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Physiological Effects of 3 Non-PBDE Brominated Flame Retardants on *Pimephales promelas* (Fathead Minnow) Exposed in Outdoor Mesocosms

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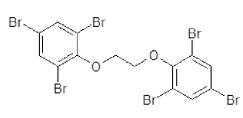
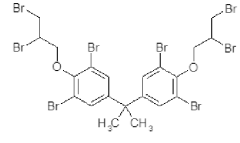
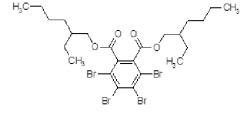
Abstract—Brominated flame retardants (BFRs) such as the polybrominated diphenyl ethers (PBDEs) are being phased out due to ecotoxicological and human health concerns. Potential substitutes 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), tetrabromobisphenol A bis(2,3-dibromopropyl ether) (TBBPA-DBPE), and bis(2-ethylhexyl)tetrabromophthalate (BEHTBP) have been identified as potential replacements for PBDEs, but a better understanding of their environmental fate and toxicological effects are needed. To accomplish this, outdoor mesocosms were treated with BTBPE, TBBPA-DBPE, and BEHTBP at concentrations similar to those currently observed in the natural environment. Caged fathead minnow (*Pimephales promelas*) were added to the mesocosms for an exposure period of 42 days followed by 28 days of depuration. Tissue and serum samples were taken to measure physical and oxidative stress, and reproductive endpoints and accumulation of the BFRs. The fathead minnows accumulated BTBPE and TBBPA-DBPE, however there were limited changes in the measured physical, biochemical and reproductive parameters.

Keywords: brominated flame retardants, fathead minnow, BTBPE, TBBPA-DBPE, BZ-54, vitellogenin, mesocosms

INTRODUCTION

One of the most well studied groups of brominated flame retardants (BFRs) are the polybrominated diphenyl ethers (PBDEs). PBDEs are also persistent, bioaccumulative and toxic (United Nations Environment Programme, 2006). As a result of these properties, strict restrictions and bans have been placed on the use of PBDEs, leading to increased demand for non-PBDE BFRs (United States Environmental Protection Agency, 2005). Several BFRs have been identified as suitable replacement compounds, including 1,2,-bis(2,4,6-

Table 1. Physical and chemical properties of test compounds.

	Compound		
	BTBPE	TBBPA-DBPE	BEHTBP
Structure			
Molecular formula	$C_{14}H_8Br_6O_2$	$C_{21}H_{20}Br_8O_2$	$C_{24}H_{34}Br_4O_4$
Molecular weight	687.64	943.62	706.15
Vapour pressure (Pa, 25°C)	3.17×10^{-8}	8.48×10^{-13}	2.28×10^{-9}
Water solubility*	6.55×10^{-7}	1.16×10^{-10}	1.98×10^{-9}
Log Kow*	9.15	11.52	11.95
Log octanol-air partition*	15.67	20.30	16.86

*Parameters were estimated using USEPA's EpiSuite Ver. 4.10 (EPA 2011).

tribromophenoxy)ethane (BTBPE), tetrabromobisphenol A bis(2,3-dibromopropyl ether) (TBBPA-DBPE), and bis(2-ethylhexyl)tetrabromophthalate (BEHTBP), a component of the commercial BZ-54 (Great Lakes Chemical Corporation) mixture. There is limited information available for these emerging BFRs regarding their environmental distribution and effects, and the potential for exposure of humans. Given that these non-PBDE flame retardants share properties similar to those of PBDEs, their environmental fate may be expected to be similar to certain mixtures of PBDE (Stapleton *et al*., 2008). This lack of knowledge, combined with recent identification of these specific compounds in environmental samples (Gauthier *et al*., 2007; Stapleton *et al*., 2008) warrants further investigation into their environmental fate and toxicity. BTBPE, TBBPA-DBPE, and BEHTBP were chosen for study based upon their recent detection in environmental media.

Studies have shown that BFRs are capable of bioaccumulating and biomagnifying in fish (Kuo *et al*., 2010), and can elicit responses in fish that include disruption to the endocrine system (Chou *et al*., 2010), reproductive system (Kuiper *et al*., 2007) and increased oxidative stress (Kling *et al*., 2008). The objectives of this study were to determine the bioavailability and physiological impacts of these replacement compounds, under semi-natural conditions to fathead minnow (*Pimephales promelas*).

