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Evaluation of the *in vitro* estrogenicity of emerging bisphenol analogs and their respective estrogenic contributions in municipal sewage sludge in China

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HIGHLIGHTS

- Eight BPs were identified in sewage sludge of waste water treatment plants.
- Eight BPs exhibited estrogenic activity in BLYES assay.
- All sewage sludge samples elicited considerable estrogenic activity.
- BPs made minor contributions to the total estrogenic activity of sewage sludge.

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ABSTRACT

There is a potential risk to the environment from persistent estrogenic compounds in sewage sludge. In this study, eight bisphenols (BPs) were identified in sewage sludge collected from wastewater treatment plants in 15 cities in China. The estrogenic potencies of the eight BPs and the estrogenic activities of sludge samples were evaluated using a bioluminescence yeast estrogen screen (BLYES) assay. All sludge samples elicited considerable estrogenic activity at a range of 2.8–4.7 ng E2 g^{-1} dry weight (dw). All BPs exhibited estrogenic activity in the BLYES assay, but there were significant differences between the potency of individual chemicals. Bisphenol AF had the highest activity, followed by tetrachlorobisphenol A, bisphenol F, bisphenol A, bisphenol E, bisphenol S and 2,4-dihydroxybenzophenone. Tetrabromobisphenol A showed weak estrogenic activity at 1×10^4 nM, but significant cytotoxicity above this concentration. The total estradiol equivalency quantities (EEQs) of BPs were in the range of 2.16–49.13 pg E2 g^{-1} dw, accounting for 0.05–1.47% of the total EEQs in sewage sludge samples. The results indicate that BPs made a minor contribution to the estrogenic activity of the investigated sewage sludge. Nevertheless, our results suggest that considerable attention should be directed to the estrogenic potentials of emerging organic pollutants because of their widespread use and their potential to persist in the environment.

1. Introduction

Bisphenols (BPs) form a large family of chemicals that are commonly used to produce polycarbonates and epoxy resins. Of these compounds, bisphenol A (BPA) is the most widely used; more than 3 million tons $year^{-1}$ of BPA is used all over the world in the manufacture of numerous consumer products (Delfosse et al., 2012). BPs, which consist of two phenolic rings joined together by a bridging carbon or other chemical structure, are structurally similar to BPA. Bisphenol B (BPB), bisphenol F (BPF), bisphenol AF (BPAF), bisphenol S (BPS), teramethylbisphenol A (TMBPA), and other bisphenols are also used as materials for polycarbonate resin. Halogenated derivatives of BPA, which have bromine or chlorine substitutes on the phenolic rings, are used as flame retardants. Tetrabromobisphenol A (TBBPA) is the most-produced brominated flame retardant (global production >150000 tons $year^{-1}$) (de Wit et al., 2010). Tetrachlorobisphenol A (TCBPA), closely related to TBBPA, is also used as a flame retardant, but is manufactured in lower quantities (global production <10000 tons $year^{-1}$) (Chu et al., 2005).

Extensive industrial production and household applications facilitate the release of BPs into the environment. Several studies have shown that BPA is released from consumer products, and that it is detectable at trace levels in food, drinking water, wastewater, air, and dusts (Vandenberg et al., 2007). TBBPA has been found in

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river sediment in Japan and China (Watanabe et al., 1983; Zhang et al., 2009), sewage sludge in Sweden and Canada (Sellström and Jansson, 1995; Alaee et al., 2003), and air samples in Sweden (Sjödin et al., 2001). TCBPA has been detected in waste paper recycling plants in Japan (Fukazawa et al., 2002), drinking water in China (Fan et al., 2013), sediment and sludge in USA (Voordeckers et al., 2002; Chu et al., 2005). BPF has been detected in surface water, sewage and sediments in Germany (Fromme et al., 2002). BPF and BPAF have recently been found in sediments and indoor dust samples obtained from North America and several Asian countries (Song et al. 2012; Liao et al., 2012a,b). Of greater significance is the fact that some studies have identified BPs, such as BPA and TBBPA, in human serum, urine, and placental tissues in Germany, Sweden and Norway (Jakobsson et al., 2002; Thomsen et al., 2002; Dekant and Völkel, 2008; Vandenberg et al., 2010). The major source of consumer exposure is likely to be through food and drinks that have been in contact with BPs-containing materials.

BPA has been identified as a weak estrogenic chemical that modifies natural endocrine functions through binding to the estrogen receptor. It has been shown to cause a range of adverse effects in laboratory animals (O'Connor and Chapin, 2003; Vandenberg et al., 2009; Chung et al., 2011). The estrogenic activity of BPA was reported to be four to six orders of magnitude weaker than that of natural estrogen, 17β-estradiol (VomSaal et al., 1998; Song et al., 2006). However, until now, little attention has been paid to the toxicity of other BPs. Because of their similar molecular structures and physicochemical properties, other BPs may also exhibit similar toxicological effects. Our recent work demonstrated that exposure to TCBPA, TBBPA, and BPAF resulted in developmental toxicity in zebrafish embryos/larvae, but only BPAF specifically showed estrogenic activity (Song et al., 2014a). Kitamura et al. (2005) compared the endocrine-disrupting activities of BPA and 19 related compounds by means of different *in vitro* and *in vivo* reporter assays. Some BPs exhibited estrogenic activity in the human breast cancer cell line MCF-7, but there were notable differences in activity (Kitamura et al., 2005). Recent studies have shown that the estrogenic activity of BPAF was about one order of magnitude stronger than does BPA (Okada et al., 2008; Matsushima et al., 2010). However, information on the toxicological consequences, environmental presence, and environmental fate of these compounds is still limited.

