Bioaccumulation, maternal transfer and elimination of polybrominated diphenyl ethers in wild frogs

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ABSTRACT
To investigate bioaccumulation, maternal transfer and elimination of polybrominated diphenyl ethers (PBDEs) in amphibians, we collected adult frogs (Rana limnocharis) from a rice field in an e-waste recycling site in China. We found that ∑PBDEs in the whole frogs and various tissues (brain, liver, testis and egg) ranged from 17.10 to 141.11 ng g⁻¹ wet weight. Various tissues exhibited a similar PBDE congener profile, which was characterized by intermediate brominated congeners (BDE-99 and BDE-153) as the largest contributors, with less lower brominated congeners (BDE-28 and BDE-47) and higher brominated congeners (BDE-209). The maternal transfer capacity of PBDEs declined with the increase in bromine numbers of PBDE congeners. We suggest that the bromine atom number (the molecular size, to some degree) might be a determining factor for the maternal transport of a PBDE congener rather than K ow (Octanol–Water partition coefficient), which expresses a compound’s lipophilicity. ∑PBDEs concentrations in frogs decreased over time during a depuration period of 54 days when these wild frogs were brought to the lab from the e-waste recycling site. The half-life of ∑PBDEs was 35 days, with about 14 days for BDE-47, and 36 and 81 days for BDE-99 and BDE-153, respectively. The data shows that the elimination of PBDEs has no essential difference from aquatic and terrestrial species.

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1. Introduction
Polybrominated diphenyl ethers (PBDEs) are wildly used in plastics, textiles, electronic appliances, building materials, etc., because of their flame retarding and hence fire safety properties. As additives, PBDEs can easily escape from the polymers they are added to, due to weak bounding to the polymers, during the processing, using and recycling process (Alaee et al., 2003; Hale et al., 2006). Because of their persistence, long distance transportation, and bioaccumulation potential, PBDEs have become ubiquitous in the environment (Hale et al., 2003). PBDEs can be bioaccumulated in animals and humans due to their high hydrophobicity. Toxicological studies have shown that prolonged exposure to PBDEs could lead to serious health consequences, such as thyroidogenic, estrogenic, and hepatic effects as well as neurodevelopmental disorders (Darnerud et al., 2001; McDonald, 2002; Costa and Giordano, 2007).

The bioaccumulation, transformation and elimination of PBDEs in animals are important for predicting chemical residues in organisms, developing environmental quality criteria and standards for protection of human health, and assessing the ecological risks of these chemicals. Currently, most studies concerning PBDEs in animals are related to aquatic and terrestrial wildlife. However, the information about the presence of PBDEs in amphibians is limited, although amphibians could be used as bioindicators of environmental stress for their unique physiological characteristics and life habit (ter Schure et al., 2002; Wu et al., 2009), ter Schure et al. (2002) reported for the first time the presence of PBDEs in wild frogs, which were collected from Sweden. Recently, Wu et al. (2009) investigated the levels, profiles and biotransfer of PBDEs in wild frogs from an e-waste recycling site in China. To our knowledge, the above two studies are only two reports concerning PBDEs in amphibians. Considering limited data available, the further effort is needed to study the presence of PBDEs in amphibians, especially concerning bioaccumulation in potential target tissues, elimination, and the difference between amphibians and aquatic and terrestrial wildlife.

Taizhou, an area of Zhejiang Province, is one of the largest e-waste recycling areas in China. In this area, there are many villages heavily involved in e-waste recycling. The e-waste recycling...
activities in these regions have led to the release of PBDEs into the surrounding environment, and Taizhou has become a PBDE-polluted area (Zhao et al., 2009). Previous studies reported high concentration levels of PBDEs in atmosphere, soils, sediments and biotic samples from this area (Liang et al., 2008; Yang et al., 2008, 2009; Han et al., 2009; Qin et al., 2009). Thus, this area provided an appropriate locale for investigating behaviors of PBDEs in wild amphibians.

In the present study, therefore, we examined the bioaccumulation and distribution of PBDEs in the whole frogs and target tissues for potentially toxicity, such as the liver, testis and brain. To assess the potential risks to frog reproduction, we also evaluated the maternal transfer of PBDEs in frogs. To fill up the gap of the elimination of PBDEs in amphibian, we examined the change of PBDE level in the whole frogs during a depuration period and calculated the apparent half-lives of PBDEs.