MATERIALS AND METHODS

Compounds

Analytical standards were provided by Wellington Laboratories (Guelph, Ontario, Canada). The physical and chemical properties of the test compounds are presented in Table 1.

Mesocosm setup

The University of Guelph Microcosm Facility (ON, Canada) consists of 30 artificial ponds of approximately 12,000 L. The mesocosms have a depth of 1.2 m and a diameter of 3.9 m, and are filled with water to a depth of approximately 1 m. The water supply for the mesocosms is an irrigation pond ($62 \times 62 \times 4$ m deep) supplied by a well located on site. Trays containing soil were added to each mesocosm such that ~50% of the bottom was covered. Prior to treatment water was circulated between the irrigation pond and the mesocosms for three weeks. Circulation was discontinued one week prior to treatment.

Sampling and experimental design

Twelve mesocosms received four treatments ($n = 3$); solvent control, BTBPE, TBBPA-DBPE and BZ-54 (a commercial flame retardant containing BEHTBP). Each treatment was applied as a single subsurface injection of the compound dissolved in 125 mL of DMSO, designed to give a nominal concentration of 500 ng (compound)/g (sediment) in the upper 5 cm of sediment. Water quality parameters were measured regularly and were within acceptable ranges for fish

Table 2. The physical and chemical parameters of the mesocosms throughout the exposure period (July 2nd to August 13th, 2008). Values presented are the mean of the measurement for the three replicates of each respective treatment and the associated standard deviation.

Treatment	Min temp. (°C) (n = 99)	Max temp. (°C) (n = 99)	pH (n = 30)	Dissolved oxygen (mg/L) (n = 99)	Alkalinity (mg/L of $CaCO_3$) (n = 12)	Hardness (mg/L of $CaCO_3$) (n = 12)	Conductivity (μS/cm) (n = 30)
Control	21 ± 1	24 ± 2	8.6 ± 0.5	14.1 ± 5.0	126 ± 34	261 ± 41	486.1 ± 106.9
BTBPE	21 ± 1	24 ± 1	8.6 ± 0.5	15.9 ± 4.9*	123 ± 29	261 ± 56	482.2 ± 93.4
TBBPA-DBPE	21 ± 1	25 ± 1	8.6 ± 0.5	14.1 ± 5.4	132 ± 21	263 ± 45	521.7 ± 90.4
BZ-54	21 ± 1	25 ± 1	8.7 ± 0.6	13.9 ± 5.5	127 ± 26	266 ± 38	502.1 ± 85.6

*Significant difference from control as calculated using Dunnett's test (α = 0.05).

health and there was no influence of treatment on any of the water quality parameters (Table 2).

Fish exposure and sampling

Fathead minnow (16 fish per mesocosm) were purchased from Silhanek Baitfish Farms (Bobcaygeon, ON, Canada) in July 2008 and acclimated for 10 days in the mesocosms prior to treatment. Minnows were held in hanging mesh enclosures, with a sediment filled plastic container at the bottom of each enclosure. Fish ($n = 3$) were randomly sampled at 7, 14, 28, 42 days, after which the remaining fish were transferred to control mesocosms and sampled during the depuration period (days 49 and 70). At each sampling day fish were anesthetized with tricaine methanesulfonate (MS-222, approximately 0.1 g/L). Measurements of fork length and total weight were taken and condition factor ($K = [\text{total wt}/\text{fork length}^3] \times 100$) was calculated. The minnows were bled following partial severance at the dorsal aorta with blood collected using a heparinized capillary tube. Blood was pooled by sex and treatment in 1 mg/mL heparin-washed 600 μL polypropylene vials until approximately 60 μL of blood was collected. Plasma was isolated by centrifugation at 3,000 g for 10 minutes and subsequently stored at –20°C. Liver and gonads were removed and weighed to calculate the liver somatic index (LSI = liver wt/[total body wt – liver wt]) and gonadal somatic index (GSI = gonad wt/[total body wt – gonad wt]). Liver and plasma were snap frozen in liquid nitrogen, and stored at –80°C until further analysis.