BPA is a significant contaminant in wastewater and biosolids from sewage treatment plants (STPs), and may affect wildlife at environmental relevant concentrations (Crain et al., 2007). In a recent study, we identified BPs, such as TCBPA, BPAF, and BPE, in sewage sludge for the first time in China (Song et al., 2014b). Because all the compounds in sewage sludge carry an intrinsic risk of off-site transport once applied to land, it is essential to provide information on their exposure risk potentials in the surrounding environment. In this study, the estrogenic potencies of BPA and other BPs found in sewage sludge were examined using a bioluminescence yeast estrogen screen (BLYES). Additionally, the contributions of BPs to the total estrogenic activities of sewage sludge were investigated by calculating estrogenic activities of BPs relative to those of sewage sludge samples. To the best of our knowledge, this is the first attempt to assess the risk of BPs in municipal sewage sludge. The results will not only show the contamination status of BPs, but also may be useful for assessing their risk in sewage sludge.

2. Materials and methods

2.1. Materials

TCBPA and BPAF were purchased from TCI (Portland, OR). 17β-estradiol (E2), BPA, and TBBPA were purchased from Sigma–Aldrich (USA). BPE, BPF, BPS, and 2,4-dihydroxybenzophenone (DHBP) were obtained from TCI (Tokyo, Japan). All chemicals had a purity of 98% or greater unless otherwise mentioned. BPs were dissolved in methanol to form stock solutions of 1000 μg mL^{-1} and were stored away from light. Ultrapure water (18.3 MU) was produced by a Milli-Q system (Millipore, Billerica, MA, USA). ENVI-Carb (0.5 g, 6 mL) and Sep-Pak C18 (1 g, 6 mL) solid-phase extraction cartridges were obtained from Supelco (St. Louis, MO, USA) and Waters (Milford, MA, USA), respectively. Other chemicals used in this study were analytical grade.

2.2. Estrogenic potency of BP chemicals

The relative estrogenic activity was determined by a bioluminescence estrogen assay. The re-engineered *Saccharomyces cerevisiae* BLYES was kindly provided by Gary S. Sayler (University of Tennessee, USA). The BLYES assay has been described in detail in Sanseverino et al. (2005)) and Eldridge et al. (2007). Briefly, a strain of BLYES was grown overnight in a modified minimal medium without leucine and uracil (YMM leu^-, ura^-) at 30 °C, and by shaking at 200 revolution(s) per minute (rpm) until an approximate optical density at 600 nm (OD600) of 1.0 was achieved. Cells were centrifuged and re-suspended in fresh YMM (leu^-, ura^-) until the OD600 was 1.0. Two hundred microliters were transferred to each well of a black 96-well microplate (Thermo Scientific, USA). Appropriate dilutions of test chemicals were added to each well. Bioluminescence was measured every hour for 12 h in a microplate reader (Varioskan Flash, Thermo Scientific). The positive control was E2, and negative controls were either medium only, or medium containing methanol.

The response of each chemical, expressed in mean relative bioluminescence units, was converted to relative response units, expressed as a percentage of the maximum response observed for E2 (% E2max). For each chemical, the response versus the log of the chemical concentration was plotted. A linear regression was determined using the points that fell on the linear portion of the curve. Each 50% effective concentration (EC50) was calculated using a linear regression formula. To allow comparison with the E2 standard, the relative potency, called the estradiol equivalency factor (EEF) was calculated as follows:

$$EEF_{chemical} = (EC50_{E2})/(EC50_{chemical}) \quad (1)$$

2.3. Estrogenic activity in sludge extracts

Sewage sludge samples were collected from municipal waste water treatment plants in 15 cities. Freshly digested sludge samples were collected at the dewatering stage, packed in aluminum foil, and sealed in polypropylene bags. They were then immediately delivered to the laboratory, where they were stored at −20 °C until analysis. All sludge samples were freeze dried, homogenized, and sieved through a stainless steel 100-mesh sieve. The sample pretreatment procedure was similar to that used in our previous study (Song et al., 2014b). Briefly, 0.3 g of each sample was placed in a 15-mL, polyethylene terephthalate centrifuge tube (Corning Inc., Corning, NY, USA) and extracted with 5 mL of methanol triply by shaking at 350 rpm for 60 min. The supernatant was collected after centrifugation at 3500×*g* for 10 min, and then passed through a 6-mL, methanol preconditioned ENVI-Carb cartridge to eliminate matrix interferences (Pang et al., 2013). Analyte residues on the ENVI-Carb cartridge were then eluted with a mixture of methanol and tetrahydrofuran/methanol (4:6, v/v). All eluates were combined and loaded onto a Sep-Pak C18 cartridge for further purification. The analytes were eluted with methanol and finally concentrated to 1 mL under a nitrogen stream.