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In vitro endocrine disruption and TCDD-like effects of three novel brominated flame retardants: TBPH, TBB, & TBCO

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HIGHLIGHTS

- The three novel brominated flame retardants are endocrine disrupting compounds.
- TBB, TBPH, and TBCO do not activate the aryl hydrocarbon receptor in the H4IIE assay.
- TBB, TBPH and TBCO increase concentrations of E2 in the H295R steroidogenesis assay.

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ABSTRACT

The novel brominated flame retardants (NBFRs), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), *Bis*(2-ethylhexyl)-2,3,4,5-tetrabromophtalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO) are components of flame retardant mixtures including Firemaster 550 and Saytex BC-48. Despite the detection of these NBFRs in environmental and biotic matrices, studies regarding their toxicological effects are poorly represented in the literature. The present study examined endocrine disruption by these three NBFRs using the yeast YES/YAS reporter assay and the mammalian H295R steroidogenesis assay. Activation of the aryl hydrocarbon receptor (AhR) was also assessed using the H4IIE reporter assay. The NBFRs produced no TCDD-like effects in the H4IIE assay or agonistic effects in the YES/YAS assays. TBB produced a maximal antiestrogenic effect of 62% at 0.5 mg L^{-1} in the YES assay while TBPH and TBCO produced maximal antiandrogenic effects of 74% and 59% at 300 mg L^{-1} and 1500 mg L^{-1}, respectively, in the YAS assay. Significant effects were also observed in the H295R assay. At 0.05 mg L^{-1}, 15 mg L^{-1}, and 15 mg L^{-1} TBB, TBPH, and TBCO exposures, respectively resulted in a 2.8-fold, 5.4-fold, and 3.3-fold increase in concentrations of E2. This is one of the first studies to demonstrate the *in vitro* endocrine disrupting potentials of TBB, TBPH, and TBCO.

1. Introduction

Brominated flame retardants (BFRs) are added to materials such as electronics, textiles, polyurethane foams, and plastics to increase their fire resistance. There are at least 175 brominated compounds that are listed as flame retardants (Alaee et al., 2003) including hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) which had the largest worldwide production volumes at 22,000 tons/year in 2003 (Covaci et al., 2011) and 170,000 tons/year in 2004, respectively (BSEF, 2013). Polybrominated diphenyl ethers (PBDEs) were once the most widely used BFRs, but several of the technical mixtures were phased-out of use in Europe, followed by several U.S. states. In an agreement between the US EPA and chemical manufacturers, the PentaBDE and OctaBDE technical mixtures were voluntarily phased out of production. The two PBDE formulations were eventually added to the list of Persistent Organic Pollutants (POPs) under the international Stockholm Convention (Ma et al., 2012) while the remaining technical mixture of PBDE, DecaBDE, will be phased out of production and importation to the U.S. by 2013.

Withdrawal of PBDEs from North American markets has led to increased production of non-PBDE BFRs including novel BFRs

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(NBFRs) (Covaci et al., 2011). Though some of these replacement NBFRs have potential for long-range atmospheric transport, environmental persistence, and bioaccumulation, their environmental concentrations and toxicological effects are poorly represented in the literature (De Wit et al., 2011). Examples of NBFRs are 2-ethylhexyl tetrabromobenzoate (TBB), *bis*-(2-ethylhexyl) tetrabromophthalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO). TBB and TBPH are components of the technical mixtures Firemaster 550 (35% TBB, 15% TBPH), Firemaster BZ-54 (70% TBB, 30% TBPH), and DP-45 (TBPH only) marketed by Chemtura Corporation (Bearr et al., 2012; Ma et al., 2012) and TBCO is a component of Saytex BC-48 marketed by Albermarle Corporation (De Wit et al., 2011). Firemaster 550, which is a technical mixture of TBPH and TBB, was used as a replacement for PentaBDE mixtures in polyurethane foams, and both compounds have been listed as high production volume chemicals by the U.S. EPA (Ma et al., 2012). From 1990 to 2006, TBPH had a U.S production volume of 450–4,500 metric tons/year (De Wit et al., 2011), but there is little data on production volumes of TBB or TBCO.

Certain NBFRs including TBB, TBPH, and TBCO have similar potentials for bioaccumulation, persistence, and long-range atmospheric transport as PBDEs and HBCDs (Gentes et al., 2012; Ma et al., 2012; Möller et al., 2011). For example, TBB and TBPH have both been detected in several environmental matrices including dust, air, and biota and have been listed as NBFRs requiring further investigation and monitoring in the Norwegian environment (Harju et al., 2009). From 2008 to 2010, as part of the Integrated Atmospheric Deposition Network, TBB and TBPH had been detected in the particle-phase at six locations near the North American Great Lakes, and in urban areas from Chicago and Cleveland (Ma et al., 2012). The study showed that atmospheric concentrations of both TBB and TBPH increased rapidly during the two-year sampling period possibly indicating that the use and/or accumulation of these NBFRs was increasing. The two compounds have also been detected in samples from the Global Atmospheric Sampling (GAPS) Network (Lee et al., 2010), in house dust in the United States (Stapleton et al., 2008), and indoor dust in New Zealand (Ali et al., 2012). TBPH and TBB have been detected in polyurethane foam in retail baby products in the United States as the second most abundant BFRs (Stapleton et al., 2011), and were detected in couch foam at 4.2% by weight of total flame retardants (Stapleton et al., 2009). Both compounds were detected in sewage sludge from wastewater treatment plants in San Francisco, California (De Wit et al., 2011), and TBPH alone was detected in environmental samples from the high arctic (Möller et al., 2011). TBB and TBPH have been detected in biota, including blubber from humpback dolphins (mean: <0.04 ng g^{-1} lw; 0.51 ± 1.3 ng g^{-1} lw) and finless porpoises (mean: 5.6 ± 17 ng g^{-1} lw; 342 ± 883 ng g^{-1} lw) from Hong-Kong, South China (Lam et al., 2009), in filter feeding bivalves (2220 ng g^{-1} lw; 1370 ng g^{-1} lw), and grazing gastropods (1740 ng g^{-1} lw; 380 ng g^{-1} lw) collected downstream from a textile manufacturing outfall (Guardia et al., 2012). TBPH has also recently been detected in 89% of sampled ring-billed gull livers collected from an industrialized section of the St. Lawrence River downstream from Montreal, Canada (Gentes et al., 2012). The ring-billed gull livers from the St. Lawrence site exhibit the greatest frequency of detection of TBPH and the greatest concentrations in any bird (17.6 ng g^{-1} ww). TBCO has been detected but was not quantifiable in herring gull eggs in the North American Great Lakes (Covaci et al., 2011), but overall few data have been collected regarding the occurrence of TBCO in environmental and biotic matrices.