2. Materials and methods

2.1. Chemicals

BDE-71, 13C-labeled BDE-209 used as surrogate standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). A standard solution of PBDE congeners (EO-5278) (Cambridge Isotope Laboratories, MA, USA) was used to quantify the following congeners: BDE-28, 47, 99, 100, 153, 154, 183 and 209. n-Hexane, methylene dichloride, and nonane were pesticide grade and purchased from Tedia (Fairfield, OH, USA). All solvents and other reagents were of pesticide grade.

2.2. Wild sampling

Fifty-eight rice frogs (R. limnocharis) were collected from a rice field (NW) (Qin et al., 2009) in an e-waste recycling site (121°13′21″E; 28°21′41″N) in Taizhou (Fig. 1) in the early May in 2009 and the early May in 2010. Twenty frogs were dissected, and livers, testes, eggs and brains were removed. Because a brain and a testis were too small to detect PBDEs concentration, several tissue samples were combined into one sample for analysis. Hence, six pooled testis samples, six pooled brain samples, 20 liver samples and 10 egg samples were obtained. These samples included 10 pairs of egg samples and liver samples from 10 female frogs. These frogs did not lay eggs because of low temperature. Also, 16 whole frogs were analyzed for PBDEs concentrations in the whole frogs. All samples were cleaned with deionized water, wrapped in aluminum foil twice, and sealed in plastic bags to minimize the possibility of contamination. Then, samples were stored at −20 °C in darkness until analysis.

2.3. Elimination experiment

The remaining 22 frogs from the rice field in the e-waste recycling site in Taizhou were used for an elimination experiment. Firstly, seven frogs were stored at −20 °C as samples of day 0 of the depuration period. The remaining 15 frogs were raised in three glass tanks (five frogs in each tank) in the laboratory at 23 ± 2 °C. The tanks contained dechlorinated water, and the frog body was partly but not completely immersed in water, which was changed every two days. The frogs were fed on Yellow mealworm (Tenebrio molitor) everyday, in which PBDEs level was below the limit of detection (LOD) using our method for detecting PBDEs. Every 18 days, five frogs from three tanks were randomly sampled to examine the PBDE levels and to calculate the half-lives to PBDE congeners, resulting in three sampling occasions on day 18, 36, and 54 of a total depuration period of 54 days. We assumed that the elimination reaction follows the first-order model to simplify the real elimination mechanism.

The values of $t_{1/2}$ were calculated using the formula:

$$t_{1/2} = -\ln(0.5)/k_{el}$$

where $k_{el}$ is the terminal elimination rate constant for the compounds in the frogs, and

$$k_{el} = -\ln(10) \cdot b$$

where $b$ is the slope of the least-squares linear regression line of the log concentrations of PBDE congeners in frogs over time.

2.4. Extraction

The whole frog and tissue samples were freeze-dried and homogenized with anhydrous sodium sulfate, then spiked with BDE-71, and 13C-labeled BDE-209. The samples were subsequently extracted by ultrasonic extraction for twice with 30 mL of n-hexane/dichloromethane (1:1, vol/vol), then the combined extracts were evaporated to dryness for gravimetric determination of extracted lipid content. The concentrated extract was cleaned by passing through a 15-mm i.d. column, which was packed, from the bottom to top, with 1 g activated silica gel, 8 g acid silica gel (40% concentrated sulfuric acid, w/w), 1 g activated silica gel, and 1 cm anhydrous sodium sulfate. The PBDE mixture was eluted with 100 mL of hexane, concentrated to 2 mL using a rotary evaporator, transferred, and finally concentrated to approximate 0.1 mL under a gentle nitrogen stream. The final extract was transferred to GC vials. Throughout the extraction, cleanup and analysis procedures, the analytes were protected from light by wrapping the containers with aluminum foil or by using amber glassware.
2.5. PBDEs analysis

