37″N	0.032
14	118°51′3″E, 28°56′24″N	–

[a] The detection limit is 0.001 μg/L. The sign - means below limit of detection.

limit was 0.001 μg/L. Each sample was analyzed in duplicate with the deviations less than 30%. The recovery rate of tested TBPs ranges from 70% to 130%.

2.4. The recombinant two-hybrid yeast bioassay

The endocrine disrupting effects of TBPs toward human ERα, AR and TRβ were evaluated by human recombinant ERα, AR and TRβ two-hybrid yeast bioassay, respectively. The yeast cells were pre-incubated overnight at 30 °C in SD/-Leu/-Trp medium. Chemicals of 5 μL and 995 μL medium with OD_{600} values around 0.75 were mixed and the mixture of 200 μL was further cultured for 2.5 h at 30 °C with OD_{600} close to 0.2–0.3. The cultured yeast cells were lysed with chloroform and the *o*-nitrophenyl-β-D-galactopyranoside of 40 μL was added into the cell lysate to initiate the enzyme reaction. After 1 h incubation, the reaction was terminated by adding 100 μL sodium carbonate (1 M). The optical density OD_{420} values were monitored by Infinite 200 PRO NanoQuant Multimode Microplate Reader (Tecan Group Ltd. Switzerland). The β-galactosidase activity was calculated following a reported protocol (Ding et al., 2017a; Li et al., 2008b).

$$U = \frac{OD_{420} - OD'_{420}}{t \times V \times OD_{600}} \times D$$

where U, t, V and D are the β-galactosidase activity, enzyme reaction time, volume and diluting factor, respectively. OD_{600} is the cell density measured at 600 nm OD_{420} and OD'_{420} are the cell density of the sample and the blank measured at 420 nm, respectively.

2.5. Acute toxicity test with luminescent bacteria

The freeze-dried bioluminescent bacterium *P. phosphoreum* and *V. fischeri* were purchased from Marina Pine Photon Technology Co. LTD (Beijing, China). The cold sterilized 2% NaCl solution of 1 mL was added to the freeze-dried powder and was incubated at 4 °C for 10 min to pre-culture *P. phosphoreum*. The solution of TBPs with different concentrations was prepared by the addition of 3% NaCl. The 100 μL TBPs solution and 2.5 μL dissolved *P. phosphoreum* was added to make the test culture. The mixture of 100 μL 3% NaCl solution and 2.5 μL dissolved *P. phosphoreum* was used as positive control. The mixture of 99 μL 3% NaCl solution, 1 μL DMSO and 2.5 μL dissolved *P. phosphoreum* was treated as negative control. The whole mixture process was incubated by magnetic oscillator at 0 °C for 15 min. The luminescence was measured with Infinite® 200 PRO multimode reader (Tecan Group Ltd., Switzerland) following a reported protocol (Deng et al., 2012; Wang et al., 2016).

The toxicity of TBPs to *V. fischeri* was determined according the reported procedure (Ding et al., 2017b). The free-dried *V. fischeri* powder was incubated at 20 °C for 15 min, and diluted using 500 μL recovery diluent. The 85 μL stock solutions of test chemicals, 15 μL osmotic pressure regulating fluid and 2.5 μL dissolved *V. fischeri* was mixed to make the test culture. The positive control was prepared with 85 μL deionized water, 15 μL osmotic pressure regulating fluid and 2.5 μL dissolved *V. fischeri*. The negative control was prepared with 84 μL deionized water, 1 μL DMSO, 15 μL osmotic pressure regulating fluid and 2.5 μL dissolved *V. fischeri*. The mixture was incubated at 20 °C for 5 min. The luminescence was measured with Infinite® 200 PRO multimode reader (Tecan Group Ltd., Switzerland).