Based on screening-level assessments using EU criteria, TBCO is a potential aquatic hazardous substance and is characterized as a potentially persistent and bioaccumulative compound (Fisk et al., 2008). TBCO is also included on the Canadian non-domestic Substances List with as much as 10 tons/year being imported into Canada (Covaci et al., 2011). Though TBCO is a potential aquatic hazard, few data on mode of action or toxic potency are available. There are limited data regarding sub-lethal toxicological studies for either TBPH or TBB; Fathead Minnow exposed to the technical mixtures, Firemaster 550 and Firemaster BZ-54 (1 mg fish/d), exhibited acute genotoxicity with DNA damage observed in liver cells (Bearr et al., 2012). In a recent investigation, rats exposed to Firemaster 550 (1000 μg/day) exhibited a 65% increase in total concentrations of thyroxine in serum and a significantly advanced pubertal onset (Patisaul et al., 2013). TBPH and TBB which are derived from *bis*(2-ethylhexyl)-phthalate (DEHP) and 2-ethylhexyl benzoate (EHB), respectively have been observed to undergo sequential debromination in photodegradation experiments (Davis and Stapleton, 2009). Total debromination of TBPH leading to the formation of di-(2-ethylhexyl) phthalate (DEHP) is possible, and requires further investigation due to DEHP's possible biological effects.

The purpose of this investigation was to generate toxicological data for TBB, TBPH, and TBCO by use of *in vitro* bioassays. The *in vitro* bioassay endpoints were based on the toxicities of structural analogs of the compounds (Fig. 1). Recent *in vitro* metabolism experiments have shown that TBPH is metabolized to mono(2-ethylhexyl) tetrabromophthalate (TBMEHP) (Roberts et al., 2012), a brominated analog of MEHP which itself has been shown to affect concentrations of steroid hormones including estradiol and testosterone in rat ovarian follicles (Inada et al., 2012; Kessler et al., 2012).

In this study, the capabilities of three NBFRs to disrupt normal endocrine functions were investigated. Potential as receptor agonists or antagonists were measured by use of the yeast estrogen screen (YES) and yeast androgen screen (YAS) reporter assays while non-receptor mediated steroidogenic effects were investigated by use of the mammalian cell model, the H295R steroidogenesis assay. Following reports of aryl hydrocarbon receptor (AhR) activity by DEHP (Kruger et al., 2008; Mankidy et al., 2013), the three NBFRs were tested for AhR binding activities by use of the H4IIE rat hepatoma cell reporter assay. To our knowledge this report presents the first data regarding these potential sub-lethal effects of TBB and TBCO.

2. Materials and methods

2.1. Chemicals

2-Ethylhexyl tetrabromobenzoate (TBB) was obtained from Wellington Laboratories (Ontario, Canada), bis-(2-ethylhexyl) tetrabromophthalate (TBPH) was obtained from Waterstone Technology (Indiana, United States), and 1,2,5,6-tetrabromocyclooctane (TBCO) was obtained from Specs (Delft, Netherlands). All single compounds were reported to be >95% pure by the manufacturer. All solvents, DMSO, EtOH, ethylacetate, and hexane, were of analytical grade and obtained from Sigma–Aldrich (Ontario, Canada).

2.2. Cell viability

Cytotoxic effects of the three NBFRs to the H4IIE and H295R cells were evaluated by use of the WST-1 assay (Roche Applied Science, Indiana, United States). Cells were propagated as mentioned below. Cytotoxicities were determined after 48 h incubation with individual NBFRs. WST-1 reagent was used to determine metabolically active cells at the end of the incubation period according to the manufacturer's recommendations.

In the YES/YAS assays, cytotoxic effects were measured by use of optical density (690 nm) (Routledge and Sumpter, 1996). After 48 h. incubation, each well was assayed for turbidity and compared to solvent control values. Cellular cytotoxicity was defined as ≥30% reduction in cell density from solvent controls.

2.3. H4IIE-luc transactivation reporter gene assay

The H4IIE-*luc* cellular assay is derived from rat hepatoma cells which have been stably transfected with a luciferase gene under control of a dioxin-responsive element (El-Fouly Richter et al., 1994; Garrison et al., 1996; Hilscherova et al., 2000). H4IIE-*luc* cells were propagated as previously described (Horii et al., 2009). Cells were incubated for 24 h prior to dosing. Test and control wells were dosed with 1% per well volume of the individual NBFRs prepared in DMSO. Luciferase activity

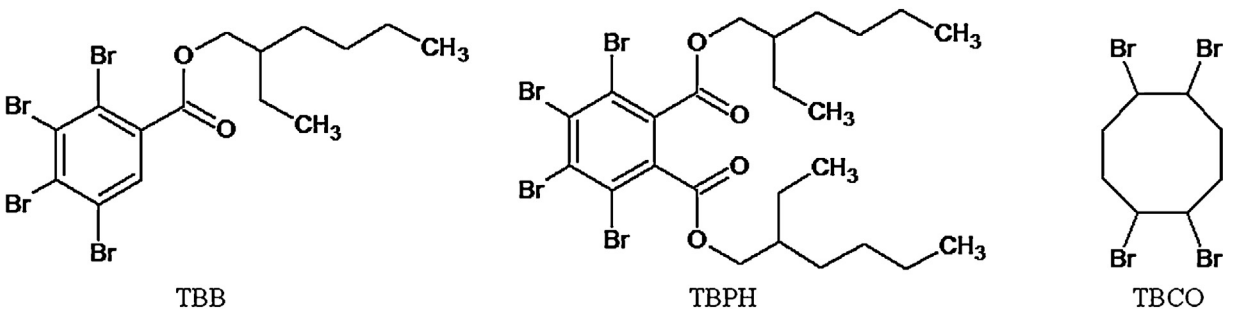

Fig. 1. Chemical structures of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate(TBB), bis(2-ethylhexyl)-3,4,5,6-tetrabromo-phthalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO).

was measured by use of the SteadylitePlus Kit (Perkin Elmer, MA, USA). The following concentrations of the test compounds were used: (TBB) 5×10^{-5}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2} mg L^{-1}, (TBPH) 0.75, 1.5, 3, 15, 30, 150 mg L^{-1}, and (TBCO) 0.3, 1.5, 3, 15, 30 mg L^{-1}. A TCDD standard curve was included in each plate to control for inter-plate variability.