All sample extracts were analyzed by Agilent 6890 series gas chromatograph coupled with Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) using negative chemical ionization (NCI) source in the selected ion monitoring (SIM) mode. The gas chromatography column was DB-5 MS fused silica capillaries (15 m, 250-μm inner diameter, 0.25-μm film thickness). The injector and interface temperature were 265 and 300 °C, respectively, and samples were injected in the pulsed splitless mode. Methane was used as the chemical ionization moderating gas and helium as the carrier gas at a flow rate of 1.0 mL min⁻¹. The GC oven temperature program was carried out as follows: start at 100 °C, held for 1 min, increased to 200 °C at 10 °C min⁻¹, and then to 300 °C at 20 °C min⁻¹, held for 20 min. Ion fragments m/z 79 and 81 were monitored for tri- to hepta-BDE congeners, m/z 488.6, 486.6 for BDE-209 and 492.6, 494.6 for 13C-labeled BDE-209.

2.6. Quality assurance/quality control

To avoid potential sample contamination, cross contamination, and PBDE degradation, proper handling was adopted from sample collection to chemical analysis. One procedural blank was run for every batch of nine samples to check the potential contamination from the analysis process. Instrumental quality control was done by regular injection of solvent blanks and standard solutions. Recoveries of surrogate standards BDE-71 and 13C-labeled BDE-209 were on average 84 ± 12% and 65 ± 8%, respectively. Analyte value of BDE-209 was corrected for the recovery of 13C-labeled BDE-209, and the other congeners were corrected for the recovery of BDE-71. The limit of detection (LOD) was defined as the concentration of analyte in the sample producing a peak with the ratio of signal to noise of 3 (peak-to-peak). Non-detect or below LOD values were estimated as half of the detection limit for the purpose of calculating totals and means. For the tissue samples, LODs ranged from 0.005 to 0.025 ng g⁻¹ wet weight for tri- to hepta-BDE, and 0.03 ng g⁻¹ wet weight for deca-BDE.

2.7. Data analysis

The differences in PBDEs concentrations among various tissues were investigated by nonparametric test, followed by the Mann-Whitney U test. The lipid-normalized PBDE concentration ratios of egg to liver (E/L ratio) were used to assess the extent of maternal transfer of hydrophobic chemicals. The relationship between E/L ratios and bromine atoms numbers was analyzed using Linear Regression. The linear regressive slopes of the elimination of PBDE congeners were compared using GLM Univariate Analysis. A value of α = 0.05 was chosen to give a significant difference. All statistical analysis was performed using SPSS software version 13.0 (SPSS Inc., Chicago, USA). Volumes and surface areas of PBDE congeners were calculated using SYBYL-X 1.0 (Tripos Inc. Co.).

3. Results and discussion

3.1. PBDEs levels, tissue distribution and profiles

Table 1 shows the values of PBDEs concentrations in the whole rice frogs. Among the 16 samples, the ∑PBDEs levels ranged from 5.78 to 53.63 ng g⁻¹ wet wt., and the mean value was 27.68 ng g⁻¹ wet wt. The mean values of PBDEs concentrations in brains, livers, testes and eggs were 17.10, 28.38, 26.19, and 141.11 ng g⁻¹ wet wt., respectively.

Wu et al. (2009) reported that the mean levels of ∑PBDEs ranged from 2.26 ng g⁻¹ wet wt. in male muscle to 22.3 ng g⁻¹ wet wt. in eggs of frogs, which were collected from a PBDEs-contaminated area in South China. ter Schure et al. (2002) reported that the mean levels of BDE-47 and BDE-99 in livers of wild frogs were 0.067 and 0.066 ng g⁻¹ wet wt., respectively. In the present study, the levels of the mean ∑PBDEs ranged from 17.10 ng g⁻¹ wet wt. in brain to 141.11 ng g⁻¹ wet wt. in eggs of the frogs, respectively. The values of BDE-47 and BDE-99 in livers were 5.62 and 11.65 ng g⁻¹ wet wt., respectively. Comparing with the two available studies, the PBDE concentrations were higher in the present study.