2.6. Acute toxicity test with C. reinhardtii

The cells of *C. reinhardtii* (Marine Ecology Laboratory, Ocean University of China) were pre-cultured with *tris*-acetate-phosphate (TAP) medium in triangular flask (Text S1). The light intensity was set at 4000 lx and the light-dark cycle was set for 12 h: 12 h. In the total reaction system of 250 mL, the initial inoculum in the mid-exponential growth phase was cultured with TAP medium and the final concentration was 100 × 104 cells/mL. The different concentrations of TBPs stock solution were added to triangular flask and the final concentration were 1, 5 and 10 mg/L, respectively. The negative control contained isovolumetric DMSO without TBPs. The cell density was counted every 24 h using a light microscope (YS2-H, Nikon, Japan). The total chlorophyll content was determined at an interval of 24 h, 48 h, 72 h and 96 h following a reported protocol (Yoshida et al., 2004). A 4 mL algae cell suspension was centrifuged under 5000 g for 5 min. After removal of the supernatant, 3.5 mL 80% ethanol was added to extract for 4 h at 20 °C in the dark. The solution was centrifuged at 8000 g for 5 min and the absorbance of the supernatant at 649 nm and 665 nm were measured by a UV–Vis spectrophotometer (Jasco V-750 Spectrophotometer, JASCO Corporation, Japan). The total chlorophyll content was calculated according to the following formula: total chlorophyll (mg/L) = $(6.10 \times OD_{665} + 20.04 \times OD_{649}) \times 0.06$.

2.7. Statistical analysis

All *in vitro* experiments were independently performed three times in triplicate and the data were expressed as mean ± standard deviation (SD) (n = 3). The significant difference is considered with $p < 0.05$. The dose-response response curves obtained from the recombinant two-hybrid yeast bioassay and toxicity test with luminescent bacteria were fitted using Graphpad Prism 7.0 software. The median effective concentration (EC50) values were calculated using Graphpad Prism 7.0 following the formula: $Y = Bottom + \frac{(Top-Bottom)}{1+10^{((\log EC50-x)\times HillSlope)}}$.

3. Results and discussion

3.1. Occurrence of TBPs in the Qiantang River

We analyzed the concentrations of 2-TBP, 2,4-DTBP and 2,6-DTBP using GC-MS method in water samples collected from 14 sampling sites of Qiantang River in April 2016. To the best of our knowledge, this the first report on the reported residues of TBPs in Qiantang River. The concentrations of 2,4-DTBP ranges from <0.001 to 0.057 μg/L and the concentration of 2-TBP and 2,6-DTBP is below the detection limit 0.001 μg/L (Table 1).

3.2. Endocrine disrupting effects of TBPs

The estrogenic, androgenic and thyroid disrupting effects evaluated by the recombinant two-hybrid yeast bioassay provided a rapid and high-throughput screening of chemicals for their agonistic/antagonistic potency toward different receptors (Li et al., 2010a; Lv et al., 2017; Zhuang et al., 2017). The positive control showed that the well-known endogenous human ERα agonist E2, AR agonist dihydrotestosterone, and TRβ agonist T3, significantly induced the β-galactosidase activity and exhibited the sub-maximal stimulatory response at 2.5×10^{-4}, 5.0×10^{-2} and 5.0×10^{-1} μM, respectively (Fig. S1, A, B, C). This result is in line with the reported values (Fent et al., 2014; Li et al., 2008a, 2010b; Zhuang et al., 2017), indicating the steadiness and accuracy of this yeast bioassay. TBPs at the concentrations ranging 5×10^{-6} - 50 μM showed no toxicity to yeast cells. Three TBPs induced no significant β-galactosidase activity (Fig. S1, A, B, C), showing that they have no agonist activity toward human ERα, AR and TRβ. The antiestrogenic activity of TBPs was determined by the co-incubation with 2.5×10^{-4} μM E2. Results showed that all tested TBPs inhibited the