2.4. YES/YAS assays

Estrogenic and androgenic activities of the three NBFRs: TBB, TBPH, and TBCO were measured *via* production of β-galactosidase and the subsequent metabolism of chlorophenol red-β-D-galactopyranoside (CPRG). All media and procedures used for the YES/YAS assays were prepared according to the original protocol (Routledge and Sumpter, 1996). 17β-estradiol and dihydrotestosterone (DHT) standards were included with each plate to control for inter-plate variability. Activity was measured at 570 nm and 690 nm by use of Eq. (1). The corrected value represents the test response corrected for potential toxicity to cells.

$$\text{Corrected value} = A570\,\text{nm} - A690\,\text{nm} \quad (1)$$

Anti-estrogenic (YES) and anti-androgenic (YAS) activities of the three NBFRs were measured by reduction in activity of β-galactosidase in yeast cells in the presence of 8.17×10^{-4} mg L^{-1} 17β-estradiol (YES), and 1.45×10^{-3} mg L^{-1} DHT (YAS). 4-Hydroxytamoxifen (3.88×10^{-9} mg L^{-1}), and hydroxyflutamide (2.92×10^{-8} mg L^{-1}) were used as E2 and DHT antagonist controls for the YES and YAS assays respectively. Concentrations of the three NBFRs which elicited the greatest inhibition (YES: 5×10^{-01}, 0.03, 30 mg L^{-1}; YAS: 5×10^{-01}, 1000, 300 mg L^{-1}; TBB, TBPH and TBCO, respectively) were used to test for recovery of activation signals of the cellular assay systems. This control was employed to test for inhibitory effects due to non-receptor mediated mechanisms. To elicit an inhibitory response, each NBFR was combined with a specific receptor agonist, E2 or DHT, then incubated with an additional volume of agonist at three different concentrations. Recoveries of activation signals were tested by use of three concentrations of E2: 2.72×10^{-4}, 8.17×10^{-4}, and 2.72×10^{-3} mg L^{-1} (YES) and three concentrations of DHT: and 2.90×10^{-4}, 1.45×10^{-3}, and 2.90×10^{-3} mg L^{-1} (YAS) (Supplemental Figs. 1 and 2).

All procedures for the anti-estrogenic and anti-androgenic assays were the same as those for the YES/YAS agonist assays described above.

2.5. H295R cell culture and exposure

The H295R human adrenocarcinoma cell line was cultured according to the standardized H295R assay protocol approved by the OECD (OECD, 2011). H295R cells were dosed with the following concentrations of the test compounds: (TBB) ranging from 5×10^{-5} to 5×10^{-2} mg L^{-1}, (TBPH) ranging from 1.5 to 30 mg L^{-1}, and (TBCO) ranging from 0.3 to 15 mg L^{-1}. Forskolin (4.11 mg L^{-1}), a strong inducer of both E2 and T production, and prochloraz (1.13 mg L^{-1}), a strong inhibitor of both E2 and T production, were used as controls in the H295R steroidogenesis assay. Final concentrations of solvent carriers did not exceed 0.1%. Conditioned media was collected following 48 h of exposure and assayed for [E2] and [T] by use of ELISA.

2.6. 17β-Estradiol and testosterone extraction and quantification by use of EIA

Extraction of E2 and T from media was performed according to an established protocol (Chang et al., 2010; Hecker at al., 2006). Concentrations of E2 and T were determined by competitive EIA according to the manufacturer's recommended method (Caymen Chemical Company, Ann Arbor, MI, USA).

2.7. Statistics

Statistical analysis for cellular assays was carried out by use of IBM SPSS Statistics software (V.20). Data was initially tested for normality by use of the Shapiro–Wilk's test and homogeneity of variance by use of Levene's test ($p > 0.05$). If assumptions of normality and homogeneity of variance were met, a one-way ANOVA was used to evaluate differences between sample treatment and solvent controls. Differences were considered significant at a p-value <0.05. In those cases where the basic assumptions for parametric statistics were not met, distribution-free tests such as Kruskal–Wallace followed by Mann–Whitney U tests were employed. All data is reported as mean ± SE.

3. Results

3.1. TCDD-Like potencies of compounds

The three NBFRs, TBB, TBPH, and TBCO caused no TCDD-like potencies in the H4IIE-*luc* bioassay (data not shown). A TCDD standard curve [2.25×10^{-7} mg L^{-1} to 4.83×10^{-5} mg L^{-1}] was used to calculate TCDD equivalents. The three NBFRs had no cytotoxic effects at the tested concentrations.

3.2. Receptor-mediated androgenic and estrogenic potencies of compounds

The three NBFRs, TBB, TBPH, and TBCO caused no estrogen-like or androgen-like potencies in the YES/YAS bioassays. A six point E2 standard curve [2.72×10^{-6} mg L^{-1} to 2.72×10^{-3} mg L^{-1}] (YES), and a seven point DHT standard curve [2.90×10^{-6} mg L^{-1} to 8.71×10^{-3} mg L^{-1}] (YAS) were used to calculate E2 and androgen equivalents. The three NBFRs had no cytotoxic effects at the tested concentrations.

3.3. Androgen receptor mediated antiandrogenic potencies of NBFRs

The three NBFRs, TBB, TBPH, and TBCO were screened for antiandrogenic potencies by use of the YAS assay. The signal from cells activated by a 1.45×10^{-3} mg L^{-1} DHT control was set at 100%. Cells co-treated with androgen antagonist control hydroxyflutamide [2.92×10^{-8} mg L^{-1}] exhibited a 52% reduction in β-galactosidase signal. The following concentrations of the test compounds were used in the YAS assay: (TBB) 5×10^{-10}, 5×10^{-8}, 5×10^{-6}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-01} mg L^{-1}, (TBPH) 3×10^{-2}, 0.3, 3, 15, 30, 150, 300, 1500 mg L^{-1}, and (TBCO) 3×10^{-3}, 3×10^{-2}, 0.3, 3, 15, 150, 300 mg L^{-1}. Each NBFR tested resulted in statistically significant inhibition of receptor mediated β-galactosidase production. At 0.5 mg L^{-1} TBB exposures resulted in a maximal antiandrogenic response of 31% inhibition of β-galactosidase production compared to the DHT control (Fig. 2A). TBPH, the brominated structural analog of the phthalate DEHP, demonstrated dose-dependent inhibition of β-galactosidase production. At 1500 mg L^{-1}TBPH exposures resulted in a maximal antiandrogenic response of 59% compared to the DHT control (Fig. 2B). TBCO responded in a dose-dependent manner and produced the greatest inhibition of β-galactosidase production of all tested compounds. At 300 mg L^{-1} TBCO exposures resulted in a maximal antiandrogenic response of 74% compared to the DHT control (Fig. 2C).

