Ecotoxicology and Environmental Safety 119 (2015) 116–122

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Hepatic oxidative stress biomarker responses in freshwater fish *Carassius auratus* exposed to four benzophenone UV filters

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ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form
10 May 2015
Accepted 11 May 2015
Available online 18 May 2015

Keywords:
UV filters
Benzophenones
Oxidative stress
Integrated biomarker response
Histological analysis

ABSTRACTS

Benzophenone (BP) type UV filters are widely used in many personal care products to protect human from UV irradiation. Despite the estrogenic potencies to fish and the environmental occurrences of BP derivatives in aquatic systems, little information is available regarding their effects on the antioxidant defense system in fish. In this work, the oxidative stress induced in livers of *Carassius auratus* was assessed using four biomarkers. The integrated biomarker response (IBR) was applied to assess the overall antioxidant status in fish. Moreover, liver tissues were also studied histologically. The changes in the activities of antioxidant enzymes and glutathione levels suggested that BPs generates oxidative stress in fish. The IBR index revealed that the hepatic oxidative toxicity followed the order BP-1 > BP-2 > BP-4 > BP-3. The histopathological analysis revealed lesions caused by BPs. This investigation provides essential information for assessing the potential ecological risk of BP-type UV filters.

1. Introduction

Benzophenones (BPs) are one of the primary components in the UV filters family and are, extensively used as sunscreen agents in personal care products (PCPs) for the protection of skin and hair from UV irradiation (Chisvert et al., 2012). In the European Union, 2-hydroxyl-4-methoxyl benzophenone (BP-3) and 2-hydroxyl-4-methoxyl benzophenone-5-sulfonic acid (BP-4) are approved to be used as UV filters in sunscreens at maximum individual concentrations of 10% and 5%, respectively, and in Japan, 2,4-dihydroxybenzophenone (BP-1), 2,2′,4,4′-tetrahydroxybenzophenone (BP-2) and 2,2′-dihydroxy-4,4′-dimethoxybenzophenone (BP-6) are also allowed in sunscreens. Currently, BPs have been classified as chemicals suspected of having endocrine disrupting effects due to increasing evidence that BPs are able to interfere with the endocrine system (Kerdivel et al., 2013).

BPs have been detected in environmental matrices such as water (Almeida et al., 2013; Wu et al., 2013; Jurado et al., 2014), soil, sediment (Zhang et al., 2011) and indoor dust (Wang et al., 2013a). The amounts found in water samples are in the ng L^{-1} range, which are not far below the doses that cause toxic effects in animals (Tarazona et al., 2010). Fish are important organisms to monitor the occurrence of persistent lipophilic contaminants (Fent et al., 2010). The presence of BPs UV filters was reported in fish from Switzerland rivers, Swiss lakes and Texas streams (Balmer et al., 2005; Mottaleb et al., 2009; Fent et al., 2010), even in marketed fish (Tsai et al., 2014). Some BP UV-filters have hormonal activity in fish. It is reported that BP-1, BP-2, BP-3 and BP-4 elicit estrogenic and anti-androgenic activities in fish (Kunz et al., 2006) *in vivo* and *in vitro* tests, and it is noteworthy that BP-1, BP-2 and 4-hydroxybenzophenone (4BP) possess estrogenic activities higher than that of BP-3 (Morohoshi et al., 2005). BP-2 could negatively affect egg production and gonadal development in fish (Weisbrod et al., 2007). In addition, induction of vitellogenin and impairment of reproduction in fish (*Japanese medaka* and *rainbow trout*), and alterations of gene expression in both adult zebrafish and eleuthero-embryos were reported with an aqueous exposure to BP-3 (Coronado et al., 2008; Bluthgen et al., 2012). Alterations of gene expression involved in hormonal pathways and steroidogenesis were also reported in zebrafish exposed to BP-4 (Zucchi et al., 2011).