Based on wet weight concentration, the level of ∑PBDEs in frog eggs was the highest among all frog samples. However, on a lipid weight basis, the concentrations in eggs and livers of frogs were comparable (see Section 3.2 below). Our results are consistent with the results on wild frogs reported by Wu et al. (2009). Several laboratory studies showed that exposure of fertilized eggs of birds could cause adverse effects on offspring, such as the inhibition in the growth, and changes in sex hormones (Fernie et al., 2005, 2006; Marteinson et al., 2011). A recent investigation reported a negative relationship between productivity and PBDEs concentration in osprey eggs, suggesting PBDEs may reduce reproductive success of ospreys (Henny et al., 2009). In addition, data concerning PBDEs in animal tests is very scarce. In this study, we reported for the first time the presence of PBDEs in frog testes. The level of mean ∑PBDEs in frog testes was almost equivalent to the levels of livers. In spite of the little information available whether PBDEs damage the function of testes, the presence of PBDEs in frog testes as well as that in frog eggs has raised a concern about potential reproductive risk of PBDEs for wild frogs.

Considering the potential of PBDEs to cause neurobehavioral deficits (McDonald, 2002; Costa and Giordano, 2007), the observed PBDE levels in frog brain may also be of concern. However,
previous studies reported the presence of PBDEs in the brains of some birds and mammals (Voorspoels et al., 2006; Gebbink et al., 2008; Isobe et al., 2009), showing that the concentrations of ∑PBDEs in brain were markedly lower than those in other tissues. This phenomenon could be explained by the protective function of the blood–brain barrier (BBB) against accumulation of PBDEs, although tissue-specific accumulation cannot be excluded. In the present study, the PBDE level in frog brains was slightly lower than the other tissues, but there was no significantly difference between brains and livers. The results may suggest that the BBB of a frog has less function to protect the brain from PBDEs than that of a bird or a mammal. To verify this supposition, a further study at a large sample size is needed.

All rice frog tissues and whole frogs exhibited a similar PBDE congener profile (Fig. 2). BDE-99, as the dominant congener, constituted 41.1–47.6% of the total PBDE concentrations, followed by BDE-47 at 18.4–24.5%, BDE-153 at 12.6–22.6%, and BDE-209 at 2.8–6.9%. Wu et al. (2009) also reported that BDE-99 was the dominant congener, while BDE-153 was the second dominant congener, followed by BDE-183. Wu et al. (2009) concluded that the frogs exhibited a unique PBDE congener profile that intermediated between aquatic and terrestrial species. PBDE congener profiles in aquatic species are characterized by lower brominated congeners as the dominated congeners, even if these are from areas with different background PBDE (Voorspoels et al., 2003; Zennegg et al., 2003; Hites et al., 2004; Meng et al., 2008; Qin et al., 2009). In our study and the study by Wu et al. (2009), therefore, the frogs exhibited a different PBDE congener profile from aquatic animal species. However, numerous studies have shown that PBDE congener profiles in terrestrial species are related to the background PBDE profile in the environment. For example, penta-BDE and octa-BDE are main PBDE pollutants in North America and Europe, correspondingly, BDE-47 and BDE-99 are the main congeners in terrestrial species including humans in these regions (Jaspers et al., 2006; Voorspoels et al., 2007). Deca-BDE congeners are main PBDE pollutants in Asia, correspondingly, higher brominated congeners are dominant contributors in terrestrial species including humans in Asia (Wang et al., 2007; Tanabe et al., 2008). Therefore, we suggest that the conclusion that the frogs exhibited a different PBDE congener profile from terrestrial species might be arbitrary to some degree in the Wu et al. study (2009). To elucidate the differences in PBDE congener profile between amphibians and aquatic/terrestrial species, a further investigation about these species in the same area is necessary.

3.2. Maternal transfer

In oviparous organisms, hydrophobic chemicals in females are transferred to eggs along with yolk proteins, which are formed in the mother liver (Russell et al., 1999; Kadokami et al., 2004). Therefore, in some studies the lipid-normalized concentration ratios of egg to liver (E/L ratio) were used to assess the extent of maternal transfer of hydrophobic chemicals (Wu et al., 2009). Following the method described by Wu et al. (2009), in the present study, we used the E/L ratio on the lipid wt. basis to assess the behavior of maternal transfer of PBDEs in frogs.

We detected PBDEs in 10 pairs of eggs and livers. On the lipid wt. basis, the E/L ratios (mean ± SD) for BDE-28, 47, 100, 99, 154, 153, 183, and 209 were 1.46 ± 0.75, 1.48 ± 0.53, 1.41 ± 0.59, 1.33 ± 0.48, 1.14 ± 0.50, 1.17 ± 0.70, 0.99 ± 0.59 and 0.67 ± 0.57, respectively. The ratio was 1.29 ± 0.50 for the total PBDEs. The E/L ratios for these congeners decreased with increasing bromine atoms (molecular size), and a linear relationship was obvious.