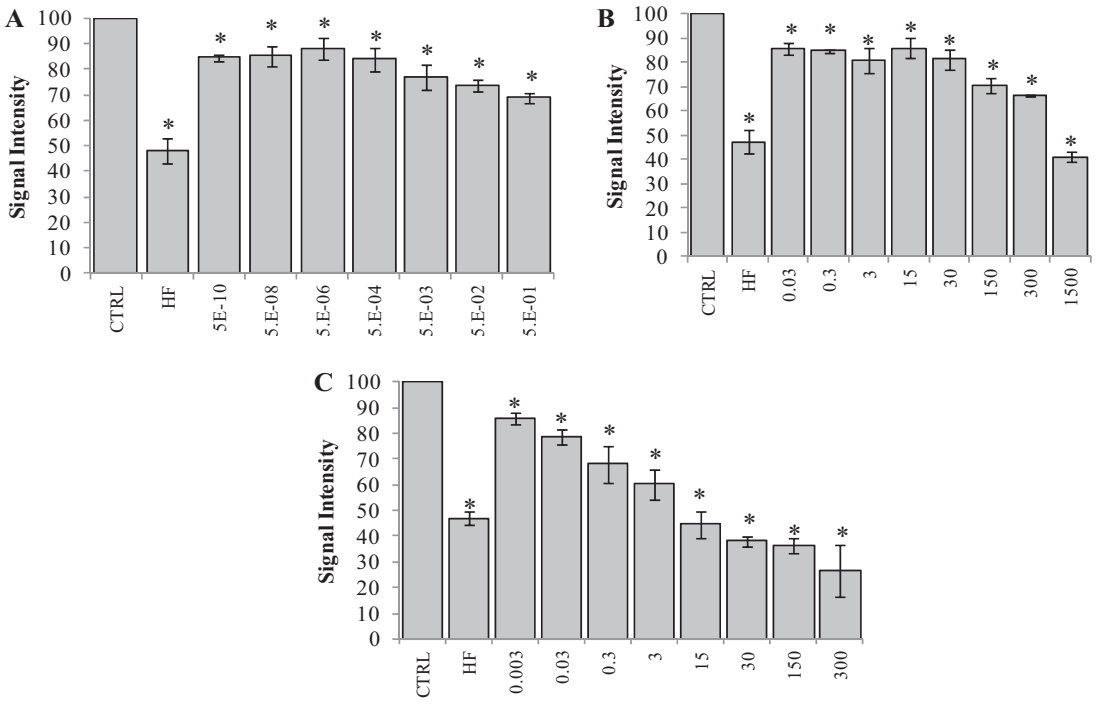

Fig. 2. Antiandrogenic potency of (A) TBB, (B) TBPH, and (C) TBCO in mg L^{-1} measured by the yeast androgen screen. Antiandrogenic potency is presented as the reduction in signal intensity (mean ± SE) compared to DHT activated control cells (CTRL). Hydroxyflutamide (HF) acted as a positive control. Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than activated controls are indicated by asterisks (*$p < 0.05$).

3.4. Estrogen receptor mediated antiestrogenic activities of compounds

The three NBFRs were screened for antiestrogenic potencies by use of the YES assay. The signal from cells activated by 8.17×10^{-4} mg L^{-1} E2 controls was set at 100%. Cells co-treated with hydroxytamoxifen [3.88×10^{-9} mg L^{-1}] exhibited a 71% reduction in β-galactosidase signal. The following concentrations of the test compounds were used in the YES assay: (TBB) 5×10^{-10}, 5×10^{-8}, 5×10^{-6}, 5×10^{-4}, 5×10^{-3}, 5×10^{-2}, 5×10^{-01} mg L^{-1},

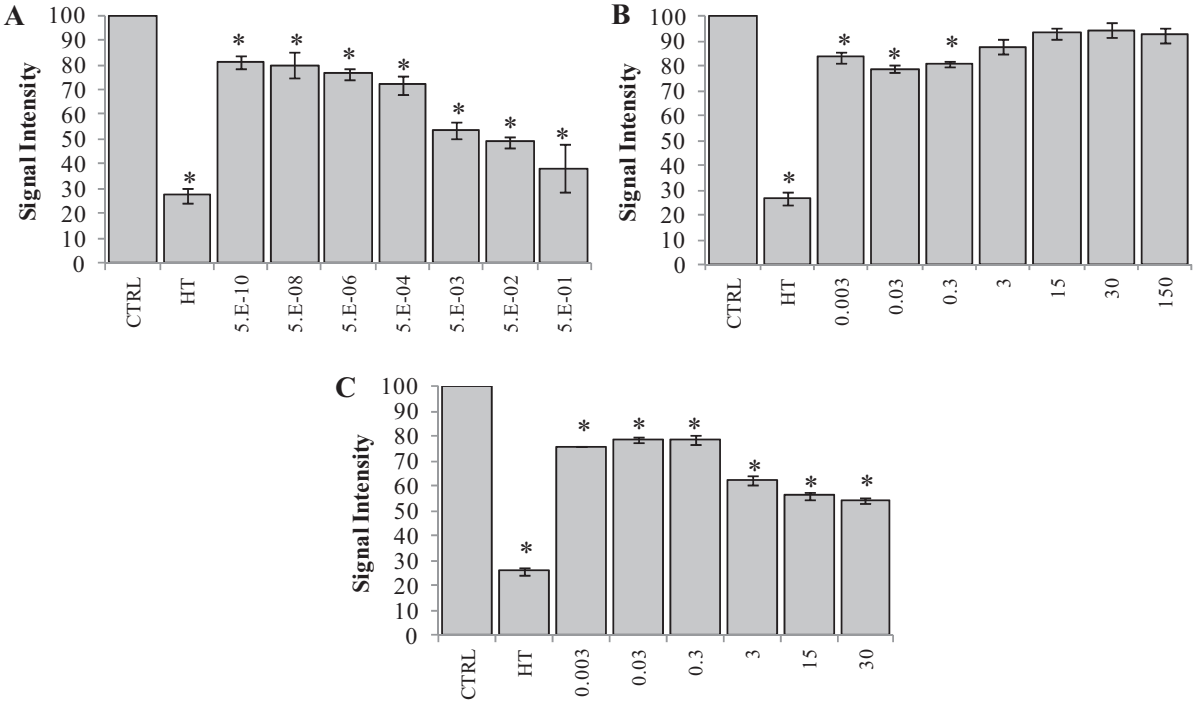

Fig. 3. Antiestrogenic potency of (A) TBB, (B) TBPH, and (C) TBCO in mg L^{-1} measured by the yeast estrogen screen. Antiestrogenic potency is presented as the reduction in signal intensity (mean ± SE) compared to E2 activated control cells (CTRL). 4-Hydroxytamoxifen (HT) acted as a positive control. Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than activated controls are indicated by asterisks (*$p < 0.05$).