BPs have even been detected in human samples, such as breast milk, urine (Asimakopoulos et al., 2014a, 2014b) and blood (Zhang et al., 2013; Vela-Soria et al., 2014). Recently, through concentration analysis of BP-3 as well as four of its metabolic derivatives, BP-1, BP-2, 2,2′-dihydroxyl-4-methoxl benzophenone (BP-8), and 4BP in urine of children and adults, BP-3 was found in nearly all urine samples from the U.S. and China (Wang and Kannan, 2013b). High urinary concentrations of BP-1 are associated with estrogen-dependent diseases, such as endometriosis in women (Kunisue et al., 2012). These findings demonstrate the apparent likelihood that BPs contained in PCPs might be emitted into the aquatic

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http://dx.doi.org/10.1016/j.ecoenv.2015.05.017
0147-6513/

environment and persistent with a great potential for bioaccumulation through food chains (Gago-Ferrero et al., 2013). Thus, the occurrence (Liao and Kannan, 2014; Tsui et al., 2014), the degradation (Beel et al., 2013; Duirk et al., 2013; Yang and Ying, 2013; Ji et al., 2013; Laurentiis et al., 2013; Xiao et al., 2013) and, particularly the potential toxicities to humans and the ecosytems of these BP UV-filters have attracted widespread attention (Hofkamp et al., 2008; Downs et al., 2014; Paredes et al., 2014; Liu et al., 2015).

Biomarkers such as enzyme activity, protein level and DNA can be used to measure the interaction between biological systems and physical, chemicals or biological environmental agents. And the evaluation of oxidative stress biomarkers is critical to the investigation of oxidative stress in organisms. Grabicova et al. found, after 42 days of UV filter 2-phenylbenzimidazole-5-sulfonicacid (PBSA) exposure in rainbow trout liver, the catalase (CAT) and superoxide dismutase (SOD) activities were not significantly different between the control group and the group that was exposed to the highest concentration of PBSA (1000 $\mu g\ L^{-1}$), and no pathological changes were obvious in the liver or gonads (Grabicova et al., 2013). Gao et al. observed increased CAT activity in the *Tetrahymena thermophile* exposed to 1.0 $\mu g\ L^{-1}$ BP-3, while no significant changes of the SOD activity (Gao et al., 2013). Therefore, we attempted to test the hypothesis whether these environmentally important benzophenones induce oxidative stress in fish. In the present study, four oxidation-related biomarkers, the activities of SOD, CAT and glutathione S-transferase (GST), and the level of glutathione (GSH) were measured to estimate the single toxicities of four common BP-type UV filters on antioxidant status in fish *Carassius auratus* (*C. auratus*). Next, integrated biomarker response (IBR) index was applied to improve their discriminatory power, and was calculated to estimate the integral effects of BPs-induced oxidative stress. Finally, liver tissues were examined histologically.

2. Materials and methods

2.1. Chemicals and reagents

BP-1 (CAS no:131-56-6, 99% purity), BP-2 (CAS no:131-55-5, 99% purity), BP-3 (CAS no:131-57-7, 99% purity) and BP-4 (CAS no:4065-45-6, 98% purity) were purchased from J&K Co. (Shanghai, China). The stock solutions were prepared in dimethylsulfoxide (DMSO). All other reagents were of analytical purity. The commercial assay kits for the analysis of the oxidative stress biomarkers were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Water quality

The water quality parameters of the tap water used for the acclimation period and the subsequent experiments were measured and are listed in Table 1.

2.3. Animals and experimental exposure

C. auratus (weight: 28.84 ± 3.35 g) were obtained commercially from a local supplier (Nanjing, China). Before the experiment, the healthy fish were acclimatized for at least 7 d in tanks containing dechlorinated and aerated tap water at 24 ± 2 °C. The fish were fed with the commercial fish food twice a day but fasted 24 h prior to biochemical analysis. During the acclimated period, the total mortality was less than 1%.