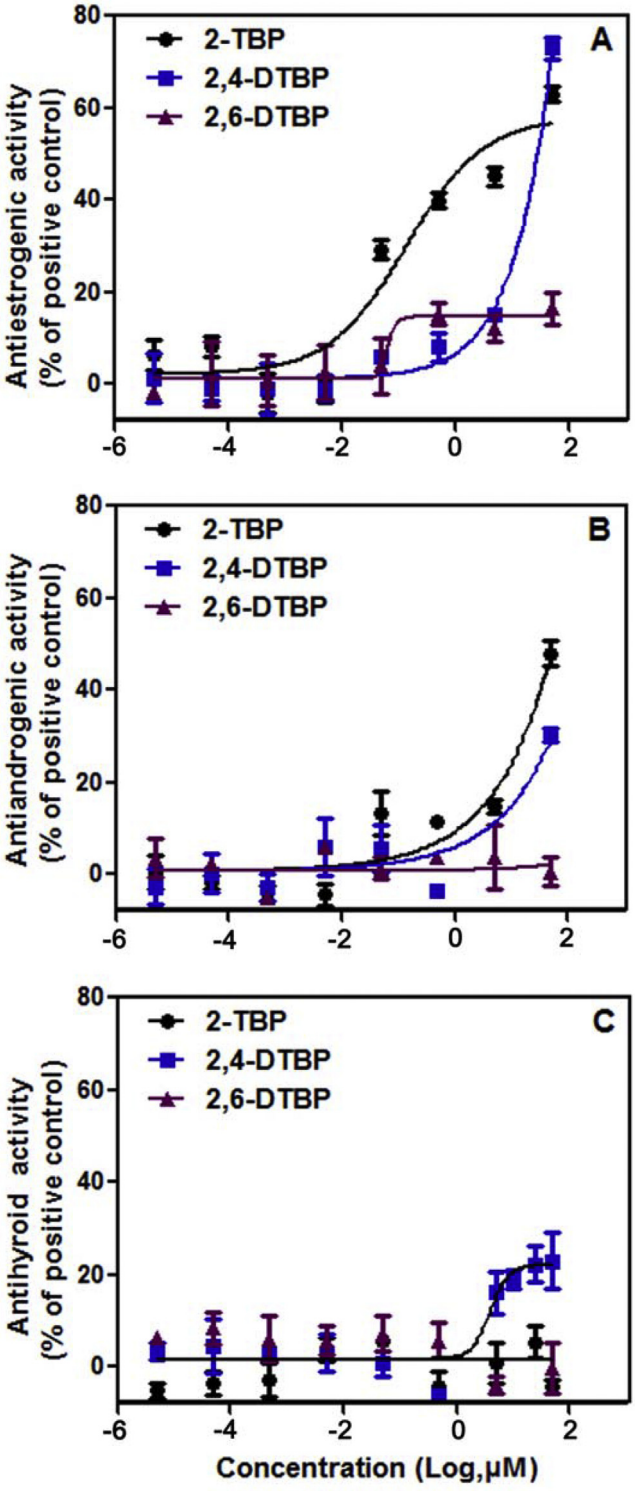

Fig. 2. (A) Antiestrogenic activity of TBPs determined by the recombinant ERα two-hybrid yeast assay. (B) Antiandrogenic activity of TBPs determined by the recombinant AR two-hybrid yeast assay. (C) Antithyroid activity of TBPs determined by the recombinant TRβ two-hybrid yeast assay. Results were expressed as the percent inhibition relative to the values observed with 2.5×10^{-4} μM E2, 5.0×10^{-2} μM DHT and 5.0×10^{-1} μM T3, respectively.

β-galactosidase activity induced by E2 (Fig. 2, A). 2-TBP at concentrations ranging from 0.050 μM to 5 μM showed a moderate antiestrogenic activity and have the potent antagonistic effect at maximum concentration (50 μM). 2,4-DTBP and 2,6-DTBP showed