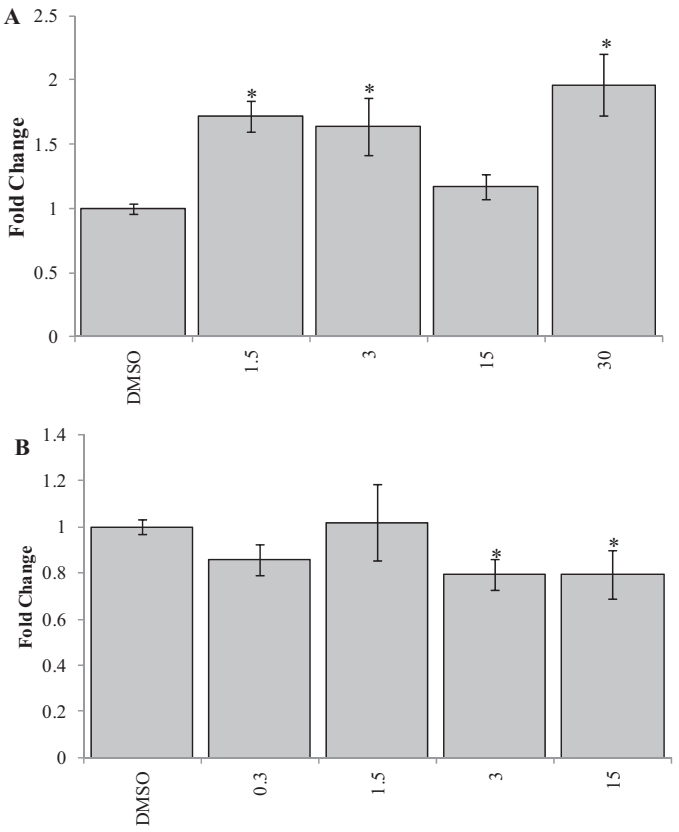

Fig. 4. Effects of (A) TBPH and (B) TBCO exposures on relative concentrations of testosterone hormone measured in the H295R cell assay. Four concentrations (mg L^{-1}) of TBPH and TBCO were tested and data are given as relative fold change in hormone production (mean ± SE) compared to solvent controls (DMSO). Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than solvent controls are indicated by asterisks ($^*p < 0.05$).

(TBPH) 3×10^{-3}, 3×10^{-2}, 0.3, 3, 15, 30, 150 mg L^{-1}, and (TBCO) 3×10^{-3}, 3×10^{-2}, 0.3, 3, 15, 30 mg L^{-1}. Each NBFR resulted in statistically significant inhibition of receptor mediated β-galactosidase production. Of the three NBFRs, TBB resulted in the greatest reduction of β-galactosidase production while responding in a dose-dependent manner. At 0.5 mg L^{-1} TBB exposures resulted in a maximal antiestrogenic response of 62% compared to the E2 control (Fig. 3A). TBPH and TBCO exposures resulted in maximal antiestrogenic responses of 21% and 46% at concentrations of 3×10^{-2} mg L^{-1} and 30 mg L^{-1}, respectively compared to E2 controls (Fig. 3B and C). TBPH exposures resulted in a reverse dose response trend where the lesser exposure concentrations resulted in the greatest inhibition.

3.5. Effects of NBFRs on testosterone synthesis

Only two of three NBFRs, TBPH and TBCO significantly affected production of testosterone in conditioned media compared to solvent controls in the H295R cellular assay. The maximal exposure concentration of TBPH, 30 mg L^{-1}, resulted in a moderate1.96-fold increase in concentrations of T compared to controls (Fig. 4A). Across four exposure concentrations TBPH exposures produced a range of 1.17–1.96-fold increase indicating limited dose-responsive behavior. At doses of 3 mg L^{-1} and 15 mg L^{-1} TBCO exposures resulted in slightly lesser concentrations of T compared to solvent controls. At 15 mg L^{-1} TBCO, the concentration of T was 0.79-fold lesser compared to solvent controls (Fig. 4B), while exposures of 0.3 and 1.5 mg L^{-1} produced no significant differences from solvent controls.

3.6. Effects of NBFRs on E2 synthesis

At all exposure doses, the three NBFRs elicited significant increases in concentrations of E2 in conditioned media compared to solvent controls. TBB exposed cells responded at a maximum of 2.82-fold change compared to solvent controls (Fig. 5A). TBPH exposure resulted in the greatest increase of concentrations of E2 eliciting a maximal response of 5.29-fold change compared to solvent controls (Fig. 5B). At 15 mg L^{-1}, TBCO elicited a maximal response of 3.29-fold change compared to solvent controls (Fig. 5C).

4. Discussion

The three NBFRs TBB, TBPH, and TBCO are components of several flame retardant technical mixtures and have been discovered in numerous environmental and biotic samples. TBPH is a brominated analog of the phthalate plasticizer DEHP, which has several associated toxicities including endocrine disruption, AhR agonism, and developmental and reproductive toxicities (Foster et al., 2001; Lyche et al., 2009). There are yet few published reports of toxicities of TBB or TBCO. Our investigation elucidated the potential biological effects with respect to the endocrine disrupting and TCDD-like properties of the three NBFRs.

Dosing concentrations of the three NBFRs were based on pilot data regarding cytotoxicity and solubility in media, which was previously generated by the authors. In this study, antagonism was defined as a dose dependent inhibitory effect that was comparable in magnitude to the inhibitory controls, hydroxyflutamide or 4-hydroxytamoxifen. Compounds that did not meet these criteria but demonstrated significant inhibitory effects were deemed potential weak antagonists. Controls for recovery of activation signals with exposures to TBB, TBPH, and TBCO showed recoveries of activation responses with the addition of three concentrations of DHT: 2.90×10^{-4}, 1.45×10^{-3}, and 2.90×10^{-3} mg L^{-1} (YAS) and E2: 2.72×10^{-4}, 8.17×10^{-4}, and 2.72×10^{-3} mg L^{-1}(YES) (Supplemental Figs. 1 and 2).

4.1. TCDD-like effects

The three NBFRs TBB, TBPH, and TBCO did not result in any TCDD-like effects at tested concentrations. DEHP has previously been described to elicite weak agonistic AhR activity (Kruger et al., 2008; Mankidy et al., 2013). Discrepancies between the TCDD-like potencies of TBPH and its structural analog DEHP are likely due to the bromine atoms at the 2, 3, 4, 5 positions. The bromine atoms increase steric hindrance and hydrophobicity which change the physico-chemical characteristics of the compound resulting in differential interaction with the AhR. To our knowledge this is the first investigation of the TCDD-like effects of TBB, TBPH, or TBCO.