![Fig. 2. The congener profiles of polybrominated diphenyl ethers (PBDEs) in various tissues of rice frogs from a contaminated site in Taizhou. Error bars represent standard deviation.](image-url)

![Fig. 3. Correlations between the lipid-normalized PBDE concentration ratios of egg to liver (E/L ratio) and bromine atom numbers, volumes, and surface areas of PBDE congeners in wild female frogs from a contaminated site in Taizhou.](image-url)
(Fig. 3). In the study by Wu et al. (2009), the mean E/L ratios for PBDE congeners were all less than 1.0 except for hepta- and two hexa-BDEs, and for the total PBDEs was 0.86. BDE-183 had the highest value in their study, and the ratio for BDE-28 and 47 were the minimum. In our study, however, the ratios for BDE-28 and BDE-47 were the maximum. With an evidently difference from our result, the plot of E/L ratios of PBDE congeners in frogs versus number of bromine atoms followed a parabolic relationship in the Wu et al. (2009) report, i.e. the E/L ratios increased with increasing the numbers of bromine atoms up to 7 and then declined as the bromine atom numbers rose. Wu et al. explained that the transport of PBDEs from maternal tissue to eggs appeared to be related to both lipophilicity, i.e. the octanol–water partition coefficient (K_{ow}), and molecular sizes of the chemicals. For tri- to hepta-BDEs (from BDE-28 to BDE-183), the maternal transfer potential was likely based on their lipophilicity because chemicals with higher log K_{ow} are expected to have higher affinity to the lipoproteins, whereas for octa– deca-BDEs (from BDE-196 to BDE-209), the transfer might be inhibited by their relatively large molecular sizes (Wu et al., 2009). In our study, however, a negative correlation between transfer ratios and bromine atom numbers was found. We cannot explain why our result differed from the result reported by Wu et al. (2009). However, our result is consistent with most studies on the maternal transfer of lipophilic compounds in the literature. Based on a study concerning a series of compounds including PBDEs, organochlorine pesticides and by-products, polybrominated biphenyls, polychlorinated biphenyls (PCBs) and methysulfonyl-(MeSO2) PCBs in glaucesc gulls, Verreault et al. (2006) concluded that the maternal transfer favored lower halogenated compounds, whereas higher halogenated compounds were less readily transferred, and consequently more selectively retained in the mother. In fact, some wild studies have demonstrated that lower brominated congeners contribute more to \( \Sigma \)PBDEs than higher brominated congeners in eggs relative to other tissues, including livers (Pirard and De Pauw, 2007; Voorspoels et al., 2007; Zhang et al., 2010). In other words, lower brominated congeners seem to be more easily transferred into eggs than higher brominated congeners. In a study on frogs, the maternal transfer of polychlorinated dibenzo-p-dioxins/dibenzofurans was also reported to be negatively correlated with the chlorine number (Kadokami et al., 2004). Similarly, a decrease of transfer ratio with the increase of a chlorination degree was found in the maternal transfer of PCBs in fish (Serrano et al., 2008). Therefore, we suggest that the bromine atom number (the molecular size, to some degree) of a PBDE congener might be a determining factor for the transport of PBDEs from maternal tissues to eggs. The larger the bromine atom number of a PBDE congener, the more difficult it is transferred to eggs (the lower E/L ratio).

In addition, some authors generally correlated transfer ratios of compounds with K_{ow} and concluded that transfer ratios were negatively correlated with K_{ow} (Kadokami et al., 2004; Verreault et al., 2006; Serrano et al., 2008). It is difficult to reasonably explain this phenomenon that the maternal transfer of a lipophilic compound is negatively correlated with K_{ow}, a parameter reflecting lipophilicity. Considering that K_{ow} values of PBDE congeners increase with bromine atom numbers, we think, the maternal transfer might only apparently correlate with K_{ow} values, and instead correlate with bromine atom numbers, i.e. the molecular size.