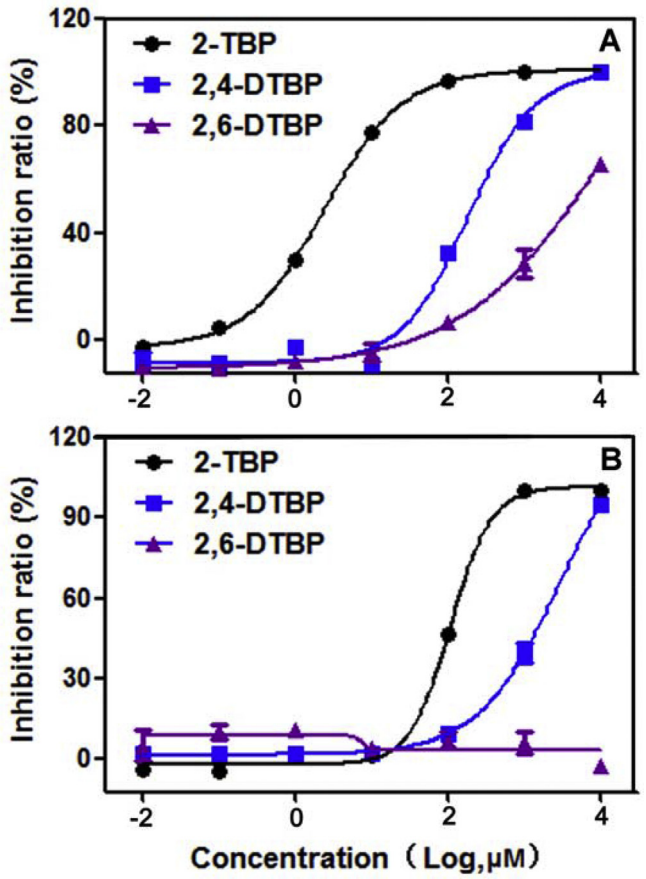

Fig. 3. The concentration-response curves of TBPs toward the light emission of *P. phosphoreum* (A) and *V. fischeri* (B).

no antagonistic effect at concentrations ranging from 5×10^{-6} to 0.05 μM 2,4-DTBP and 2,6-DTBP gradually showed antagonistic effect with increasing concentrations. 2,4-DTBP was previously identified as a ERα antagonist using a human ERα binding assay (Akahori et al., 2008), in line with our results. 2-TBP and 2,4-DTBP were reported to have antiestrogenic activity on hepatic ER by competitive binding studies using cytosolic preparations of rainbow trout livers (Tollefsen and Nilsen, 2008).

Three TBPs were further co-incubated with DHT (5×10^{-2} μM) and the co-exposure to individual TBP and DHT induced the β-galactosidase activity, (Fig. 2, B), showing the antiandrogenic potency of TBPs toward human AR. 2-TBP and 2,4-DTBP were AR antagonists at concentrations higher than 0.5 μM 2-TBP was a weak AR antagonist in line with a previous report (Li et al., 2010a). 2,6-DTBP showed no antiandrogenic activity at all tested concentrations (5×10^{-6} nM - 50 μM) (Fig. 2, B). The antithyroid activity of TBPs was determined by the co-incubation with 5×10^{-1} μM T3. 2,4-DTBP elicited a weak antithyroid activity with <30% inhibition (Fig. 2, C).

Many estrogenic disrupting phenolic compounds were reported to cause adverse effects on organisms, including the increased incidence of endometriosis and breast cancer in woman, the decreased uterine weight in rats and the disrupted ectopic expression of vitellogenin in fish (Kim et al., 2003; Kunisue et al., 2012; Liu et al., 2010; Safe, 1995; Zhang et al., 2017). Exposure to androgenic phenolic pollutants may cause adverse effects on prostate and testis development and subsequently influences adult spermatogenic capacity and male reproduction (Lassen et al., 2014; Uzumcu et al., 2004). Several phenols showed endocrine disrupting