4.2. (Anti) androgenic effects

DEHP is a known endocrine disruptor with several toxic effects that can act *via* antiandrogenic mechanisms (Lyche et al., 2009). It has been previously detailed that the antiandrogenic toxicity of DEHP is moderated through its mono ester metabolite MEHP (Kessler et al., 2012). Several studies have shown that MEHP exerts little affinity for the androgen receptor and does not produce androgen receptor mediated effects (Piché et al., 2012). MEHP likely exerts its antiandrogenic effects by blocking activities of enzymes of the steroidogenic pathway and through the inhibition of cholesterol transportation (Piché et al., 2012). Unlike its

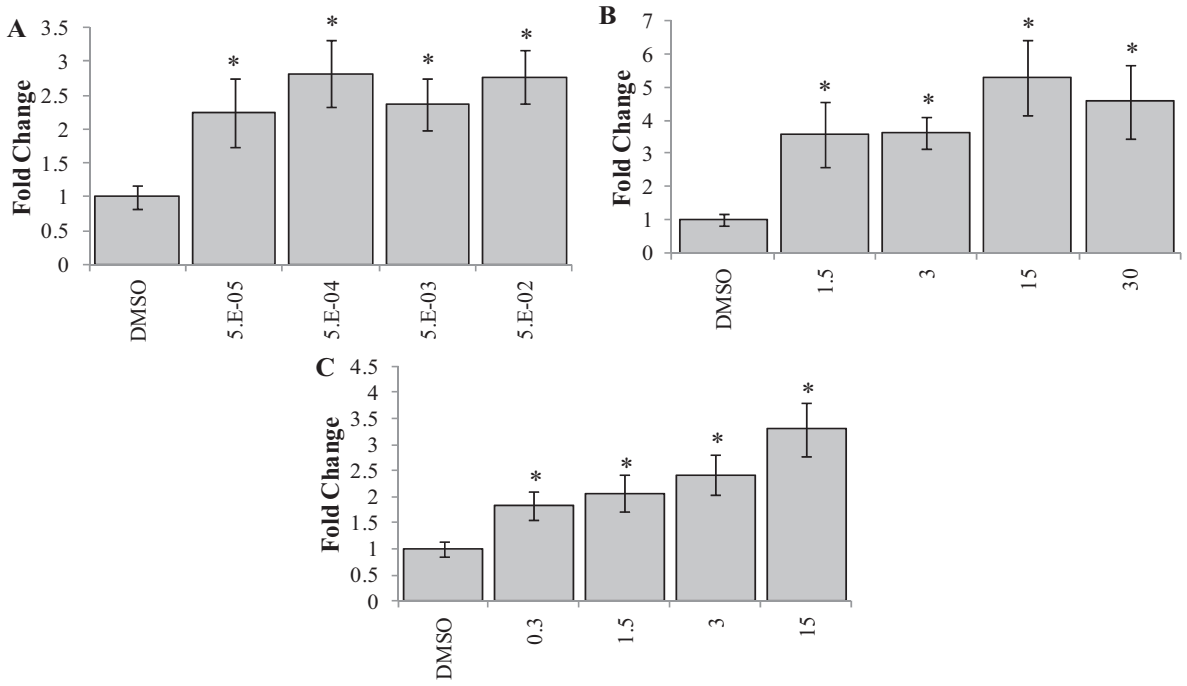

Fig. 5. Effects of (A) TBB, (B) TBPH, and (C) TBCO exposures on relative 17-β-estradiol hormone concentrations measured in the H295R cell assay. Four concentrations (mg L^{-1}) of each NBFR were tested and data are given as relative fold change in hormone production (mean ± SE) compared to solvent controls (DMSO). Each assay contained four wells per NBFR exposure concentration and a total of four assays were used for analysis. Exposure concentrations that resulted in effects that were significantly different than solvent controls are indicated by asterisks ($^*p < 0.05$).

mono ester metabolite, *in vitro* androgenic screening of DEHP has demonstrated that the unmetabolized phthalate might bind to the androgen and estrogen receptors (David, 2006; Piché et al., 2012). TBPH demonstrated no agonistic effects in the YAS assay (data not shown), but produced significant antiandrogenic effects. Contrary to previous studies (Ezechiáš et al., 2012) TBPH produced antagonistic effects greater than hydroxyflutamide, and responded in a dose dependant trend (Fig. 2B). Differences from previous *in vitro* investigations can be attributed to differences in exposure doses in the yeast system. Previous investigations which have used mammalian cellular assays have demonstrated the inability of DEHP to bind with the androgen receptor, though this might be due to rapid biotransformation of DEHP to its metabolite MEHP. Yeast cells might have different mechanisms and/or rates of metabolism of DEHP than mammalian cells, which might help to explain the observed antagonistic effects of DEHPs brominated analog, TBPH.

The results presented here represent some of the first data regarding potential androgenic effects of TBB and TBCO. The weak antagonistic response of TBB might be due to limitations in dosing concentrations which were restricted by the concentrations of the stock solutions and cytotoxicity. TBCO can be characterized as an androgen receptor antagonist; the compound responded in a dose-dependent manner and had a significantly greater antagonistic response than hydroxyflutamide at $2.92 \times 10^{-8} \times \text{mg} \times \text{L}^{-1}$ (Fig. 2C). Further exposure and investigations of mechanisms are required to confirm the potential antiandrogenic effects of TBCO.

4.3. (Anti) estrogenic effects

By use of the aforementioned characteristics of an antagonist, the three NBFRs can be classified as weak estrogen antagonists. The antagonistic effects of the three compounds indicated weak antagonism, while only TBB and TBPH responded in dose-dependent trends (Fig. 3A–C). Contrary to previous *in vitro* investigations in which no antagonistic effects were observed (Ezechiáš et al., 2012), TBPH exposures resulted in weak antagonistic effects. The discrepancies between these data and previous investigations might be due to differences in exposure concentrations. The rationale of the reverse TBPH dose-response is unknown, though initial cytotoxicity experiments showed no significant increase in cellular cytotoxicity at greater concentrations. The weak antagonistic effects of TBPH indicate that TBPH differs from DEHP in its interaction with the estrogen receptor.

The results of this investigation are the first to indicate the potential for antagonism of TBB and TBCO with the ER. Further *in vitro* investigations and *in vivo* assays are required to elucidate the potential mechanisms of toxicity and gauge potential organismal effects.