### 3.3. Elimination of PBDEs

During a depuration period of 54 days, the \( \Sigma \)PBDEs concentrations in whole rice frogs decreased over time (Fig. 4), and the half-life of \( \Sigma \)PBDEs was calculated at 35 days. The elimination rate varied among different PBDE congeners. For example, BDE-47 fell sharply during the first 18 days, and then, almost kept unchanged.

So, the \( t_{1/2} \) of BDE-47 was within 18 days. BDE-99 decreased at a similar rate during the whole depuration period, and its \( t_{1/2} \) was 36 days. BDE-153 remained relatively unaltered during the first 18 days, and then decreased slowly, and its \( t_{1/2} \) was 81 days. As a result, the levels of BDE-209 and other congeners were too low to calculate their \( t_{1/2} \) values. The debromination of PBDEs, especially higher brominated congeners, was observed in many experiments (Tomy et al., 2004; Stapleton et al., 2006; Huwe and Smith, 2007), and possibly affected the calculation of half-lives in the elimination experiment. However, in the present study, the effect of the debromination of higher brominated congeners was likely negligible because higher brominated congeners constituted less than 10% of the \( \Sigma \)PBDEs in frogs. Therefore, the values of half-lives for PBDE congeners obtained in our study were accurate and reliable.

In previous studies, the half-lives among different PBDE congeners were also reported to vary at a large range. Tomy et al. (2004) reported that the half-lives of BDE-47, 99 and 153 were 39, 87 and 115 days, respectively, in the juvenile Lake Trout with dietary exposure (~2.5 ng g⁻¹ per BDE congener). In Drout-Lard et al. (2007), the half-lives of PBDEs in adult male American kestrel were expected to be on the order of 72, 175 and 572 days for BDE-47, 99 and 153, respectively. Staskal et al. (2005) reported that after an oral-exposure, a large majority of BDE-47 was excreted in mice within the first few days, the initial depuration \( t_{1/2} \) was 1.5 days, and then a slower elimination ensued with the terminal half-life was approximately 23 days.

The whole depuration process of BDE-47 in mice was exactly similar to our finding. von Meyerinck et al. (1990) reported that after a single oral dose of PBDE Bromkal 70 of 300 mg kg⁻¹ body weight, the half-lives of Br₁₀-DE, Br₁₁-DE1 and Br₁₀-DE2 in female and male rats were 29.9, 47.4, 90.9 days and 19.1, 36.8, 119.1 days, respectively. From the above studies, we could draw a conclusion that the half-lives of PBDE congeners range in a particular range.

Although the half-lives among different PBDE congeners varied at a large range in previous reports, all studies reported a consistent order of the half-life, i.e. BDE-153 > BDE-99 > BDE-47. In the present study, the order of the half-lives for individual PBDE congeners in frogs was conform to the common order observed in fish, birds and rodents. We also found that log of half-lives of BDE-47, 99, 153 are linearly related to the bromine atom numbers (Fig. 6). In another word, the half-lives became longer with the increase of bromine atom numbers in BDE-47, 99 and 153.

![Fig. 4. Decrease in the level of polybrominated diphenyl ethers (PBDEs) in whole frogs during a depuration period of 54 days. Error bars represent standard deviation.](image-url)
4. Conclusion

We found that the PBDEs levels in the wild frogs from an e-waste recycling site in Taizhou were higher than the reported levels in previous studies, showing a heavy PBDEs pollution in this e-waste recycling area in China. Frogs are great bio-accumulators of PBDEs and possibly good bio-indicators too. All frog tissues exhibited a similar PBDE congener profile, with the intermediate brominated congeners (BDE-99 and BDE-153) as the largest contributors. In addition, we found that the transfer capacity of PBDEs from maternal tissues to eggs declined with the increase in bromine numbers, suggesting that the bromine number, even the molecular size, of a PBDE congener is a determining factor for the maternal transport of PBDEs rather than lipophilicity. Also, we studied PBDEs elimination in frogs for the first time, and found that PBDEs concentrations in frogs decreased over time during a depuration period of 54 days and the elimination rates were different among different congeners, BDE-47 > BDE99 > BDE153. Compared with the data on the half-lives of PBDEs in other species, the elimination of PBDEs in frogs seems to not essentially different from those observed in other species.

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