Biochemical measures

Fatty acyl-CoA oxidase activity (FAO)

The FAO activity-assay was used to quantify the production of the reactive oxygen species H_2O_2, which is specifically generated by an enzyme unique to peroxisomal β-oxidation, fatty acyl-CoA, using methods described previously by Oakes *et al.* (2003). Activity of fatty acyl-CoA oxidase was expressed both as nmoles H_2O_2/g liver and nmoles H_2O_2/min/mg protein.

Thiobarbituric acid reactive substances assay (TBARS)

The TBARS assay was used to quantify oxidative damage by measuring peroxidative damage to lipids that occurs with reactive oxygen species generation, in fish liver samples as previously described by Oakes and Van Der Kraak (2003). The concentration of lipid peroxides was expressed as nmols MDA per g tissue (wet wt).

Vitellogenin ELISA assay

The concentration of vitellogenin was measured using a fathead minnow ELISA assay developed at the University of Waterloo using the methods outlined in Parks *et al.* (1999).

Sex steroids

Circulating levels of gonadal sex steroids, 17β-estradiol (E2) and 11-ketotestosterone (11KT), were measured in plasma directly by radioimmunoassay (RIA) as described in McMaster *et al.* (1992).

Tissue residue analysis

The fathead minnows were extracted and processed in a trace organic contaminant cleanroom with a charcoal and HEPA filtered environment and a negative pressure foyer. Samples were extracted in 33 mL cells on a Dionex ASE 300 (Automated Solvent Extractor). Extracts were purified using gel permeation chromatography. Analyses were performed on a Agilent 6890 series GC system with a Restek 1614 15 m, 0.25 mmID, 0.10 μm column, coupled to an Agilent 5973 MS in negative CI mode monitoring for 79, 81, 357, 385, 463 and 470.

Statistics

Male, female and immature fish were analyzed separately. Effects of the exposure to the chemical for each endpoint, relative to the controls were analyzed using one way analysis of variance (ANOVA), followed by the Holm-Sidak pair-wise multiple comparison procedure. Data that did not meet the assumptions of normality and equal variance were log transformed. Differences were considered significant at $p < 0.05$. Given the small and unequal sample sizes, differences between the uptake and depuration period within treatments were compared using a two tailed unequal variance *t*-test (Welch-Satterthwaite test) as described by Ruxton (2006).

RESULTS

Fish BFR concentration

Concentrations of BTBPE and TBBPA-DBPE in the whole body extracts of fathead minnows ranged from 15 to 37,007 ng/g lipid and 5300 to 170,000 ng/g lipid, respectively (Fig. 1). There was a slight, non-significant decrease in concentration during the depuration period. BEHTBP was only measurable in the fish at day 7, after which brominated transformation products were detected (data not shown).

Physical responses

The condition factor of immature BZ-54 exposed fish was significantly altered compared to the control and BTBPE treatments during the uptake period (ANOVA, $p = 0.012$) but not for females (ANOVA, $p = 0.684$) or males (ANOVA, $p = 0.431$) (Table 3). The LSI was unaltered between treatments for all sexes and exposure periods, while the male fish from the control and BZ-54 treatments had a significantly larger LSI during the uptake compared to the depuration period. The GSI was unchanged between treatments for all sexes and exposure periods. Male fish from the control treatment and female fish from the BTBPE treatment showed a significant decrease in GSI during the depuration period.