4.4. Effects on testosterone production in the H295R steroidogenesis assay

TBB did not demonstrate statistically significant changes in concentrations of T (data not shown) at any of the tested concentrations. TBPH is a structural analog to the plasticizer DEHP which is ubiquitously found in the environment and causes several toxic effects including male reproductive abnormalities in animal models (Lyche et al., 2009). It is hypothesized that several of the toxic effects of DEHP are mediated through interactions and disruption of endocrine homeostasis (Lyche et al., 2009; Piché et al., 2012). The results of TBPH exposures, though significantly different than controls, represent a weak increase in concentrations of T (Fig. 4A). These results are contrary to existing data for DEHP (Mankidy et al., 2013; Parks et al., 2000; Piché et al., 2012) and might be attributed to the bromine atoms attached to the phthalate moiety, differences in exposure concentrations, or differences in the cellular physiology of the assay system. For example the observed reductions in concentrations of testosterone in DEHP exposed cells are partially moderated through the activation of the PPARα (peroxisome proliferator activated receptor) nuclear receptors. Activation of PPARα *via* exposure to DEHP has been linked to decreases in concentrations of T. Experimentation with PPARα null mice resulted in

lesser reductions of concentrations of testosterone than in their wild-type counterparts (Corton and Lapinskas, 2005; Gazouli et al., 2002). Though PPARα affects the concentration of T *in vivo* and *in vitro*, PPARs in general have differential tissue and species specific expression patterns (Rusyn et al., 2006). For example, DEHP has demonstrated limited effects on the liver in humans, which include hepatic tumors, due to the limited expression, and/or truncated or mutant variations of PPARα (Boelsterli, 2007; Rusyn et al., 2006). These differences between *in vitro* experimentation and cellular physiologies might account for differences in results.

Exposure of H295R cells to TBCO resulted in a statistically significant decrease in concentrations of T at the two greatest concentrations (Fig. 4B). Similar to the TBPH exposures, TBCO elicited a weak response in the H295R system. This is the first data regarding the potential androgen disrupting effects of TBCO. From this preliminary data, further investigations into TBCO's endocrine disrupting potentials are warranted.

4.5. Effects on estrogen production in the H295R steroidogenesis assay

The three NBFRs significantly increased synthesis of E2 in the H295R system. TBPH exposures resulted in the greatest increase of concentrations of E2 (Fig. 5B), though of the three compounds only TBCO responded as a dose-dependent trend (Fig. 5C). These results for TBPH exposures are in accordance with previous *in vitro* exposures of DEHP which demonstrated the compounds potential endocrine disrupting effects (Hokanson et al., 2006; Mankidy et al., 2013).

A greater understanding of the effects/mechanisms of the three NBFRs can be achieved in the comparison of the two specific assay systems, the YES and H295R. The YES system represent a receptor mediated endpoint that is relegated to those elements that have been transfected into the cells, specifically the human estrogen receptor (hER) (Routledge and Sumpter, 1996), while the H295R cellular system inherently expresses the complete biosynthetic pathway of E2. The data from the YES assay shows that the NBFRs do not interact with the estrogen receptor in an agonistic fashion; a hypothesis for TBPH that is supported by investigations into toxicities of DEHP (Piché et al., 2012). While data from the H295R assays suggests that the three compounds target the biosynthetic pathway of E2. Indeed MEHP, the metabolite of DEHP is known to affect aromatase, a major enzyme in E2 synthesis (Hokanson et al., 2006).

Due to the analogous structures of TBPH and DEHP, many of the limited toxicological investigations currently focus on potential androgenic disruption. To our knowledge the results from the YES and H295R assays represent some of the first data regarding potential estrogen specific mechanisms of endocrine disruption of TBB, TBPH, and TBCO in an *in vitro* system.

Conflict of interest

The authors declare that there were no conflicts of interest in production of this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2013.09.009.

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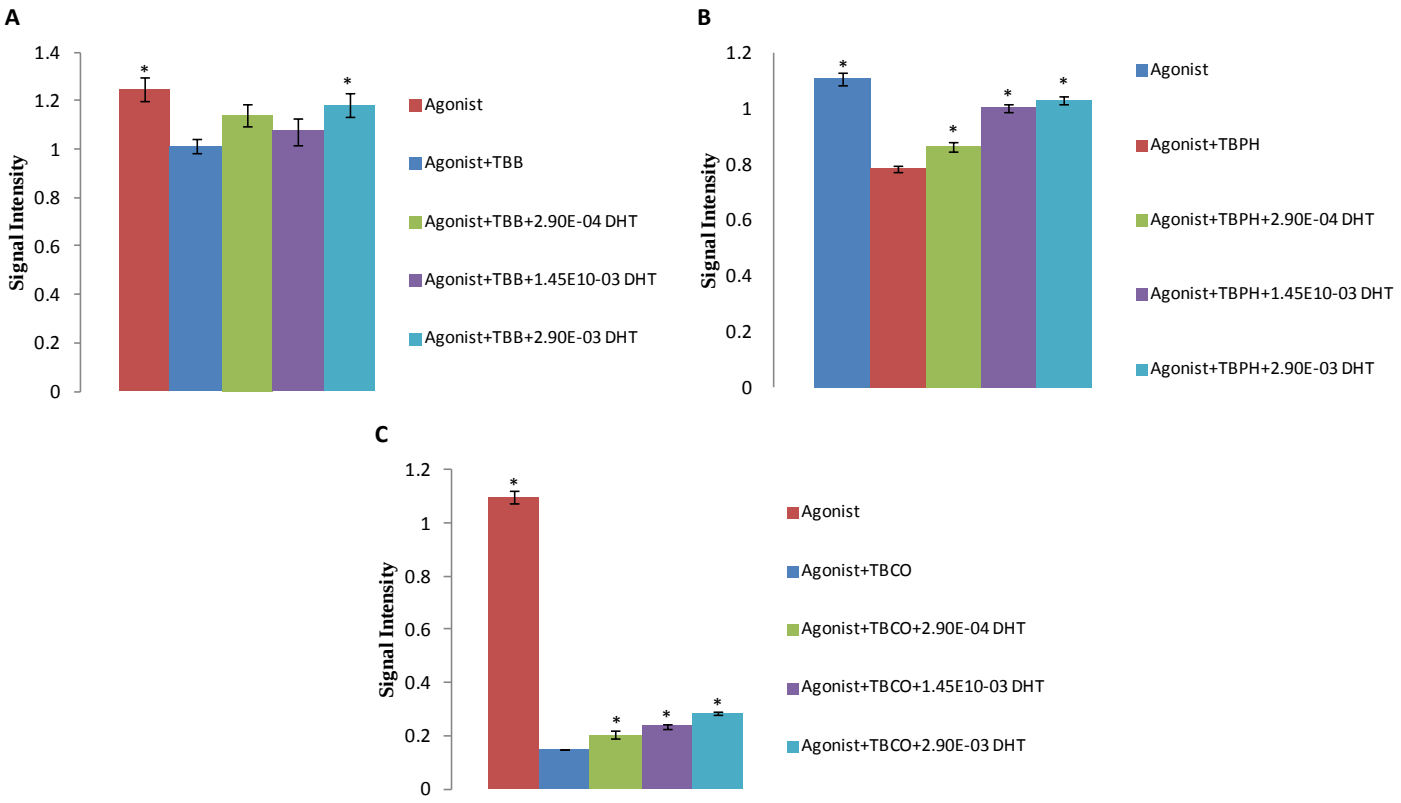
Supplemental Table 1. Physiochemical properties of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), *Bis*(2-ethylhexyl)-2,3,4,5-tetrabromophtalate (TBPH), and 1,2,5,6-tetrabromocyclooctane (TBCO)