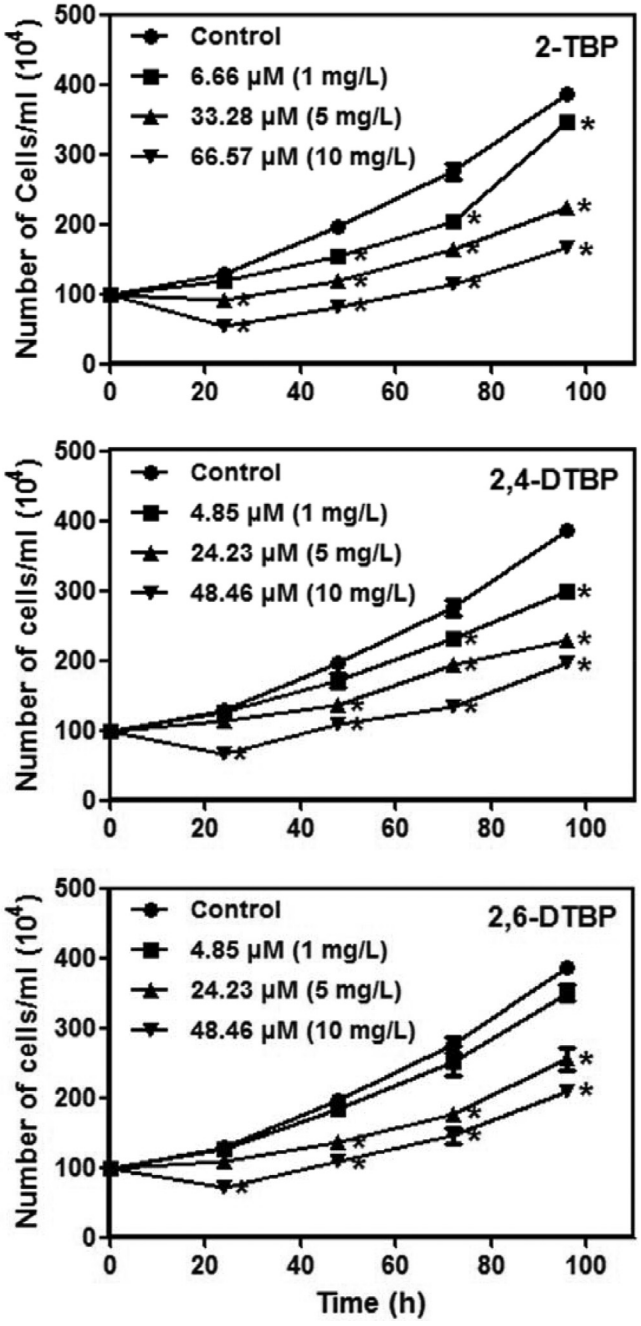

Fig. 4. The inhibition effects of TBPs on the growth of *C. reinhardtii*. The significant difference is considered with $p < 0.05$.

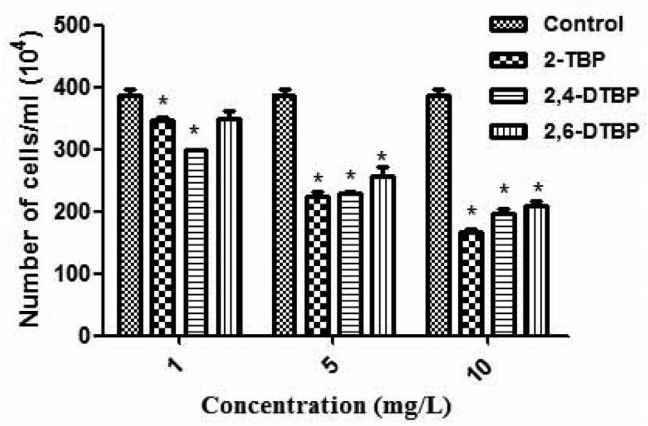

Fig. 5. Growth of *C. reinhardtii* exposed to TBPs for 96 h. The significant difference is considered with $p < 0.05$.

effect on thyroid hormones during pregnancy of women (Aker et al., 2016). Since 2-TBP, 2,4-DTBP and 2,6-DTBP showed antagonistic effect toward ERα, AR and TRβ, they may pose further human health effects. More attention should be paid to the potential risk of their endocrine disrupting effects.