Oxidative stress

Oxidative damage, as quantified by hepatic TBARS, was unchanged between

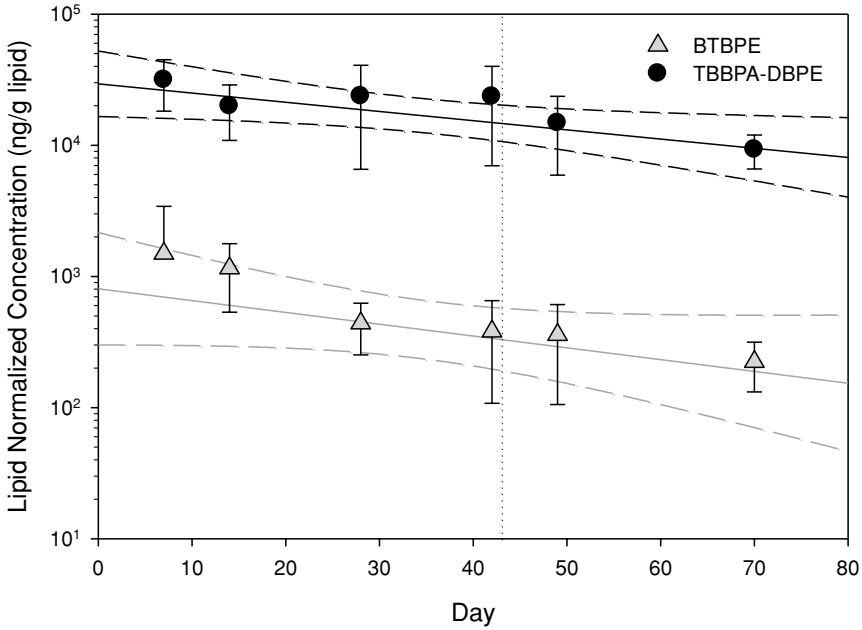


Fig. 1. Lipid normalized concentration of BTBPE (triangles) and TBBPA-DBPE (circles) in whole body fathead minnow extracts, each value is the mean of three fish and the standard deviation. The vertical dotted line separates the uptake period (days 0–42) and the depuration period (days 42–70).

treatments, but was significantly increased in BZ-54 exposed immature fish during the uptake period (Table 4). The FAO activity was not significantly altered between treatments for all sexes and exposure periods.

Reproductive responses

Concentrations of vitellogenin, E2 and 11-KT were not significantly altered by exposure to BFR in any sex (data not shown). However there was a large, but non-significant increase in concentrations of vitellogenin for each sex in the BTBPE treatment compared to the controls Fig. 2.

DISCUSSION

Concentrations of BTBPE and TBBPA-DBPE were elevated in the fathead minnows throughout the uptake and depuration period. The dissipation of BEHTBP after 14 days and the presence of several brominated transformation products suggest metabolism is occurring. Modest changes were observed in the physical endpoints; the condition factor in immature BZ-54 treated fish was significantly different from the control treatment during the uptake period. A pattern of

Table 3. Physical endpoints for male, female and immature fathead minnows during the uptake (day 0–42) and depuration periods (day 42–70). Values are mean ± standard deviation (*n*). Significant differences ($p < 0.05$) between the uptake and depuration period for each endpoint were detected using unequal variation *t*-test and are indicated by *, while differences between treatments were determined by Holm-Sidak pairwise comparisons and are indicated by capital letters; the absence of capital letters indicates no significant difference.

	Condition factor		Liver somatic index		Gonadal somatic index	
	Uptake	Depuration	Uptake	Depuration	Uptake	Depuration
Male						
Control	0.93 ± 0.1 (8)	0.95 ± 0.1 (6)	1.47 ± 0.4 (8)	1.07 ± 0.2 (6)*	1.00 ± 0.3 (4)	0.48 ± 0.3 (6)*
BTBPE	1.14 ± 0.4 (6)	0.93 ± 0.1 (4)	1.61 ± 0.9 (6)	0.96 ± 0.3 (4)	0.88 ± 0.6 (6)	0.45 ± 0.2 (3)
TBBPA-DBPE	1.02 ± 0.1 (7)	0.89 ± 0.1 (3)	1.90 ± 0.8 (7)	1.36 ± 0.4 (3)	0.62 ± 0.4 (5)	0.36 ± 0.1 (3)
BZ-54	1.00 ± 0.1 (11)	0.99 ± 0.1 (4)	2.16 ± 0.8 (11)	1.13 ± 0.1 (4)*	0.88 ± 0.6 (7)	0.29 ± 0.2 (4)
Female						
Control	0.94 ± 0.1 (7)	0.99 ± 0.1 (3)	1.60 ± 0.7 (7)	1.49 ± 0.0 (3)	2.48 ± 2.3 (7)	(0)
BTBPE	0.91 ± 0.3 (11)	0.85 ± 0.1 (9)	2.14 ± 1.4 (10)	0.87 ± 0.3 (9)*	4.59 ± 3.8 (11)	1.34 ± 0.4 (9)*
TBBPA-DBPE	0.96 ± 0.0 (4)	0.92 ± 0.1 (9)	1.64 ± 0.6 (4)	0.94 ± 0.7 (9)	2.10 ± 0.7 (4)	1.69 ± 1.1 (9)
BZ-54	0.97 (1)	0.83 ± 0.1 (3)	0.70 (1)	2.03 ± 1.9 (2)	1.21 (1)	0.83 ± 0.2 (3)
Immature						
Control	0.88 ± 0.1 (21)^{A}	0.90 ± 0.1 (9)	1.29 ± 0.4 (21)	1.03 ± 0.5 (9)	—	—
BTBPE	0.88 ± 0.1 (19)^{A}	0.90 ± 0.1 (4)	1.15 ± 0.5 (19)	1.06 ± 0.4 (4)	—	—
TBBPA-DBPE	0.95 ± 0.2 (19)^{AB}	(0)	1.25 ± 0.3 (19)	(0)	—	—
BZ-54	1.00 ± 0.1 (18)^{B}	0.93 ± 0.1 (5)	1.39 ± 0.6 (18)	1.12 ± 0.4 (5)	—	—