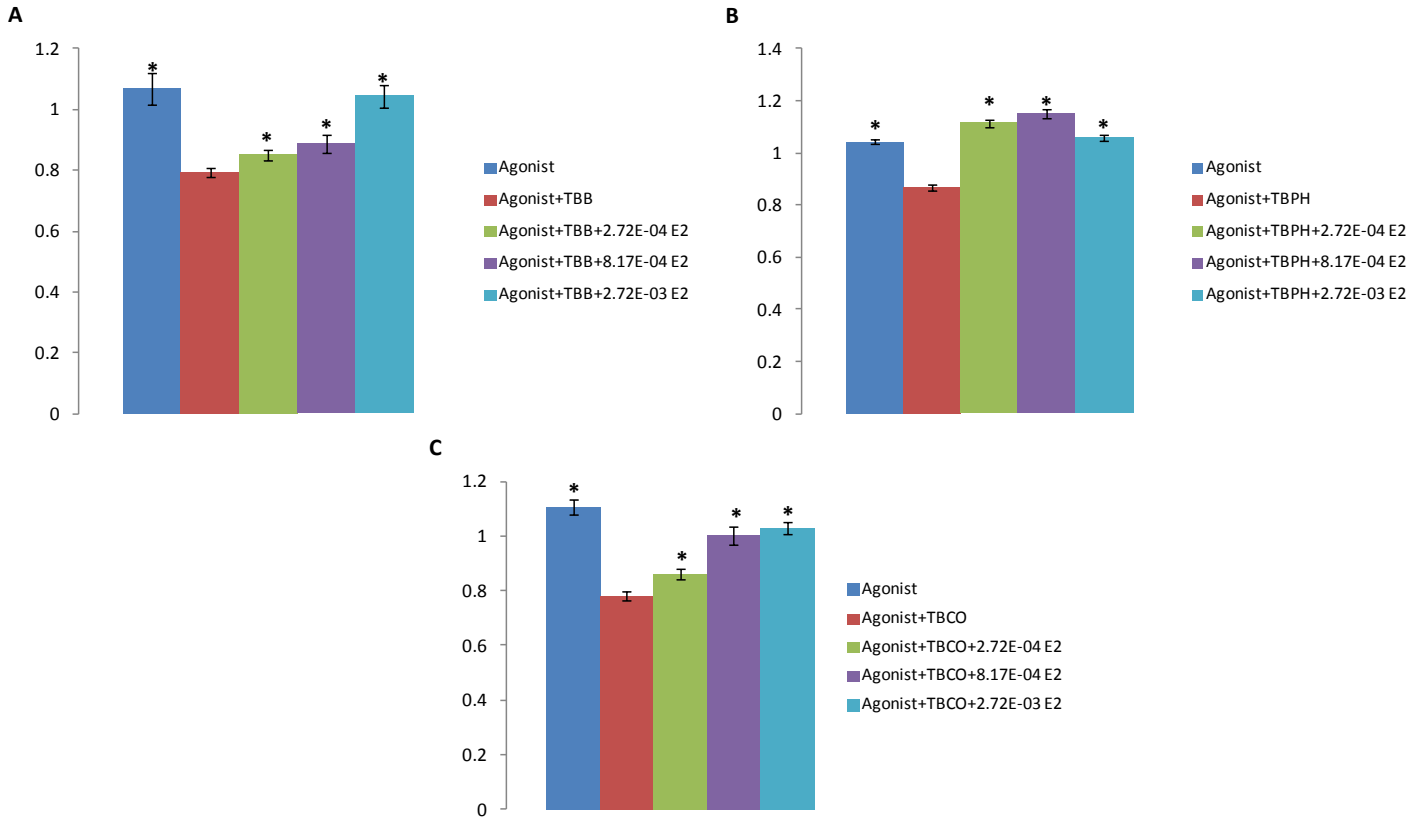
Chemical Name	TBB	TBPH	TBCO
Molecular Weight	549.93	706.15	427.80
Solubility (mg/L)	1.14×10^{-5} [c]	1.98×10^{-9} [b]	0.06915[b]
Log K_{OW}	8.8[c]	11.95[a]	5.24[a]

[a] Estimated from: KowWIN v1.68 (U.S. EPA)
[b] Estimated from: WSKow v1.42 (U.S. EPA)
[c] Bearr et al., 2010



Supplemental Fig 1. The control for recovery of signal activity of (A) TBB at $5x10^{-01}$ mg L^{-1}, (B) TBPH at 1000 mg L^{-1}, and (C) TBCO at 300 mg L^{-1} measured by the yeast androgen screen (YAS). A baseline agonist (DHT) concentration of $1.45x\ 10^{-3}$ mg L^{-1} was added to each well with increasing concentrations added to demonstrate the recovery of signal activity. Activity is presented as mean± SE. Each assay contained four wells per NBFR exposure concentration. Exposures that resulted in effects that were significantly different than inhibition controls (agonist+NBFR) are indicated by asterisks(*$p<0.05$).

***** The magnitude of inhibition in recovery experiments is different than that reported in the research article (Fig. 2) due to changes in yeast growth media



Supplemental Fig 2. The control for recovery of signal activity of (A) TBB at $5x10^{-01}$ mg L^{-1}, (B) TBPH at 0.03 mg L^{-1}, and (C) TBCO at 30 mg L^{-1} measured by the yeast estrogen screen (YES). A baseline agonist (E2) concentration of $8.17x\ 10^{-4}$ mg L^{-1} was added to each well with increasing concentrations added to demonstrate the recovery of signal activity. Activity is presented as mean± SE. Each assay contained four wells per NBFR exposure concentration. Exposures that resulted in effects that were significantly different than inhibition controls (agonist+NBFR) are indicated by asterisks(*$p<0.05$).

***** The magnitude of inhibition in recovery experiments is different than that reported in the research article (Fig. 3) due to changes in yeast growth media