3.3. Acute toxicity of TBPs on aquatic organisms

3.3.1. Luminescent bacteria toxicity in response to TBPs

The ecotoxicity of TBPs were evaluated using *P. phosphoreum* and *V. fischeri*. These two bioluminescent bacterium were used widely as bioluminescent indicators of various contaminants (Deng et al., 2012; Osano et al., 2002). TBPs at concentrations ranging from 0.01 μM to 1×10^4 μM inhibited the light emission of *P. phosphoreum* and *V. fischeri* in a concentration-dependent manner (Fig. 3). The inhibition ratio of 2-TBP toward *P. phosphoreum* was 30.3% ± 0.2% at 1 μM and reached 96.9% ± 0.4% at 100 μM. 2,4-DTBP and 2,6-DTBP at concentrations higher than 10 μM showed significant toxicity toward *P. phosphoreum* and their inhibition ratios were 99.7% ± 0.2% and 65.9% ± 0.1%, respectively. The calculated EC50 values of 2-TBP and 2,4-DTBP toward *P. phosphoreum* were 2.37 μM and 181.8 μM, indicating an increased acute toxicity ranking as 2,6-DTBP < 2,4-DTBP < 2-TBP. 2-TBP and 2,4-DTBP began to exhibit significant toxicity at the concentration of 10 μM and 100 μM, respectively, and their maximum inhibition ratios were over 95%. 2,6-DTBP showed no significant toxicity and the inhibition ratio was less than 10%. As for the acute toxicity toward *V. fischeri*, 2,6-DTBP exhibited no acute toxicity whereas 2-TBP and 2,4-DTBP showed toxic effect at concentrations higher than 10 μM. The EC50 value of 2-TBP and 2,4-DTBP were 107.2 μM and 2830 μM, respectively. Compared with *V. fischeri*, *P. phosphoreum* showed higher susceptibility to TBPs. According to the acute toxic class (Table S3), 2-TBP was toxic to *P. phosphoreum*, and harmful to *V. fischeri*. 2,4-DTBP was harmful to *P. phosphoreum* and not harmful to *V. fischeri*. 2,6-DTBP was not harmful to both *P. phosphoreum* and *V. fischeri*.

3.3.2. Acute toxicity of TBPs to C. reinhardtii

Considering the diversity of various aquatic organisms susceptible to different toxic contaminants, we further evaluated the aquatic toxicity of TBPs toward freshwater green algae *C. reinhardtii*. This algal species was used widely as indicator organisms for testing aquatic toxicity of pollutants (Domingos et al., 2011; Jamers et al., 2013). The growth of *C. reinhardtii* was significantly affected after the exposure to TBPs (1, 5, 10 mg/L) with increasing concentrations for 24, 48, 72 and 96 h (Fig. 4). At higher exposure concentrations or longer exposure time, the inhibition of growth of *C. reinhardtii* was increased. At the initial 24 h, the exposure to TBPs at 1 mg/L caused no significant change toward *C. reinhardtii* in comparison with the control group. The significant growth inhibition was observed after 48 h exposure of 2-TBP, 72 h exposure of 2,4-DTBP. After 96 h exposure to TBPs with increasing concentrations, the inhibition of growth of *C. reinhardtii* was increased (Fig. 5). We also investigated the toxic effect of TBPs on chlorophyll content of *C. reinhardtii*. TBPs decreased the chlorophyll content in a dose-dependent manner in comparison with the negative control (Fig. 6). At concentration of 1 mg/L, only 2-TBP significantly reduced the chlorophyll content after 48 h exposure. TBPs at 10 mg/L