Table 4. Oxidative stress endpoints in male, female and immature fathead minnows during the uptake (day 0–42) and depuration periods (day 42–70). Values are mean ± standard deviation (n). Significant differences ($p < 0.05$) between the uptake and depuration period for each endpoint were detected using unequal variation t-test and are indicated by *, while differences between treatments were determined by Holm-Sidak pairwise comparisons and are indicated by capital letters; the absence of capital letters indicates no significant difference.

	TBARS (nmoles MDA/g liver)		FAO/Liver (nmoles H_2O_2/g liver)		FAO/Protein (nmoles H_2O_2/min/mg protein)	
	Uptake	Depuration	Uptake	Depuration	Uptake	Depuration
Male						
Control	382.58 ± 374.9 (7)	481.71 ± 363.3 (6)	22.26 ± 16.5 (5)	53.18 ± 51.7 (6)	888.28 ± 950.9 (5)	1540.49 ± 1653.5 (6)
BTBPE	713.99 ± 885.8 (6)	1826.68 ± 1907.9 (7)	22.98 ± 24.5 (5)	971.84 ± 1101.7 (5)	670.69 ± 602.0 (5)	9732.21 ± 11920.5 (3)
TBBPA-DBPE	706.19 ± 1006.6 (7)	96.51 ± 16.4 (3)	106.38 ± 82.4 (7)	253.12 ± 201.0 (3)	4388.12 ± 4608.3 (7)	3671.53 ± 1247.5 (3)
BZ-54	949.51 ± 1130.3 (10)	133.56 (1)	43.85 ± 61.4 (12)	461.87 ± 731.6 (4)	1335.51 ± 1412.0 (12)	7488.84 ± 13642.0 (4)
Female						
Control	152.30 ± 54.7 (7)	103.97 ± 57.0 (3)	47.78 ± 52.4 (7)	61.49 ± 75.8 (2)	804.94 ± 695.4 (7)	2125.57 ± 2815.1 (2)
BTBPE	286.54 ± 701.4 (11)	542.44 ± 1021.9 (7)	42.60 ± 54.1 (10)	211.40 ± 290.5 (6)	1455.82 ± 2980.7 (10)	2530.75 ± 3382.7 (6)
TBBPA-DBPE	154.62 ± 123.3 (4)	640.65 ± 883.3 (4)	124.16 ± 145.3 (3)	428.35 ± 387.7 (3)	1574.97 ± 406.3 (3)	10040.79 ± 9350.4 (3)
BZ-54	4088.80 (1)	1528.91 ± 347.3 (3)	(0)	241.34 (1)	(0)	3893.65 (1)
Immature						
Control	312.18 ± 521.2 (21)	131.35 ± 44.5 (8)	52.30 ± 72.2 (16)	47.29 ± 43.5 (7)	1968.59 ± 3786.8 (16)	1390.69 ± 2378.2 (7)
BTBPE	292.98 ± 582.6 (17)	549.60 ± 683.1 (7)	135.95 ± 294.1 (14)	99.73 ± 89.6 (4)	1389.36 ± 2464.2 (14)	2209.11 ± 2478.9 (4)
TBBPA-DBPE	1142.68 ± 1277.4 (18)	(0)	60.00 ± 74.5 (13)	(0)	1537.77 ± 2249.1 (13)	(0)
BZ-54	1036.44 ± 1256.1 (19)	137.60 ± 5.2 (2)*	99.47 ± 122.1 (16)	100.41 (1)	2021.73 ± 2591.0 (16)	1318.89 (1)