After acclimatization, fifteen acclimated fish were transferred to each aquarium containing 30 L dechlorinated and aerated water. The solubility of BP-3 is the most smallest than others, and the saturated solubility of BP-3 is 5.1 mg L^{-1} (Gago-Ferrero et al., 2013). Thus the lower concentration 0.5 mg L^{-1} were chose as close to worst-case environmental concentrations, the higher one of 5 mg L^{-1} as a pharmacological concentration. BPs were analyzed on an Agilent 1200 High Performance Liquid Chromatograph (HPLC) equipped with a Diode Array Detector. A Zorbax 300 SB-C18 column (4.6 × 150 mm, 5 μm) was used for the separation at 30 °C. The mobile phase was 0.3% formic acid in water (A) and methanol (B) with an isocratic elution of 20:80 (v/v). The injection volume was 100 μL, the flow rate was set at 1 mL min^{-1}, and the detection wavelength was 290 nm. The actual exposure concentrations of BP-1, BP-2, BP-3 and BP-4 in low dose groups are 0.48 ± 0.03, 0.48 ± 0.05, 0.48 ± 0.03 and 0.49 ± 0.03 mg L^{-1}, respectively, and in high dose groups 4.70 ± 0.47, 4.78 ± 0.40, 4.76 ± 0.32 and 4.90 ± 0.40 mg L^{-1}, respectively.

Every two days, a half of water in the tanks was exchanged with fresh water containing the same concentrations of BPs to maintain the constant concentrations during the experiments. The final DMSO concentration was 0.005% in all tanks except the aqueous control. The protocols for fish maintaining, experimentation and sacrifice were approved by the Ethics Committee of Nanjing University (Qu et al., 2014).

Table 1
Water quality parameters of the water used for acclimation and subsequent experiments.

Water quality parameters			
pH	7.25 ± 0.25	K^+	2.34 ± 0.05 mg/L
Conductivity	350.6 ± 12.5 μs/cm	Mg^{2+}	7.75 ± 0.02 mg/L
Total hardness	146.5 ± 9.3 mg $CaCO_3$/L	Ca^{2+}	42.05 ± 0.82 mg/L
Alkalinity	40.5 ± 5.0 mg $CaCO_3$/L	Cl^-	28.1 ± 1.2 mg/L
Na^+	12.2 ± 0.2 mg/L	DO (dissolved oxygen)	6.5 ± 0.5 mg O_2/L

2.4. Sample preparation and biochemical assessments

4 fish were randomly sampled from each group on days 7, 14 and 28, and then killed by a blow to the head and dissected for liver in each treatment. Subsequently, the liver of each fish was placed on an ice plate and rinsed with cold physiological saline (0.9% NaCl), next weighed, and homogenized with cold physiological saline using an Ultra Turrax homogenizer (IKA, Germany). The homogenates were centrifuged by Eppendorf 5417R centrifuge (Eppendorf, Germany) at 4000 rpm at 4 °C for 15 min, to get the supernatants for further biochemical analysis. Four biomarkers, including the activities of SOD, CAT and GST, and GSH level, were analyzed. The SOD activity was measured based on the inhibition of cytochrome *c* caused by the superoxide radical (McCord and Fridovich, 1969). The CAT activity was evaluated by monitoring residual H_2O_2 absorbance at 405 nm (Góth, 1991). The GST activity was measured using GST assay kit by a spectrophotometer with 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione-reduced (GSH) as substrates at 412 nm (Han et al., 2013). The GSH level was evaluated following the procedure of Jollow et al. (1974) with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as the reagent. DTNB was reduced by free sulfhydryl groups of GSH to form yellow-colored 5-thio-nitrobenzoic acid. The activities of antioxidant enzymes were normalized by total protein and represented as a relative percentage of the control. Total proteins were determined using the Bradford method (Bradford, 1976).

2.5. Integrated biomarker response (IBR)

A battery of biomarkers is often used to evaluate the effects of