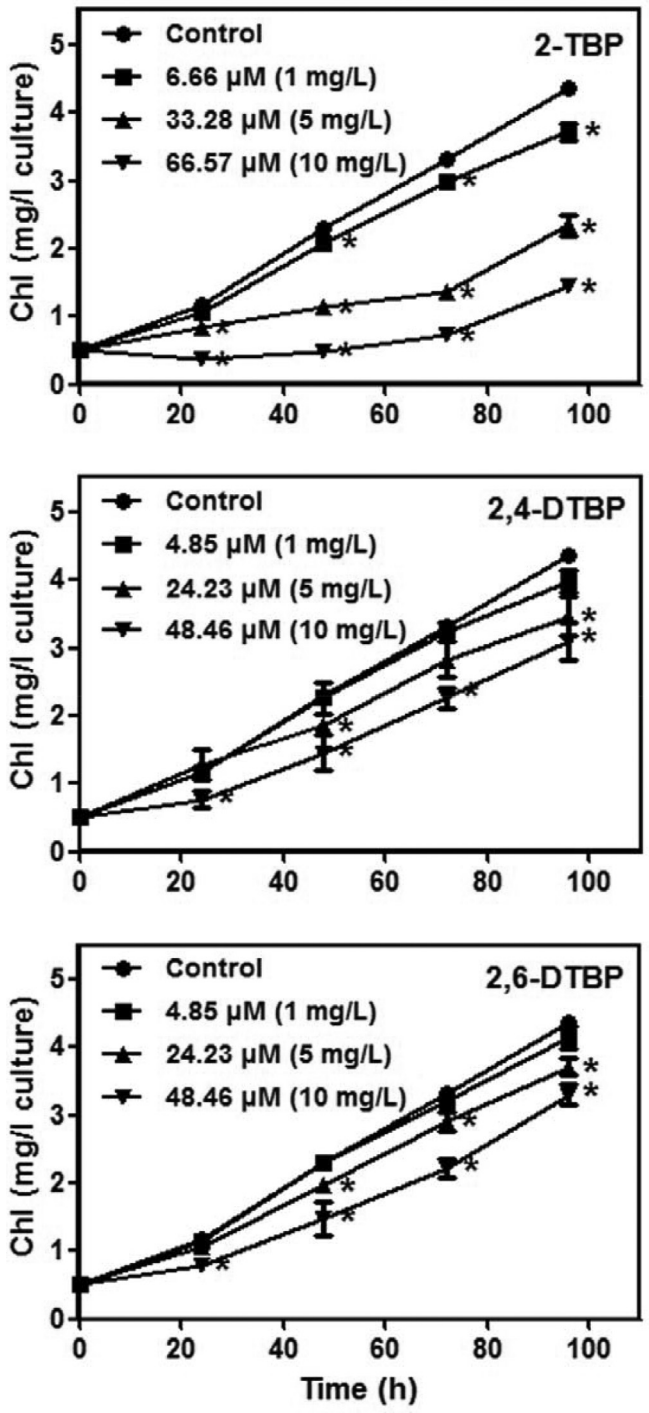

Fig. 6. The inhibition effects of TBPs on total chlorophyll content of *C. reinhardtii*. The significant difference is considered with $p < 0.05$.

showed significant inhibitory effects from the initial 24 h. 2-TBP, 2,4-DTBP and 2,6-DTBP had aquatic toxic effect on green algae, indicating their potential risk to the aquatic ecosystems.

4. Conclusions

The detection of emerging phenolic contaminant, 2,4-DTBP with concentrations at the μg/L level was reported the first time in drinking water source of the Qiantang River Basin, East China. The ramifications of this monitored result are not fully known, but potential human and ecological effects of TBPs warrant further studies. TBPs exhibited antagonistic effects toward human ERα, AR and TRβ in a concentration-dependent manner and showed acute aquatic toxicity toward *P. phosphoreum*, *V. fischeri* and *C. reinhardtii*. 2-TBP posed the most significant risk toward bioluminescent bacterium. TBPs have the structural changes in the substituted side chain and these variations in the molecular structure may contribute to their different toxicities. Future relevant risk assessment and structural design of environmentally benign novel TBPs-related compounds should take the structure-activity relationship into full account. Our study point out the potential risk of TBPs and is essential for the comprehensive evaluation of their risk toward human health and ecosystem. Future monitoring efforts should be made toward these phenolic compounds in the drinking water source of the Qiantang River. Such data will facilitate further risk assessment of TBPs and related contaminants in this important watershed.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

The authors thank the financial support from the National Natural Science Foundation of China (No. 21477113, 21621005), the Major Research Plan of the National Natural Science Foundation of China (No. 91643107), the Guangzhou Key Laboratory of Environmental Exposure and Health (No. GZKLEEH201605) and the Fundamental Research Funds for the Central Universities (No. 2018QNA6013). We thank Dr. Erik Jan Dubbink (Erasmus University, Holland) for the pGBT9 plasmid and Prof. Zijian Wang (Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China) for the human recombinant AR and TRβ yeast strain. We acknowledge Prof. Oliver J. Hao (University of Maryland) for the assistance in this project.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.04.117.

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