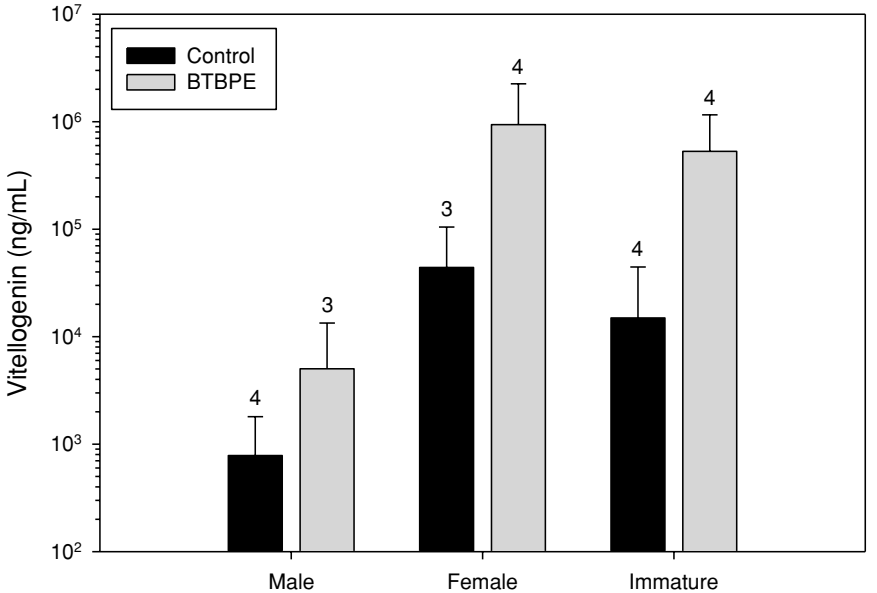


Fig. 2. Concentration of vitellogenin in fish from the control and BTBPE treatments during the uptake phase. The numbers above the bars are the number of fish pooled for that sample.

reduced gonadal development associated with greater energy storage in liver and body tissue has been documented in wild fish populations exposed to environmental toxicants (Wartman *et al*., 2009), but changes in the condition factor, LSI and GSI were not consistently changed with respect to treatment or sex in our study. Reduction in circulating sex steroid hormones is a generalized response to a number of potential toxicants and corresponds with decreased gonad size. Our study did not find any correlation with changes in sex steroid concentration and gonad size; however the small sample size in this study limited the ability to detect significant trends in hormone production.

Male fish do not normally have measurable quantities of circulating vitellogenin, however their livers are capable of synthesizing vitellogenin in response to stimulation with exogenous estrogens. Due to their structural properties, some BFRs have been shown to have estrogenic properties (Kovarich *et al*., 2011). While not statistically significant, the elevated concentration of vitellogenin in BTBPE exposed fathead minnows could have ecological implications (Kidd *et al*., 2007).

Oxidative stress, as quantified by the TBARS assay, was significantly increased in immature fish exposed to BZ-54 during the uptake phase. Male and female fish from the BZ-54 treatment also had increased oxidative damage during the uptake phase, but due to the small sample size ($n = 1$), significant comparisons could not be made. In a fathead minnow feeding study, Bearr *et al*. (2010) found

dietary exposure to BZ-54 significantly increased DNA damage in liver cells. The authors suggest that oxidative stress, as the result of peroxisome proliferator-activated receptor activation, could be responsible for the observed DNA damage. Our study demonstrates that BZ-54 is capable of inducing oxidative stress in fathead minnows.

This study demonstrates that these BFRs are bioavailable to fathead minnows under semi-natural conditions, and in the case of BTBPE and TBBPA-DBPE they appear to be persistent in fish. While there was significant uptake of these BFRs, the apparent physiological effects were limited, in part due to the small sample sizes and limited volumes of plasma which may have contributed to difficulty in detecting significant differences. The increased oxidative stress and vitellogenin induction in response to the exposure to environmentally relevant concentrations of these BFRs warrants further investigation.

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